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Studies on methotrexate cytotoxicity and apoptosis in human B-lymphocyte cell lines: with emphasis on 5,10-methylenetetrahydrofolate reductase 677C→T genotype and folate status

A dissertation presented for the degree of

Doctor of Philosophy (PhD) at the University of Dublin, Trinity College

By

Muriel Gallagher

Department of Clinical Medicine

October 2011

Thesis 9507

To my parents,

Mary and Tommy

Declaration

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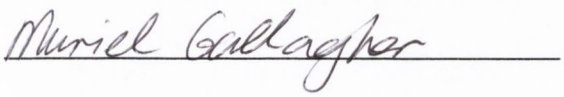

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List of abbreviations

5-formyl-THF	(6S)-5-Formyl-5,6,7,8-tetrahydrofolic acid
5hmC	5-hydroxymet□yl cytosine
5meC	5-methyl cytosine
5-methyl-THF	(6S)-5-Methyl-5,6,7,8-tetrahydrofolic acid
7-OH-Mtx	7-hydroxymethotrexate
ADA	Adenosine deaminase
ADE	Adverse drug event
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
AGGC	N-Acetyl-S-geranylgeranyl-L-cysteine
AICAR	Amino-imidazolecarboxamide ribosyl-5-phosphate
AICARFT	AICAR formyltransferase
ALL	Acute lymphoblastic leukemia
AMP	Adenosine monophosphate
APRT	Amidophosphoribosyl transferase
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCRP	Breast cancer resistance protein
BER	Base-excision repair
BHMT	Betaine-homocysteine methyltransferase
BMI	Body mass index
BSA	Bovine serum albumin
CA	*Candida albicans*
CAD	Caspase-activated deoxyribonuclease
CBS	Cystationine-β synthase
CCR	Coriell Cell Repositories
CDK	cyclin-dependent kinase
cDNA	Complementary DNA
CHD	Coronary heart disease
CHO	Chinese ham□ter ovary
CLA	Cutaneous lymphocyte-associated antigen
CRC	Colorectal cancer
cSHMT	Cytoplasmic serine hydroxymethyltransferase
dcSAM	Decarboxylated SAM
DD	Death domain
dFBS	Dialysed foetal bovine serum
DFE	Dietary folate equivalents
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTPs	Deoxy Trinucleotidephosphates

dTMP	Deoxythymidine monophosphate (or thymidylate)
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
dUTPase	Deoxyuridine triphosphate nucleotidohydrolase
DVT	Deep vein thrombosis
DYRK2	Dual-specificity tyrosine-phosphorylation-regulated kinase 2
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPIC	European Prospective Investigation into Cancer and Nutrition
FA	Folic acid
FAD	Flavin adenine dinucleotide
FBS	Foetal bovine serum
FDH	10-formyl THF dehydrogenase
FIGLU	Formiminoglutamic acid
FITC-AnnV	Fluorescein isothiocyanate-labelled Annexin V
FITC-dUTP	Fluorescein isothiocyanate-labelled dUTP
FPGS	Folylpolyglutamate synthase
FR	Folate receptor
FSAI	Food Safety Authority of Ireland
FSC	Forward scatter
FTHFS	10-formyl THF synthase
GAR	Glycinamide ribonucleotide
GARFT	GAR formyltransferase
GCP	Glutamate carboxypeptidase II
GCS	Glycine cleavage system
GGH	γ-glutamyl hydrolase
GNMT	Glycine N-methyl transferase
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphat□
GWAS	Genome-wide association study
Hcy	Homocysteine
HEp-2	Human laryngeal squamous carcinoma cells
HIPK2	Homeodomain-interacting protein kinase-2
HPGRT	Hypoxanthine-guanine phosphoribo□yl transferase
HPLC	High-performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
Hx	Hypoxanthine
IAP	Inhibitor of apoptosis protein
ICAM-1	Intercellular adhesion molecule 1
ICMT	Isoprenylcysteine carboxymethyltransferase
IFN	Interferon
IHD	Ischemic heart disease
IL	Interleukin
IMP	Inosine monophosphate
MAPK	Mitogen-activated protein kinase
MAT	Methionine adenosyltransferase

MBP	Myelin basic protein
MCP-1	Monocyte chemoattractant protein 1
MEFs	Mouse embryonic fibroblasts
MFT	Mitochondrial folate transporter
MMP	Matrix metalloprotease
MMR	Mismatch repair
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
MS	Methionine synthase
mSHMT	Mitochondrial serine hydroxymethyltransferase
MTHFC	Methenyl THF cyclohydrolase
MTHFD	Methylene THF dehydrogenase
MTHFR	5,10-methylenetetrahydrofolate reductase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), inner salt
Mtx	Methotrexate
NAC	N-acetyl-L-cysteine
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced form)
NHANES	National health and nutrition examination survey
NK	Natural killer
NSCLC	Non-small cell lung cancer
NTDs	Neural tube defects
OCM	One-carbon metabolism
ODC	Ornithine decarboxylase
OR	Odds ratio
pABA	Para-aminobenzoic acid
pABG	Para-aminobenzoglutamate
PARP	Poly-ADP-ribose polymerase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCFT	Proton-coupled folate transporter
PcG	Polycomb
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEMT	Phosphatidylethanolamine methyltransferase
PES	Phenazine ethosulfate
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PI	Propidium iodide
PKCδ	Protein kinase C δ
PP2A	Protein phosphatase 2A
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
RBC	Red blood cell

