Altered expression of methylenetetrahydrofolate reductase modifies response to methotrexate and 5-fluorouracil in mice

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This thesis is dedicated to my family

for their endless support, encouragement, pride and love

throughout my life

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ABSTRACT

Folates are essential cofactors that are required for the synthesis of nucleotides, the precursors of DNA and RNA. Two widely used anti-metabolite chemotherapeutic drugs, anti-folate methotrexate (MTX) and the pyrimidine antagonist 5-fluorouracil (5-FU), inhibit DNA and RNA synthesis and induce apoptosis, through their effects on the folate pathway. A common polymorphism (677C→T) in methylenetetrahydrofolate reductase (MTHFR), a critical folate-metabolizing enzyme in nucleotide synthesis, may modify the chemosensitivity of MTX and 5-FU.

In this thesis, two mouse models (a well-characterized mouse model deficient in MTHFR (*Mthfr*$^{-/-}$ and *Mthfr*$^{+/-}$) and a new mouse model that overexpresses MTHFR (*MTHFR-Tg*)) were used to investigate the effect of MTHFR expression on response to MTX and 5-FU. The latter mouse model, *MTHFR-Tg*, was generated and characterized in this thesis. *MTHFR-Tg* mice had an increase in methionine in brain, a decrease in cysteine in duodenum, an increase in glutathione in liver and decreases in 10-formyltetrahydrofolate levels in liver and duodenum.

Mthfr-deficient and *MTHFR-Tg* mice and their wild-type littermates were injected with MTX or 5-FU (with saline as a control) and assessed for hematological parameters (hematocrit, hemoglobin, red and white blood cell numbers), plasma homocysteine levels, serum nephrotoxicity and hepatotoxicity markers, splenic dUTP/dTTP ratios and apoptosis.

MTHFR overexpression increased the effect of MTX on hematopoietic cells, through reduced DNA synthesis and deoxyribonucleotide imbalance (higher dUTP/dTTP ratios)-induced splenic apoptosis, with a protection against MTX-induced hyperhomocysteinemia. MTHFR deficiency had similar effects on MTX-treated

hematopoietic cells, through hyperhomocysteinemia-induced splenic apoptosis, with enhanced MTX-induced hyperhomocysteinemia and nephrotoxicity.

Furthermore, in transformed mouse embryonic fibroblasts, MTHFR overexpression was protective against MTX-induced apoptosis, possibly through its protective effect against hyperhomocysteinemia.

Lastly, MTHFR overexpression enhanced 5-FU-induced leucopoenia and splenic apoptosis while reducing plasma Hcy levels.

The major problem with MTX and 5-FU therapies is the variability in therapeutic outcome and toxicity which is the primary reason for discontinuation of therapy. Our studies illustrate the critical effect of MTHFR expression on the chemosensitivity to MTX and 5-FU, and the importance of pharmacogenetic testing for the *MTHFR* polymorphism and possibly polymorphisms in other folate-metabolizing enzymes to maximize efficacy and minimize toxicity.

RÉSUMÉ

Les folates sont essentiels pour la synthèse des nucléotides. Deux anti-métabolites, le méthotrexate (MTX) et le 5-fluorouracile (5-FU), inhibent la synthèse des acides nucléiques et induisent l'apoptose. Un polymorphisme fréquent (677C→T) de la méthylènetétrahydrofolate réductase (MTHFR) peut modifier la chimiosensibilité au MTX et au 5-FU.

Nous avons étudié l'effet de l'expression de MTHFR sur la réponse au MTX et au 5-FU en utilisant des souris qui sont déficientes en MTHFR (*Mthfr*$^{-/-}$ et *Mthfr*$^{+/-}$) ou qui surexpriment MTHFR (*MTHFR-Tg*). Les souris *MTHFR-Tg* ont un niveau élevé de méthionine dans le cerveau, une diminution de la cystéine dans le duodénum, une augmentation de la glutathione hépatique et moins de 10-formyltétrahydrofolate dans le foie et le duodénum.

Les souris *Mthfr*$^{-/-}$, *Mthfr*$^{+/-}$ et *MTHFR-Tg*, de même que leur fraterie, ont été injectés avec du MTX ou du 5-FU. Plusieurs paramètres hématologiques ont été évalués, ainsi que l'homocystéine plasmatique, des marqueurs de néphrotoxicité et d'hépatotoxicité, les rapports dUTP/dTTP spléniques et l'apoptose.

La surexpression de MTHFR limite l'hyperhomocystéinémie induite par le MTX. De plus, elle accroît l'effet du MTX sur les cellules hématopoïétiques. Cela découle de la réduction de la synthèse d'ADN et de l'apoptose splénique suivant le déséquilibre dUTP/dTTP. Une déficience en MTHFR a des effets comparables sur les cellules hématopoïétiques traitées au MTX, étant donné l'apoptose splénique résultant de l'hyperhomocystéinémie. La néphrotoxicité induite par le MTX est exacerbée, de même que l'hyperhomocystéinémie.

Nous avons aussi transformé des lignées primaires de fibroblastes embryonnaires dérivées de nos souris. La surexpression de MTHFR a un effet protecteur contre l'apoptose induite par le MTX, possiblement parce que l'hyperhomocystéinémie est limitée.

Finalement, la surexpression de MTHFR augmente la leucopénie induite par le 5-FU ainsi que l'apoptose splénique et réduit les niveaux d'Hcy plasmatiques.

La variabilité du résultat thérapeutique et de la toxicité complique les thérapies impliquant le MTX et le 5-FU. Nos travaux illustrent l'effet de l'expression de MTHFR sur la chimiosensibilité au MTX et au 5-FU, ainsi que l'importance de tester le polymorphisme de *MTHFR* et possiblement les polymorphismes d'autres enzymes qui métabolisent les folates, afin de maximiser l'efficacité des anti-métabolites et d'en minimiser la toxicité.

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THESIS FORMAT

This thesis is a manuscript-based thesis. In this thesis, there are five chapters. The first chapter is a review of the relevant literature. Chapter II has been accepted by The Journal of Pharmacogenetics and Genomics. Chapter III and IV will be submitted for publication, following the publication of Chapter II. All data chapters are linked by their connecting texts. The last chapter is the discussion.

CONTRIBUTION OF AUTHORS

For chapters II, III and IV, the candidate designed and performed the majority of the experiments, analyzed and interpreted the data, and wrote the manuscripts under the supervision of Dr. Rima Rozen.

In Chapter II, the candidate performed the experiments for the generation and the characterization of the transgenic mice under the guidance of Daniel Leclerc. Daniel Leclerc designed all the required intermediate constructs to make the final construct, the primers for genotyping and the probe for Southern Blotting. The candidate performed all animal experiments, genotyping, backcrossing onto the C57Bl/6 background for 15 generations, methotrexate injections, and tissue and blood collection. Andrea Lawrance performed the HPLC measurements. Liyuan Deng performed the TLC measurements. Hana Friedman performed the site-specific recombination of the final construct with the *HPRT* destination vector. The electroporation to embryonic stem cells and blastocyst injection was done in Dr. Alan Peterson's laboratory. Natalia and Sergey Krupenko performed the folate measurements. Stepan Melynk and Jill James performed the folate-related metabolite measurements. Michel Tremblay performed all thiol measurements. Complete blood cell counts and toxicity parameter measurements were performed by the Animal Resource Center of McGill University. Marilene Paquet performed the total necropsies of the transgenic mice.

In Chapter III, the candidate performed all animal experiments, genotyping, methotrexate injections, tissue collection and sectioning, and apoptosis measurements. The candidate performed all tissue culture experiments, passage of the cells, administration of methotrexate, protein extraction and apoptosis measurements. The extractions of mouse

embryonic fibroblasts were performed with the help of Laura Pickell and Ermenia Di Pietro, and these cells were transformed by Timothy Johns in Dr. Eric Shoubridge's laboratory.

In Chapter IV, the candidate performed all animal experiments, genotyping, 5-fluorouracil injections, tissue and blood collection, tissue sectioning and apoptosis measurements. Michel Tremblay performed all thiol measurements. Complete blood cell counts were performed by the Animal Resource Center of McGill University.

ABBREVIATIONS

AI	apoptotic index
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AICARFT	aminoimidazole carboxamide formyl transferase
ALL	acute lymphoblastic leukemia
AST	aspartate aminotransferase
BHMT	betaine homocysteine methyltransferase
BUN	blood urea nitrogen
CBS	cystathionine β-synthase
CML	chronic myelogenous leukemia
Cys	cysteine
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DMG	dimethylglycine
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate

dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
FAICAR	formylaminoimidazole-4-carboxamide ribonucleotide
5-FdUMP	5-fluorodeoxyuridine monophosphate
5-FdUTP	5-fluorodeoxyuridine triphosphate
FPGS	folylpolyglutamate synthetase
FR	folate receptor
5-FU	5-fluorouracil
5-FUTP	5-fluorouridine triphosphate
GARFT	glycinamide ribonucleotide formyl transferase
GGH	pteroylpolyglutamate hydrolase (γ-glutamyl hydrolase)
GSH	glutathione
Hb	hemoglobin
Hct	hematocrit
HPLC	high performance liquid chromatography
HPRT	hypoxanthine guanine phoshoribosyltransferase
IMP	inositol monophosphate
LD	lethal dose
Met	methionine
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
MTX	methotrexate
NHL	non-Hodgkin’s lymphoma

OMP	orotidine monophosphate
PRPP	phosphoribosylpyrophosphate
RA	rheumatoid arthritis
RBC	red blood cells
RFC	reduced folate carrier
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SHMT	serine hydroxymethyltransferase
tHcy	total homocysteine
THF	tetrahydrofolate
TLC	thin layer chromatography
TS	thymidylate synthase
TUNEL	the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
WBC	white blood cells

CONVENTIONS

In this thesis, mouse genes are written in lowercase italics (e.g. *Mthfr*) and human genes are written in uppercase italics (e.g. *MTHFR*). The names of the proteins are not italicized for both mouse and human and are written in uppercase (e.g. MTHFR).

CHAPTER I.

LITERATURE REVIEW

1.1 FOLATES AND THEIR METABOLISM

1.1.1 Overview

Folates are a family of B-vitamins, which are water-soluble pteridine compounds. The naturally occurring forms are predominantly folate polyglutamates that are mostly found in liver, yeast, egg yolks and green vegetables. Folic acid is a highly stable, supplemental folate used to fortify food and multivitamins. The biologically active form of folate is tetrahydrofolate (THF); 5-methylTHF is the predominant form of folate found in tissues and serum. N^5- and N^{10}-substituted folate derivatives play a key role in the biosynthesis of purines, thymidylate, amino acids, and in the degradation of histidine [1-3] (see **Figure 1.1**). Serine is a primary source of folate-activated one-carbon units [4].

Dietary folate polyglutamates are hydrolyzed by pteroylpolyglutamate hydrolase (γ-glutamyl hydrolase; GGH) in the intestinal brush border and are absorbed as folate monoglutamates [5,6]. They are transported across the cell membrane by the reduced folate carrier (RFC) or membrane-associated folate binding proteins which are also called folate receptors (FRs). RFC is ubiquitously expressed, whereas the FR is variably expressed in tissues [7-10]. RFC mediates a bidirectional low affinity, high capacity system for the uptake of reduced folates (such as 5-methylTHF and 5-formylTHF, also known as leucovorin) and folate antagonists (anti-folates, such as methotrexate) at high concentrations and neutral pH [11-14]. The FR mediates a unidirectional high affinity, low capacity system for the uptake of exogenous folate (such as folic acid) at low concentrations and acidic pH [10,15]. Within cells, folates are polyglutamated by folylpolyglutamate synthetase (FPGS) to prevent their efflux, and achieve the optimal concentration of intracellular folate for activity of folate-dependent enzymes [16]. Lysosomal GGH regulates intracellular folate polyglutamate levels.

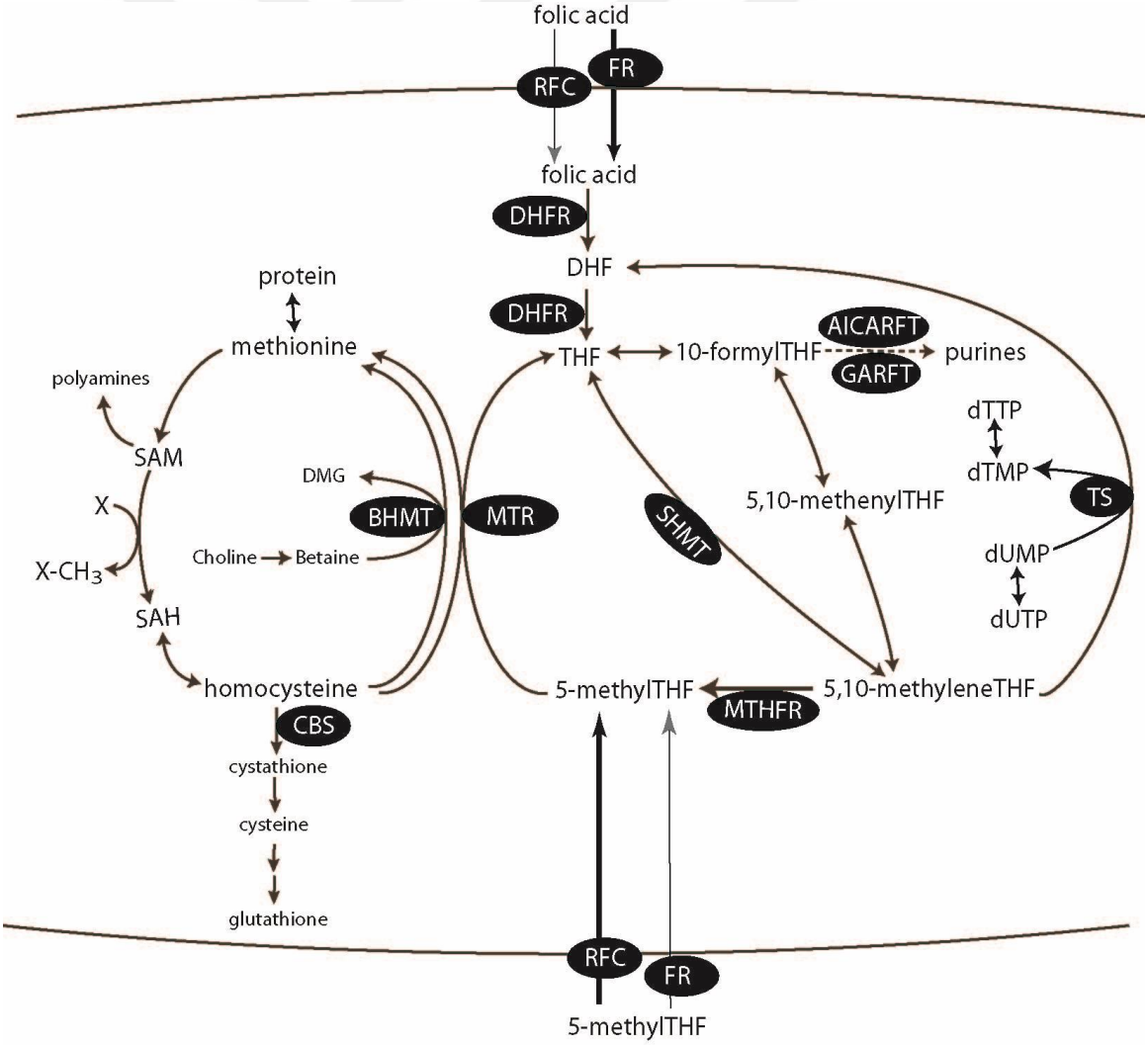
Folate monoglutamates can pass into circulation through the basolateral membrane of the intestinal epithelium. The major circulating form of folate, 5-methylTHF monoglutamate, reaches liver, the storage organ for folate polyglutamates, via the portal circulation [1,3].

Folic acid is reduced first to dihydrofolate (DHF), then to THF, by dihydrofolate reductase (DHFR). THF is converted into 5,10-methyleneTHF by serine hydroxymethyltransferase (SHMT) with the addition of a carbon unit from serine. Then methylenetetrahydrofolate reductase (MTHFR), using FAD and NADPH as cofactors, catalyses the irreversible reduction of 5,10-methyleneTHF to 5-methylTHF. The 5,10-methyleneTHF serves as a source of one-carbon units for the conversion of dUMP to dTMP by thymidylate synthase (TS). It may also be reduced to 10-formylTHF, which is used for *de novo* purine synthesis, catalyzed by glycinamide ribonucleotide formyl transferase (GARFT) and aminoimidazole carboxamide formyl transferase (AICARFT). The product of MTHFR, 5-methylTHF, serves as the methyl donor for the remethylation of homocysteine (Hcy) to methionine (Met), which is catalyzed by methionine synthase (MTR). MTR uses methylcobalamin as a cofactor and requires activation by MTR reductase (MTRR). Met is converted to S-adenosylmethionine (SAM) by methionine adenosyltransferase. SAM is considered a universal methyl donor; it is used in the methylation of DNA, RNA, proteins, histones, neurotransmitters, hormones and phospholipids [17] and in the synthesis of polyamines, which are necessary for cellular growth and replication [18]. S-adenosylhomocysteine (SAH), the by-product of these methylation reactions, is subsequently hydrolyzed, to produce Hcy by SAH hydrolase. In liver, kidney, brain, intestine, and pancreas [19], Hcy may also undergo transsulfuration to generate cysteine and glutathione. The first step is catalyzed by cystathionine β-synthase (CBS), which is allosterically activated by SAM and uses pyridoxal phosphate as a cofactor [1,3,20].

Since the major circulating form of folate is 5-methylTHF and the MTHFR reaction is irreversible, intracellular folates must pass through the MTR reaction in order to regenerate THF, a reaction which requires methylcobalamin and 5-methylTHF as methyl donors. If there is a deficiency in MTR activity, because of mutations or cobalamin deficiency, intracellular folates are trapped as 5-methylTHF and cannot be used for purine and pyrimidine synthesis, resulting with megaloblastic anemia. In the liver and kidney, Hcy remethylation can be also catalyzed by betaine homocysteine methyltransferase (BHMT), which requires betaine (dietary betaine or the oxidation product of choline) as a methyl donor [1,3].

Figure 1.1 Folate Metabolism

Abbreviations: the reduced folate carrier (RFC), folate receptor (FR), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), dimethylglycine (DMG), dihydrofolate reductase (DHFR), serine hydroxymethyltransferase (SHMT), methylenetetrahydrofolate reductase (MTHFR), thymidylate synthase (TS), glycinamide ribonucleotide formyl transferase (GARFT), aminoimidazole carboxamide formyl transferase (AICARFT), methionine synthase (MTR) and betaine homocysteine methyltransferase (BHMT), cystathionine β-synthase (CBS).



1.1.2 Nucleotide synthesis

Nucleic acids are polymers of nucleotides, which are joined by phosphodiester linkages between the 5′OH of one pentose sugar and the 3′OH of the next. Nucleotides are a combination of a pentose sugar (ribose or deoxyribose), a nitrogenous base (purine or pyrimidine), and one or more phosphate groups [21] (see **Table 1.1**).

Nucleotides have several important roles in cellular metabolism. They are chemical energy carriers in metabolic transactions (ATP), essential chemical links for hormonal response (cellular secondary messengers such as cAMP and cGMP), structural components of enzyme cofactors (NAD, FAD, SAM), and subunits of nucleic acids (DNA and RNA) for carrying genetic information [21].

Folate cofactors are required for biosynthesis of purines and thymidine, the precursors for nucleotides in DNA and RNA synthesis.

In *de novo* purine biosynthesis, the initial building block of purines is ribose 5′-phosphate, which is converted to phosphoribosylpyrophosphate (PRPP) by PRPP synthetase. Subsequent reactions add carbon or nitrogen elements to build up the purine ring. The C8 carbon is provided by 10-formylTHF which is incorporated into glycinamide ribonucleotide to form formylglycinamide ribonucleotide by GARFT. The C2 carbon is also provided by 10-formylTHF which is incorporated into 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) to form formylaminoimidazole-4-carboxamide ribonucleotide (FAICAR) by AICARFT. Inositol monophosphate (IMP) is produced from FAICAR and can be converted to adenosine monophosphate (AMP) or guanosine monophosphate (GMP) [22].

In *de novo* pyrimidine biosynthesis, the initial building block of pyrimidines is glutamine, which is converted to carbamoylphosphate and aspartic acid, by

carbamoylphosphate synthetase (rate limiting step). After three more steps, orotic acid is produced and is converted to orotidine monophosphate (OMP), with addition of PRPP, by orotate phosphoribosyltransferase. Finally, uridine monophosphate (UMP) is produced by OMP decarboxylase. UMP is phosphorylated to UDP by nucleoside monophosphate kinase (as are other deoxyribo/ribonucleotide monophosphates) and to UTP by nucleoside diphosphate kinase (as are other deoxyribo/ribonucleotide diphosphates). CTP can be produced from UTP by CTP synthetase. Conversion between ribo- and deoxyribonucleotide diphosphates (UDP to dUDP, CDP to dCDP, ADP to dATP, GDP to dGTP) is catalyzed by ribonucleotide reductase, which is the rate-limiting enzyme of entire process of DNA synthesis. Unlike the synthesis of dAMP, dGMP and dCMP, the *de novo* synthesis of dTMP involves only deoxyribonucleotides. The conversion of dUMP to dTMP is catalyzed by TS which uses 5,10-methyleneTHF as a methyl donor [22].

Folates, as methyl donors, are critical for DNA synthesis, repair and the maintenance of DNA integrity and stability. Several studies have shown that folate deficiency decreases DNA integrity and increases DNA damage, through increased uracil misincorporation into DNA and chromosomal breaks. These conditions ultimately enhance carcinogenesis [23-25].

Folate deficiency can induce an imbalance in deoxyribonucleotide pools, with generation of higher levels of dUMP at the expense of dTMP (an increase in the dUMP/dTMP ratio), due to the decreased supply of the TS substrate, 5,10-methyleneTHF. This can lead to DNA polymerase-mediated dUTP misincorporation into DNA [26,27]. Uracil is excised from DNA by uracil-DNA glycosylase and without sufficient dTTP to fill in the abasic site, a single-strand break can result. Repair of two adjacent uracils on opposite strands can result in double-strand breaks and chromosome instability [28]. Unrepaired double-strand breaks may lead to the loss of function of tumor suppressor genes.

Additionally, during the repair of double-strand breaks, errors may occur that result in translocations. They result in genetic instability, DNA fragmentation and sensitivity to DNA damaging agents, which may enhance cellular transformation and carcinogenesis [29].

Table 1.1 Nucleotide and Nucleic Acid Nomenclature

Based on Table 10-1 of Lehninger [21]

Base	Nucleoside Pentose (ribose or deoxyribose) +Base (purine or pyrimidine)	Nucleotide Pentose (ribose or deoxyribose) +Base (purine or pyrimidine)+ Phosphate group	Nucleic acid
Purines			
Adenine	Adenosine	Adenylate	RNA
	Deoxyadenosine	Deoxyadenylate	DNA
Guanine	Guanosine	Guanylate	RNA
	Deoxyguanosine	Deoxyguanylate	DNA
Pyrimidines			
Cytosine	Cytidine	Cytidylate	RNA
	Deoxycytidine	Deoxycytidylate	DNA
Thymine	Thymidine/	Thymidylate/	
	Deoxythymidine	Deoxythymidylate	DNA
Uracil	Uridine	Uridylate	RNA

1.1.3 Methylation

Folates, as methyl donors, hold a key position in DNA synthesis and repair, as mentioned in the above section, as well as in DNA methylation, regulation of gene expression, and overall maintenance of DNA integrity and stability.

Epigenetic events, DNA methylation (addition of a methyl group at position C5 of cytosine within CpG dinucleotide repeats by DNA methyltransferases) and post-translational modifications of histones (histone (de)methylation and (de)acetylation), alter gene expression, genomic stability, cellular growth, development and function (tissue differentiation and aging), without changes in DNA coding sequence that are heritable through cell division [30,31].

Aberrant DNA methylation was associated with several diseases, such as imprinting disorders, repeat-instability diseases, and mainly cancer. Alterations in gene expression and genomic instability by genome wide hypomethylation and hypermethylation of CpG islands (in promoter regions or the first exon of most of the genes and usually unmethylated in normal somatic cells) may enhance carcinogenesis [31,32].

Site-specific alterations in DNA methylation within critical genes are implicated in carcinogenesis. This may include hypomethylation/activation of protooncogenes or hypermethylation/inactivation of tumor suppressor genes. In addition, methylated cytosines represent mutational hotspots; 5-methylcytosine is more prone to hydrolytic deamination than unmethylated cytosine. This can cause a G/T mismatch and subsequently, if unrepaired, to a C→T transition mutation [33,34]. Additionally, DNA hypomethylation in the coding regions of critical genes can be deleterious. Unmethylated cytosines can undergo deamination to uracil and as previously discussed, uracil removal can cause DNA strand

breaks, mutations, genomic instability and ultimately carcinogenesis [35-37]. DNA hypomethylation, as a consequence of uracil misincorporation (loss of the methyl group in thymine, by replacing thymine with uracil), was shown to alter sequence-specific transcription factor binding and gene expression [38].

DNA methylation can alter gene expression directly, by inhibiting transcription factor binding and indirectly, by affecting chromatin accessibility. Methylated promoter regions can induce recruitment of methyl-cytosine-binding proteins that associate with histone deacetylases. In addition, DNA methyltransferases can recruit histone deacetylases. Deacetylation of histones results in condensation of chromatin, which limits the access of transcription factors to promoter regions of nearby genes, and leads to transcriptional silencing of genes [30,39,40].

DNA methylation can be modified by dietary methionine, choline, or folate deficiency [37,41] and/or polymorphisms in folate metabolizing enzymes, such as MTHFR [42]. Hepatocarcinogenesis through alterations in DNA methylation was demonstrated in rats fed folate/methyl-deficient diets [43]. In addition, folate deficiency with or without MTHFR deficiency favors the development of colorectal tumors through genomic hypomethylation [24,25,41]. *In vivo* folate deficiency induces hypomethylation in the coding region of *p53*, an important regulator of DNA repair, and causes DNA strand breaks within *p53* gene [35,36]. Also *in vitro* and *in vivo* imbalance in deoxyribonucleotide pools and uracil misincorporation promote error-prone DNA synthesis via reduced DNA repair as discussed above [26,44,45]. Both mechanisms may enhance carcinogenesis in folate deficiency.

In addition to DNA, the methylation of other macromolecules can be affected by folate status. For example, the demethylation of tRNA and snRNA has been demonstrated as a result of folate/methyl-deficient diets [46-48]. Additionally, in central nervous system,

synthesis of membrane phospholipids, myelin basic protein, and neurotransmitters require methylation; therefore alteration in methylation can be one of the potential mechanisms in the occurrence of neuropsychiatric disorders [49].

1.1.4 Homocysteine

Alterations in folate metabolism can change not only the methylation profile but also Hcy levels through changes in 5-methylTHF availability. Hcy is a neurotoxic and cardiotoxic sulfur-containing amino acid and stands at a checkpoint that interconnects one-carbon-transferring reactions with the metabolism of sulfur-containing amino acids.

Hyperhomocysteinemia has been shown to be associated with cardiovascular disease, cerebrovascular disease, stroke and venous thrombosis [50-53], as a consequence of Hcy-induced endothelial cell injury, smooth muscle cell proliferation, endothelial dysfunction, endoplasmic reticulum stress, oxidative stress, lipid peroxidation, inflammation and prothrombotic effects [54]. Additionally, Hcy has an excitatory effect on neurons; therefore hyperhomocysteinemia may cause neurological disorders [55].

1.1.5 Apoptosis

Apoptosis, or programmed cell death, is essential for various developmental processes as well as the elimination of damaged cells in order to maintain cellular homeostasis. Apoptosis can be induced by several stimulating factors leading to the activation of cysteine aspartate protease (caspase) family members. There are 13 mammalian members of this family; all of them are synthesized and secreted in the procaspase form and are activated by proteolytic cleavage. The specific caspases are activated, depending on the apoptotic stimuli

and tissue. Activated initiator caspases (caspase-2, -8, -9 and -10) activate effector caspases (caspase-3, -6 and -7), which are responsible for destruction of cytoskeletal and structural proteins. This destruction causes the morphologic characteristics of apoptotic cells: cellular and nuclear shrinkage, nuclear chromatin condensation and membrane breakdown, DNA fragmentation, loss of contact, formation and phagocytosis of apoptotic bodies by neighbouring cells [56-59].

In vitro and *in vivo* folate deficiency inhibits cellular proliferation, disturbs cell cycling, causes genetic damage and eventually results in apoptosis [44,45]. Imbalances in deoxyribonucleotide pools have shown to stimulate protease activation, which initiates apoptosis [60]. DNA damage activates the mitochondrial intrinsic apoptosis pathway to prevent accumulation of damaged cells, as a protective mechanism against carcinogenesis and the mechanism of action of most chemotherapeutic drugs [56,59,61].

Furthermore, folate deficiency induces apoptosis, via *p53* upregulation and accumulation of uracil and p53 protein, in mice splenic erythroblasts and *in vitro* in several cell lines [62,63]. Depending on the number of double-strand breaks caused by uracil misincorporation, p53 can activate proapoptotic genes, such as BAX [64]. p53 induces apoptosis via extrinsic pathway (by stimulating expression of death receptors) or intrinsic pathway (by activating genes that encode mitochondrial membrane permeabilization proteins) [65]. In addition to folate deficiency, choline deficiency also induces apoptosis via *p53* upregulation [66] or decreasing phosphatidylcholine levels, which are required for normal cell cycle progression [67]. In rat hepatocytes, folate/methionine/choline deficiency has been shown to induce apoptosis via endonuclease-mediated DNA cleavage; the surviving cells undergo malignant transformation [45,68]. Aberrant DNA synthesis and repair may

cause malignant transformation through inactivation of proapoptotic pathways [65]. On the other hand, folate-deficient cells that are proficient in DNA repair have been shown to undergo apoptosis [63].

In addition, folate deficiency causes hyperhomocysteinemia, which has a pro-apoptotic effect via an increase in oxidative stress [69]. Homocysteine induces overproduction of hydrogen peroxide, activation of NF-κB, activation of caspase-12 (via stimulating endoplasmic reticulum stress), and impairs the nitric oxide synthase pathway [70-72].

1.1.6 *MTHFR* and *Mthfr*

MTHFR is a key folate-metabolizing enzyme in regulating the distribution of one-carbon groups between methylation reactions and nucleic acid synthesis.

1.1.6.1 Gene

The molecular characterization of *MTHFR* began with a cloned 2.2-kb human cDNA, containing 11 coding exons. It is similar to the cDNA/gene structure of that in mouse, with 90% identity in the coding sequences [73,74]. The mouse and human MTHFR amino acid sequences are approximately 90% identical [75]. The human gene has been mapped to chromosomal region 1p36.3 [73] and the mouse gene has been mapped to the syntenic region in mouse, distal chromosome 4 [76].

The regulation of *MTHFR* is complex; the gene has at least two promoters, which have multiple transcriptional start sites that are used to preferentially express different protein isoforms [77]. The *MTHFR* promoters are TATA-less but contain multiple potential

Sp1 [78] and NF-κB binding sites. NF-κB activation results in an increase in *Mthfr* mRNA [79].

MTHFR has three transcripts, 2.8 kb, 7.5 kb, and 9.5 kb. *Mthfr* has four transcripts, 2.8 kb, 3 kb, 6.3 kb, and 7.4 kb. Both transcript sizes and expression levels are tissue specific [77].

Mammalian MTHFR is an FAD-dependent cytosolic enzyme and functions with NADPH (a reducing agent). It has an N-terminal catalytic domain (FAD, NADPH, and 5,10-methyleneTHF binding) and a C-terminal regulatory domain (SAM binding). It is a dimeric protein with subunits of either 77 kDa or 70 kDa [80]. The 2.2-kb human cDNA encodes the polypeptide of 70 kDa [74]. An upstream translational start site and alternative splicing of *MTHFR* result in an active enzyme of 77 kDa. Therefore clusters of transcriptional start sites and alternative splicing in exon 1 enable translation of two protein isoforms, through 2 different initiation codons. The major subunit in most human tissues is 77 kDa [77].

Mammalian MTHFR is ubiquitously expressed and its expression is most intense in testis, intermediate in brain and kidney, and lower in the other tissues. Tissue-specific expression can be regulated at the 3' end of the gene through polyadenylation site choice [77].

Mammalian MTHFR activity is negatively regulated by DHF or SAM binding [81,82]. SAM binding is reversible by SAH binding and induced by MTHFR phosphorylation [83,84].

1.1.6.2 Severe MTHFR deficiency

Severe MTHFR deficiency, an autosomal recessive congenital metabolic disease, is the most common inborn error of folate metabolism. It is characterized by 0%-20% residual enzyme activity and is associated with hyperhomocysteinemia, homocystinuria, hypo- or normo-methioninemia, and altered folate distribution, as well as developmental delay, and severe neurological and vascular abnormalities [1,3].

1.1.6.3 Mild MTHFR deficiency

Two common polymorphisms of the human MTHFR enzyme cause mild MTHFR deficiency: 677C→T, which leads to the substitution (missense mutation) of alanine to valine (in the N-terminal catalytic domain) at codon 222, exon 4 [74] and 1298A→C, which leads to the replacement of glutamate by alanine (in the C-terminal regulatory domain) at codon 429, exon 7 [85,86].

The homozygous 677C→T variant, with ~30% of the wild-type activity, has a prevalence of ~10-15% in the North American Caucasian population. Heterozygotes have ~60% of the wild-type activity and form ~40% of the population [74,87].

The Ala222Val MTHFR is particularly sensitive to heat treatment (46°C, 5 minutes) and is called thermolabile. The residual activity after heating in *MTHFR* 677TT individuals is 30% of wild-type [74]. Thermolabile MTHFR has an enhanced tendency to dissociate into monomers and to lose its FAD cofactor on dilution; the resulting loss of activity is slowed in the presence of 5-methylTHF or SAM [83]. The distribution of folate derivatives is altered in *MTHFR* 677TT individuals. Whereas most red blood cell (RBC) folate is normally found as 5-methylTHF, individuals with thermolabile MTHFR have formylated folates in addition to methylated folates in their RBCs [88]. In addition, genomic DNA methylation in

peripheral blood mononuclear cells is significantly lower in *MTHFR* 677TT individuals with low folate status. This may be due to the reduction in 5-methylTHF and SAM levels or the increase in SAH (an inhibitor of SAM-dependent methyltransferases) levels. In *MTHFR* 677TT individuals, genomic DNA methylation is directly correlated with 5-methylTHF levels in RBCs (an indicator of long-term folate status) and inversely correlated with plasma Hcy levels [89,90]. *MTHFR* 677TT individuals who maintain adequate folate levels have normal Hcy levels, because adequate 5-methylTHF levels may stabilize the enzyme and enable it to function normally. When folate status is low, they have mild hyperhomocysteinemia [91].

The homozygous 1298A→C variant, with ~60% of the wild-type activity, has a prevalence of ~12% in Caucasians [92].

In contrast to the Ala222Val MTHFR, the Glu429Ala variant is biochemically indistinguishable from the wild-type enzyme and is not thermolabile [83]. Similar to the *MTHFR* 677C→T polymorphism, the *MTHFR* 1298A→C polymorphism has been associated with lower MTHFR activity in lymphocytes, but not associated with decreased plasma folate levels or increased plasma Hcy levels. However, individuals who are heterozygous for both polymorphisms have higher plasma Hcy levels and reduced plasma folate levels [93]. Interestingly, there are higher RBC folate levels in individuals with the *MTHFR* 1298A→C polymorphism [94].

Mild MTHFR deficiency is associated with an increased risk for several common multifactorial conditions, including neural tube defects, congenital heart defects, cardiovascular disease, neuropsychiatric disorders, and pregnancy complications. These may be due to altered folate distribution and hyperhomocysteinemia (reviewed in [87]).

However, when folate levels are sufficient and alcohol intake is low, the *MTHFR* 677TT genotype is associated with a decreased (2-fold) risk for colorectal carcinoma and acute lymphoblastic leukemia, probably due to the adequate deoxyribonucleotide pools for DNA synthesis/repair through increased availability of 5,10-methyleneTHF for thymidylate synthesis [95,96]. In addition, mild MTHFR deficiency may also affect the response to certain medications including methotrexate, 5-fluorouracil and valproic acid [97-99].

1.1.6.4 *Mthfr* knockout mouse

Mthfr knockout mice have been generated on the BALB/c background and characterized in our laboratory [100]. Homozygous knockout mice (*Mthfr*$^{-/-}$) provide a model for severe MTHFR deficiency. They have significant developmental delay, cerebellar defects, hyperhomocysteinemia (10-fold increase compared to controls), low plasma total folate levels, low plasma, liver and brain 5-methylTHF levels, significant DNA hypomethylation in brain and ovaries, increased SAH in liver, brain, testes and ovaries and decreased SAM levels in brain, testes and ovaries. Only a small proportion of homozygous offspring survive to adulthood and they are mostly infertile. Heterozygous knockout mice (*Mthfr*$^{+/-}$) provide a good model for *MTHFR* 677TT individuals. They look normal and have reduced enzyme activity (60% of wild type), mild hyperhomocysteinemia (1.6-fold higher than wild-types), lower liver 5-methylTHF levels than wild-types, DNA hypomethylation in brain and ovaries, increased SAH in liver and brain and decreased SAM in testes and ovaries [100,101]. Abnormal lipid deposition in the proximal aorta was shown in both homozygous and heterozygous knockout mice, confirming the correlation between hyperhomocysteinemia and atherosclerotic vasculopathies [100]. In addition, they had

endothelial dysfunction [102] and an increase in the vascular stiffness in mesenteric vessels [103]. Furthermore, *Mthfr*$^{+/-}$ mothers had an increase in the risk of fetal loss, and their embryos had increases in the risk of intrauterine growth retardation and congenital heart defects [104].

BALB/c *Mthfr* knockout mice were backcrossed onto the C57Bl/6 background for 15 generations. C57Bl/6 *Mthfr*$^{-/-}$ mice have similar phenotype, but a higher survival rate compared with BALB/c *Mthfr*$^{-/-}$ mice (unpublished data).

1.2 FOLATE-METABOLIZING ENZYME INHIBITORS

1.2.1 Overview

Anti-metabolites are catalytically inactive compounds that inhibit enzyme activity due to their similarity to substrate structures. Several chemotherapeutic drugs are antimetabolites that target DNA and RNA synthesis by disrupting the biosynthesis of purines and pyrimidines in rapidly proliferating cancer cells. They can also be incorporated into macromolecules, such as DNA and RNA, and inhibit their normal function [105]. Three groups of chemotherapeutic anti-metabolites are: folate antagonists (anti-folates), purine antagonists and pyrimidine antagonists.

Anti-folates have a similar structure to folic acid and therefore compete with folate cofactors for folate-dependent active enzyme sites. The first clinically used anti-folate was aminopterin (2,4-diamino-pteroylglutamate), which was replaced by the better and less toxic methotrexate (MTX) (N^{10} –methyl derivative of folic acid) [106]. Aminopterin and MTX both compete with folic acid for DHFR, the primary target of anti-folates that is highly

expressed in tumors [106,107]. These compounds inhibit DNA synthesis through the depletion of THF pools and are used in the treatment of leukemia [108]. In 1950, Farber *et al.* showed that a folate analogue caused a decrease in leukemia cell counts [109] and, since then, anti-folates have been commonly used in the treatment of leukemia due to their anti-proliferative action. Among all anti-folates, MTX is the most commonly used for both hematological malignancies and solid tumors.

There are two classifications of anti-folates: classical analogs (transported by RFC and require polyglutamation, such as MTX) and non-classical analogs (lipophilic, do not use transport systems and do not require polyglutamation, such as trimetrexate) [108,110]. Intracellular polyglutamation by FPGS is crucial for classical anti-folates to achieve cellular retention, increase their half-life and affinity for their target enzymes. GGH facilitates their efflux from the cell by removing polyglutamates [108,111].

Purine antagonists (such as thioguanine, thiazofurin and arabinofuranosyl-fluoroadenine) target IMP dehydrogenase and hypoxanthine guanine phoshoribosyltransferase (HPRT; enzyme of the purine salvage pathway) and inhibit guanine nucleotide synthesis in tumor cells, which have upregulation in biosynthesis of guanine nucleotides. The most commonly used purine antagonist is 6-mercaptopurine [112].

Pyrimidine antagonists (such as 5-fluorouracil (5-FU), gemcitabine and cytarabine) inhibit DNA synthesis, cause cell cycle arrest in the S-phase and induce apoptosis. In 1954, enhanced uracil utilization in malignant cells showed that uracil synthesis could be a potential chemotherapeutic target [113]. Since then, pyrimidine antagonists have become an important group of chemotherapeutics. The most commonly used pyrimidine antagonist is 5-FU [105].

1.2.2 Methotrexate (MTX)

1.2.2.1 Mechanism of its action and its therapeutic spectrum

Methotrexate (MTX) is a widely used anti-folate in the treatment of leukemias, solid tumors, graft-versus-host disease (GVHD), rheumatoid arthritis (RA) and psoriasis. It is an inhibitor of dihydrofolate reductase (DHFR), the enzyme required to maintain the active tetrahydrofolate pool. As a consequence of this inhibition, 5,10-methyleneTHF (TS substrate) and 10-formylTHF (GARFT and AICARFT substrate), the essential carbon donors in the biosynthesis of thymidylate and purines, respectively, are depleted resulting in the inhibition of DNA and RNA synthesis. The efficacy of MTX is attributed to its effect on nucleotide synthesis [108,114]. MTX is transported into cells mainly through RFC and competes with folic acid for DHFR binding and causes the accumulation of DHF by blocking DHF to THF conversion. Both the depletion of intracellular reduced folates (THF pools) and the direct reversible inhibition of its primary intracellular target, DHFR (K_i=0.004 nM) inhibit DNA synthesis, cause cell cycle arrest in the S-phase and induce apoptosis [106,108,115].

MTX cytotoxicity depends on its polyglutamation. Within cells, MTX is polyglutamated, a modification that enhances its intracellular retention and affinity for folate-dependent enzymes. MTX polyglutamates inhibit TS (K_i=47 nM), GARFT (K_i=2500 nM) and AICARFT (K_i=56 nM) [108]. In addition, inhibition of DHFR by MTX causes accumulation of DHF; DHF polyglutamates are capable of inhibiting TS, GARFT and AICARFT [115]. MTX competes with other folates (5-methylTHF, folic acid, leucovorin) for both cellular uptake, DHFR binding and polyglutamation. MTX is the least avid substrate

for polyglutamation among folates, so an alteration in polyglutamation will affect MTX to a greater extent [116].

The selectivity of MTX for malignant cells is a result of enhanced transport into rapidly proliferating cells that also have higher FPGS activity (and therefore increased polyglutamation). Furthermore, bone marrow and intestinal epithelium cells use circulating hypoxanthine through purine salvage pathway to obtain their purine requirement. In contrast, tumor cells mainly use active *de novo* biosynthesis of purines, the enzymes of which (GARFT and AICARFT) are targeted by MTX and DHF polyglutamates [106].

Another important factor in MTX cytotoxicity is the activity of TS (dUTP/dTTP ratio). In the conversion of dUMP to dTMP by TS, DHF is produced. In order to regenerate the active THF, DHFR activity is required. In the presence of inhibited DHFR, THF depletion rate depends on TS activity [106]. MTX treatment can significantly increase intracellular dUTP/dTTP ratios: through depletion of its cosubstrate 5,10-methyleneTHF or through direct inhibition of TS. Alterations in the deoxyribonucleotide balance was shown to induce uracil misincorporation into DNA [117], increase the uracil content of DNA [118], resulting in DNA strand breaks [119], error-prone DNA repair [120], decreased cell replication [121] and cell cycle arrest [44].

THF is the source of biologically active folate cofactors in one-carbon metabolism, and include 5-methylTHF, a methyl donor in Hcy remethylation. MTX treatment reduces 5-methylTHF pools and results in hyperhomocysteinemia (due to decreased Hcy remethylation) [122,123], DNA hypomethylation (due to decreased synthesis of SAM) [124,125] and choline deficiency (due to increased demand of choline for Hcy remethylation)

[126]. Low circulatory levels of 5-methylTHF are a marker of MTX cytotoxicity [116]. High Hcy levels could also be a marker of the pharmacodynamic effect of MTX [127].

In addition to its well-known chemotherapeutic effect, MTX is the most commonly used disease-modifying anti-rheumatic drug through its effect on purine synthesis. Inhibition of *de novo* purine biosynthesis by inhibiting AICARFT results in accumulation of AICAR which induces release of adenosine, a potent anti-inflammatory agent [116,128,129].

The pharmacokinetics of MTX is important for its clinical response. MTX is the only chemotherapeutic drug where routine monitoring of its plasma concentration is essential for limiting toxicity and predicting better therapeutic outcomes [130,131]. Circulating RBC MTX polyglutamates are also correlated with its therapeutic outcome [132]. Furthermore, since MTX polyglutamates are stored in the liver and bone marrow precursors [116], higher lymphoblastic MTX polyglutamate concentrations are associated with better therapeutic outcome in the treatment of leukemia [133].

The toxic effects of MTX are first observed in the oral mucosa (as mucositis), gastrointestinal mucosa (as diarrhea), hepatocytes (as an increase in liver enzymes), bone marrow cells (as myelosuppression: anemia, leucopoenia, thrombocytopenia), and testicular germ cells (as a decrease in sperm counts) [116]. Other MTX-related complications include neurotoxicity (as seizures) and nephrotoxicity (as an increase in serum creatinine and urea) [134]. If plasma MTX levels are low (< 10 μM), 70-90% of it is excreted in the urine. If plasma MTX levels are high (>10 μM), 20-50% of it is converted to 7-OH-MTX, which is an inhibitor of DHFR, TS and AICARFT, and may precipitate intrarenally, leading to renal failure. Since the major route of elimination of MTX is through the kidney, defective renal function will cause prolonged retention of MTX and increase its toxicity [108,135].

Leucovorin (folinic acid) is administered with MTX to decrease MTX-related toxicities by providing reduced folates for 1C transfers and competing with MTX for cellular uptake [136]. Selective salvage of normal cells with leucovorin use is possible, because of their lower metabolic rate of activity compared to malignant cells [116].

MTX resistance can occur by a variety of mechanisms. It can be caused by decreased ability to transport MTX into the cells (such as in RFC polymorphism), an increase in DHFR activity, an alteration in the DHFR's affinity for MTX or the altered MTX polyglutamation (reduced polyglutamation due to FPGS polymorphism, or increased degradation due to GGH polymorphism, or increased efflux from the cells) [108,137]. Multidrug resistance-associated proteins (MRPs) are responsible for efflux of MTX, folic acid, and leucovorin from the cells. Inhibition of MRPs results in intracellular accumulation of MTX polyglutamates and increased MTX response. In contrast, increased expression of MRPs is associated with poor clinical response to MTX [138].

1.2.2.2 Methotrexate and mild MTHFR deficiency in malignant diseases

Response to MTX can be influenced by polymorphisms in genes encoding folate-dependent enzymes. Among these polymorphisms, the *MTHFR* 677C→T polymorphism is the most widely studied [139,140].

Recent studies have suggested an association between the *MTHFR* 677C→T polymorphism and increased MTX toxicity and decreased efficacy [97,98,141-143].