RCC1	Regulator of chromatin condensation 1
RFC	Reduced folate carrier
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memoria Institute 1640 medium
RR	Relative risk
RREB-1	Ras responsive element-binding protein 1
RT-PCR	Reverse transcriptase polymerase chain reaction
SAH	S-adenosyl homocysteine
SAHH	S-adenosyl homocysteine hydrolase
SA	S-adenosyl methionine
SAMC	Human mitochondrial SAM carrier
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHMT	Serine hydroxymethyltransferase
SNPs	Single nucleotide polymorphisms
SSC	Side scatter
SUMO	Small ubiquitin-like modifier
TBE	Tris-Borate-EDTA
TBST	Tris buffered saline with tween-20
Tc	Cytotoxic T-lymphocyte
TdT	Terminal deoxynucleotidyl transferase
TEMED	N,N,N',N'-Tetramethylethylenediamine
TeT	Tetanus toxoid
Th	Helper T-lymphocyte
THF	Tetrahydrofolate
Thy	Thymidine
TLCK	N-tosyl-L-lysine chloromethyl ketone hydrochloride
TLD	Thymineless death
TNF-α	Tumour necrosis factor α
TPCK	N-p-tosl-L-phenylalanine chloromethyl ketone
Treg	Regulatory T-lymphocyte
tRNA	Transfer RNA
TrxG	Trithorax
TS	Thymidlyate synthase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UDG	Uracil DNA glycosylase
VCAM-1	Vascular cell adhesion mlecule 1
WHO	World Health Organisation
Z-IETD-fmk	Z-IETD-fluoromethylketone
Z-LEHD-fmk	Z-LEHD-fluoromethylketone
Z-VAD-fmk	Z-VAD-fluoromethylketone

Summary

Methotrexate (Mtx) is an antifolate drug which is widely used in treatment of cancer and of autoimmune and inflammatory conditions. The main mechanism by which Mtx operates is inhibition of dihydrofolate reductase (DHFR), thereby preventing the regeneration of the active folate cofactor THF, leading to a blockade of intracellular folate metabolism. Folates are required for many cellular reactions, most notably synthesis of DNA precursor molecules and for generation of the universal methyl-group donor, S-adenosylmethionine (SAM). Mtx therefore interferes with a metabolic pathway which is linked to many diverse cellular processes. Despite appreciation of the drug's clinical efficacy, the precise mechanism by which Mtx has its effects within the cell is incompletely understood. A key area of interest is the potential influence of pharmacogenomic factors on individual responses to Mtx. There is a common 677C→T polymorphism in MTHFR, an enzyme which occupies a key regulatory point in the folate metabolic pathway. The minor allele of this polymorphism is associated with reduced MTHFR activity, increased plasma homocysteine (Hcy) and altered distribution of folate cofactors within the cell. Several clinical association studies have suggested that the MTHFR 677C→T polymorphism may affect Mtx efficacy or toxicity, but there is a lack of biochemical evidence to test these reports.

The MTHFR genotype of several transformed B-lymphocyte cell lines was determined by PCR genotyping. The influence of MTHFR 677C→T genotype on response to Mtx was investigated by examination of cell proliferation and cell viability after exposure to the drug. Cell proliferation and cell viability were assessed by trypan blue (TB) staining and cell counting using a haemocytometer, and by use of the MTS cell proliferation assay. Cell lines of TT genotype had a lower proliferation rate compared to cells of CC genotype in the control condition and in the Mtx-treated condition, as assessed by cell counting. Cell lines of TT genotype had lower cell viability compared to CC genotype in the control condition, but there was no difference in cell viability by genotype after Mtx exposure. As assessed by the MTS assay, cell lines of TT genotype had a lower proliferation rate compared to cells of CC genotype. This difference was most significant at the lowest concentration of Mtx used, and was reduced at higher Mtx concentrations. These results indicate that while there was a difference in proliferation rate and viability attributable to MTHFR 677C→T genotype under the control condition, it was diminished by the potent effect of Mtx on these cell lines.

The ability of the folate species 5-formyl THF, 5-methyl THF and folic acid (FA) to prevent the anti-proliferative effect of Mtx was examined using the MTS cell proliferation assay. Both 5-formyl THF and 5-methyl THF had a dose-dependent blocking effect on Mtx, but FA did not block the

effect of Mtx. In order to determine the contribution of inhibition of dTMP and purine synthesis to the action of Mtx, the ability of exogenous thymidine (Thy) and/or hypoxanthine (Hx) to prevent the anti-proliferative effect of Mtx was also examined. Addition of Thy partially restored cell proliferation in the presence of Mtx, but addition of Hx had no effect. The addition of a combination of Thy and Hx fully restored cell proliferation to control levels, indicating that inhibition of both dTMP and purine synthesis is required for Mtx to have its effect.

The effect of FA and riboflavin availability in the growth medium on cell proliferation, cell viability and homocysteine (Hcy) efflux was examined in relation to MTHFR 677C→T genotype. In standard growth medium containing both of these nutrients, cell growth and viability were maintained over a 72h period and there was very little Hcy efflux measured. In folate-free medium, cell growth continued during the same time frame, although there was a progressive decline in cell viability and increased Hcy release. Deficiency of both FA and riboflavin caused a rapid decline in live cell number and viability, and a comparatively greater release of Hcy. Cells of TT genotype were more sensitive to deficiency of FA or both nutrients, as demonstrated by a lower live cell count and lower cell viability compared to the CC cell line.

The apoptotic effect of Mtx in transformed B-lymphocyte cell lines was investigated by several methods. Western blot was employed for detection of protein markers of apoptosis. Flow cytometry was used to detect morphological changes associated with apoptosis and to quantify apoptosis by cell cycle analysis and Annexin V/PI staining. Cell lines of TT genotype were found to have a greater level of apoptosis than CC cell lines after 24h exposure to Mtx, when the effect of the drug was not yet significant. There was no difference by genotype detected after 48h incubation time. Mtx-induced apoptosis was prevented by addition of 5-formyl THF and 5-methyl THF, but not FA. Addition of exogenous Thy partially prevented Mtx-induced apoptosis, but exogenous Hx had no effect. A combination of Thy plus Hx completely prevented Mtx-induced apoptosis. The mechanism of Mtx-induced apoptosis was investigated by use of inhibitors of caspases and serine proteases. Mtx-induced apoptosis was found to be largely caspase-dependent, but caspase inhibition did not completely prevent apoptosis. Inhibition of chymotrypsin-like serine protease activity was also found to play a part in Mtx-induced apoptosis. Serine protease inhibition was found to inhibit apoptotic DNA degradation to a greater extent than phosphatidylserine exposure, indicating that serine protease enzymes may be involved in a particular subset of cellular apoptotic responses.