With regards to increased MTX toxicity, MTX-treated chronic myelogenous leukemia (CML) *MTHFR* 677TT patients undergoing bone marrow transplantation had a higher risk of

oral mucositis and delayed hematological recovery compared to the *MTHFR* 677CC patients [97]. Similarly, all *MTHFR* 677TT patients receiving cyclophosphamide, MTX, and 5-FU (CMF) treatment for early breast cancer exhibited grade 4 toxicity (severe myelosuppression) [98]. Also in a study of MTX toxicity in ovarian cancer patients, Toffoli *et al.* found that the *MTHFR* 677TT genotype was associated with a higher incidence of grade 3-4 toxicity which was associated with MTX-induced hyperhomocysteinemia [143]. Additionally, *MTHFR* 677TT patients with acute lymphoblastic leukemia (ALL) or acute promyelocytic leukemia showed greater myelosuppression and hepatotoxicity than the other genotype groups [144]. These authors suggested that the reduced folate levels induced by MTX treatment may be further reduced in *MTHFR* 677TT patients, who already have altered folate distribution. The decreased amount of folates available for deoxyribonucleotide synthesis in these patients may cause more severe MTX-induced DNA damage and therefore myelosuppression (grade 3-4) and delayed oral mucosa healing [97,143]. In addition to the association of MTX-induced hyperhomocysteinemia with myelosuppression as discussed above, Quinn *et al*. have shown that MTX-induced hyperhomocysteinemia was also associated with neurotoxicity in children with cancer [145]. In contrast to this study, Kishi *et al.* could not find any correlation between MTX-induced hyperhomocysteinemia and neurotoxicity in children with ALL receiving high-dose MTX therapy [146]. Hyperhomocysteinemia through MTHFR deficiency was correlated with chronic allograft nephropathy in renal transplant patients receiving MTX for GVHD prophylaxis [147,148]. Furthermore, *MTHFR* 677TT adult patients with non-Hodgkin's lymphoma (NHL) receiving MTX chemotherapy had a higher risk of severe oral mucositis, hepatotoxicity and myelosuppression [149]. The authors suggested that in these patients, MTX-induced toxicity could be associated with increased Hcy levels through their MTHFR deficiency. In addition, in ALL and malignant lymphoma

patients, the *MTHFR* 677C→T polymorphism was associated with residual levels of MTX after 48 hours (the terminal elimination phase) which was linked with MTX toxicity [150]. In another study of patients with hematological malignancy or aplastic anemia receiving MTX after allogenic stem cell transplantation, individuals with the *MTHFR* 677TT genotype had higher total billirubin and aspartic transaminase levels (indicators of hepatotoxicity) and delayed platelet recovery [151].

With regards to decreased MTX efficacy, the *MTHFR* 677C→T polymorphism was associated with increased risk of relapse following MTX-induced remission in childhood ALL. Also there was a significant association between the *MTHFR* 677T allele and increased risk of relapse and decreased possibility of event-free and disease-free survival without any difference in MTX toxicity [152,153]. A similar effect was shown in breast cancer cells transfected with either the *MTHFR* 677T allele or anti-sense *MTHFR*. Their chemosensitivity to MTX was significantly decreased compared to cells expressing the wild-type *MTHFR* [142]. In addition, in patients with hematological malignancy or aplastic anemia receiving MTX after allogenic stem cell transplantation, *MTHFR* 677TT patients had a higher percentage of one-year cumulative treatment-related mortality and a lower percentage of one-year overall survival compared with other genotypes [151]. Also *MTHFR* 677TT adult patients with NHL receiving MTX chemotherapy had a decreased possibility of event-free survival [149]. These authors suggested that increased 5,10-methyleneTHF levels due to MTHFR deficiency counteracts the anti-folate chemotherapeutic effect of MTX and therefore, its effect on DNA synthesis.

There are some contradictorary studies that suggest the *MTHFR* 677TT genotype is associated with increased efficacy and therefore increased toxicity. They propose that the

low folate levels found in *MTHFR* 677TT patients exacerbate the anti-folate effect of MTX (therefore increasing its efficacy and also its toxicity). MTX-treated CML *MTHFR* 677TT patients undergoing bone marrow transplantation had a decreased risk of acute GVHD and an increased risk of oral mucositis [154,155]. Also the *MTHFR* 677C→T polymorphism increased the *in vitro* sensitivity to MTX in childhood ALL. *MTHFR* 677TT patients required a lower concentration of MTX to inhibit 50% of TS activity (TSI_{50}, an index of efficacy) compared to *MTHFR* 677CT and *MTHFR* 677CC patients [156]. In addition, donors having the *MTHFR* 677TT or *MTHFR* 677CT genotype had a lower incidence of GVHD after allogenic hematopoietic stem cell transplantation treated with MTX, suggesting better clinical response in these patients [157].

Also there are some contradictorary studies that suggest the *MTHFR* 677TT genotype is associated with decreased efficacy and therefore decreased toxicity. In a study of ALL, the *MTHFR* 677T allele was associated with lower rates of grade 3 leucopoenia and liver toxicity. They suggested that reduced sensitivity to MTX, through counteracting effect of MTHFR deficiency, was correlated with reduced MTX toxicity [153,158].

On the other hand, a study of children with NHL showed no effect of the *MTHFR* 677C→T polymorphism on either prognosis or toxicity. They suggested that leucovorin stabilizes the thermolabile MTHFR and if folate levels are greater than 15.4 nmol/L, leucovorin neutralizes the effect of the *MTHFR* 677TT genotype [159].

In addition to the *MTHFR* 677C→T polymorphism, the *MTHFR* 1298A→C polymorphism also affects MTX response. In CML patients undergoing hematopoietic cell transplantation, the *MTHFR* 1298C allele was associated with an increased risk of acute GVHD and a decreased risk of oral mucositis [154,155]. These authors suggested that the

MTHFR 1298CC genotype was associated with lower MTX efficacy and therefore decreased MTX toxicity due to higher RBC folate levels in those patients [94]. Also a study of childhood ALL showed that the *MTHFR* 1298AC genotype was associated with decreased sensitivity to MTX [160].

In contrast to other efficacy studies, Robien *et al.* showed a significant association of the *MTHFR* 1298A→C polymorphism with decreased risk of relapse after hematopoietic cell transplantation in CML patients receiving MTX treatment. In addition, patients with the *MTHFR* 677CC/1298CC genotype had the lowest risk of relapse. However, they showed no correlation between the *MTHFR* 677C→T polymorphism and the risk for relapse [161]. Also in contrast to other toxicity studies, in adult NHL patients, the *MTHFR* 1298CC genotype was associated with severe oral mucositis [149].

1.2.2.3 Methotrexate and mild MTHFR deficiency in rheumatoid arthritis

MTX is the most commonly used antirheumatic drug which is being prescribed to half a million rheumatoid arthritis (RA) patients worldwide. There is significant inter-patient variability in its efficacy (with good clinical response achieved in only 50% of patients) as well as its toxicity, which is the primary reason underlying discontinuation of MTX treatment and occurs in about 30% of RA patients [116,162].

Several studies have examined the effect of folate supplementation on toxicity in RA patients treated with MTX. Two studies demonstrated that in unsupplemented patients, the *MTHFR* 677T allele was associated with greater MTX toxicity [163,164]. A similar study showed that the *MTHFR* 677TT and *MTHFR* 677CT genotypes were associated with higher

rates of discontinuation of MTX treatment due to elevated liver enzyme levels (hepatotoxicity) mediated by hyperhomocysteinemia [165]. These authors suggested that the association between the *MTHFR* 677T allele and greater MTX toxicity may be explained by the greater folate depletion through MTHFR deficiency. In folate supplemented patients, both hepatotoxicity and hyperhomocysteinemia were significantly reduced, without significant loss of efficacy [165]. Other studies have confirmed the observation that folic acid supplementation reduces the risk of MTX-induced toxicity in RA [166-169] and other autoimmune diseases [170]. It has been proposed that stabilization of the thermolabile variant of MTHFR enzyme by folate supplementation mediates this protective effect. On the other hand, some studies have shown that both the efficacy and the toxicity of MTX were reduced in RA patients supplemented with either folic acid or folinic acid through competition for the folate-binding site of DHFR and cellular uptake, respectively [171,172].

There are some studies that contradict the studies discussed above. Even in the absence of folate supplementation in MTX-treated patients, no association was observed between the *MTHFR* 677C→T polymorphism and MTX toxicity [173,174]. However, Haagsma *et al*. suggested that MTX-induced hyperhomocysteinemia is greater in the *MTHFR* 677CT genotype which may be related with an increased risk of gastrointestinal MTX toxicity [173]. On the other hand, toxicity has been observed in folate supplemented RA patients receiving MTX; specifically that the *MTHFR* 677TT genotype was associated with central nervous system side effects [175].

In addition to the *MTHFR* 677C→T polymorphism, the *MTHFR* 1298A→C polymorphism also affects MTX response in RA patients. In several studies on RA patients receiving MTX without folate supplementation, the *MTHFR* 1298AA genotype was

associated with the requirement of a higher MTX dose at advanced stages of RA treatment, whereas the *MTHFR* 1298CC genotype was associated with better efficacy and greater improvement in disease control. The patients with the *MTHFR* 677C-1298C haplotype were receiving lower doses of MTX than those without the haplotype whereas the patients with the *MTHFR* 677T-1298A haplotype had an increased risk of side effects than those without the haplotype [163,164,176]. As discussed above, although the *MTHFR* 1298A→C polymorphism is associated with lower MTHFR activity in lymphocytes, it is not associated with decreased plasma folate levels or increased plasma Hcy levels. *MTHFR* 1298CC patients have greater MTX sensitivity, possibly because of the adequate intracellular folate levels in lymphocytes for immunosuppression, without greater toxicity, because toxicity is associated with lower plasma folate levels and increased plasma Hcy levels [93]. On the other hand, in a study of RA patients receiving MTX with folate supplementation, the *MTHFR* 1298CC genotype was associated with a significant decrease in MTX-induced toxicity, although these patients had higher plasma Hcy levels (independent of their serum folate levels); therefore the authors suggested that there is no association between plasma Hcy levels and MTX-induced toxicity [177]. Overall, RA patients with the *MTHFR* 1298CC genotype may be more responsive to MTX treatment and protected against its toxicity through MTHFR deficiency. In contrast, Wessels *et al.* showed that in RA patients receiving MTX with folate supplementation, the *MTHFR* 1298A allele was associated with better clinical response; this improvement was even better with addition of *MTHFR* 677C to the haplotype. Additionally, the *MTHFR* 1298CC genotype was associated with greater MTX toxicity [178].

The effect of MTHFR genotype has also been examined in another autoimmune disease such as psoriasis. For instance, a study showed that in psoriasis patients receiving MTX without folate supplementation, the *MTHFR* 1298C allele and the *MTHFR* 677CT/1298AC haplotype carriers developed less MTX-induced hepatotoxicity. They suggested that increase in 5,10-methylene THF through MTHFR deficiency counteracts with MTX-induced THF reduction, which is correlated with MTX-related toxicity [179].

1.2.2.4 Methotrexate and other polymorphisms in folate-metabolizing enzymes

As mentioned in the above section, MTX leads to significant changes in intracellular folate derivatives, which are used as cofactors of various folate-metabolizing enzymes. Polymorphisms in these enzymes (such as TS, DHFR, MTHFD1, AICARFT, SHMT, MTRR, and FPGS) and drug transporters (such as RFC) will affect its mechanism of action, and therefore its clinical response and toxicity [134].

Since DHFR is the major target enzyme of MTX, polymorphisms in *DHFR* may modify its efficacy and toxicity. The *DHFR* 216G allele was associated with better event-free survival and increased MTX-induced neutropenia, whereas the *DHFR* 317TT genotype (these patients have higher *DHFR* expression) was associated with reduced event-free survival in childhood ALL patients receiving MTX therapy. MTX cytotoxicity depends on DHFR and TS inhibition and their expression is upregulated by *Cyclin D1*. ALL patients having all event-predisposing genotypes in *Cyclin D1*, *DHFR*, *TS* had significant reduction in

event-free survival. The combined genotype effect and MTX-related toxicity was inversely proportional to the number of event-predisposing genotypes [180,181].

Additionally, the *RFC* 80G→A polymorphism (these patients have higher plasma MTX levels) was associated with poor outcome in childhood ALL patients receiving MTX chemotherapy [182,183]. Also in ALL and malignant lymphoma patients, the *RFC* 80G→A polymorphism was associated with MTX-induced hepatotoxicity [150]. In contrast, in children with ALL and malignant lymphoma, MTX-induced gastrointestinal toxicity was correlated with the *RFC* 80G allele [184].

Moreover, the *TS* *3R/*3R genotype (homozygous for the 5′UTR 28 bp triple repeat allele; these patients have higher TS activity) was associated with increased risk of hematological and central nervous system relapse in ALL patients [185]. Also the *TS* *3R/*3R genotype was associated with poor outcome and decreased risk of event-free survival in ALL patients receiving MTX chemotherapy [186,187] whereas *TS* *2R/*2R genotype (homozygous for the 5′UTR 28 bp double repeat allele; these patients have lower TS activity) was associated with higher sensitivity to MTX and higher MTX toxicity [188]. In addition, the combined effect of the *TS* *2R/*2R genotype and the *TS* 1494del6 genotype was also associated with increased risk of MTX-induced toxicity (oral mucositis) [155]. Similar to the *TS* *3R/*3R genotype, the *MTHFD1* 1958A allele was associated with decreased possibility of event-free survival and disease-free survival in ALL patients receiving MTX treatment. The combined effect of the *TS* *3R/*3R genotype and either the *MTHFR* 677T allele or the *MTHFD1* 1958A allele resulted in greater reduction of event-free survival in ALL patients [153].

Furthermore, another study on childhood ALL, showed that the *MTRR* 66G allele (these patients have low MTRR activity) was associated with decreased sensitivity to MTX whereas the *SHMT* 1420TT genotype (these patients have increased SHMT expression) was associated with an increase in MTX resistance. They suggested that low MTRR activity prevents Hcy remethylation to Met, and since the MTHFR reaction is irreversible, 5-methylTHF is trapped resulting with inhibition of purine and thymidylate synthesis. In contrast, increased SHMT expression favors DNA synthesis, which is counter to the action of MTX, and reducing Hcy remethylation to Met [160].

Lastly, the *FPGS* 452C→T polymorphism (increased MTX polyglutamation) was associated with grade 2 thrombocytopenia in children with ALL receiving MTX treatment [189].

The effects of these polymorphisms have also been investigated in the response of RA patients receiving MTX. In folate supplemented patients, the *MTHFR* 677TT genotype and the *SHMT* 1420CC genotype (these patients have reduced plasma and RBC folate levels) were associated with central nervous system side effects whereas the *AICARFT* 347GG genotype (these patients have reduced *de novo* purine synthesis) was associated with gastrointestinal side effects, and the *TS* *2R/*2R genotype and the *SHMT* 1420CC genotype were associated with alopecia. Weisman *et al.* showed that homozygosity for at least one of the *MTHFR* 677T, the *SHMT* 1420C, the *AICARFT* 347G or the *TS* *2R alleles were associated with greater toxicity of MTX [175].

Moreover, in a study on RA patients receiving MTX without folate supplementation, the *TS* *3R/*3R genotype (these patients have higher TS activity) was associated with poor outcome and required higher MTX doses whereas the *TS* 3′UTR 6 bp deletion genotype

(these patients have lower *TS* mRNA expression) was associated with greater improvement and better outcome [190]. Kumagai *et al.* suggested that an increase in TS expression lowered the MTX efficacy, while a decrease in TS expression resulted in greater sensitivity to MTX [174]. In addition, the *RFC* 80AA genotype (these patients have higher plasma folate and MTX levels, and RBC MTX polyglutamates) and the *AICARFT* 347GG genotype were associated with better outcome in RA patients receiving MTX therapy. Dervieux *et al.* showed that homozygosity for at least one of the *RFC* 80A, the *AICARFT* 347G, or the *TS* *2R alleles were associated with greater response to MTX, because of increased levels of MTX polyglutamates [190,191].

Studies of psoriasis patients receiving MTX without folate supplementation demonstrated the *RFC* 80A allele and the *TS* 3′UTR 6 bp deletion were associated with MTX-related toxicity, the *RFC* 80A allele and the *AICARFT* 347GG genotype were associated with MTX discontinuation because of MTX-related toxicity whereas the *TS** 3R allele was associated with poor outcome, and the *TS* *3R/*3R genotype was associated with greater MTX toxicity (hepatotoxicity). Furthermore, MTX–induced toxicities were attenuated or eliminated by folate supplementation [179]. These authors suggested that greater MTX toxicity in the *TS* *3R/*3R genotype can be explained by greater MTX-induced plasma Hcy increase because of reduced 5-methylTHF availability, as a consequence of greater 5,10-methylene THF depletion through an increase in TS activity.

1.2.2.5 Methotrexate and rodent studies

Studies on rodents have been used to investigate the chemosensitivity of MTX. As discussed earlier, rapidly proliferating cells (bone marrow cells, gastrointestinal epithelium and hepatocytes) are major targets of MTX. Toxic effects of MTX on mouse bone marrow cells were shown by exposing these cells to different MTX concentrations [192]. Also MTX-induced apoptosis through an increase in cleaved caspase-3-positive cells was shown in rat intestinal Peyer's patches and non-patch epithelium [193]. In addition, daily low dose MTX administration to young mice resulted in cellular depression of the lymphoid tissues, testes, and skin whereas higher doses of MTX resulted in acute to subacute hematopoietic and gastrointestinal damage, and also osteoporosis in older mice [194]. Additionally, daily long-term low dose MTX administration to mice resulted in fatty metamorphosis (hepatotoxicity) [195]. Furthermore, chronic high dose MTX treatment in rats caused severe hematopoietic depression in the spleen and bone marrow, ulcerative gastrointestinal lesions, fatty metamorphosis in liver, hemosiderosis in the spleen and liver, and pulmonary lesions [196].

MTX treatment results in decreased SAM and betaine levels in rat livers, due to the decrease in Hcy remethylation, through decreased 5-methyl THF availability. In addition, increased consumption of choline to supply betaine for Hcy methylation causes choline deficiency. Rat liver contains high levels of choline oxidase for betaine synthesis, therefore is less susceptible to MTX-induced hepatotoxicity through choline deficiency, compared to humans [197,198]. Both choline deficiency and MTX treatment cause fatty infiltration in rat livers. MTX-treated choline-deficient rats had even greater decreases in SAM levels and greater increases in SAH and triacylglycerol levels compared to choline deficiency alone, because MTX-induced choline deficiency potentiates existing choline deficiency. Choline is

a precursor of phosphatidylcholine, which is required in synthesis of VLDL. VLDL transfers triglycerides from liver to peripheral tissues, so reduced VLDL levels, through choline deficiency, result in accumulation of triglycerides (fatty infiltration) in liver. MTX-induced hepatic fatty infiltration through choline deficiency results in hepatocyte damage, therefore choline can overcome this hepatotoxicity [126,199,200].

Another study in rat livers has shown that there were decreases in total folate and 5-methylTHF levels and an increase in formylTHF levels following MTX treatment. This effect is exacerbated in choline-deficient animals. Total folate and THF levels were greatly reduced in the livers of MTX-treated choline-deficient, whereas formylTHF levels were increased 3-fold, compared to controls [201]. Also chronic MTX therapy increases urinary and fecal folate levels [202]. In addition, a study showed that MTX impairs the capacity of incorporating folate in both liver and brain in rats. Similar to liver, total folate levels were significantly reduced in brain and also there was significant DNA hypomethylation [203].

The folate depletion resulting from MTX treatment affects methionine cycle intermediates. A study in rats showed that there was a 2-fold increase in Hcy levels in serum, liver and kidney, and a decrease in SAM levels in liver and kidney, following MTX treatment. Also MTX treatment reduced the SAM/SAH ratio [204]. Furthermore, Hilton *et al.* showed a decrease in SAM levels and an increase in SAH levels in livers of MTX-treated leukemic mice. 5-methylTHF rescued this MTX effect [205].

As discussed earlier, MTX is a potent anti-inflammatory drug widely used in the treatment of RA. Its anti-inflammatory effect is associated with its capacity to promote intracellular accumulation of AICAR and adenosine release. In a mouse model of inflammation, Cronstein *et al.* showed the expected increase in adenosine release, AICAR

accumulation, and inhibition in leukocyte accumulation following MTX treatment [128]. Baggott *et al.* suggested that anti-inflammatory efficacy of MTX depends on AICARFT inhibition which produces immunosuppression [129].

1.2.2.6 Methotrexate and its effect on apoptosis

MTX induces apoptosis via activation of caspases-9, -2, and -3 and causes DNA fragmentation, nuclear condensation, and destruction of cytoskeletal and structural proteins [206]. DNA damage induced by deoxyribonucleotide pool imbalance activates the mitochondrial intrinsic apoptosis pathway [59,60]. Released mitochondrial cytochrome C binds to apoptotic protease activating factor-1 to activate procaspase-9. Active caspase-9 activates caspase-3, leading to DNA fragmentation and cell death [58,207].

Similar to other chemotherapeutic drugs, MTX cytotoxicity is mediated by apoptosis [208]. In addition to activation of the mitochondrial intrinsic apoptosis pathway through DNA damage, MTX induces apoptosis through activation of death-inducing ligand/receptor systems, such as CD95 (APO-1/FAS) in human leukemia T-cell lines [209,210]. Activated FAS binds to FAS-associated death domain containing protein (FADD) and activates procaspase-8 which subsequently activates caspase-3. Also, it was shown *in vitro* that apoptosis induced by DNA damage through thymidine depletion is mediated by FAS signalling [211]. Therefore, MTX induces apoptosis through DNA damage and thymidine depletion by inhibiting thymidine and purine synthesis via the inhibition of DHFR, TS and AICARFT [115].

On the other hand, MTX's anti-inflammatory effect is attributed to its proliferative inhibition of activated T lymphocytes, which is mediated by apoptosis through a CD95-independent pathway [212] and activation of IL-2 or IL-4 [213,214]. Furthermore, it was shown that adenosine induces apoptosis in mouse thymocytes [215].

1.2.3 5-Fluorouracil (5-FU)

1.2.3.1 Mechanism of its action and its therapeutic spectrum

5-FU is a widely used pyrimidine antagonist in the treatment of gastrointestinal and genitourinary tract tumors. It is an inhibitor of thymidylate synthase (TS), a rate-limiting enzyme of DNA synthesis and repair. 5-FU, a fluorinated uracil, blocks DNA synthesis, causes cell cycle arrest in the S-phase and induces apoptosis [105]. 5-FU is converted to 3 metabolites: 5-fluorodeoxyuridine monophosphate (5-FdUMP), 5-fluorodeoxyuridine triphosphate (5-FdUTP) and 5-fluorouridine triphosphate (5-FUTP). The metabolite 5-FdUMP competes with dUMP for the nucleotide binding site of TS. It forms a stable ternary complex with TS and 5,10-methyleneTHF to block dUMP binding and inhibit TS activity, dTMP synthesis, and ultimately DNA synthesis due to the imbalance in deoxyribonucleotide pools [105]. In addition, the misincorporation of 5-FdUTP and/or accumulated dUTP into DNA leads to double strand breaks which induce p53-dependent apoptosis, as discussed earlier [64]. Furthermore, 5-FUTP can be incorporated into RNA, which may cause impairment in RNA processing and function, and also induces p53-dependent apoptosis [105].

Leucovorin (folinic acid), a precursor of 5,10-methyleneTHF, enhances 5-FU cytotoxicity by increasing the formation and stability of the 5,10-methyleneTHF-TS-5-

FdUMP ternary complex [142]. Coadministration of leucovorin with 5-FU resulted in enhanced therapeutic response in gastrointestinal tumor patients [216]. Also in advanced/colorectal carcinoma patients, leucovorin enhanced the antitumor effect of 5-FU [217,218]. In addition, a study on head and neck cancer patients showed that 5-FU-responding patients had significantly higher tumoral 5,10-methyleneTHF levels compared to 5-FU-nonresponding patients [219]. These studies demonstrate the importance of 5,10-methyleneTHF for the formation and stability of the ternary complex, optimal TS inhibition and 5-FU cytotoxicity.

Similar to 5,10-methyleneTHF, TS is an important component in the ternary complex; therefore it is essential for 5-FU cytotoxicity. Low TS expression was related with increased 5-FU sensitivity in several human tumor cell lines [220], colorectal cancer [221] and breast cancer patients [222]. In addition, human colon cancer cell lines expressing antisense *TS* cDNA had increased chemosensitivity to 5-FU [223]. Furthermore, in several types of cancers, higher intratumoral *TS* expression was associated with lower response rate, shorter survival and worse prognosis (reviewed in [224]). As a summary, increased TS inhibition, decreased TS stimulation and dissociation of the ternary complex via combination with leucovorin will achieve better 5-FU efficacy [225].