It has been suggested that Mtx may partially mediate its cellular effects by inhibition of transmethylation reactions and subsequent effects on cell signalling proteins. The effect of AGGC,

a competitive inhibitor of ICMT on the anti-proliferative and apoptotic effect of Mtx was examined. AGGC was cytotoxic at concentrations greater than 200μM. However, a non-toxic concentration of AGGC partially restored cell proliferation in the presence of Mtx. AGGC had an inherent apoptotic effect, but nonetheless partially inhibited Mtx-induced apoptosis. The effect of AGGC was more potent when FA was present in the cell culture medium compared to folate-free culture medium. These observations support the idea that Mtx may interfere with intracellular signalling events involving methylation reactions.

1 Chapter 1: Introduction

Pharmacogenomics is the effort to understand how individual genetic differences influence the efficacy and toxicity of drug treatments. In current clinical practice, drugs are administered based on standard dosage protocols. It is clear that not every patient responds in the same way to a particular drug, and toxic effects of drug treatment are a significant problem. This is particularly the case in cancer treatment, where the chemotherapeutic drugs used can have severe side effects due to their cytotoxic nature. Toxicity is also a major problem for individuals who are on long term therapy for chronic conditions such as rheumatoid arthritis (RA) and other autoimmune diseases. The aim of pharmacogenomic research is to realize an era of 'personalised medicine' where treatment and dosage can be tailored to an individual's needs, based on information about their genetic makeup or specific biomarkers of disease. The aim is to produce the best possible treatment effect with the least side effects, avoiding the need for trial and error as is currently the case. This approach is still in its infancy, although the benefits of a more precise biological understanding of disease processes are beginning to be translated to clinical practice. Notably in cancer treatment, a number of drugs have been developed in response to specific genetic mutations found in tumour cells (Chin et al. 2011).

This approach to medical research and practice comes in the context of a wider research interest in gene-environment interactions and their role in health and disease. Rare genetic changes are known to be responsible for many severe and rare diseases, but there are also many relatively common genetic polymorphisms that have the potential to influence metabolism and disease risk. Individual response to a particular environmental factor will be modified by genetic differences. Conversely, environmental factors can have an influence on gene expression metabolism and potentially alter the effects of other factors. For example, alcohol use has been shown to interfere with folate (vitamin B9) absorption by reducing expression of a specific folate transport protein, and folate deficiency exacerbates alcohol-related liver disease (Halsted et al. 2002).

Diet is a major route of exposure to environmental influences. Folate is an example of a nutrient which is known to influence several health outcomes over and above the consequences of severe deficiency of the vitamin. Low folate status is a risk factor for cardiovascular disease, and periconceptional supplementation with folic acid is known to reduce the incidence of neural tube defects (NTDs) (MRC 1991). Folate status also has an influence on cancer risk, although the role of this nutrient is thought to be complex and dependent on cancer type and stage (Yang et al. 2009). Some countries have introduced mandatory fortification of the food supply with folic acid, but the effect and ethics of supplementing the food supply of a whole population are debated, as

the total and long-term effects are not known (Mason 2009). The contribution of folate status to several disease states is the subject of intense research, as are genetic polymorphisms which influence folate metabolism.

This thesis addresses a pharmacogenomic question in relation to folate metabolism and the efficacy of the antifolate drug methotrexate, the first-line drug used in treatment certain cancers and inflammatory diseases.

1.1 Folate structure

Folate is a general term used to refer to the water soluble vitamin B9 and its derivatives. Folates are essential for the various cellular reactions which involve transfer of one carbon units, collectively known as one-carbon metabolism (OCM). These reactions include synthesis of methionine, purine ring synthesis, thymidylate (dTMP) synthesis, serine-glycine interconversion, histidine catabolism and formation of methyl group donors that are necessary for all cellular methylation events.

Folic acid was originally isolated from spinach leaves in the 1940's, and was therefore given its name from the latin word for leaf, 'folia' (Mitchell et al. 1988). The basic structure of the folate molecule consists of a pteridine ring with an aminobenzoylglutamic acid attached. The structure of folic acid is shown in figure 1.1. Further glutamate residues can be attached to the γ-carboxyl end of the molecule, forming polyglutamated derivatives of varying length. The term 'folic acid' correctly refers to the form in which the pteridine ring is fully oxidized, but the biologically active form is the fully reduced derivative 5,6,7,8,-tetrahydrofolate (THF). 'Folate' is thus used as a general term which includes folic acid and its other biologically active derivatives. THF can be substituted with one-carbon units at the N-5 and N-10 positions of the pteridine ring, forming a range of folate molecules which are important intermediates in the biosynthetic reactions of one carbon metabolism (OCM) (Lucock 2000). The N-5 postion can be substituted with a methyl, formyl or formimino group, the N-10 position can be substituted with a formyl group, and bridging of these two positions can be achieved by a methylene or methenyl one carbon unit. THF thus acts as a carrier mediating transfer of one-carbon units which are required to form structural components of many molecules. The structures of 5-methyl-THF, 5-formyl-THF and 5,10-methylene-THF are shown in figure 1.1.