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in the catabolism of 5-FU. It converts 5-FU to the inactive 5,6-dihydro5-FU. DPD is responsible for 80% of 5-FU elimination, a process which occurs mainly in the liver [137]. Low DPD activity was associated with grade 3-4 5-FU toxicity in cancer patients [226]. In addition, several cancer studies found an inverse correlation between DPD levels and 5-FU response (reviewed in [224]).

The main toxic effects of 5-FU are on rapidly proliferating tissues, such as bone marrow (as myelosuppression: anemia, leucopoenia, thrombocytopenia) and gastrointestinal mucosa (as diarrhea, stomatitis, vomiting and nausea). Dermatological toxicities (as "hand-foot" syndrome), neurological toxicities (as seizures) and cardiac toxicities (as chest pain, changes in ECG and cardiac enzymes) may also occur [136,227]. A decrease in Hcy levels was shown when 5-FU therapy was combined with leucovorin [228].

5-FU resistance can occur by a variety of mechanisms [225]. It can be caused by decreased accumulation of activated 5-FU metabolites due to decreased activation or increased inactivation. Also, target-associated resistance may occur via a decreased effect on RNA, decreased stability of the ternary complex, decreased availability or polyglutamation of 5,10-methyleneTHF, decreased binding of 5-FdUMP to TS or *TS* gene amplification. Lastly, there may be pharmacokinetic resistance due to the inability to reach to tumor, altered 5-FU distribution or increased elimination. Overall, the major mechanism of 5-FU resistance is *TS* gene amplification [229,230]. High-grade, undifferentiated tumors have higher TS mRNA levels [231].

1.2.3.2 5-Fluorouracil and mild MTHFR deficiency

Two studies suggest that *MTHFR* deficiency may be a potential therapeutic target for enhancing the chemosensitivity to 5-FU. Sohn *et al.* suggested that an accumulation of 5,10-methyleneTHF resulting from the *MTHFR* 677 C→T polymorphism might increase 5-FU cytotoxicity by increasing the formation and stability of the 5,10-methyleneTHF-TS-5-FdUMP ternary complex [142]. They showed that colon and breast cancer cells transfected with either the *MTHFR* 677T allele or anti-sense *MTHFR* had an increase in

chemosensitivity to 5-FU compared to cells expressing the wild-type *MTHFR*. In addition, the growth rate of the colon cancer cell xenografts expressing the *MTHFR* 677T allele in nude mice was inhibited more effectively by 5-FU compared to xenografts expressing the wild-type *MTHFR* [142]. In a similar study, antisense inhibition of *MTHFR* was associated with increased chemosensitivity to 5-FU (greater decreases in the growth rate, % of viable cells and tumor volume) both in human cancer cell lines and in nude mice [232].

Genetic *MTHFR* deficiency may also influence response to 5-FU; several studies of advanced/metastatic colorectal cancer patients reported that the *MTHFR* 677T allele was associated with higher response rates to 5-FU [233-235]. In contrast to these studies, in rectosigmoid colon cancer patients, the *MTHFR* 677CC genotype was associated with longer survival. They suggested that DNA hypomethylation in *MTHFR* 677TT patients affect their gene expression profile for survival [236].

Two recent studies of breast cancer and colorectal cancer patients receiving combined chemotherapy with 5-FU showed no association between the *MTHFR* 677C→T polymorphism and 5-FU chemosensitivity [237,238]. Another recent study on colorectal cancer patients receiving combined chemotherapy with 5-FU showed that the *MTHFR* 677C→T polymorphism was not a significant prognostic factor, although there was a trend towards better 5-FU response in polymorphic patients [239]. These authors suggested that combined chemotherapy (irinotecan or oxaliplatin) with 5-FU could be masking the genotype effect on 5-FU response. The earlier studies, which demonstrated a positive association between the *MTHFR* 677T allele and 5-FU response, were carried out on patients receiving 5-FU/leucovorin alone.

The *MTHFR* 1298CC polymorphism may also modify 5-FU response. Etienne *et al.* showed that the *MTHFR* 1298CC genotype was associated with the worst prognosis in advanced colorectal cancer patients [234]. In contrast, in human cancer cell lines, the *MTHFR* 1298C allele was associated with a greater 5-FU efficacy whereas 5-FU sensitivity was not linked to the *MTHFR* 677C→T polymorphism [240].

1.2.3.3 5-Fluorouracil and other polymorphisms in folate-metabolizing enzymes

As discussed earlier, TS is the major target enzyme of 5-FU, therefore polymorphisms in *TS* may modify its chemosensitivity.

TS expression has been shown to be negatively correlated with the clinical response to 5-FU. In colorectal cancer patients receiving 5-FU therapy, the *TS* *3R/*3R genotype (homozygosity for the 5′UTR 28 bp triple repeat allele resulting in higher TS mRNA and protein levels) was associated with lower response rate to 5-FU and a decrease in survival [241-243]. In rectal cancer patients, the *TS* *2R allele (the 5′UTR 28 bp double repeat allele resulting in lower TS mRNA and protein levels) was associated with increased tumor downstaging and improved 3-year disease-free survival [244]. Also gastric and colorectal cancer patients with the *TS* *3R/*3R genotype had reduced survival compared with those having either the *TS* *2R/*2R or *TS* *2R/*3R genotypes [245-247]. In addition to gastrointestinal cancers, Shintani *et al.* associated low TS mRNA levels with better disease-free survival in non-small-cell lung cancer patients [248].

Furthermore, loss of heterozygosity (LOH) at the *TS* locus in tumors was associated with reduced survival and worse response to 5-FU in advanced colorectal cancer patients with the *TS* *3R/loss genotype. This study illustrates the importance of genotyping of tumor tissue [249].

In contrast, in colorectal patients, some studies linked high TS expression with longer survival and increased 5-FU sensitivity [250-252]. Others showed no association between the *TS* 5′UTR genotype and response to 5-FU therapy in metastatic colorectal cancer patients [253-256] and in 19 human cancer cell lines [240].

With regards to toxicity, in colorectal cancer patients, the *TS* *2R allele was associated with a higher risk of grade 3-4 5-FU toxicity [242,257]. Furthermore, low TS expression levels in normal colonic mucosa in colorectal cancer patients were associated with higher risk of 5-FU-related side-effects and treatment discontinuation [258].

Another variant in *TS*, a G→C polymorphism within the triple repeat sequence, results in reduced TS expression. Human colon cancer cells having the *TS* *3RG/*3RG genotype (a high TS expression genotype) were less sensitive to 5-FU [259]. In addition, the genotypes with low expression (*TS* *2R/*2R, *2R/*3RC, and *3RC/*3RC) were associated with longer survival and better clinical outcome both in colorectal cancer patients [260] and in advanced gastric cancer patients [261].

Another polymorphism in *TS*, the *TS* 3′UTR 6 bp deletion results in lower TS mRNA expression and was associated with increased sensitivity to 5-FU in advanced gastric cancer patients [262]. In contrast, the *TS* 3′UTR 6 bp deletion genotype was associated with reduced response to 5-FU in advanced colorectal cancer patients [263].

In addition, the *TS* *3R/6 bp deletion haplotype was associated with better survival in colorectal patients [264]. In colorectal cancer patients, the combined genotype effect of the *TS* *3R/*3R and any *TS* 3′UTR genotype or the *TS* *2R/*3R and the *TS* 3′UTR 6 bp/6 bp (no 6 bp deletion) were associated with longer disease-free survival and overall survival [265]. Moreover, in colorectal cancer patients, the *TS* *2R/6 bp haplotype was associated with higher risk of 5-FU toxicity [257].

In summary, *TS* expression levels, tumoral LOH at the *TS l*ocus and alterations in folate status (the *TS* *3R/*3R genotype was correlated with low folate status) could influence tumor response to 5-FU and its toxicity (reviewed in [221]).

In addition to the effects of *MTHFR* and *TS* polymorphisms on 5-FU chemosensitivity, alteration in FPGS levels may also modify 5-FU response. The chemosensitivity of colon cancer cells having FPGS overexpression (expressing the sense FPGS cDNA) was significantly enhanced, whereas the chemosensitivity of colon cancer cells having FPGS inhibition (expressing the anti-sense FPGS cDNA) was decreased. Sohn *et al.* suggested that increased intracellular 5,10-methyleneTHF polyglutamates through FPGS overexpression might enhance 5-FU cytotoxicity by increasing the formation and stability of the 5,10-methyleneTHF-TS-5-FdUMP ternary complex; therefore FPGS gene transfer may enhance the chemosensitivity to 5-FU [266].

1.2.3.4 5-Fluorouracil and rodent studies

Studies on rodents have been used to investigate the chemosensitivity of 5-FU. Two studies of human tumor xenografts in nude mice showed an inverse correlation between 5-

FU sensitivity and DPD levels whereas there was no correlation between 5-FU sensitivity and TS levels [267,268]. Additionally, rodent studies where 5-FU given in combination with 5,10-methyleneTHF or leucovorin result in better 5-FU response compared to 5-FU alone [269,270]. In folate deficient mice, it was shown that folate status at the time of initiation of 5-FU/leucovorin treatment and the timing of leucovorin administration may influence the antitumor activity of 5-FU [271]. Also two studies on nude mice showing enhanced 5-FU response when combined with MTHFR deficiency (via *MTHFR* 677 C→T polymorphism or antisense inhibition of *MTHFR*) were discussed earlier [142,232].

1.2.3.5 5-Fluorouracil and its effect on apoptosis

5-FU induces DNA damage via inhibition of TS by its metabolite 5-FdUMP and incorporation of its metabolite 5-FdUTP into DNA and induces RNA damage via incorporation of its metabolite 5-FUTP into RNA. In response to 5-FU-induced DNA and RNA damage, activated *p53* induces cell cycle arrest or triggers apoptosis by upregulating pro-apoptotic *FAS* and *BAX* or downregulating anti-apoptotic *BCL2* [272]. Loss of *p53* function was associated with reduction in chemosensitivity to 5-FU and resistance to 5-FU-induced apoptosis [273,274]. In addition, ferredoxin contributes to p53-mediated apoptosis via mitochondrial oxidative stress production. Targeted disruption of the ferredoxin reductase gene in human colon cancer cells resulted in decreased sensitivity to 5-FU-induced apoptosis [275].

Several studies on different cancers demonstrated an association between *p53* mutation and resistance to 5-FU [224]. Patients most likely to benefit from 5-FU therapy had

c-myc amplification and wild-type *p53* in their primary colorectal tumor. Wild-type *p53* causes feedback regulation of TS protein synthesis by binding TS mRNA, but mutated *p53* is characterized with TS overexpression (because of no feedback), and was related with poor response to 5-FU [224].

Similar to the effect of MTX on apoptosis as discussed earlier, 5-FU induces *FAS* and the production of death-inducing signaling complex (DISC), which is comprised of FAS, FAS ligand, FADD, and caspase-8. Activated caspase-8 activates effector caspases such as caspase-3, leading to apoptosis [272]. In thymidylate deficiency, apoptosis induced by DNA damage occurs through FAS signaling [211]. In addition, 5-FU induces mitochondrial intrinsic pathway through DNA damage. Released mitochondrial cytochrome C binds to apoptotic protease activating factor-1 to activate procaspase-9. Active caspase-9 activates caspase-3, leading to apoptosis. Also active caspase-8 leads to the cleavage of Bid and regulates mitochondrial cytochrome C release [272].

Furthermore, 5-FU-induced intracellular deoxyribonucleotide pool imbalance (through dUTP and 5dUTP misincorporation into DNA) triggers endonuclease-mediated DNA cleavage, resulting in DNA double-strand breaks and ultimately apoptosis [276,277]. Poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme and a substrate for caspase-3, is activated by binding to DNA single- or double-strand breaks [278]. Also, as discussed earlier, depending on the number of DNA double-strand breaks, p53 can activate proapoptotic genes, such as BAX [64].

Lastly, in intestinal epithelial and colon cancer cells, 5-FU-induced apoptosis is p53-dependent through the effect of 5-FU on RNA metabolism [273,279]. In support of this, *in vitro* inducible TS expression and thymidine can inhibit p53-dependent apoptosis induced by

anti-folates, but not by 5-FU [274] whereas uridine can inhibit p53-dependent apoptosis induced by 5-FU via inhibiting incorporation of 5-FUTP into RNA [279,280].

1.3 THESIS RATIONALE

The *MTHFR* 677C→T polymorphism has been shown to modify the response to MTX and 5-FU through altered distribution of folates. However the effect of MTHFR deficiency on the clinical outcome and its proposed mechanisms are not clear. In addition, dietary folate can influence the chemosensitivity of these chemotherapeutic drugs. Understanding the mechanisms that affect the action of these drugs will be useful for enhancing their chemosensitivity. In this thesis, animal models will be used to provide further information on the role of MTHFR and the impact of MTHFR expression on response to MTX and 5-FU, in a controlled environment.

In Chapter II, an *MTHFR*-overexpressing transgenic mouse line will be generated and the effect of MTHFR overexpression and deficiency on MTX response will be investigated in mice.

In Chapter III, the effect of MTHFR overexpression and deficiency on MTX-induced apoptosis will also be examined in mice.

In Chapter IV, the effect of MTHFR overexpression on 5-FU response will be investigated in mice.

CHAPTER II

Altered expression of methylenetetrahydrofolate reductase modifies response to methotrexate in mice

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2.1 ABSTRACT

Folates provide one-carbon units for nucleotide synthesis and methylation reactions. A common polymorphism (677C→T) in methylenetetrahydrofolate reductase (MTHFR) encodes an enzyme with reduced activity. Response to the anti-folate methotrexate (MTX) may be modified in 677TT individuals since MTHFR converts nonmethylated folates, utilized for thymidine and purine synthesis, to 5-methyltetrahydrofolate, utilized in homocysteine remethylation to methionine. To study potential interactions between MTHFR activity and MTX, we examined the impact of decreased and increased MTHFR expression on MTX response in mice.

Mthfr-deficient (*Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$) and wild-type (*Mthfr*$^{+/+}$) mice were injected with MTX or saline and assessed for hematological parameters (hematocrit, hemoglobin, red and white blood cell numbers), plasma homocysteine, nephrotoxicity, hepatotoxicity and splenic dUTP/dTTP ratios. *MTHFR*-overexpressing transgenic mice (*MTHFR-Tg*) were generated, metabolites and folate distributions were measured, and response to MTX was assessed.

MTX-treated *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice displayed hyperhomocysteinemia and decreased hematocrit, hemoglobin and red blood cell numbers compared to wild-type animals. *Mthfr*$^{-/-}$ mice also showed increased nephrotoxicity and hepatotoxicity. *MTHFR-Tg* mice were generated and confirmed to have increased expression of MTHFR with altered distributions of folate and thiols in a tissue-specific manner. Following MTX treatment, *MTHFR-Tg* mice exhibited the same decreases in hematological parameters as *Mthfr*-deficient mice, and significantly decreased thymidine synthesis (higher

dUTP/dTTP ratios) compared to wild-type mice, but they were protected from MTX-induced hyperhomocysteinemia.

Under- and over- expression of MTHFR increase MTX-induced myelosuppression but have distinct effects on plasma homocysteine and nephrotoxicity. Pharmacogenetic analysis of polymorphisms in folate-dependent enzymes may be useful in optimization of MTX therapy.

2.2 INTRODUCTION

Folate, an essential vitamin for synthesis, repair and methylation of DNA, helps to maintain genome integrity. Methylenetetrahydrofolate reductase (MTHFR) may play a key role in regulating distribution of one-carbon moieties between nucleotide synthesis and methylation reactions (**Figure 2.1**). MTHFR catalyzes the irreversible reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF). The substrate of MTHFR provides carbon units for conversion of dUMP to dTMP by thymidylate synthase (TS), or is converted to 10-formyltetrahydrofolate (10-formylTHF) for purine synthesis. The product of MTHFR serves as the methyl donor for remethylation of homocysteine to methionine; methionine is converted to S-adenosylmethionine (SAM) for use in numerous methylation reactions. S-adenosylhomocysteine (SAH), the by-product of methylation reactions, is subsequently hydrolyzed, thus regenerating homocysteine, a potentially cytotoxic amino acid. In addition to remethylation, homocysteine may undergo transsulfuration to generate cysteine and glutathione.

The polymorphism in MTHFR (677C→T) results in the production of a thermolabile enzyme with reduced activity [74]. Individuals with mild MTHFR deficiency, which affects 10 – 15% of many Caucasian populations, have lower levels of methylTHF [88], elevated plasma Hcy [74] and global DNA hypomethylation [90]. 677TT individuals are also at higher risk for neural tube defects, vascular disease and pregnancy complications [87] and are at lower risk for certain forms of leukemia and colorectal cancer [95,96]. Furthermore, since mild MTHFR deficiency disrupts the flux of one-carbon moieties, there is recent interest in examining the ways in which 677TT individuals respond to drugs that target folate metabolism, such as methotrexate [87].

Methotrexate (MTX) is widely used in treatment of leukemia, solid tumors, and rheumatoid arthritis. It is an inhibitor of dihydrofolate reductase (DHFR), the enzyme required to maintain the active tetrahydrofolate (THF) pool. As a consequence of this inhibition, 5,10-methyleneTHF and 10-formylTHF, the carbon donors in biosynthesis of thymidylate and purines, respectively, are depleted resulting in inhibition of DNA and RNA synthesis, cell cycle arrest and apoptosis [114]. 5-MethylTHF pools are also affected by MTX and their depletion results in hyperhomocysteinemia and DNA hypomethylation [123-125].

Response to MTX can be influenced by polymorphisms in genes encoding folate-dependent enzymes [139]. The most common MTX side effect is myelosuppression, characterized by anemia (reduced hematocrit, hemoglobin and number of red blood cells), leucopoenia (reduced number of white blood cells) and thrombocytopenia (reduced number of platelets) [97]. Other MTX-related complications include neurotoxicity, renal dysfunction, hepatotoxicity, and gastrointestinal toxicity [134]. Recent studies have suggested an association between the MTHFR polymorphism and increased MTX

toxicity or decreased efficacy [97,98,141-143]. Among bone marrow transplant patients treated with MTX, 677TT patients had a higher risk of oral mucositis and delayed platelet recovery compared to 677CC patients [97]. 677TT patients experienced severe myelotoxicity following cyclophosphamide, MTX, and 5-fluorouracil treatment for early breast cancer [98], and this genotype group with acute lymphoblastic leukemia or acute promyelocytic leukemia was more likely to display myelosuppression and hepatotoxicity following MTX treatment [144].

In this study, we investigated the effects of altered MTHFR activity on MTX response *in vivo*, using mouse models. In earlier work, we had generated *Mthfr*-deficient mice and showed that *Mthfr*$^{-/-}$ and *Mthfr*$^{+/-}$ mice have altered distribution of folates, hyperhomocysteinemia and decreased DNA methylation [100,101]; these findings are similar to those observed in 677TT individuals. Since MTX interferes with DNA synthesis and increases homocysteine levels, we hypothesized that *Mthfr* deficiency might enhance MTX-dependent cytotoxicity on hematopoietic cells and enhance the increase in homocysteine. To further test the impact of MTHFR expression on MTX response, we generated an *MTHFR*-overexpressing transgenic mouse line (*MTHFR-Tg*). We hypothesized that, in response to MTX, *MTHFR-Tg* mice would have reduced availability of 5,10-methyleneTHF for DNA synthesis and therefore an adverse hematopoietic profile. We found that MTX-treated *Mthfr*- deficient and *MTHFR-Tg* mice were both adversely affected in terms of proliferation of the hematopoietic system, compared to their wild-type littermates, although they responded differently with respect to homocysteine elevation and nephrotoxicity.

2.3 MATERIALS AND METHODS

Methotrexate response

Mice

Experimentation received approval from the Animal Care Committee of the Montreal Children's Hospital according to the recommendations of the Canadian Council on Animal Care. Mice were fed standard mouse chow (Laboratory diet 5001, Agribrands Purina).

Adult male mice were injected intraperitoneally once every three days for two weeks (four injections total) with either saline or MTX (Faulding). The doses of MTX , either 10 mg/kg (LD10) or 20 mg/kg (LD20), have been demonstrated to produce toxicity without death [281]. Weight loss and general appearance were evaluated after each injection. Twenty-four hours after the last injection, mice were sacrificed for cardiac blood and tissues.

The first study examined the effect of *Mthfr* deficiency on response to MTX. Adult male *Mthfr*$^{-/-}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice were used; these mice were the result of 15 generations of backcrossing to C57Bl/6 from BALB/c, the original background strain [100]. C57Bl/6 *Mthfr*$^{-/-}$ mice have a higher survival rate compared with BALB/c *Mthfr*$^{-/-}$ mice (unpublished data).

The second study examined the effect of *MTHFR* overexpression on response to MTX. *MTHFR-Tg*, generated as described below, and wild-type littermates were the result of at least 6 generations backcrossing to C57Bl/6 from their original mixed background (129/Sv and C57Bl/6).

Hematological parameters

Cardiac blood was collected in potassium–EDTA tubes. Complete blood cell counts were obtained through the Animal Resource Center of McGill University.

Measurements of plasma total homocysteine (tHcy)

Cardiac blood was collected in potassium–EDTA tubes. Plasma was collected after centrifugation (6000xg, 6 minutes, 4°C) and stored at -70°C. Measurements were performed by HPLC [282].

Serum aspartate aminotransferase and blood urea nitrogen levels

Cardiac blood was collected in serum gel tubes. Serum was collected after centrifugation (4000xg, 10 minutes, room temperature) and analyzed on the day of collection by a clinical chemistry automatic analyzer at the Animal Resource Center of McGill University.

Evaluation of nucleotide pools

Free deoxyribonucleotides were separated by HPLC as previously described [283] with slight modifications. Briefly, frozen spleen was ground to powder, treated with 0.6 M trichloroacetic acid, neutralized with trioctylamine and injected onto an Econosphere C18 column (particle size 5 μm, length 250 x 4.6mm, Waters instrument Part No. 70071, Mandel, Montreal, Canada). Separation of dUTP and dTTP was achieved by isocratic elution with 100% Buffer A (0.1 M $NH_4H_2PO_4$, 0.33 M KCl, 0.25% methanol, pH 5.35) for 12 minutes followed by a linear gradient to 25% Buffer B (0.1 M $NH_4H_2PO_4$, 0.4 M KCl, 20% methanol, pH 5) for 18 minutes followed by a linear gradient to 80% Buffer B for 10 minutes, then 10 minutes of 80% Buffer B, followed by re-equilibration with 100% Buffer A for 20 minutes. Flow rate for the entire run was 0.8 mL/min. UV detection monitored

peaks at wavelengths of 254 nm and 280 nm. Peaks were assigned by co-elution with known standards.