1.2 Folate absorption

Mammalian cells lack the capacity to synthesize folate, so it must be obtained from the diet. Folate obtained from natural dietary sources consists mainly of polyglutamated tetrahydrofolates

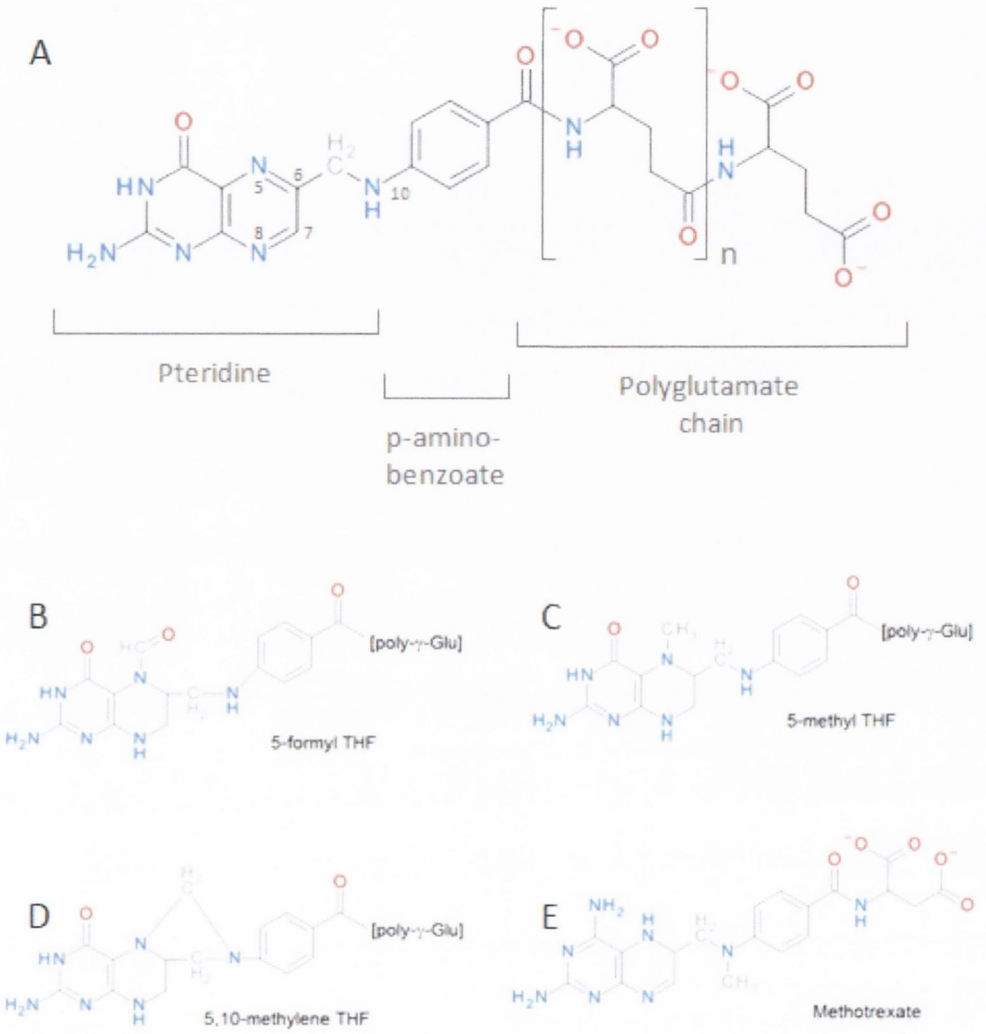

Figure 1.1 Folate molecular structure

A: Molecular structure of folic acid. The N-5, C-6, C-7, and N-8 positions that are reduced in the physiologically active form tetrahydrofolate (THF) are marked, as are the N-5 and N-10 positions where one-carbon groups are added. B: 5-formyl-THF C: 5-methyl THF D: 5,10,-methylene THF E: Methotrexate.

in a variety of one carbon substituted forms. Polyglutamated folates cannot be absorbed directly, but are broken down to the monoglutamate form in the gut by the action of glutamate carboxypeptidase II (GCP), also known as conjugase (Rosenberg et al. 1969). This enzyme has a pH optimum of 6.5 (Chandler et al. 1986), which approximates the pH in the proximal jejunum. It is thought that the activity of GCP in the gut is sufficient to effectively convert dietary folate polyglutamates to the monoglutamate form based on several studies. For example, a study compared folate absorption from spinach which contains 60% polyglutamated folate, to spinach which had been treated to convert the naturally present folates to the monoglutamate form. The results demonstrated that the extent of glutamation of the ingested folate did not affect bioavailability (Konings et al. 2002). Monoglutamated folate thus formed in the gut lumen is absorbed into enterocytes, and from there it is released into the hepatic portal vein. Absorbed folate is subject to extensive 'first pass' metabolism whereby much of the folate is taken up by the liver. Once absorbed into liver cells, folate can either be released into the bile where it is subject to enterohepatic circulation and reabsorption, converted to a polyglutamated form and retained within liver cells, or released into the bloodstream (Zhao et al. 2009a). The main form of folate which enters the systemic circulation is monoglutamated 5-methyl-THF.

Folate monoglutamates are transported into peripheral tissue cells by the reduced folate carrier (RFC), described further in section 1.4. Once inside the cell, additional glutamate residues are conjugated to the folate molecule by folylpolyglutamate synthase (FPGS). This alteration of the folate molecule has two important effects; polyglutamated folates cannot be passively transported across membranes so this modification leads to retention in the cytosol, and polyglutamation also augments the affinity of folate cofactors for folate-requiring enzymes. The number of polyglutamate residues in folates extracted from rat liver was found to vary between 3-8 depending on presence of folate in the diet, with longer chain polyglutamates found when folate intake was restricted (Cassady et al. 1980). Lowe et al. (1993) found similar effects in Chinese hamster ovary cells expressing varying levels of FPGS. When levels of folate in the culture medium were low, folate accumulation was dependent on folate availability and was not limited by FPGS activity. At high concentrations of folate, the mean length of polyglutamate chains was shorter and retention of folate was limited by the capacity of FPGS (Lowe et al. 1993). FPGS adds glutamate residues in a sequential manner (Cichowicz et al. 1981), so when folate availability is high there is increased competition of intracellular folates for the enzyme, resulting in a shorter average polyglutamate chain length. Polyglutamation also increases the affinity of folates for biosynthetic enzymes involved in one-carbon metabolism (Matthews et al. 1987). For example, kinetic studies of 5,10-methylenetetrahydrofolate reductase (MTHFR) isolated from pig liver

found that addition of one polyglutamate residue to the MTHFR substrate 5,10-methylene THF increased the Vmax for the reaction compared to the monoglutamate form. Addition of further glutamate residues further enhanced efficiency of the reaction by reducing the Km value (Matthews and Baugh 1980). The enzyme γ-glutamyl hydrolase (GGH) is a lysosomal exopeptidase which is capable of sequential removal of glutamate residues from folates in vitro (Yao et al. 1996). However, it is not known if this enzyme plays a role in regulating polyglutamation in vitro, as its activity in the cytoplasm has not been described.