MTHFR-overexpressing transgenic mice

Generation

A *MTHFR* cDNA encoding the shorter isoform (70 kDa) regulated by the CMV promoter was inserted into an *HPRT* targeting vector by Gateway cloning technology (Invitrogen) [284]. The parent plasmid, pIRES-MTHFR (*MTHFR* coding sequence inserted into the pIRES vector), was digested with *NsiI* and *BsrGI* into 2 segments: 6 kb (mouse CMV promoter, *MTHFR* cDNA, polyadenylation site and ampicillin resistance) and 1.3 kb (non-*MTHFR* sequences). The 6 kb fragment was self-ligated and transferred into *E. coli* Top 10 F'. Positive ampicillin-resistant colonies were selected by restriction digestion and sequencing of junction areas. A positive clone, designated pCMVMR, was digested with *NruI* and *XhoI* to isolate a 4.9 kb segment (CMV promoter, MTHFR cDNA and polyadenylation site), which was ligated into pENTR 1A (Gateway entry vector). pENTR1A carries the attL1 and attL2 recombination sites for efficient recombination with a Gateway-based *Hprt* targeting vector which carries attR1 and attR2 recombination sites [284]. The ligation product was transformed into *E. coli* Top 10 F'. Positive kanamycin-resistant colonies were selected by restriction digestion and sequencing of junction areas. The resulting construct was pENTRMR. Site-specific recombination of pENTRMR with the HPRT destination vector [284], at the att sites, generated the final construct pHPRTMR, which was transferred into *E. coli* Top 10 F'. Ampicillin-resistant colonies were selected and analyzed by restriction digestion and sequencing of junction areas. To increase homologous recombination efficiency, pHPRTMR was linearized with *PvuI* before electroporation into *Hprt*- ES cells. Homologous recombination

simultaneously restored the deleted *HPRT* locus in *Hprt-* ES cells and inserted a single copy of the *MTHFR* cDNA into the *HPRT* 5' flanking region. Restoration of *HPRT* expression was confirmed by HAT selection of ES clones. Six ES clones were further analyzed by PCR amplification of the 2 kb segment covering the *MTHFR* coding sequence, using the two primers: 5'-GAG GGC AGT GCC AGC AGT GG-3' and 5'-GGA GGA GGA AGG CGG GAC AGG-3'. The integrity of functional sequences upstream and downstream of the MTHFR cDNA was also tested by PCR. Amplification of segments upstream of the *MTHFR* cDNA segment (in the CMV promoter) was performed with all possible combinations of 4 primers (2 sense, 2 antisense): 5'-GGG CCA GAT ATA CGC GTT GAC AT-3', 5'-GAT TAT TGA CTA GTT ATT AAT AGT AAT CAA-3', 5'-ATC GGG TAT ATA CCT CAA GGC G -3' and 5'-ATG TAT TGA ATG CCA TTT ACC GGG-3'. PCR amplification of a segment downstream of *MTHFR* cDNA, and encompassing the polyadenylation site, was done using the 2 primers: 5'-CGT CCA TCT GGT CAG AAA AGA CAA T -3' and 5'-AAG GAT TAT TTT ACT CCT TTA ACG TAG -3'. Of the 6 clones selected, all demonstrated MTHFR overexpression by enzymatic assays [285]. Three of these positive ES clones were injected into C57Bl/6 blastocysts and two chimeric male mice derived from one clone successfully transmitted the MTHFR transgene to the next generation (germline female mice). Germline female mice were mated with male C57Bl/6 mice and offspring were backcrossed onto the C57Bl/6 background for at least 5 generations.

PCR-based genotyping

For identification of mice with a transgene, mice were genotyped by PCR using a human *MTHFR* cDNA [75] exon 1 (GenBank GI:4336807) sense primer 5'-GGA ATC TGG TGA CAA GTG GTT-3' and an antisense primer 5'-CTG CCA TCC GGT CAA

ACC TT-3' encompassing the last 3 bp of exon 1 and the first 17 bp of exon 2 (GenBank GI:4336808). With these primers, the transgene as well as the mouse *Mthfr*-pseudogene (*Mthfr*-ps) [286] are amplified (95 bp). However, only the transgene is digested with *EcoRI* to yield bands of 66 and 29 bp (**Figure 2.6A**, left panel).

To distinguish between heterozygosity and homozygosity for the transgene in females, a PCR-based method with the following 3 primers was used: a sense sequence 5'-GGG CCC TGA GGC GCG GGA T-3' in human *HPRT* exon 1 (GenBank GI:54288828) that is present in the transgene, a sense sequence 5'-TTG AAG GTC AAC TGC ATA GTG AGT T-3' in mouse *Hprt* intron 1 (GenBank GI:38348687) that is absent in the transgene and an antisense sequence 5'-CCA AAC TCC AAA TCA TAA AAG CCA TT-3' in mouse *Hprt* intron 1 (GenBank GI:3834868), that is present in the transgene and in the endogenous *Hprt* gene. The expected sizes of PCR fragments are 151 bp, 151 + 95 bp, and 95 bp for wild-type, heterozygous and homozygous transgenic females, respectively, as shown in **Figure 2.6A**, right panel.

Southern blotting

Genomic DNA was digested with *DraIII* and *ScaI*, *ApaLI* and *ScaI*, or *PshAI*, separated by agarose electrophoresis, and blotted. Blots were probed with an 847 bp DIG-labeled DNA segment specific for a portion of the CMV promoter and *MTHFR* exons 1 and 2. The DNA probe was generated using primers 5'-CCT ACT TGG CAG TAC ATC TAC GTA t -3' and 5'-GCT TAG CTT TGT GCA GAT GGC CC- 3', with the PCR DIG Probe synthesis kit (Roche). Images were generated by exposure to lumi-film chemiluminescent detection film (Roche) (**Figure 2.6B**).

RT-PCR, Western blotting and MTHFR enzyme activity assays

After RNA isolation using the Trizol procedure (Invitrogen) and treatment with DNase (Invitrogen), RT was performed with SuperScript II Reverse Transcriptase (Invitrogen) and RT-PCR was performed using primers in exon 2 and exon 5; mouse *Gapdh* served as the internal control [100]. Intensity of the *Mthfr* band relative to *Gapdh* was used to estimate mRNA levels.

Protein extracts from cells and tissues were prepared as described [100]. For Western blotting, 50-100 μg proteins were run on SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose membranes at 70 V, 2 hours at 4°C. Nonspecific binding sites were blocked with 2% skim milk in Tris-buffered saline/Tween buffer overnight at 4°C. Blots were incubated with rabbit antiserum against purified MTHFR [74] or β-Actin (control). Intensity of the MTHFR band relative to β-Actin was used to estimate protein levels. An activity assay, which assays MTHFR in the physiologically reverse direction, was performed using radiolabeled ^{14}C- 5-methylTHF monoglutamate as substrate and menadione as electron acceptor, as previously described [285].

Evaluation of global DNA methylation

HPLC with UV detection [287] or TLC (Thin Layer Chromatography) [288] was used to calculate the ratio of methylated cytosines/ (methylated cytosines + unmethylated cytosines). For HPLC, 10 μg of RNA-free genomic DNA was treated with nuclease P1 and calf intestinal alkaline phosphatase (CIAP). Bases were separated on a column using buffers containing 0.01M $NH_4H_2PO_4$ and either 2.5% (pH 5.3) or 10% (pH 5.1) methanol, allowing quantification of relative amounts of each base.

TLC was performed as previously described [288]. 5 μg of RNA-free genomic DNA was digested with *Msp I* (a methyl-cytosine insensitive enzyme), treated with CIAP, end-labeled with [γ-32P]ATP, hydrolyzed to the nucleotide bases by nuclease P1, spotted on a cellulose TLC plate and developed in isobutyric-acid-water-ammonium hydroxide (66:33:1). Images were quantified by phosphorimagery.

Measurement of folate-related metabolites

Levels of SAM, SAM/SAH, adenosine, methionine, homocysteine, GSH, cysteinylglycine and cysteine in liver, brain and duodenum were measured by HPLC using electrochemical (coulometric) detection [289].

Measurement of folate derivatives

Levels of 5,10-methyleneTHF, 5-methylTHF, 10-formylTHF, dihydrofolate and total folate in plasma, liver, brain and duodenum were measured by a ternary complex assay [290].

Statistical analysis

Two-factor analysis of variance (ANOVA) and independent-sample *t*-tests were performed using SPSS for WINDOWS software, version 11.0. *P*-values <0.05 were considered significant.

2.4 RESULTS

Effects of MTHFR deficiency on MTX treatment

Plasma thiols

Mice treated with MTX (10 mg/kg) had higher plasma homocysteine and homocysteine/cysteine ratios, compared to mice injected with saline (**Figure 2.2A** and **2.2C**). *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice had elevated homocysteine compared to *Mthfr*$^{+/+}$ mice, as previously described [100]. A genotype effect was also evident in cysteine levels, specifically between *Mthfr*$^{-/-}$ and *Mthfr*$^{+/+}$ in both treatment groups (**Figure 2.2B**).

Hematological parameters

This experiment was carried out using 10 mg/kg and 20 mg/kg MTX, with similar trends. However, genotype differences were more pronounced at 20 mg/kg. Hematocrit, hemoglobin and RBC numbers in MTX-treated mice were significantly lower compared to those in saline-treated mice (**Figure 2.3**, data shown for MTX 20 mg/kg). Compared to saline-treated animals, MTX treatment significantly decreased hematocrit, hemoglobin and RBC numbers in *Mthfr*$^{-/-}$ and *Mthfr*$^{+/-}$ mice, but not in *Mthfr*$^{+/+}$ mice. In addition, MTX-treated *Mthfr*$^{-/-}$ mice had lower hematocrit, hemoglobin, numbers of RBC and WBC compared to *Mthfr*$^{+/+}$ mice.

dUTP/dTTP ratios

Since hematopoietic cells are formed and destroyed in spleen, we examined this tissue for nucleotide levels to determine if imbalances in nucleotide pools, due to altered distributions of folate derivatives, could have contributed to reduced proliferation and decreased numbers of hematopoietic cells. MTX-treated *Mthfr*$^{+/+}$ mice had significantly higher dUTP/dTTP ratios, compared to saline-treated *Mthfr*$^{+/+}$ mice (**Figure 2.4**); this finding suggests that MTX reduced the levels of 5,10-methyleneTHF that is required for dUMP conversion to dTMP. *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice had nonsignificantly lower dUTP/dTTP ratios compared to their wild-type littermates. Although this result was not

statistically significant, it is consistent with the reported increase in nonmethyl folates, which include the MTHFR substrate 5,10-methyleneTHF, in MTHFR-deficient humans and mice [88,101].

Toxicity

MTX can cause significant toxicity to kidney and liver. Serum blood urea nitrogen (BUN) and aspartate aminotransferase (AST) levels were measured as markers of nephrotoxicity and hepatotoxicity, respectively. Compared to saline controls, MTX-treated mice had significantly higher serum levels of BUN (**Figure 2.5A**) and borderline significant increases in AST (**Figure 2.5B; p=0.08**). *Mthfr*$^{-/-}$ mice had the highest levels of BUN and AST levels, especially following MTX treatment. Significant differences between *Mthfr*$^{-/-}$ mice and their MTHFR-expressing littermates were observed for BUN and AST.

Generation and characterization of MTHFR-overexpressing transgenic mice

Generation

To overexpress MTHFR, we constructed an insertion targeting vector, in which the human MTHFR cDNA, driven by the ubiquitous CMV promoter, was inserted into the *Hprt* locus [284]. Intermediate constructs (pIRES-MTHFR, pCMVMR or pENTRMR) were transfected into cultured cells (N2a and RAW264.7 cell lines) to confirm MTHFR overexpression by Western blotting and enzyme assays (data not shown). The final construct pHPRTMR was transfected into *Hprt*- embryonic stem (ES) cells and multiple HAT- resistant clones were recovered. Six clones were further analyzed and all contained the transfected construct as demonstrated by amplification of a 2 kb segment that covered the MTHFR coding sequence, segments upstream of the

MTHFR cDNA (in the CMV promoter) and a segment encompassing the polyadenylation site. These 6 clones were also shown to have a 2-fold increase in enzyme activity. Three clones were injected into blastocysts and 2 chimeric male mice generated from one cell line transmitted the MTHFR transgene, giving rise to germline females.

To identify transgene-bearing mice, we used a PCR method in which the MTHFR transgene and the *Mthfr* pseudogene (*Mthfr*-ps), a retrotransposon with high homology to a mouse *Mthfr* cDNA segment [286], are both amplified (95 bp). PCR products were incubated with *EcoRI* that cuts the transgene (66 bp+29 bp), but not the pseudogene (**Figure 2.6A**, left panel). With this method, the *Mthfr*-ps provides a positive control for amplification and the *EcoRI*- digested fragments indicate the presence of the transgene. The endogenous gene does not amplify under these conditions, because the 3' end of the antisense primer encompasses sequences in exon 1 and the rest of the oligonucleotide binds to exon 2. Distinction between heterozygosity and homozygosity for the transgene is performed with a 3-primer PCR (**Figure 2.6A**, right panel).

We also performed Southern blotting of DNA from homozygous transgenic female mice to confirm that the expected recombination event had occurred at *Hprt* and to identify positive animals in subsequent generations (**Figure 2.6B** and **2.6C**). Bands of the expected sizes were observed for transgenic animals and not in wild-type mice.

Confirmation of overexpression

Germline female mice were mated with C57Bl/6 mice and in male progeny, MTHFR overexpression was confirmed in various tissues (**Figure 2.7**). All studies were performed on male mice, to avoid confounding by X inactivation, since the transgene is inserted into the X-linked *Hprt* locus. MTHFR overexpression was first confirmed by semi-quantitative RT-PCR in liver and kidney (**Figure 2.7A**). There was an approximate

2.5-fold increase in MTHFR mRNA, normalized to *Gapdh*, in liver and a 1.4-fold increase in kidney. Western blotting demonstrated that immunoreactive MTHFR protein levels, relative to β-Actin, were higher in tested tissues of *MTHFR-Tg* mice compared to those in wild-type mice. Increases were 1.8-fold in liver, 4.5-fold in kidney, 1.9-fold in intestine, 2.5-fold in brain and 2.5-fold in heart (**Figure 2.7B**). Immunohistochemistry was also performed for liver, kidney and intestine of wild-type and transgenic mice, and relative signal intensities were consistent with the results from Western blotting (data not shown).

MTHFR enzyme activity was increased 2.8-fold in liver, 2-fold in kidney, 1.3-fold in intestine, 1.7-fold in brain and 2.4-fold in heart (**Figure 2.7C**). Results for enzyme activity were consistent with the increases in protein levels on Western blots. Although there was some variability, transgenic mice appeared to have a 2- to 3-fold average increase in MTHFR expression, with somewhat lower increases in intestine.

Phenotype

Both heterozygous and homozygous *MTHFR-Tg* mice were viable, healthy and grew normally. Mating of *Tg/+* females with C57Bl/6 mice produced offspring with the expected 1:1 proportions of wild-type: transgenic mice (*n*=133:133). Wild-type: transgenic ratios for male and female offspring were similar (*n*= 65:65 for males and *n*= 68:68 for females). These data suggest that there was no significant fetal loss of *MTHFR-Tg* animals. Matings of heterozygotes produced viable homozygous *MTHFR-Tg* females in expected proportions.

There were no gross pathological changes in 3, 12 and 18 month-old male *MTHFR-Tg* mice following total necropsy and examination of over 30 tissues. Complete

blood count and routine biochemical assessments were similar to those of wild-type littermates (3 mice per genotype in each age group).

Metabolite levels

Measurements of reduced folate pools (**Table 2.1**) and of relevant tissue metabolites (**Table 2.2**) in *MTHFR-Tg* and wild-type littermates provided evidence for altered fluxes of one-carbon units. Transgenic mice did not show changes in folate distributions in plasma, where most of the folate is in the form of 5-methylTHF, the MTHFR product (**Table 2.1**). Brain, which has low quantities of total folate compared to liver and intestine, also did not display any genotype-specific differences. However, in liver and duodenum, *MTHFR-Tg* mice had significantly lower levels of 10-formylTHF. Evaluation of metabolites in the homocysteine remethylation and transsulfuration pathways revealed some genotype-specific and tissue-specific differences (**Table 2.2**). *MTHFR-Tg* mice had an increase of methionine in brain, an increase of glutathione in liver, and a decrease of cysteine in duodenum.

The decrease in 10-formylTHF is consistent with enhanced conversion of nonmethylTHF to methylTHF by MTHFR. Increased availability of methylTHF would drive methionine synthesis by homocysteine remethylation, as shown by the significant increase of methionine in brain and a nonsignificant increase in liver.

MTHFR-Tg and wild-type littermates were also examined for deoxyribonucleotide pools (dUTP/dTTP ratios) in liver, duodenum and brain. Global DNA methylation was assessed in liver, duodenum and brain by 2 methods: HPLC and TLC. There were no significant genotype differences in these parameters (data not shown, n= 5 per genotype).

Effects of MTHFR overexpression on MTX treatment

Plasma thiols

Mice treated with MTX (10 mg/kg) had higher plasma homocysteine compared to mice injected with saline (**Figure 2.8**). However, mice with MTHFR overexpression had lower plasma homocysteine levels and lower homocysteine/cysteine ratios following MTX treatment compared to their MTX-treated wild-type littermates.

Hematological parameters

Hematocrit, hemoglobin and RBC numbers in MTX (10 mg/kg or 20 mg/kg)-treated mice were significantly lower compared to those in saline-treated mice (**Figure 2.9**, data shown for MTX 10 mg/kg). Compared to saline-treated mice, these three parameters were significantly reduced in MTX-treated *MTHFR-Tg* mice, but unaffected in MTX-treated wild-type mice. Furthermore, in MTX-treated mice, MTHFR overexpression was associated with lower hematocrit, hemoglobin and RBC numbers compared to wild-type littermates.

dUTP/dTTP ratios

MTX treatment significantly increased dUTP/dTTP ratios in the spleen of both genotype groups, suggesting that there was decreased folate-dependent conversion of dUMP to dTMP due to the drug (**Figure 2.10**). This observation is similar to the trend discussed above for MTX-treated *Mthfr*-deficient mice. However, these ratios were significantly higher in *MTHFR-Tg* mice compared to wild-type littermates, in both the saline- and MTX-treated groups, suggesting that MTHFR overexpression also compromises the conversion of dUMP to dTMP. This observation is consistent with enhanced conversion of 5,10-methyleneTHF to 5-methylTHF through MTHFR overexpression, which decreases availability of 5,10-methyleneTHF for thymidine synthesis.

Toxicity

There was no significant treatment or genotype difference in serum BUN or AST (data not shown).

2.5 DISCUSSION

Genetic disruptions in folate metabolism may influence the efficacy or toxicity of chemotherapeutics that target enzymes in folate metabolism. To understand the regulation of one-carbon metabolism and the interaction between MTHFR activity and MTX, we used a well-characterized mouse model deficient in MTHFR (*Mthfr*$^{-/-}$ and *Mthfr*$^{+/-}$) and generated a new model that overexpresses MTHFR (*MTHFR-Tg*).

By inhibiting DHFR, MTX limits the reduction of dihydrofolate to THF, thereby disrupting all THF-dependent reactions [114]. Two of these reactions, thymidine and purine synthesis, are reduced due to decreased availability of 5,10-methyleneTHF and 10-formylTHF, respectively, and result in an inhibition of DNA synthesis. MTHFR activity may affect MTX response since it regulates the distribution of one-carbon groups between thymidylate synthesis and methylation reactions. Several studies have suggested that MTX-induced myelosuppression may be exacerbated in individuals carrying the *MTHFR* 677C→T polymorphism [97,98,143,144]. In contrast, a study of this polymorphism and MTX response in breast cells *in vitro* concluded that the reduction in MTHFR activity was associated with reduced sensitivity to MTX [142]. Our results are consistent with the former findings since MTX-treated *Mthfr*$^{-/-}$ and *Mthfr*$^{+/-}$ mice exhibited significant myelosuppression (reduction in hematocrit, hemoglobin and RBC numbers compared with saline-treated mice), whereas *Mthfr*$^{+/+}$ mice did not show this response. Since the product of the MTHFR reaction, 5-methylTHF, is the primary

circulatory form of folate, the low circulating folate levels in 677TT patients or in *Mthfr*-deficient mice may be compounded by the additional folate-lowering effect of MTX treatment. This folate deficiency may cause significant DNA damage or reduce DNA synthesis, particularly in rapidly proliferating hematopoietic cells. We have previously reported that *Mthfr*$^{-/-}$ mice have lower plasma total folate levels (25% of wild-type) and a lower proportion of 5-methylTHF in plasma, liver and brain [101].

An alternate mechanism for enhanced myelosuppression in *Mthfr*-deficient mice is increased apoptosis of hematopoietic cells. Apoptosis may be triggered by the high circulating homocysteine levels in *Mthfr*$^{-/-}$ and *Mthfr*$^{+/-}$ mice. Several studies have demonstrated a pro-apoptotic effect of homocysteine in cortical neurons and cultured lymphocytes, possibly through an increase in oxidative stress [69,291] and we have directly demonstrated increased apoptosis in brain of *Mthfr*$^{-/-}$ mice [292]. Studies are underway to examine interaction between MTX and MTHFR deficiency in promoting apoptosis in mice.

By reducing total folate, MTX inhibits methyleneTHF-dependent thymidine synthesis. Our finding of increased splenic dUTP/dTTP ratios following MTX treatment in wild-type mice and in *MTHFR-Tg* mice is consistent with this argument. A reduction in MTHFR activity might be expected to counteract this effect since MTHFR deficiency increases nonmethylfolate, which would include methyleneTHF. Nucleotide ratios tended to decrease in *Mthfr*-deficient mice, albeit nonsignificantly, but this imbalance did not seem to play a major role in hematopoiesis since *Mthfr*-deficient mice were more dramatically affected than wild-type animals following MTX treatment.

Toxicity is a major concern in the use of MTX. Nephrotoxicity (assessed by BUN levels) was observed in all groups following MTX treatment and was particularly marked in *Mthfr*$^{-/-}$ mice. MTX-treated and saline-treated *Mthfr*$^{-/-}$ mice also displayed greater hepatotoxicity (assessed by serum AST levels), compared to their littermates. These findings are consistent with studies reporting a correlation between folate depletion-induced hyperhomocysteinemia and hepatotoxicity in *MTHFR* 677TT patients [144,165].

To further explore the role of MTHFR, we created MTHFR-overexpressing mice. Overexpression was demonstrated in several tissues, with 2- to 3-fold increases in mRNA, protein and enzyme activity. The consistent increases in all three parameters suggest that there was little or no post-transcriptional regulation of MTHFR. Enhanced MTHFR expression should lead to increased 5-methylTHF formation at the expense of 5,10-methyleneTHF and other nonmethylTHF derivatives, such as 10-formylTHF (which is synthesized from 5,10-methyleneTHF). Our observed decreases in hepatic and intestinal 10-formylTHF in *MTHFR-Tg* mice are consistent with this altered distribution pattern. As mentioned above, the folate redistribution in *Mthfr*-deficient mice [101] is in the reverse direction to that seen in these *MTHFR-Tg* animals. Together, our data confirm the important role that MTHFR plays in maintaining the balance between folates destined for nucleotide synthesis and those destined for methionine synthesis.

Methionine is formed through homocysteine remethylation, a process which utilizes 5-methylTHF. Enhanced homocysteine remethylation was observed in the *MTHFR-Tg* mice, as demonstrated by elevated brain methionine. Although methionine elevations were not observed in liver or intestine, there was evidence of enhanced homocysteine transsulfuration to glutathione in liver, a process which is regulated by SAM. Since SAM activates cystathionine-β-synthase, the first enzyme in the

transsulfuration pathway [20], increased production of methionine and SAM in these tissues should enhance homocysteine transsulfuration to generate cysteine and glutathione. Rodents preferentially convert cysteine to glutathione; methionine-enriched diets in mice resulted in increased plasma glutathione levels without changes in cysteine [293]. The observations in that study are consistent with our observation of increased glutathione in liver. The decrease in cysteine in the intestine is consistent with enhanced conversion of cysteine to glutathione although we did not observe a change in glutathione levels in this tissue. Brain does not have an active transsulfuration pathway [19], as indicated by the relatively low baseline levels of cysteine in brain compared to other tissues, and the absence of a change in transsulfuration metabolites in the transgenic mice. In our earlier study of mice with decreased MTHFR expression (*Mthfr*$^{-/-}$ and *Mthfr*$^{+/-}$), we observed the reverse pattern *i.e.*, a decrease in methionine and glutathione with an increase in cysteine in liver [294].

We did not observe significant decreases in 5,10-methyleneTHF levels in transgenic mice. Nonetheless, there were two manifestations of methyleneTHF reductions: the decrease in 10-formylTHF, as mentioned above, and the increase in the dUTP/dTTP ratio in spleen, indicating compromised activity of thymidylate synthase presumably due to the decrease in its substrate 5,10-methyleneTHF. The altered nucleotide ratios in the spleen of transgenic mice were observed in untreated and MTX-treated mice.

MTX treatment resulted in the expected decreases in several hematopoietic parameters (hematocrit, hemoglobin and RBC numbers) in both *MTHFR-Tg* and wild-type mice. *MTHFR-Tg* mice responded similarly to *Mthfr*$^{-/-}$ mice, with greater

reductions in these parameters compared to wild-type littermates. This finding, as well as the pronounced MTX-induced dUTP/dTTP ratio increase, may be due to the inherent reduction in nonmethylTHF in MTHFR-overexpressing mice. These decreases in thymidine synthesis and in proliferation of hematopoietic cells in transgenic mice suggest that increased MTHFR expression may enhance chemosensitivity to MTX.