The regulation of intracellular folate levels is mainly achieved through a homeostatic mechanism which limits folate accumulation. The main circulating form of folate, 5-methyl THF is a poor substrate for FPGS (Chen et al. 1996b), and must be converted to the preferred substrate THF before it can be retained within the cell. 5-methyl THF is demethylated by the enzyme methionine synthase (MS), which converts 5-methyl THF and homocysteine (Hcy) to methionine and THF. Polyglutamated 5-methyl THF has a much higher affinity for MS than the monoglutamate form (Coward et al. 1975), with the result that sufficient cellular levels of polyglutamated 5-methyl THF will prevent demethylation and therefore intracellular retention of additional 5-methyl THF monoglutamate. MS also requires vitamin B12 (cobalamin) as a cofactor (Goulding et al. 1997), and vitamin B12 deficiency can result in low MS activity and consequent trapping of cellular folates in 5-methyl THF form (Shane and Stokstad 1985). Excess 5-methyl THF monoglutamate is transported out of the cell. High folate intake increases plasma folate levels, but this does not necessarily translate to an increase in intracellular folates if tissues are already saturated. Clifford et al. (1990) measured the folate content in serum and body tissues of groups of rats which were fed on diets containing increasing amounts of folic acid over a 25-day period. Tissue folate levels were saturable at high folate intakes, while serum folate reached much higher levels than any of the tissues tested (Clifford et al. 1990).

1.3 Turnover, catabolism, and excretion of folate

Studies of whole body folate turnover indicate that folate within the body can be modelled as separate pools, characterised by rapid or slow turnover (Gregory III and Quinlivan 2002). The rapid folate pool has a half-life of approximately 31h (Krumdieck et al. 1978), and is likely to reflect folate which is absorbed or metabolised into tissue folate pools. There is a second pool of folate with a long half-life of 100 days or more (Krumdieck et al. 1978; Gregory and Scott 1996), which represents folate incorporated into cellular metabolic pools. A subsequent study of the kinetics of human folate metabolism using radiolabelled folate estimated a mean residence time of 119 days for folate incorporated into body tissues (Lin et al. 2004), in close agreement with the

earlier studies mentioned above. In cases of normal dietary intake, very little folate is excreted. Folate which is released into the bile is reabsorbed, and although free folate is filtered by the kidney most is reabsorbed from the glomerular filtrate in the proximal renal tubule. Approximately 2-5μg of folate per day is excreted in the urine, but in cases of high intake this level increases (Shinton 1972). Folate catabolism also occurs in vivo, whereby folates are cleaved at the carbon-9 to nitrogen-10 bond producing pterin and para-aminobenzoglutamate (pABG) (Kownacki-Brown et al. 1993). pABG is subsequently acetylated by arylamine N-acetyltransferase (Minchin 1995) forming acetyl-pABG, which was found to be the main excreted folate catabolite in humans (Caudill et al. 1998) and in rat (McNulty et al. 1993). Geoghegan et al. developed a HPLC method which could detect both intact folates and folate catabolites in rat urine, and determined that free pABG in urine is due to nonspecific deacetylation of acetyl-pABG rather than incomplete acetylation in vivo (Geoghegan et al. 1995).

1.4 Folate transport proteins

The folate molecule is water-soluble, and therefore does not readily cross biological membranes. A number of folate transport proteins exist to move folate into and out of cells and intracellular compartments. These have different mechanisms and optimum conditions for folate transport, and the expression of each transport protein varies by cell type. Uptake of folate into a particular tissue depends on the type of folate transporters expressed and the plasma folate concentration.

The reduced folate carrier (RFC) is encoded by the SLC19A1 gene on chromosome 21q22.3 (Moscow et al. 1995). It is a member of the SLC superfamily of solute carriers. RFC is an integral membrane protein with 12 trans-membrane domains (Liu and Matherly 2002). It transports 5-methyl THF and 5-formyl THF with a Km in the range of 2-7 μM, at a pH optimum of 7.4. The affinity for folic acid is much lower, in the range of 150-200 μM (Zhao et al. 2009a). The transport of folate by the RFC is coupled to transport of organic phosphates in the opposite direction (Sierra et al. 1995). The concentration of organic phosphates is higher within cells than in the plasma, and this transport down a concentration gradient provides energy for folate transport. The RFC is ubiquitously expressed, and due to its pH optimum is the main route of folate absorption into systemic tissues (Whetstine et al. 2002). Knockout of the RFC gene in mice is embryonic lethal (Zhao et al. 2001). A small number of mice homozygous for the knockout of RFC could be brought to term by injecting the mother with folic acid, but subsequently died within 12 days of birth. This study demonstrates the importance of the RFC to normal physiological function.

The study of a rare hereditary defect in folate absorption led to the identification of the proton-coupled folate transporter (PCFT). The PCFT is another member of the SLC superfamily, encoded

by the SLC64A1 gene on chromosome 17q 11.2 (Qiu et al. 2006). In contrast to the RFC, the pH optimum for folate transport by the PCFT is pH 5.5 and at neutral pH the transport of folate is negligible (Nakai et al. 2007). The Km of the human PCFT for folic acid is 1.3μM at pH 5.5 (Qiu et al. 2006). Studies of rodent PCFT proteins have shown that the Km values for folic acid and 5-methyl THF are similar (Qiu et al. 2007). The PCFT is a folate-proton symporter, with a net transfer of positive charge accompanying folate transport. The initial study which characterised the PCFT also showed that folate transport was dependent on the presence of a transmembrane proton gradient (Qiu et al. 2006). The PCFT is expressed in the small intestine, kidney, liver, placenta, and to a lesser degree retina and brain (Qiu et al. 2006; Qiu et al. 2007).