A major side effect of MTX, hyperhomocysteinemia, is a risk factor for coronary artery disease, stroke and neurologic impairment [146,295]. In our study, MTHFR overexpression was protective against MTX-induced homocysteine increases; MTX treatment increased plasma homocysteine levels in wild-type mice but not in *MTHFR-Tg* littermates. In addition, *MTHFR-Tg* mice did not have elevations in markers of nephrotoxicity or hepatotoxicity, in contrast to our observations in *Mthfr*$^{-/-}$ mice.

There were no differences in homocysteine levels or in hematopoietic parameters between untreated wild-type and transgenic mice. This finding suggests that the 2- to 3-fold increase in MTHFR activity alone was not enough to disturb these functions. However, the combined stress of genetic disruption and drug treatment led to more significant effects on homocysteine remethylation and to a greater inhibition of proliferation. In summary, an increase in MTHFR expression during MTX treatment may enhance chemosensitivity of hematopoietic cells to MTX without enhancing nephrotoxicity or hyperhomocysteinemia. A decrease in MTHFR expression during MTX treatment has similar effects on chemosensitivity but is associated with enhanced toxicity and hyperhomocysteinemia.

There is significant inter-patient variability in response to MTX treatment, and MTX toxicity is often the reason for discontinuation of therapy. Pharmacogenetic analysis of polymorphisms in folate-metabolizing enzymes may provide a useful tool to

predict MTX efficacy or toxicity and thereby maximize the benefit: risk ratio. Our studies illustrate the critical role of MTHFR in MTX chemosensitivity and the importance of pharmacogenetic testing for the *MTHFR* polymorphism and possibly other polymorphisms in folate metabolism.

2.6 ACKNOWLEDGMENTS

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Table 2.1 Folate derivatives in plasma, liver, duodenum and brain of wild-type (Wt) and transgenic (Tg) mice

	Plasma		Liver		Duodenum		Brain	
Folate derivative	Wt	Tg	Wt	Tg	Wt	Tg	Wt	Tg
5,10-methyleneTHF +THF	2.6	2.0	90.1	89.7	75.0	51.9	4.2	4.9
	(0.5)	(0.3)	(4.6)	(7.7)	(23.3)	(10.8)	(0.1)	(0.3)
5-methylTHF	27.6	28.7	36.5	40.2	25.5	13.0	1.8	2.0
	(1.0)	(1.7)	(1.9)	(2.7)	(7)	(2.3)	(0.2)	(0.2)
10-formylTHF	1.1	1.3	16.9	**10.3***	14.0	**4.4***	0.04	0.06
	(0.2)	(0.4)	(1.7)	(1.5)	(4.2)	(0.6)	(0.02)	(0.06)
DHF+F	3.6	3.7	3.8	6.8	0.4	0.7	0.10	0.20
	(0.9)	(0.7)	(1.5)	(1.9)	(0.2)	(0.2)	(0.05)	(0.06)
Total folate	34.9	35.6	147.2	147.0	114.9	70.0	6.1	7.2
	(0.8)	(2.4)	(5.0)	(9.5)	(34.0)	(12.9)	(0.3)	(0.5)

Values are mean of 5 mice per genotype, with standard deviation in parentheses. Plasma units, pmol/ml; tissue units, pmol/mg protein.

*$P<0.05$ for comparison between Tg and Wt in the same tissue.

10-FormylTHF levels in liver and duodenum were significantly decreased in Tg compared to Wt littermates.

THF, Tetrahydrofolate; DHF, Dihydrofolate; F, Folate.

Table 2.2 Metabolites in liver, duodenum and brain of wild-type and transgenic mice

	Liver		Duodenum		Brain	
Metabolite	Wt	Tg	Wt	Tg	Wt	Tg
SAH (pmol/mg protein)	219.2 (20.1)	263.3 (26.6)	209.5 (29.2)	254.7 (43)	110.8 (7.2)	140.6 (12.7)
SAM (pmol/mg protein)	492.9 (98.7)	531.8 (76.6)	630.2 (151.7)	687.0 (126.4)	472.6 (40.5)	459.0 (107.7)
SAM/SAH	2.4 (0.6)	2.1 (0.3)	2.1 (0.3)	3.0 (0.7)	4.4 (0.5)	2.9 (0.7)
Adenosine (pmol/mg protein)	872.0 (83.5)	869.9 (77.9)	903.8 (70.6)	898.6 (78.2)	1629.6 (51.0)	1662.0 (45.9)
Methionine (nmol/mg protein)	35.2 (4.4)	45.9 (10.4)	83.3 (8.8)	73.0 (10.8)	45.3 (6.8)	**66.3*** (5.6)
Homocysteine (nmol/mg protein)	0.60 (0.03)	0.70 (0.06)	2.40 (0.30)	2.10 (0.30)	0.30 (0.04)	0.40 (0.03)
GSH (nmol/mg protein)	39.1 (2.8)	**46.9*** (1.1)	28.7 (6.3)	25.6 (2.5)	22.6 (1.1)	20.9 (0.6)
CysGly (nmol/mg protein)	4.1 (0.5)	4.2 (0.4)	13.4 (1.0)	12.5 (1.7)	19.6 (0.9)	16.8 (1.0)
Cysteine (nmol/mg protein)	12.6 (1.2)	12.3 (1.3)	50.6 (7.6)	**26.0*** (1.2)	3.9 (0.2)	5.1 (0.9)

Values are mean of 5 mice per genotype with standard deviation is in parentheses.

*$P<0.05$ for comparison between Tg and Wt in the same tissue.

Methionine levels in brain were increased, glutathione levels in liver were increased, and cysteine levels in duodenum were decreased in Tg compared to Wt littermates.

SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; GSH, Glutathione; CysGly, Cysteinylglycine.

Figure 2.1 Folate metabolic pathway. MTHFR, methylenetetrahydrofolate reductase; DHFR, dihydrofolate reductase; TS, thymidylate synthase; THF, tetrahydrofolate; DHF, dihydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

Figure 2.2 Plasma levels of total homocysteine (tHcy) (A), cysteine (Cys) (B), homocysteine/cysteine (Hcy/Cys) ratios (C) and glutathione (GSH) (D) of *Mthfr*$^{+/+}$ (horizontal lines), *Mthfr*$^{+/-}$ (diagonal lines) and *Mthfr*$^{-/-}$ (vertical lines) mice treated with saline or MTX (10 mg/kg).

Values represent the mean (± S.E.M.) of 5-20 animals per group.

*, # $P<0.05$, two-factor ANOVA for treatment and genotype, respectively. MTX-treated mice had significantly higher plasma Hcy and Hcy/Cys ratios compared to saline-treated mice.

There was a significant difference in plasma Hcy and cysteine levels, and Hcy/Cys ratios among *Mthfr*$^{-/-}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice.

†$P<0.05$, *t*-test (*Mthfr*$^{-/-}$ versus *Mthfr*$^{+/+}$, within treatment group)

∫ $P<0.05$, *t*-test (*Mthfr*$^{+/-}$ versus *Mthfr*$^{+/+}$, within treatment group)

Figure 2.3 Hematocrit (A), hemoglobin (B), number of red blood cells (RBC) (C) and number of white blood cells (WBC) (D) of *Mthfr*$^{+/+}$ (horizontal lines), *Mthfr*$^{+/-}$ (diagonal lines) and *Mthfr*$^{-/-}$ (vertical lines) mice treated with saline or MTX (20 mg/kg).

Values represent the mean (± S.E.M.) of 5-8 animals per group.

*, ¶ $P<0.01$, two-factor ANOVA for treatment and interaction between treatment and genotype, respectively. Hematocrit, hemoglobin, and RBC numbers were significantly lower in MTX-treated mice compared to saline-treated mice.

‡$P<0.005$, *t*-test (MTX versus saline, genotype constant). *Mthfr*$^{-/-}$ and *Mthfr*$^{+/-}$ mice are affected by MTX; *Mthfr*$^{+/+}$ mice are not.

†$P<0.05$, *t*-test (*Mthfr*$^{-/-}$ versus *Mthfr*$^{+/+}$, within treatment group)

∫ $P<0.05$, *t*-test (*Mthfr*$^{+/-}$ versus *Mthfr*$^{+/+}$, within treatment group)

Figure 2.4 dUTP/dTTP ratios in the spleen of *Mthfr*$^{+/+}$ (horizontal lines), *Mthfr*$^{+/-}$ (diagonal lines) and *Mthfr*$^{-/-}$ (vertical lines) mice treated with saline or MTX (20 mg/kg).

Values represent the mean (± S.E.M.) of 5-8 animals per group.

There was no significant treatment or genotype difference in dUTP/dTTP ratios among *Mthfr*$^{-/-}$, *Mthfr*$^{+/-}$ or *Mthfr*$^{+/+}$ mice by two-factor ANOVA.

‡ $P<0.05$, *t*-test (MTX versus saline, genotype constant). MTX-treated *Mthfr*$^{+/+}$ mice had significantly higher dUTP/dTTP ratios compared to saline-treated *Mthfr*$^{+/+}$ mice.

Figure 2.5 Serum BUN levels (A) and AST levels (B) of *Mthfr*$^{+/+}$ (horizontal lines), *Mthfr*$^{+/-}$ (diagonal lines) and *Mthfr*$^{-/-}$ (vertical lines) mice treated with saline or MTX (10 mg/kg).

Values represent the mean (± S.E.M.) of 5-20 animals per group.

*, # $P<0.05$, two-factor ANOVA for treatment and genotype, respectively. MTX increases serum BUN levels, compared to saline-treated mice. There was a significant genotype difference in BUN and AST levels among *Mthfr*$^{-/-}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice following both saline and MTX treatment.

‡ $P<0.05$, *t*-test (MTX versus saline, genotype constant).

†$P<0.05$, *t*-test (*Mthfr*$^{-/-}$ versus *Mthfr*$^{+/+}$, within treatment group)

Figure 2.6 Methodologies for determination of genotypes. (A) PCR-based genotyping. **In the left panel**, genomic DNA from male transgenic (Tg) and wild-type (Wt) mice was amplified using 2 primers that generate a band of the same size (95 bp) in the *MTHFR* transgene and in the *Mthfr-ps* pseudogene. *EcoR1* cuts the transgene amplicon (66 bp+29 bp), but not the pseudogene. The expected bands were obtained for Wt (95 bp) and Tg (66 bp+29 bp). **In the right panel**, the expected bands were obtained for Wt (151 bp), heterozygous (Tg/+) (151 bp+95 bp) and homozygous female transgenic mice (Tg/Tg) (95 bp). For both panels, controls (C) were obtained from PCR reactions performed without genomic DNA. (B) Southern blot analysis of the *MTHFR* cDNA insertion into the *Hprt* locus. Digestion of genomic DNA with *DraIII* + *ScaI* generated restriction fragments of 7.1 kb, 3.5 kb and 4.0 kb. *ApaLI* + *ScaI* digestion generated bands of 7.5 kb, 3.9 kb + 3.5 kb, and 3.8 kb representing Tg, Wt, and ps alleles, respectively. Bands of 7.2 kb and 12.4 kb + 1.9 kb, after *PshAI* digestion, represent Tg and Wt alleles, respectively. The expected sizes of bands are indicated on the right; molecular weight markers are shown on the left. (C) Schematic representations of the expected bands from the transgene on the X chromosome, the endogenous *Mthfr* gene on chromosome 4 and *Mthfr-ps* on chromosome 5. Positions of the probe used for hybridization are shown for each chromosomal segment. On the X chromosome, the striped region of the transgene indicates the region of homology with the targeting vector.

Figure 2.7 Confirmation of MTHFR overexpression. (A) Semi-quantitative RT-PCR analysis of MTHFR expression in liver and kidney of *MTHFR-Tg* mice and wild-type littermates. RT-PCR was performed using primers spanning *MTHFR* exons 2 – 5, with mouse *Gapdh* as the internal control. Pictures shown for liver and kidney were generated from different gels. (B) Western blot analysis of MTHFR expression in various tissues of *MTHFR-Tg* mice and wild-type littermates. Equal amounts of protein (50 μg) were loaded in each lane, and rabbit antiserum against purified porcine liver MTHFR was used to probe MTHFR, with β-Actin as the internal control. There are 2 isoforms of MTHFR (77 kDa and 70 kDa) in some tissues, as previously reported [77]. The 2.2 kb human cDNA encodes the polypeptide of 70 kDa. Autoradiographs from liver, kidney and heart were generated from one gel, and autoradiographs from intestine and brain were generated from another gel. (C) The MTHFR enzyme assay, which assays MTHFR in the physiologically reverse direction, was performed using radiolabeled 5-methylTHF monoglutamate as substrate and menadione as electron acceptor. Specific MTHFR activity was calculated for each sample; values are mean of 3 – 5 mice ± S.E.M. Filled bars depict values for wild-type mice; unfilled bars, Tg littermates.

Figure 2.8 Plasma levels of total homocysteine (tHcy) (A), cysteine (Cys) (B), homocysteine/cysteine (Hcy/Cys) ratios (C) and glutathione (GSH) (D) of wild-type mice (filled bars) and transgenic littermates (unfilled bars) treated with saline or MTX (10 mg/kg).

Values represent the mean (± S.E.M.) of 7 animals per group.

* $P<0.05$, two-factor ANOVA for treatment. MTX-treated mice had significantly higher plasma Hcy compared to saline-treated mice.

# $P<0.01$, two-factor ANOVA for genotype. There was a significant difference in Hcy/Cys ratios between *MTHFR- Tg* mice and their wild-type littermates.

† $P<0.05$, *t*-test (TG versus WT, within MTX group). Following MTX treatment, MTHFR overexpression is associated with lower plasma Hcy and Hcy/Cys ratios.

Figure 2.9 Hematocrit (A), hemoglobin (B), number of red blood cells (RBC) (C) and number of white blood cells (WBC) (D) of wild-type mice (filled bars) and transgenic littermates (unfilled bars) treated with saline or MTX (10 mg/kg).

Values represent the mean (± S.E.M.) of 7 animals per group.

* $P<0.01$, two-factor ANOVA for treatment. Hematocrit, hemoglobin, and number of RBCs were significantly lower in MTX-treated mice compared to saline-treated mice.

‡$P<0.001$, *t*-test (MTX versus saline treatment, genotype constant). Hematocrit, hemoglobin and RBC numbers in MTX-treated *MTHFR-Tg* mice were adversely affected compared to their saline-treated littermates.

† $P<0.05$, *t*-test (TG versus WT, within MTX group). Following MTX treatment, *MTHFR-Tg* mice have lower hematocrit, hemoglobin and RBC numbers than their wild-type littermates.

Figure 2.10 dUTP/dTTP ratios in the spleen of wild-type mice (filled bars) and transgenic littermates (unfilled bars) treated with saline or MTX (10 mg/kg).

Values represent the mean (± S.E.M.) of 5 animals per group.

*, # $P<0.05$, two-factor ANOVA for treatment and genotype.

MTX increases dUTP/dTTP ratios, compared to saline-treated mice. dUTP/dTTP ratios in *MTHFR-Tg* are significantly increased compared to their wild-type littermates in both saline-treated and MTX-treated groups.

CONNECTING TEXT-Chapter II-III

The results of Chapter II demonstrate that both under- and over-expression of MTHFR affect the chemosensitivity of anti-folate MTX in mice. Following MTX treatment, both *Mthfr*-deficient and *MTHFR-Tg* mice had greater decreases in hematocrit, hemoglobin and RBC numbers compared to their wild-type littermates. However, *Mthfr*-deficient mice had greater increases in plasma Hcy levels and nephrotoxicity, and *MTHFR-Tg* mice had a protection against MTX-induced Hcy increase.

The efficacy of MTX is attributed to its effect on DNA synthesis and apoptosis, through intracellular folate pool depletion. As shown in Chapter II, *MTHFR-Tg* mice have significantly decreased thymidine and purine synthesis (higher dUTP/dTTP ratios and decreased 10-formyl THF levels, respectively) compared to their wild-type littermates. Also it has been shown that *Mthfr*-deficient mice have altered distribution of folates and decreased total folate levels compared to their wild-type littermates [100,101]. We hypothesize that enhanced MTX-induced myelosuppression in these mice can be due to deoxyribonucleotide pool imbalance-induced apoptosis [45] or hyperhomocysteinemia-induced apoptosis [69] in rapidly proliferating hematopoietic cells. In the following chapter, we examine the level of apoptosis in the major hematolytic organ, spleen, in order to explain the impaired hematopoietic profile in these mice following MTX treatment. Furthermore, we hypothesize that overexpression of MTHFR may be protective against MTX-induced apoptosis, through its protective effect against MTX-induced Hcy increase. Therefore, in the following chapter, we also examine the level of apoptosis in transformed mouse embryonic fibroblasts derived from *MTHFR-Tg* embryos and their wild-type littermates.

CHAPTER III

Altered expression of methylenetetrahydrofolate reductase modifies the effect of methotrexate on apoptosis in mice

Basak Celtikci, Qing Wu and Rima Rozen

3.1 ABSTRACT

Folates maintain cellular homeostasis through their essential role in DNA synthesis and repair. Apoptosis also maintains cellular homeostasis and is a major mechanism of cytotoxicity for many chemotherapeutics. The anti-folate methotrexate (MTX) is a widely used chemotherapeutic which inhibits DNA synthesis and induces apoptosis. Changes in activity of a critical folate-metabolizing enzyme, methylenetetrahydrofolate reductase (MTHFR), might alter the chemosensitivity to MTX, since its substrate is used in DNA synthesis and its product is used in remethylation of homocysteine, which induces apoptosis. As we previously reported, altered expression of MTHFR enhanced MTX-induced myelosuppression. However, MTHFR deficiency enhanced MTX-induced hyperhomocysteinemia while MTHFR overexpression was protective against this toxicity.

To explain this impaired hematopoietic profile in mice with altered MTHFR expression, we evaluated MTX-induced apoptosis in the major hematolytic organ, spleen, using TUNEL staining and caspase-3/7 activity assays. We found that altered expression of MTHFR in mice significantly increased the number of TUNEL-positive cells and caspase-3/7 activity in MTX-treated spleen, compared to their wild-type littermates; this is consistent with their enhanced myelosuppression. We also evaluated MTX-induced apoptosis in transformed mouse embryonic fibroblasts (tMEFs) derived from MTHFR-overexpressing transgenic embryos and their wild-type littermates, using Western blot analysis of active Caspase-3 and caspase-3/7 activity assays. Our results showed that in MTX-treated tMEFs, MTHFR overexpression was associated with reductions in active Caspase-3 levels and caspase-3/7 activity (compared to wild-type); this reduction may be

due to the protection against hyperhomocysteinemia. Our results support pharmacogenetic testing for the *MTHFR* polymorphism and possibly polymorphisms in other folate-metabolizing enzymes in clinical settings.

3.2 INTRODUCTION

Folates, as methyl donors, are critical cofactors for DNA synthesis and repair, and the maintenance of DNA integrity and stability. Similarly, apoptosis or programmed cell death is essential for cellular homeostasis and the maintenance of DNA integrity and stability. It is also a protective mechanism against DNA damage induced by chemotherapeutic drugs and critical for their anti-proliferative action [208]. Methylenetetrahydrofolate reductase (MTHFR) is a key regulating enzyme in folate interconversion and stands at a checkpoint that interconnects synthesis of DNA precursors with homocysteine metabolism and methylation reactions. It catalyses the irreversible reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF). The substrate of MTHFR serves as a source of carbon units for the conversion of dUMP to dTMP by thymidylate synthase (TS), or it is converted to 10-formyltetrahydrofolate (10-formylTHF) for *de novo* purine synthesis. The product of MTHFR serves as the methyl donor for the remethylation of homocysteine (Hcy) to methionine (Met); Met is converted to S-adenosylmethionine (SAM), a universal methyl donor. Mild MTHFR deficiency, through a common *MTHFR* polymorphism at bp 677, decreases 5-methylTHF, resulting in hyperhomocysteinemia and global DNA hypomethylation [42,74], and may modify response to methotrexate (MTX) [97,143].

MTX is an anti-folate chemotherapeutic drug that inhibits dihydrofolate reductase, the enzyme that converts dihydrofolate to THF. As a consequence of this inhibition, 5,10-methyleneTHF and 10-formylTHF, the essential carbon donors in the biosynthesis of thymidylate and purines, respectively, are depleted resulting in inhibition of DNA and RNA synthesis [114]. MTX induces uracil misincorporation into DNA [117] and ultimately, DNA strand breaks [119]. MTX-induced nucleotide pool imbalance causes DNA damage and triggers the mitochondrial intrinsic apoptosis pathway, activates caspase-9, and then caspase-3 [60,206]. In addition, MTX-induced 5-methylTHF depletion results in hyperhomocysteinemia and DNA hypomethylation [122,124,125].

Hyperhomocysteinemia may induce apoptosis, potentially through a mechanism involving oxidative stress [54]. It was shown *in vitro* that MTHFR deficiency enhanced apoptosis [232], possibly through hyperhomocysteinemia-induced oxidative stress [69] or DNA hypomethylation affecting cell survival-related genes [90]. In addition, increased apoptosis was also observed in brain of *Mthfr*-knockout (*Mthfr*$^{-/-}$) mice [292].

In earlier work, we reported that MTHFR overexpression enhanced the chemosensitivity to MTX of hematopoietic cells with a protective effect against MTX-induced hyperhomocysteinemia whereas MTHFR deficiency had similar effects on hematopoietic cells, but was associated with enhanced hyperhomocysteinemia [296]. In our current study, we investigate the effect of altered MTHFR expression on MTX-induced apoptosis in spleen, as a potential mechanism for the enhanced myelosuppression observed in our earlier study, and in transformed mouse embryonic fibroblasts (tMEFs).

3.3 MATERIALS AND METHODS

TUNEL staining

Splenic apoptosis was assessed by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay using In Situ Cell Death Detection Kit, POD (Roche Diagnostics). Briefly, deparaffinized sections were treated with 3% H_2O_2 (room temperature, 10 minutes). After washing with phosphate-buffered saline (PBS) 3 times, sections were incubated in TUNEL reaction mixture (TdT and fluorescein-labeled nucleotide mixture; 37°C, 1 hour). Additional PBS washes were followed by incubation with anti-fluorescein antibody conjugated with horseradish peroxidase (37°C, 30 minutes). Signal conversion was completed after incubation with DAB substrate (Sigma-Aldrich) (room temperature, 10 minutes). Sections were counterstained with a 0.5% methyl green solution for 5 minutes. Northern Eclipse software was used for quantification of TUNEL-positive cells (cells with brown DAB-stained nuclei). A total of 5 randomly chosen fields (40x magnification) in at least 3 different sections per spleen were assessed in 5 animals per genotype per treatment group. Apoptotic index (AI) was calculated as the number of TUNEL-positive cells in the defined germinal center area. Counts were confirmed by a second researcher blinded to genotype and treatment status.

Transformed mouse embryonic fibroblast (tMEF) cell culture and MTX response

MEFs were extracted from embryonic day 14.5 embryos in tissue culture conditions. They were transformed by transfecting with a retroviral construct, carrying the E6E7 region of human papillomavirus type 16 [297]. tMEFs were passaged at least 20 times before starting experiments to ensure homogeneity. They were plated at 600,000 cells into 10 cm petri dishes. After 24 hours, medium was changed and either

10μM MTX diluted in PBS or PBS alone (as a control) was added to medium (DMEM with 10%FBS and 100 μg/mL penicillin and streptomycin). Cells were harvested 48 hours after MTX administration [298].

Preparation of total protein from cells

tMEFs were washed with PBS, trypsinized, and then washed again with PBS and centrifuged at 2000xg for 10 minutes at room temperature. Each pellet was suspended in 50 μL ice-cold lysis buffer (50mmol/L potassium phosphate, 0.3 mmol/L EDTA, pH 8.0) containing a protease inhibitor (Complete Mini, Roche). Lysates were alternately incubated on dry ice and wet ice for 2 minutes and repeated 3 times. They were then centrifuged at 18000xg for 30 minutes at 4°C. Supernatants were collected and protein concentrations were determined using the Bio-Rad protein assay solution (Bio-Rad).

Active Caspase-3 Western blotting

100 μg protein from cell extracts were run on SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose membranes at 70 V for 2 hours at 4°C. Nonspecific binding sites were blocked with 2% skim milk in Tris-buffered saline/Tween buffer for 2 hours at room temperature. Blots were incubated with rabbit antiserum against active Caspase-3 (Chemicon) or β-Actin (loading control) overnight at 4°C. Secondary antibody was a peroxidase-coupled anti-rabbit IgG (Amersham Biosciences). ECL Plus Western Blotting Detection Reagents (Amersham Biosciences) were used for signal detection. Signals were quantified with Quantity One 4.1.0 software. The intensity of the active Caspase-3 band relative to the β-Actin band was used as an estimate of protein levels. Cell experiments were performed at least 3 times.

MTHFR Western blotting

MTHFR Western blotting was performed as previously described [296]. ECL Plus Western Blotting Detection Reagents (Amersham Biosciences) were used for signal detection. Signals were quantified with Quantity One 4.1.0 software. The intensity of the MTHFR band relative to the β-Actin band was used as an estimate of protein levels. Cell experiments were performed at least 3 times.

Caspase-3/7 activity assays

The Caspase-Glo 3/7 Assay kit (Promega) was used to measure caspase-3/7 activity in cells and frozen spleens. Protein extracts from cells were prepared as described above and protein extracts from spleens were prepared as previously described [299]. According to the manufacturer's instructions, 2 μg protein in a total of 20μL lysis buffer was incubated with 20μL substrate for one hour and the signal was detected using a luminometer. Protein extracts from spleens of 5 animals per genotype per treatment group or cells of 2 animals per genotype per treatment group were assayed in duplicate and the mean of these two values was used for each animal.

Statistical analysis

Two-factor analysis of variance (ANOVA) and independent-sample *t*-tests were performed using SPSS for WINDOWS software, version 11.0. *P*-values <0.05 were considered significant.

3.4 RESULTS

Apoptotic index and caspase-3/7 activity in the spleen of MTX-treated mice were significantly higher compared to those in saline-treated mice (**Figure 3.1** and **3.2**). Genotype effects were also evident in both of these parameters in both treatment groups. Saline-treated

Mthfr$^{-/-}$ mice had greater increases in caspase-3/7 activity compared to saline-treated *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice, and saline-treated *Mthfr*$^{+/-}$ mice had greater increases in caspase-3/7 activity compared to saline-treated *Mthfr*$^{+/+}$ mice. MTX-treated *Mthfr*$^{-/-}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice had significant increases in both of these parameters compared to their saline-treated littermates. In addition, MTX-treated *Mthfr*$^{-/-}$ mice had greater increases in both of these parameters compared to MTX-treated *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice. Although there was no genotype difference in apoptotic index between *Mthfr*$^{+/-}$ and *Mthfr*$^{+/+}$ mice, *Mthfr*$^{+/-}$ mice had greater increases in caspase-3/7 activity compared to *Mthfr*$^{+/+}$ mice, following MTX treatment.

Similar to *Mthfr*-deficient mice and their wild-type littermates, the apoptotic index and caspase-3/7 activity in the spleen of MTX-treated *MTHFR-Tg* and *MTHFR*-Wt mice were significantly higher compared to those in saline-treated mice (**Figure 3.3** and **3.4**). A genotype effect was also evident in caspase-3/7 activity in both treatment groups. In addition, similar to *Mthfr*$^{-/-}$ mice, MTX-treated *MTHFR-Tg* mice had greater increases in both of these parameters compared to MTX-treated *MTHFR*-Wt mice.

In our previous report [296], we found that MTX treatment significantly increased dUTP/dTTP ratios in the spleen of both *MTHFR-Tg* and *MTHFR*-Wt mice. In addition, *MTHFR-Tg* mice had significantly higher dUTP/dTTP ratios compared to *MTHFR*-Wt mice. We examined the relationship between the nucleotide ratios and apoptosis, and found that the dUTP/dTTP ratios in MTX-treated *MTHFR-Tg* mice were significantly positively correlated with increased apoptotic index in spleen (**Figure 3.5**).

tMEFs derived from *MTHFR-Tg* embryos showed the expected MTHFR overexpression compared to tMEFs derived from *MTHFR*-Wt embryos in both treatment groups. MTHFR levels, relative to β-Actin, were 2-fold higher in tMEFs derived from

MTHFR-Tg embryos compared to those in tMEFs derived from *MTHFR*-Wt embryos in both treatment groups (**Figure 3.6A**). MTX-treated tMEFs derived from *MTHFR-Tg* embryos had 1.5-fold decreases in active Caspase-3 levels compared to MTX-treated tMEFs derived from *MTHFR*-Wt embryos. There was no difference in active Caspase-3 levels between *MTHFR-Tg* and *MTHFR*-Wt cells in the control group. MTX-treated tMEFs derived from *MTHFR*-Wt embryos had 3.5 fold increases in active Caspase-3 levels, compared to the control treatment whereas MTX-treated tMEFs derived from *MTHFR-Tg* embryos had 2.5 fold increases in active Caspase-3 levels, compared to the control treatment (**Figure 3.6B**).

Results for caspase-3/7 activity were consistent with the increases in active Caspase-3 levels in tMEFs derived from *MTHFR*-Wt embryos. tMEFs derived from *MTHFR*-Wt embryos had 2.5-fold increases in caspase-3/7 activity compared to tMEFs derived from *MTHFR-Tg* embryos in both treatment groups (in control group, 50694 versus 20786; in MTX group, 218615 versus 93654 relative light units, respectively). In addition, MTX-treated tMEFs derived from both *MTHFR-Tg* and *MTHFR*-Wt embryos had 4.5-fold increases in caspase-3/7 activity, compared to the control treatment.

Although tMEFs derived from $Mthfr^{-/-}$ and $Mthfr^{+/-}$ embryos showed the expected decrease in MTHFR levels compared to tMEFs derived from $Mthfr^{+/+}$ embryos in both treatment groups, their active Caspase-3 levels did not show consistent changes following MTX treatment (data not shown).

3.5 DISCUSSION

Pharmacogenetics investigates how genetic variation interacts with drug response. Investigating genetic variations in folate metabolism is a promising field and essential for

more efficient anti-folate therapy. The cytotoxicity of the anti-folate MTX is attributed to its inhibitory effect on DNA synthesis and its pro-apoptotic effect, and is associated with its inhibitory effect on folate-metabolizing enzymes. Alteration in expression of MTHFR, a critical folate-metabolizing enzyme, may modify the chemosensitivity to MTX.

We previously reported that *MTHFR-Tg* mice have decreased 10-formylTHF levels and elevated dUTP/dTTP ratios [296]. Therefore, they have reduced DNA synthesis and repair, and increased DNA damage through their deoxyribonucleotide pool imbalances. Following MTX treatment, *MTHFR-Tg* mice had enhanced MTX-induced myelosuppression (greater reduction in proliferation of hematopoietic cells) [296]. In this study, we showed that MTHFR overexpression was associated with enhanced MTX-induced apoptosis in the major hematolytic organ, spleen. There was a significant positive correlation between dUTP/dTTP ratios and apoptotic index in the spleen of *MTHFR-Tg* mice; these observations suggested that the increased MTX-induced apoptosis may be due to the deoxyribonucleotide pool imbalances with consequent DNA damage.

In addition, we previously reported that *Mthfr*$^{-/-}$ mice have altered distribution of folates, hyperhomocysteinemia and DNA hypomethylation [100]. Similar to *MTHFR-Tg* mice, *Mthfr*$^{-/-}$ mice had enhanced MTX-induced myelosuppression, but greater MTX-induced hyperhomocysteinemia [296]. In this study, we showed that *Mthfr*$^{-/-}$ mice had enhanced MTX-induced apoptosis in spleen, possibly through their enhanced hyperhomocysteinemia.

Both i*n vitro* and *in vivo* MTHFR deficiency was associated with increased apoptosis possibly through hyperhomocysteinemia or DNA hypomethylation of cell survival-related genes [232,292]. In MTX-treated *Mthfr*-deficient mice, the MTX-induced Hcy increase and DNA hypomethylation should be greater because of the greater reduction in 5-methylTHF availability for Hcy remethylation and SAM synthesis. However, in MTX-treated *MTHFR-Tg* mice, the protective effect against MTX-induced Hcy increase through MTHFR overexpression could also be protective against MTX-induced apoptosis. tMEFs derived from *MTHFR-Tg* embryos had significant protection against MTX-induced apoptosis via reduction in their active Caspase-3 levels and caspase-3/7 activity compared to tMEFs derived from *MTHFR*-Wt embryos. The distinct effects of MTHFR overexpression on apoptosis in splenocytes and fibroblasts could be due to the different proliferative capacity of these cells. Imbalances in deoxyribonucleotide pools through MTHFR overexpression should have more pronounced effect on apoptosis in splenocytes, because their high proliferation rate (high demand on nucleotide synthesis) makes them more sensitive to imbalances in deoxyribonucleotide pools compared to fibroblast cells.

Our study demonstrates the importance of screening for genetic variations in folate-metabolizing enzymes prior to MTX therapy, for predicting better therapeutic outcomes and preventing MTX-induced toxicity.

3.6 ACKNOWLEDGMENTS

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Figure 3.1 Examples of TUNEL-stained sections of the germinal centers of spleen of *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice treated with saline or MTX (20 mg/kg) at 40x magnification. Apoptotic cells are stained brown.

Figure 3.2 Quantification of TUNEL staining in the germinal centers of spleen (A), Caspase-3/7 activity in the spleen (B) of *Mthfr*$^{+/+}$ (horizontal lines), *Mthfr*$^{+/-}$ (diagonal lines) and *Mthfr*$^{-/-}$ (vertical lines) mice treated with saline or MTX (20 mg/kg).

Values represent the mean (± SEM) of 5 animals per group.

*, #, ¶ $P \leq 0.001$, two-factor ANOVA for treatment, genotype and interaction between treatment and genotype, respectively. MTX-treated mice had significantly higher apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity compared to saline-treated mice.

‡$P \leq 0.02$, *t*-test (MTX versus saline treatment, genotype constant). Apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity in MTX-treated *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice were adversely affected compared to their saline-treated littermates.

† $P<0.005$, *t*-test (*Mthfr*$^{-/-}$ versus *Mthfr*$^{+/+}$, within treatment group). Following MTX treatment, *Mthfr*$^{-/-}$ mice have higher apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity than *Mthfr*$^{+/+}$ mice. Also saline-treated *Mthfr*$^{-/-}$ mice have higher caspase-3/7 activity than *Mthfr*$^{+/+}$ mice.

∫ $P<0.005$, *t*-test (*Mthfr*$^{+/-}$ versus *Mthfr*$^{+/+}$, within treatment group) *Mthfr*$^{+/-}$ mice have higher caspase-3/7 activity than *Mthfr*$^{+/+}$ mice in both treatment groups.

Figure 3.3 Examples of TUNEL-stained sections of the germinal centers of spleen of wild-type mice and transgenic littermates treated with saline or MTX (20 mg/kg) at 40x magnification. Apoptotic cells are stained brown.

Figure 3.4 Quantification of TUNEL staining in the germinal centers of spleen (A), Caspase-3/7 activity in the spleen (B) of wild-type mice (filled bars) and transgenic littermates (unfilled bars) treated with saline or MTX (20 mg/kg).

Values represent the mean (± SEM) of 5 animals per group.

*, #, ¶ $P \leq 0.02$, two-factor ANOVA for treatment, genotype and interaction between treatment and genotype, respectively. MTX-treated mice had significantly higher apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity compared to saline-treated mice.

‡$P<0.01$, *t*-test (MTX versus saline treatment, genotype constant). Apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity in MTX-treated *MTHFR-Tg* and *MTHFR*-Wt mice were adversely affected compared to their saline-treated littermates.

† $P<0.05$, *t*-test (TG versus WT, within MTX group). Following MTX treatment, *MTHFR-Tg* mice have higher apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity than *MTHFR*-Wt mice.

Figure 3.5 The correlation of dUTP/dTTP ratios with apoptotic index in the spleen of transgenic mice.

Figure 3.6 Western blot analyses of MTHFR (A) and active Caspase-3 (B) in transformed mouse embryonic fibroblasts derived from *MTHFR-Tg* and *MTHFR*-Wt embryos. Equal amounts of protein (100 μg) were loaded in each lane. Rabbit antiserum against purified porcine liver MTHFR was used to probe MTHFR and rabbit antiserum against active Caspase-3 was used to probe active Caspase-3, with β-Actin as the internal control.

CONNECTING TEXT-Chapter III-IV

The results of Chapter II and III demonstrate that alteration in MTHFR expression affects response to the anti-folate MTX. We investigated the effect of MTHFR on MTX efficacy (inhibition of DNA synthesis in hematopoietic cells and stimulation of apoptosis in spleen and transformed mouse embryonic fibroblasts) and MTX toxicity (e.g. hyperhomocysteinemia and nephrotoxicity).

We hypothesize that if alteration in MTHFR expression in mice can affect the chemosensitivity to MTX, it may also affect the chemosensitivity to other anti-metabolite drugs that are involved in folate metabolism, such as pyrimidine antagonist 5-FU. Similar to MTX, 5-FU inhibits DNA and RNA synthesis, causes cell cycle arrest in the S-phase and induces apoptosis.

In Chapter IV, we investigate the effect of MTHFR overexpression on response to 5-FU, in terms of inhibition of DNA synthesis in hematopoietic cells and stimulation of apoptosis in spleen.

CHAPTER IV

Overexpression of methylenetetrahydrofolate reductase modifies response to 5-fluorouracil in mice

Basak Celtikci, Qing Wu and Rima Rozen

4.1 ABSTRACT

Folates are important cofactors in the synthesis of purines and thymidylate. The pyrimidine antagonist 5-fluorouracil (5-FU) is a widely used chemotherapeutic drug that targets thymidylate synthase and has other inhibitory effects on DNA and RNA synthesis. A common polymorphism in methylenetetrahydrofolate reductase (MTHFR), a key folate-metabolizing enzyme, may modify response to 5-FU, since MTHFR converts 5,10-methylenetetrahydrofolate (carbon donor in dTMP synthesis) to 5-methyltetrahydrofolate (carbon donor in homocysteine remethylation).

We previously showed that over- and under-expression of MTHFR in mice increased MTX-induced myelosuppression and apoptosis in spleen. In this study, we examined the effect of MTHFR overexpression on 5-FU response in mice. *MTHFR*-overexpressing transgenic mice (*MTHFR-Tg*) and wild-type littermates (*MTHFR*-Wt) were injected with 5-FU or saline and assessed for hematological parameters (hematocrit, hemoglobin, red and white blood cell numbers) and plasma homocysteine.

Following 5-FU treatment, *MTHFR-Tg* mice exhibited decreases only in white blood cell numbers whereas *MTHFR*-Wt mice had decreases in all hematological parameters compared to saline-treated mice; the decrease in white blood cells was greater in *MTHFR-Tg* mice compared to *MTHFR*-Wt mice. To explain the 5-FU-induced impaired hematopoietic profile, we evaluated 5-FU-induced apoptosis in the major hematolytic organ, spleen, using TUNEL staining and caspase-3/7 activity assays. MTHFR overexpression significantly increased the number of TUNEL-positive cells and caspase-3/7 activity in 5-FU-treated spleen (compared to *MTHFR*-Wt mice), consistent with greater 5-FU-induced leucopoenia.

Our results illustrate that pharmacogenetic analysis of genetic polymorphisms in folate metabolism may be useful in improving therapeutic outcome.

4.2 INTRODUCTION

Folates are essential cofactors for DNA and RNA synthesis. Because of this critical role, inhibition of folate-dependent enzymes is the mechanism of action of many chemotherapeutic drugs, such as the pyrimidine antagonist 5-fluorouracil (5-FU) and the anti-folate methotrexate (MTX). Methylenetetrahydrofolate reductase (MTHFR), a key enzyme in folate metabolism, converts 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF) (**Figure 4.1**). 5,10-methyleneTHF is the source of carbon units for the conversion of dUMP to dTMP by thymidylate synthase (TS) or it can be converted to 10-formyltetrahydrofolate (10-formylTHF) for *de novo* purine synthesis. 5-methylTHF, the primary circulatory folate, is a methyl donor for the remethylation of homocysteine (Hcy) to methionine (Met) which is converted to S-adenosylmethionine (SAM), a universal methyl donor. A common *MTHFR* polymorphism (677C→T) results in mild MTHFR deficiency and has been suggested to modify response to chemotherapeutic drugs, 5-FU and MTX [97,98,143,233-235].

5-FU is a widely used pyrimidine antagonist in the treatment of gastrointestinal and breast carcinoma. It inhibits DNA and RNA synthesis, causes cell cycle arrest in the S-phase and induces apoptosis. Its metabolite, 5-fluorodeoxyuridine monophosphate (5-FdUMP), forms a stable ternary complex with TS and 5,10-methyleneTHF to inhibit TS activity. Its other metabolites, 5-fluorodeoxyuridine triphosphate (5-FdUTP) and 5-fluorouridine triphosphate (5-FUTP), can incorporate into DNA and RNA, respectively [105]. Similar to

other chemotherapeutic drugs, common 5-FU-induced toxicities are myelosuppression, mucositis, alopecia, diarrhea and vomiting [136].

Both the *MTHFR* 677T allele and inhibition of *MTHFR* expression by anti-sense were associated with an increase in chemosensitivity to 5-FU in human cancer cell lines and in nude mice [142,232]. In addition, some studies in colorectal cancer patients showed that the *MTHFR* 677T allele was associated with higher response rate to 5-FU [233-235]. These studies suggested that an accumulation of 5,10-methyleneTHF resulting from MTHFR deficiency may increase the cytotoxic effect of 5-FU by increasing the formation and stability of the ternary complex. In contrast, some studies on breast and colorectal cancer patients showed no association or an inverse correlation between the *MTHFR* 677C→T polymorphism and 5-FU chemosensitivity [236-239]. The authors showing the inverse correlation suggested that DNA hypomethylation in *MTHFR* 677TT patients may affect their gene expression profile for survival.

To address the mechanisms by which MTHFR expression can impact on the response to anti-folate medication, we recently generated mice which overexpress MTHFR [296] and studied their response to MTX; we also used mice with MTHFR deficiency which had been generated in earlier work [100]. We showed that both over- and underexpression of MTHFR resulted in nucleotide pool imbalances (due to altered distribution of folate derivatives) and increased MTX-induced myelosuppression and apoptosis in spleen, but exhibited different effects on plasma Hcy levels. In this report, we examine the effect of MTHFR overexpression on 5-FU response. We hypothesize that MTHFR overexpression may reduce carbon supply for the TS reaction and enhance the inhibitory effect of 5-FU on DNA synthesis in rapidly proliferating hematopoietic cells.

4.3 MATERIALS AND METHODS

Mice

All mouse work received approval from the Animal Care Committee of the Montreal Children's Hospital according to the recommendations of the Canadian Council on Animal Care. Mice were fed standard mouse chow (Laboratory rodent diet 5001, Agribrands Purina).

The generation and characterization of *MTHFR*-overexpressing mice (*MTHFR-Tg*) was previously reported [296]. The *MTHFR-Tg* mice and their wild-type littermates (*MTHFR*-Wt) were the result of at least 10 generations backcrossing to C57Bl/6 from their original mixed background (129/Sv and C57Bl/6).

Adult male *MTHFR-Tg* mice and their wild-type male littermates were injected intraperitoneally for 4 consecutive days with either saline (Abbott Laboratories) or 5-FU (Mayne). The dose of 5-FU used (50 mg/kg) is in the range between LD10 and LD20 and has been demonstrated to produce toxic effects without death [232]. Weight loss and general appearance were evaluated after each injection. Twenty-four hours after the last injection, mice were euthanized with CO_2. Cardiac blood samples were collected. Half of the spleen was snap frozen in liquid nitrogen and half was fixed in 4% paraformaldehyde. Fixed tissue was removed to 70% ethanol after 24 hours, then processed and embedded.

Hematological parameters

Cardiac blood samples were collected in potassium–EDTA tubes. Complete blood cell counts were performed on an automatic blood cell counter by the Animal Resource Center of McGill University.

Measurements of plasma total homocysteine (tHcy)

Cardiac blood samples were collected in potassium–EDTA tubes. Plasma was collected after centrifugation at 6000xg for 6 minutes at 4°C and stored at -70°C. Measurements were performed by HPLC [282].

Evaluation of levels of apoptosis

TUNEL staining

Splenic apoptosis was assessed by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay using In Situ Cell Death Detection Kit, POD (Roche Diagnostics). Briefly, deparaffinized sections were treated with 3% H_2O_2 (room temperature, 10 minutes). After washing with phosphate-buffered saline (PBS) 3 times, sections were incubated in TUNEL reaction mixture (TdT and fluorescein-labeled nucleotide mixture; 37°C, 1 hour). Additional PBS washes were followed by incubation with anti-fluorescein antibody conjugated with horseradish peroxidase (37°C, 30 minutes). Signal conversion was completed after incubation with DAB substrate (Sigma-Aldrich) (room temperature, 10 minutes). Sections were counterstained with a 0.5% methyl green solution for 5 minutes. Northern Eclipse software was used for quantification of TUNEL-positive cells (cells with brown DAB-stained nuclei). A total of 5 randomly chosen fields (40x magnification) in at least 3 different sections per spleen were assessed in 5 animals per genotype per treatment group. Apoptotic index (AI) was calculated as the number of TUNEL-positive cells in the defined germinal center area. Counts were confirmed by a second researcher blinded to genotype and treatment status.

Caspase-3/7 activity assays

The Caspase-Glo 3/7 Assay kit (Promega) was used to measure caspase-3/7 activities in frozen spleens. Protein extracts were prepared as previously described [299].

According to the manufacturer's instructions, 2 μg protein in a total of 20μL lysis buffer was incubated with 20μL substrate for one hour and the signal was detected using a luminometer. Protein extracts from spleens of 10 animals per genotype per treatment group were assayed in duplicate and the mean of these two values was used for each animal.

Statistical analysis

Two-factor analysis of variance (ANOVA) and independent-sample *t*-tests were performed using SPSS for WINDOWS software, version 11.0. *P*-values <0.05 were considered significant.

4.4 RESULTS

Plasma thiols

5-FU-treated mice had significantly reduced plasma tHcy, Hcy/Cys and glutathione levels compared to saline-treated mice (**Figure 4.2**). In addition, 5-FU-treated *MTHFR-Tg* mice had significant decreases in plasma Hcy and Hcy/Cys levels compared to their saline-treated littermates. In contrast, treatment did not affect these parameters in *MTHFR*-Wt mice, although they had significant decreases in glutathione levels compared to their saline-treated littermates.

Hematological parameters

Hematocrit, hemoglobin, RBC and WBC numbers in 5-FU-treated mice were significantly lower compared to those in saline-treated mice (**Figure 4.3**). *MTHFR*-Wt mice had significant decreases in all four hematological parameters compared to their saline-treated littermates. In contrast, 5-FU treatment only affected WBC numbers in *MTHFR-Tg* mice. Following 5-FU treatment, the WBC numbers were also significantly lower in

MTHFR-Tg mice than in *MTHFR*-Wt mice. There was no genotype difference following saline treatment.

Apoptosis

Both apoptotic index and caspase-3/7 activity in 5-FU-treated mice were significantly higher than the values in saline-treated mice (**Figure 4.4** and **4.5**). Although 5-FU treatment increased apoptosis in both genotype groups, the increase was more pronounced in the *MTHFR-Tg* mice. The level of apoptosis was significantly higher in 5-FU-treated *MTHFR-Tg* mice compared to 5-FU-treated *MTHFR*-Wt mice.