The major site of folate absorption in the body is the duodenum and proximal jejunum. The pH in this region of the gut is acidic, and therefore favours folate absorption by the PCFT because of its acidic pH optimum (Nakai et al. 2007). Both the RFC (Wang et al. 2001) and PCFT are expressed in this region, and until the identification of the PCFT, folate absorption from the gut was attributed to the RFC. Experiments carried out in rat had demonstrated that folate uptake from the gut occurred at both acidic and neutral pH, by a saturable mechanism which exhibited different Km at pH 5.5 and pH 7.4 (Zimmerman et al. 1989), but it was not clear if this was due to pH dependent alterations in affinity of the transport mechanism for folate, or involved two separate carriers. Later work which studied uptake of folate in intestinal epithelial cells in which RFC function had been disrupted showed that deletion of the gene prevented transport of folate at neutral pH, but folate transport was still observed at acidic pH (Wang et al. 2005). The identification of the PCFT provides an explanation for these observations, and a more complete understanding of the route of folate absorption from the gut. The PCFT is now regarded as the main route for folate transport from the gut into enterocytes, and the primary function of the RFC as the route of uptake of folate from the bloodstream into systemic cells (Zhao et al. 2009a).

Folates can also be transported by low affinity membrane carrier proteins which are capable of transporting a variety of organic anions. These include proteins belonging to the multidrug resistance-associated protein (MRP) family, SLC2 family, and breast cancer resistance protein (BRCP). The MRP transporters have Km for folic acid and reduced folates in the milimolar range, and can serve to move folate out of tissues, from areas of high to low folate concentration (Kruh and Belinsky 2003). MRP transporters can only transport monoglutamated folates (Zeng et al. 2001). MRP2 has been shown to mediate biliary excretion of the folate analogue methotrexate in rats (Masuda et al. 1997), and since these proteins can also transport folates, it is possible that they could be involved in export of folate into the bile. The PCFT has been shown to preferentially localise to the apical membrane of intestinal epithelial cells (Subramanian et al. 2008) and renal

epithelial cells (Nakai et al. 2007). MRP proteins are present on basolateral membranes in several tissues (Kruh and Belinsky 2003), and could potentially provide a route of transport for folate monoglutamates into the circulation. The SLC2 proteins OAT-K1 and OAT-K2 are expressed in the apical membrane of renal epithelial cells (Motohashi et al. 2002), and could therefore be involved in uptake of folate from the glomerular filtrate.

There are four types of folate receptors (FR), designated FRα, FRβ, FRγ and FRδ. Each receptor is encoded by a separate gene found within a 140 kilobase region of chromosome 11, region 11q13.3-q13.5 (Ragoussis et al. 1992). The FRs are glycophosphoinositol-anchored membrane proteins, and are high affinity folate binding proteins with Kd in the region of 1-10 nM (Zhao et al. 2009a). All the folate receptors bind folic acid with Kd in the nanomolar range (Elnakat and Ratnam 2004). FRα binds the circulating form of folate, 5-methyl THF with a similar affinity to folic acid, but the affinity of FRβ for 5-methyl THF is approximately 50-fold lower (Wang et al. 1992). The folate receptors can facilitate folate uptake into cells by receptor mediated endocytosis, a process which had been recognised before the identification of the FR genes (Kamen et al. 1988). Early studies showed that receptor-bound folate is dissociated from the FR by acidification of the resulting endosome, and subsequently exported into the cytosol by a carrier mediated process, but at this stage the PCFT had not yet been identified (Kamen et al. 1991). A recent study used HeLa cells which expressed FRα and had either normal expression of PCFT (PCFT +/+) or were homozygous negative for PCFT (PCFT -/-). It was found that PCFT -/- cells could take up folate by FRα-mediated endocytosis, but folate remained within the endosome. In contrast, folate taken up by PCFT +/+ cells was transported into the cytoplasm (Zhao et al. 2009b). This evidence demonstrates that the PCFT has a role in folate uptake in addition to its function in environments where extracellular pH is low.

In normal tissues the different FRs are expressed in a highly tissue-specific manner, and are also located to particular subcellular areas. FRα is expressed at the apical membrane of epithelial cells in the kidney proximal tubule, choroid plexus, retina, uterus and placenta (Weitman et al. 1992b), and the basolateral membrane of retinal epithelial cells (Chancy et al. 2000). FRβ is expressed during normal haematopoesis in cells of neutrophil lineage(Ross et al. 1999), and also in placenta (Ratnam et al. 1989). FRα appears to be particularly important in embryonic development, as FRα homozygous knockout mice are embryonic lethal with a phenotype involving failure of neural tube closure (Piedrahita et al. 1999). The same study found that mice lacking FRβ developed normally. Little is known about the biological function of FRγ. The cDNA for FRγ was first isolated from spleen and bone marrow cells from leukemic patients (Shen et al. 1994), and FRγ was later shown to be a secreted protein due to lack of a GPI anchor (Shen et al. 1995). The existence of

FRδ was originally predicted based on gene sequence (Spiegelstein et al. 2000), but the first report of the expression of the protein was not until 2007 when it was identified in regulatory T (Treg) cells by Yamaguchi et al. (Yamaguchi et al. 2007). This study found that expression of FRδ was higher in Treg cells than in other T-cell types, and antibody staining for FRδ could be used to distinguish these from other T-cell populations. FRδ was found to be functionally relevant in Treg cells because blockade of FRδ caused a reduction in the levels of Treg cells in mice.