4.5 DISCUSSION

Pharmacogenetics explores the influence of genetic polymorphisms on drug toxicity and efficacy. An important class of chemotherapeutic drugs is anti-metabolites, such as anti-folates, purine and pyrimidine antagonists, which target folate-dependent enzymes. The pyrimidine antagonist 5-FU and the anti-folate MTX are both widely used chemotherapeutic drugs that inhibit both DNA and RNA synthesis. To understand the impact of disturbances in folate-dependent enzymes, such as MTHFR, on the response to these drugs, we employed mouse models. We had previously generated *Mthfr*-deficient mice (which have altered folate distribution and hyperhomocysteinemia) [100] as well as *MTHFR*-overexpressing mice (which have decreased 10-formylTHF and increased dUTP/dTTP ratio, indicating compromised TS activity) [296]. In earlier work, we examined the effect of altered MTHFR expression on response to MTX [296]. MTX-treated *Mthfr*-deficient and *MTHFR-Tg* mice were both adversely affected in terms of hematopoiesis and splenic apoptosis, compared to

their wild-type littermates. In addition, *Mthfr*-deficient mice had enhanced MTX-induced hyperhomocysteinemia whereas *MTHFR-Tg* mice were protected from this toxicity.

In this study, we examined the effect of MTHFR overexpression on response to 5-FU. 5-FU specifically affects the reaction involving MTHFR's substrate, 5,10-methyleneTHF whereas MTX inhibits dihydrofolate reductase and effectively reduces all the folate forms. 5-FU may decrease Hcy levels possibly through increased availability of 5-methylTHF via accumulation of TS cosubstrate 5,10-methyleneTHF. In contrast, MTX increases plasma Hcy levels by reducing the availability of 5-methylTHF [122,123]. MTHFR overexpression further decreased 5-FU-induced reduction in plasma Hcy levels whereas it was protective against MTX-induced hyperhomocysteinemia through increased availability of 5-methylTHF. On the other hand, 5-FU inhibits thymidine synthesis, and ultimately DNA synthesis through its inhibitory effect on TS activity and its incorporation into DNA. Also MTX inhibits thymidine and purine synthesis through decreased availability of 5,10-methyleneTHF and 10-formylTHF, respectively, and result in an inhibition of DNA synthesis. Therefore if reduced availability of 5,10-methyleneTHF and 10-formylTHF for DNA synthesis, due to MTHFR overexpression and MTX, is the cause of the reduced proliferation and decreased hematopoiesis, similar results should be obtained using a direct inhibitor of TS, 5-FU. Overall, 5-FU-treatment resulted in significant decreases in hematocrit, hemoglobin and RBC numbers, similar to that seen with MTX treatment; in addition, there were significant changes in numbers of WBC and platelets (data not shown). Furthermore, 5-FU-treated *MTHFR-Tg* mice experienced a significantly greater decrease in WBC numbers, compared to their wild-type littermates. As we previously reported, MTX treatment showed marked reductions in RBC numbers in *MTHFR-Tg* mice, with nonsignificant reductions in WBC numbers. In contrast, 5-FU treatment resulted in marked

decreases in WBC numbers in *MTHFR-Tg* mice compared to *MTHFR*-Wt mice, with no genotype differences for RBC numbers. The distinct cellular sensitivity profiles between 5-FU and MTX could be due to the different life spans of the affected cells. WBC have a life span of 1 to 3 days; they are usually the first of the blood cells to be affected but their production usually recovers quickly after treatment, whereas RBC have a relatively long life (120 days) [136]. Therefore MTX treatment may have allowed the WBC to recover in *MTHFR-Tg* mice, because MTX injections were given every 3 days. 5-FU was administered on consecutive days, so the WBC did not have sufficient time to recover.

Since WBC number is the first affected parameter in chemotherapy, it is associated with efficacy (attributed to its anti-proliferative effect) [136]. Although in clinical studies, the effect of MTHFR deficiency on response to 5-FU and its proposed mechanisms are not clear, *MTHFR-Tg* mice showed enhanced chemosensitivity to 5-FU with respect to WBC. Consistent with greater 5-FU-induced leucopoenia, overexpression of MTHFR significantly increased apoptosis in 5-FU-treated spleen. This can be explained by enhanced TS inhibition of 5-FU through compromised TS activity in *MTHFR-Tg* mice. Similar to its effect on MTX response, during 5-FU treatment, MTHFR overexpression further decreases thymidine synthesis and hematopoiesis via reduced DNA synthesis and possibly deoxyribonucleotide pool imbalance-induced apoptosis in spleen, therefore increases the inhibitory effect of 5-FU on nucleotide synthesis in rapidly proliferating hematopoietic cells, especially WBC.

In summary, an increase in MTHFR expression during 5-FU treatment may enhance chemosensitivity to 5-FU while reducing plasma Hcy levels. Our studies illustrate the importance of understanding how genetic disruptions in folate metabolism can affect response to 5-FU and MTX; therefore the importance of choosing the right chemotherapy regimen in the right dose, based on selected genotypical information.

4.6 ACKNOWLEDGMENTS

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Figure 4.1 Folate metabolic pathway. MTHFR, methylenetetrahydrofolate reductase; DHFR, dihydrofolate reductase; TS, thymidylate synthase; THF, tetrahydrofolate; DHF, dihydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

Figure 4.2 Plasma levels of total homocysteine (tHcy) (A), cysteine (Cys) (B), homocysteine/cysteine (Hcy/Cys) ratios (C) and glutathione (GSH) (D) of wild-type mice (filled bars) and transgenic littermates (unfilled bars) treated with saline or 5-FU.

Values represent the mean (± S.E.M.) of 10 animals per group.

* $P<0.05$, two-factor ANOVA for treatment. 5-FU-treated mice had significantly lower plasma Hcy levels, Hcy/Cys ratios and GSH levels compared to saline-treated mice.

‡$P<0.05$, *t*-test (5-FU versus saline treatment, genotype constant). Plasma Hcy and Hcy/Cys ratios in 5-FU-treated *MTHFR-Tg* mice were lower compared to values in saline-treated littermates. Plasma GSH levels in 5-FU-treated *MTHFR*-Wt mice were lower compared to those in saline-treated littermates.

Figure 4.3 Hematocrit (A), hemoglobin (B), number of red blood cells (RBC) (C) and number of white blood cells (WBC) (D) of wild-type mice (filled bars) and transgenic littermates (unfilled bars) treated with saline or 5-FU.

Values represent the mean (± SEM) of 10 animals per group.

* $P<0.001$, two-factor ANOVA for treatment. Hematocrit, hemoglobin, RBC and WBC numbers in 5-FU-treated mice were significantly lower compared to saline-treated mice.

‡$P<0.001$, *t*-test (5-FU versus saline treatment, genotype constant). Hematocrit, hemoglobin, RBC and WBC numbers in 5-FU-treated *MTHFR*-Wt mice were lower compared to their saline-treated littermates. WBC numbers in 5-FU-treated *MTHFR-Tg* mice were lower compared to their saline-treated littermates.

† $P<0.05$, *t*-test (TG versus WT, within 5-FU group). Following 5-FU treatment, *MTHFR-Tg* mice have lower WBC numbers than *MTHFR*-Wt mice; other parameters did not show a genotype difference.

Figure 4.4 Examples of TUNEL-stained sections of the germinal centers of spleen of wild-type mice and transgenic littermates treated with saline or 5-FU at 40x magnification. Apoptotic cells are stained brown.

Figure 4.5 Quantification of TUNEL staining in the germinal centers of spleen (A), Caspase-3/7 activity in the spleen (B) of wild-type mice (filled bars) and transgenic littermates (unfilled bars) treated with saline or 5-FU.

Values represent the mean (± SEM) of 5 animals for (A), 10 animals for (B) per group.

*, # $P<0.01$, two-factor ANOVA for treatment and genotype. 5-FU-treated mice had significantly higher apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity compared to saline-treated mice.

‡$P<0.01$, *t*-test (5-FU versus saline treatment, genotype constant). Apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity in 5-FU-treated *MTHFR-Tg* and *MTHFR*-Wt mice were higher compared to their saline-treated littermates.

† $P<0.05$, *t*-test (TG versus WT, within 5-FU group). Following 5-FU treatment, *MTHFR-Tg* mice have higher apoptotic index (TUNEL-positive cells/area) and caspase-3/7 activity than *MTHFR*-Wt mice.

CHAPTER V.

DISCUSSION

5.1 MTHFR OVEREXPRESSION

MTHFR is a key folate-metabolizing enzyme for regulating the balance between nucleotide synthesis and methylation reactions, two essential components of cellular homeostasis. In this thesis, to provide further information on the role of *MTHFR* and folate metabolism, including their role in the cellular response to MTX and 5-FU, an *MTHFR*-overexpressing transgenic mouse line was generated and characterized. *MTHFR* cDNA was introduced into a targeting vector that underwent homologous recombination into the *Hprt* locus. The main advantage of the *Hprt* targeting system is that ubiquitous expression of *Hprt* provides a favorable chromatin environment for the transgene expression. On the other hand, *Hprt* is on the X chromosome, so it presents as a single copy in males and is selectively inactivated by random X-inactivation in females. Therefore, only *MTHFR-Tg* males obtained from the mating of *MTHFR-Tg* females with C57Bl/6 males could be used in experiments for testing the effect of the transgene, to avoid ambiguities related to random X chromosome inactivation in females. This is a limiting factor for backcrossing and also for designing experiments. In addition to the advantage of ubiquitous expression of *Hprt*, the regulation of *MTHFR* cDNA is by the ubiquitous CMV promoter which allows for MTHFR overexpression in all tissues of *MTHFR*-Tg mice.

Mthfr$^{+/-}$ mice have reduced enzyme activity (60% of wild type), mild hyperhomocysteinemia (1.6-fold higher than wild-type), low liver 5-methylTHF levels and DNA hypomethylation [100]. They exhibit atherosclerotic vasculopathies [100], endothelial dysfunction [102] and increased vascular stiffness [103]. *Mthfr*$^{+/-}$ females are at higher risk for fetal loss and their embryos are at risk for intrauterine growth retardation and congenital heart defects [104]. These findings may be associated with their hyperhomocysteinemia. On

the other hand, *MTHFR-Tg* mice have a 2-3 fold increase in MTHFR expression, no differences in DNA methylation and plasma Hcy levels, and no gross pathological changes. However, *MTHFR-Tg* mice have increased methionine (the SAM precursor) in brain through increased 5-methylTHF availability for Hcy remethylation. Also, they have enhanced transsulfuration presenting as increased glutathione levels in the liver and decreased cysteine levels in the duodenum; these changes may be due to the activation of CBS by SAM. Furthermore, they have decreased 10-formylTHF levels in the liver and the duodenum and higher dUTP/dTTP ratios in the spleen, suggesting a reduction in purine and thymidylate synthesis. Compared with *Mthfr*$^{+/-}$ mice, the phenotype of *MTHFR-Tg* mice is less dramatic; this may be due to the many levels of regulation in folate metabolism. Therefore, MTHFR overexpression may have limited effects under normal conditions. It is possible that under conditions which disturb folate metabolism, such as MTX and 5-FU treatment, the effect of MTHFR overexpression may become more prominent.

5.2 RESPONSE TO MTX AND 5-FU

The anti-metabolite chemotherapeutic drugs MTX and 5-FU achieve their cytotoxicity through the inhibition of folate-metabolizing enzymes. Therefore, the genes controlling their activity can modify the response to these drugs. The most frequently studied folate-metabolizing enzymes are MTHFR and TS. Since the *MTHFR* 677C→T polymorphism significantly affects enzyme activity and occurs with a high allelic frequency, its role in pharmacogenetics has important health implications.

5. 2.1 DOSING REGIMEN

The primary reason for discontinuation of MTX and 5-FU therapy is their toxicity. In this thesis, the MTX doses used, 10 mg/kg (LD10) and 20 mg/kg (LD20) every 3 days for 2 weeks [281], and the 5-FU dose used, four daily doses of 50 mg/kg (LD10-LD20) [232], were previously demonstrated to produce toxic effects without mortality in mice.

Unpublished data from a previous study in our lab showed increased MTX-induced apoptosis in several tissues of *Mthfr*$^{+/-}$ mice (BALB/c background) at the dose of 5 mg/kg. There were no significant genotype and treatment differences in plasma Hcy levels, hematological parameters, and markers of hepatotoxicity and nephrotoxicity. For this reason, the doses chosen in this thesis were higher in order to observe genotype and treatment effects in these parameters.

In clinical studies of leukemia or solid neoplasias, high dose (30mg/m^2) MTX therapy is routinely used whereas in RA or psoriasis patients, long term low dose (7-15 mg/week) MTX therapy is employed [115]. In this thesis, the MTX treatment was used only to show MTX-induced toxic effects and comprised an intermediate dose and short term treatment. However, in other rodent studies which showed MTX-induced hepatotoxicity or altered folate-related metabolites, the dosing regimens were comparable to the doses used in RA patients: 0.1mg/kg/day or 0.2 mg/kg/day for 30 days [197] or for 2 weeks [126], or 0.35 mg/kg/day for 10 days [204]. Also multiple MTX-induced toxicities have been shown with doses of 0.125 to 2 mg/kg, five times/week for 24 months [196] or 0.25-2 mg/kg or 3-6 mg/kg, five times/week for 12-18 months [194]. In addition, in leukemic mice, MTX was used at five daily doses of 25 mg/kg in combination with 20 mg/kg 5-methylTHF [205]. Although there are some contradictorary studies, most of the clinical studies have

demonstrated that MTX given in combination with leucovorin prevents toxicity due to folate depletion [166].

Although 5-FU is a first line drug in colorectal carcinoma, its response rate is only 10-15% when it is used alone, not in combination with other drugs. Similar to MTX, its response rate can be increased when combined with leucovorin and also oxaliplatin or irinotecan [105].

Similar to the MTX treatment, the 5-FU treatment used in this thesis represented an intermediate dose and short term treatment, and was used to show 5-FU-induced toxic effects in mice. In other studies, the toxic effect of 5-FU on bone marrow cells of mice was shown by a single injection at 150 mg/kg [300] or at the dose of 50 mg/kg/day for 7 days [301]. In addition, the antitumor activity of 5-FU in mice was shown at a dose of 60 mg/kg, three times every 4 days [267], or 20 mg/kg/day without leucovorin [232] or 40 mg/kg/day (in combination with leucovorin 4 mg/kg/day), five days/week, for 2 weeks [142] or 3 weeks [302].

5.2.2 THE EFFECT OF ALTERED EXPRESSION OF MTHFR

In this thesis, the susceptibility of *MTHFR-Tg* and *Mthfr*-deficient mice to toxicity induced by MTX and also the susceptibility of *MTHFR-Tg* mice to toxicity induced by 5-FU was examined.

MTHFR overexpression was protective against MTX-induced plasma Hcy increases and further reduced the 5-FU-induced plasma Hcy decreases. In both cases, this effect was most likely mediated by increased availability of 5-methylTHF. Furthermore, differences in

hematological parameters were likely a consequence of decreased availability of 5,10-methyleneTHF and 10-formylTHF for DNA synthesis. Compared to wild-type littermates, MTX-treated *MTHFR-Tg* mice had greater decreases in hematocrit, hemoglobin and RBC numbers whereas 5-FU-treated *MTHFR-Tg* had greater decreases in WBC numbers. WBC have the shortest life span, and are therefore the first hematopoietic cell type affected, and recovered, in chemotherapy. The dosing schedule in the MTX study allowed them to recover; however the daily 5-FU treatment did not. *Mthfr*$^{-/-}$ were adversely affected in all four parameters, with greater decreases in hematocrit, hemoglobin and numbers of RBC and WBC, following MTX treatment. They also exhibited enhanced MTX-induced hyperhomocysteinemia and nephrotoxicity, whereas the *MTHFR-Tg* mice did not.

The reduction in the proliferation of hematopoietic cells can be explained by the increase in splenic apoptosis following both MTX and 5-FU treatment. Following MTX treatment, increased apoptosis in *MTHFR-Tg* mice may be due to their imbalances in deoxyribonucleotide pools, as shown by their higher dUTP/dTTP ratios in spleen. On the other hand, following MTX treatment, increased apoptosis in *Mthfr*$^{-/-}$ mice may be due to their greater increases in plasma Hcy levels. Supporting this mechanism, MTHFR overexpression was protective against MTX-induced apoptosis in transformed mouse embryonic fibroblasts, possibly through its protective effect against plasma Hcy increase. Imbalances in deoxyribonucleotide pools should have more pronounced effect on apoptosis in the spleen of *MTHFR-Tg* mice, because the spleen, with rapidly proliferating cells, should be more sensitive to imbalances in deoxyribonucleotide pools compared to fibroblast cells. Although deoxyribonucleotide pool imbalances may represent the mechanism for the enhanced 5-FU-induced apoptosis in the spleen of *MTHFR-Tg* mice, as we showed in MTX-

treated mice, we were unable to carry out these measurements. The chromatogram was uninterpretable due to the presence of an additional, interfering peak, presumed to be 5-FdUTP. Attempts to resolve this problem were unsuccessful.

As a final point, increased response to 5-FU has been shown in *MTHFR* 677TT patients, possibly through their increased availability of 5,10-methyleneTHF for the formation and stability of the ternary complex [233-235]. Therefore, it would be important and relevant to clinical studies to investigate the effect of 5-FU in *Mthfr*-deficient mice. This would augment the data obtained in this thesis for *MTHFR-Tg* mice.

5.3 FUTURE DIRECTIONS

One improvement in the experimental designs of both the MTX and 5-FU studies would be the administration of leucovorin together with these drugs. Most clinical studies demonstrate that use of leucovorin can compensate the effect of genetic alterations of folate distribution which are observed in *MTHFR*-overexpressing and *Mthfr*-deficient mice. However, some studies have not observed a positive effect of leucovorin and have attributed this to its negative effects on efficacy and on the variable effect of dietary folate. Controlled studies in mice may clarify these confounding factors.

In addition, it would be interesting to examine the synergistic effect of MTX and 5-FU together in *MTHFR-Tg* and *Mthfr*-deficient mice. It has been shown that MTX enhances the chemotherapeutic effect of 5-FU if MTX is administered prior to 5-FU. Inhibition of purine synthesis by MTX results in higher PRPP levels which induces the conversion of 5-FU to 5-FUMP. This ultimately increases the incorporation of 5-FUMP into RNA [105]. Investigating the effect of altered MTHFR expression on this synergistic effect can confirm

studies which have reported increased toxicity in *MTHFR* 677TT patients receiving the CMF (cyclophosphamide, MTX and 5-FU) protocol [98]. Examination of the synergistic effects of these drugs in *MTHFR-Tg* mice would augment the observations presented in this thesis, that MTHFR overexpression potentially enhances the efficacy of these drugs when they are administered alone.

It would also be interesting to utilize the *MTHFR*-overexpressing and *Mthfr*-deficient mice to study a variety of other drugs that interfere with folate metabolism. For example, fenofibrate, an anti-lipidemic drug, induces hyperhomocysteinemia which counteracts its cardioprotective effect [303,304]. Sulfasalazine, an anti-rheumatic drug, is usually used in combination with MTX and has been shown to cause greater increases in plasma Hcy levels than MTX alone [173]. Phenytoin, an anti-epileptic drug, decreases hepatic MTHFR activity in mice [305] and causes greater increases in plasma Hcy levels and greater decreases in plasma folate levels in *MTHFR* 677TT patients compared to other genotype groups [306]. Another anti-convulsant drug, valproic acid, is also commonly used in the treatment of epilepsy and is associated with increased Hcy levels and decreased folate levels [99,307]. Levodopa, an anti-parkinson drug, requires methylation to convert to its effective form, dopamine. Following levodopa treatment, high SAM demand reduces 5-methylTHF availability and increases plasma Hcy levels; *MTHFR* 677TT patients are especially at risk [308-310].

5.4 IMPLICATIONS

One of the major side-effects of MTX is hyperhomocysteinemia, an established independent risk factor for cardiovascular disease and neurological disorders [146,295].

MTHFR overexpression was protective against MTX-induced hyperhomocysteinemia; therefore it would be capable of reversing the side effects of other treatments that increase plasma Hcy levels including those discussed above.

MTX's efficacy is widely attributed to its effect on reduction of nucleotide synthesis. MTHFR overexpression decreases nucleotide synthesis by depleting 5,10-methyleneTHF and therefore increasing the ratio of dUTP to dTTP. Therefore, enhancing MTHFR may be a potential target for increasing the chemosensivity to MTX and also for new anti-folate drug discoveries.

The doses of MTX and 5-FU required for effective control of disease activity differ among patients and there is no reliable test to predict their efficacy or toxicity. Pharmacogenetic analysis of genetic polymorphisms in the folate-metabolizing enzymes may provide a useful tool to predict their efficacy and/or toxicity in order to maximize the benefit: risk ratio. By pretreatment screening of patients, we can decrease the morbidity related to toxicity, decrease the expense of toxicity monitoring (complete blood counts and serum liver enzyme levels), and select the patients who will be more likely to have better clinical response.

Investigating genetic variability in folate metabolism in the framework of pharmacogenetics is a promising field. Recent studies have shown the potential for targeting therapy based on patients' genotypes with improved therapeutic outcomes and reduced drug-related toxicity. The process of chemotyping, that is, choosing the right chemotherapy regimen in the right dose, based on selected genotypical and phenotypical information, should enter medical practice.

5.5 LIST OF CONTRIBUTIONS

1. A mouse line overexpressing *MTHFR* (*MTHFR-Tg*) was generated and identified by PCR-based genotyping and Southern blotting. MTHFR overexpression was confirmed by RT-PCR, Western blotting, and enzyme activity assays.

2. *MTHFR-Tg* mice had significant differences in several metabolites: increased methionine in brain, decreased cysteine in duodenum, increased glutathione in liver and decreased levels of 10-formylTHF in liver and duodenum.

3. MTX treatment significantly decreased hematocrit, hemoglobin and number of RBC in *Mthfr*$^{-/-}$ and *Mthfr*$^{+/-}$ mice. MTX-treated *Mthfr*$^{-/-}$ mice had greater decreases in hematocrit, hemoglobin and numbers of RBC and WBC compared to their wild-type littermates.

4. MTX-treated *Mthfr*$^{-/-}$ mice had greater MTX-induced nephrotoxicity compared to their wild-type littermates.

5. Both MTX-treated and saline-treated *Mthfr*$^{-/-}$ mice had greater hepatotoxicity compared to their wild-type littermates.

6. MTX-treated *MTHFR-Tg* mice had lower plasma Hcy levels and Hcy/Cys ratios compared to their wild-type littermates.

7. MTX-treated *MTHFR-Tg* mice had greater decreases in hematocrit, hemoglobin and number of RBC compared to their wild-type littermates.

8. *MTHFR-Tg* mice had higher dUTP/dTTP ratios compared to their wild-type littermates, in both the saline- and MTX-treated groups. This confirms the important role of MTHFR in regulating nucleotide pools and proliferation.

9. Following MTX treatment, the apoptotic index and caspase-3/7 activities were higher in the spleen of *Mthfr*$^{-/-}$ mice, compared to their wild-type littermates.

10. Following MTX treatment, the apoptotic index and caspase-3/7 activities were higher in the spleen of *MTHFR-Tg* mice, compared to their wild-type littermates. Increased dUTP/dTTP ratios in *MTHFR-Tg* mice were significantly correlated with increased levels of apoptosis in spleen.

11. Following MTX treatment, transformed mouse embryonic fibroblasts (tMEFs) derived from *MTHFR-Tg* embryos had lower active Caspase-3 levels and caspase-3/7 activities compared to tMEFs derived from *MTHFR*-Wt embryos.

12. Following 5-FU treatment, there were reductions in plasma Hcy and GSH levels and Hcy/Cys ratios compared to saline-treated mice.

13. 5-FU-treated *MTHFR-Tg* mice had lower Hcy levels and Hcy/Cys ratios compared to their saline-treated littermates.

14. 5-FU-treated *MTHFR-Tg* mice had greater decreases in number of WBC compared to their wild-type littermates.

15. 5-FU-treated *MTHFR-Tg* had higher apoptotic indices and caspase-3/7 activities in spleen compared to their wild-type littermates.

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APPENDIX

COMPLIANCE FORMS