FRs are also expressed in many types of tumour tissue, including ovary, kidney, endometrium, lung, breast, bladder, pancreas (Parker et al. 2005), and leukemic cells (Shen et al. 1994), at a considerably higher level than in healthy tissue. Antibodies to FRα have been found in serum from patients with breast and ovarian cancer, suggesting that an immune response to tumour cells expressing FRα can occur (Knutson et al. 2006). Expression of folate receptors by tumour cells may provide a useful mechanism for specific direction of chemotherapy, and research into the possibility of using FRs to target folate-conjugated drugs to cancer cells is ongoing (Zhao et al. 2008).

1.5 Reactions of one-carbon metabolism

The overall process of OCM can be envisaged as a flow of one carbon units obtained from catabolism of substrate molecules, first to form substituted THF species, and then to their final use in biosynthetic reactions. THF thus acts as a carrier allowing one carbon units to be converted between different oxidation states in the intermediate steps of this process. There are two major fates of one-carbon units involved in OCM; biosynthesis of DNA precursors (purines and thymidine monophosphate), and formation of the essential amino acid methionine and S-adenosyl methionine (SAM), the primary methyl donor for transmethylation reactions within cells. Methylation steps are involved in synthesis of a variety of biomolecules, two examples include creatinine (synthesised in the liver) and the phospholipid phosphatidylcholine. SAM is also a methyl donor for transmethylation reactions such as methylation of DNA, RNA, proteins, and other small molecules (Christman 2003). A schematic diagram of the biosynthetic reactions of OCM is presented in figure 1.2.

As discussed above, the folate molecule itself is obtained from the diet, and enters the cell as 5-methyl-THF. 5-methyl THF is not a good substrate for FPGS (Chen et al. 1996b), so in order for polyglutamation and intracellular retention to occur, it must first be demethylated. This occurs by the action of MS, which transfers the methyl group from 5-methyl THF to homocysteine, producing methionine and free THF. This is the route by which THF enters the cellular folate pool, and it is subsequently polyglutamated and converted to a variety of different substituted forms.

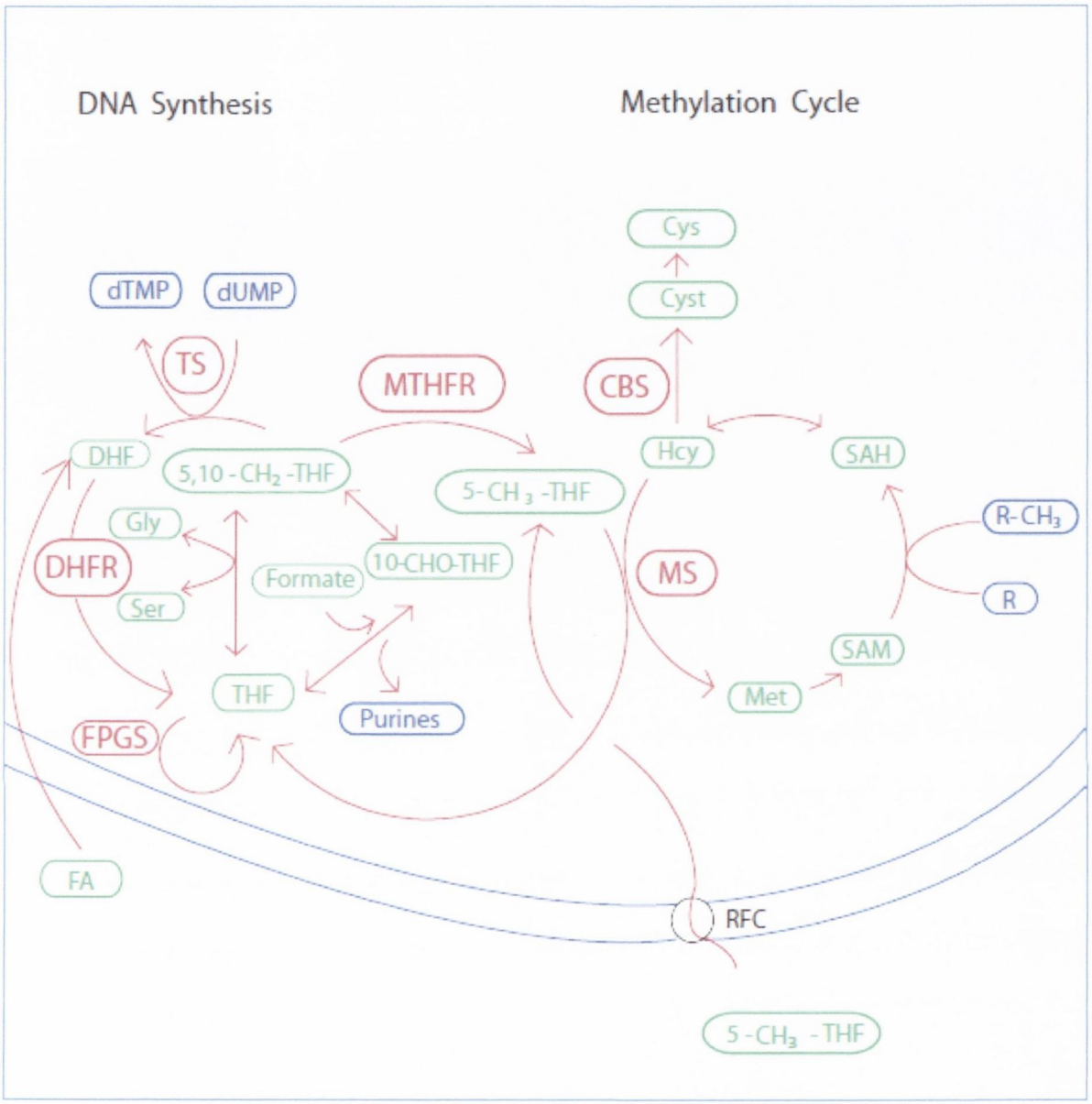

Figure 1.2: Schematic diagram of the reactions of one-carbon metabolism

5,10-CH2-THF 5,10-methylene THF; **5-CH3-THF**, 5-methyl THF; **10-CHO-THF**, 10-formyl THF; **THF**, unsubstitued THF; **Cyst**, cystathionine; **Cys**, cysteine; **Hcy**, homocysteine; **SAH**, S-adenosyl homocysteine; **SAM**, S-adenosyl methionine; **Met**, methionine, **Ser**, serine; **Gly**, glycine; **FA**, folic acid; **RFC**, reduced folate carrier; **R**, unmethylated substrate; **R-CH3**, methylated product; **dUMP**, deoxyuridine monophosphate; **dTMP**, deoxythymidine monophosphate; **TS**, thymidilate synthase; **DHFR**, dihydrofolate reductase; **FPGS**, folate polyglutamate synthase; **MTHFR**, 5,10-methylene THF reductase; **MS**, methionine synthase; **CBS**, cystathionine β synthase.

The one carbon units which form the substituted groups on the THF backbone originate from catabolism of serine, glycine, formate, histidine, sarcosine and dimethylglycine.

1.5.1 Subcellular compartmentalisation of one-carbon metabolism

Cellular folates are evenly distributed between the cytosol and mitochondria (Cook and Blair 1979), and approximately 10% of cellular folate is found in the nucleus (Shin et al. 1976). The nature of the one carbon substitutes differs between cytosol and mitochondria, with methyl-THF predominating in the cytosol and formyl folates the most prevalent form in the mitochondria (Carl et al. 1995). The reactions of one-carbon metabolism occur in different contexts in the different compartments of the cell (Appling 1991). A diagram of subcellular folate compartmentalisation is provided in figure 1.3. In the cytosol, one-carbon substituted folates are used for purine and thymidylate (dTMP) synthesis, and for production of the methyl donor SAM. To a large extent, the addition of these one-carbon units to THF occurs in the mitochondria. Transport of reduced folate from the cytosol into mitochondria occurs by a carrier mediated process, through the mitochondrial folate transporter (MFT) (Titus and Moran 2000). The exact form of folate carried by this transporter is not known, but it is thought to be monoglutamated THF (Tibbetts and Appling 2010). Folates are polyglutamated by a mitochondrial form of FPGS. Mitochondrial folates tend to have longer polyglutamate chains than those in the cytoplasm, promoting retention of folate cofactors within this compartment (Lin et al. 1993). Polyglutamated folates cannot be transported across the mitochondrial membrane, so effectively cellular folates are split into a cytosolic and a mitochondrial folate pool. It is the flux of the one-carbon donors serine, glycine, and formate which links cytoplasmic and mitochondrial one-carbon metabolism. Transporters for these one-carbon donors have not yet been molecularly characterised (Tibbetts and Appling 2010).

Within the mitochondria, one carbon units can be added to THF from either serine, glycine or to a lesser extent sarcosine or dimethylglycine. Serine hydroxymethyltransferase (SHMT) catalyses the addition of the β-carbon of serine to THF, forming glycine and 5,10-methylene THF. The major site of this reaction is in the mitochondria, but it also occurs in the cytoplasm. There are two forms of SHMT which are encoded by separate genes. The gene *SHMT1* produces a cytoplasmic form of the enzyme (cSHMT) and the *SHMT2* gene produces a mitochondrial form (mSHMT). The two SHMT proteins are similar in structure, sharing 63% amino acid sequence identity (Garrow et al. 1993). Analysis of a range of human tissue samples by northern blot and quantification of mRNA levels showed that *SHMT1* is expressed in a tissue specific manner, with high levels in liver, kidney and skeletal muscle, in contrast to *SHMT2* which is expressed to a similar level in all tissues (Girgis et al. 1998). Recently it has been shown that *SHMT2* encodes two alternate transcripts,

one of which is localised to the mitochondria and another which lacks exon 1 and is localised to the cytoplasm and nucleus (Anderson and Stover 2009). This demonstrates that SHMT activity is present in both cytoplasm and mitochondria of all tissue types, the mitochondrial SHMT activity encoded by *SHMT2*, and cytoplasmic SHMT activity encoded by either *SHMT1* or the alternate transcript from *SHMT2*.

One carbon groups are obtained from glycine by the glycine cleavage system (GCS), which is exclusively located to the inner mitochondrial membrane in mammalian cells (Yoshida and Kikuchi 1970). The location of this enzyme system is tissue specific, and it has been detected in liver, kidney and brain in vertebrates (Kikuchi et al. 2008). The GCS consists of a multienzyme complex which oxidises glycine to form CO_2, ammonia, and 5,10-methylene THF. The enzyme activities involved are the P-protein which catalyses decarboxylation of glycine, the H-protein which is a hydrogen carrier for the reaction, the T-protein which catalyses transfer of the one carbon unit to THF and the L-protein, a dehydrogenase which regenerates the active form of the H-protein (Kikuchi et al. 2008). The GCS is a major pathway of glycine catabolism, and defects in this enzyme system cause congenital hyperglycinemia (Koyata and Hiraga 1991). The 5,10-methylene THF which is derived from the GCS is a significant source of one carbon units for purine and dTMP synthesis.

Sarcosine and dimethylglycine are intermediates in the pathway of oxidative choline catabolism, which occurs primarily in the mitochondria of liver cells (Lewis et al. 1978). 5,10-methylene THF is formed by transfer of one carbon units from sarcosine by sarcosine dehydrogenase, or from dimethylglycine by dimethylglycine dehydrogenase. These enzymes were first purified from rat liver (Frisell and Mackenzie 1962), and were later identified to be folate-binding proteins and flavin-dependent enzymes (Wittwer and Wagner 1981a).

The reactions of OCM which introduce one-carbon units into the system largely occur in the mitochondria by the processes discussed above. The oxidative environment in the mitochondria preferentially directs interconversion of one-carbon substituted folates towards the formation of formate. Formate generated in the mitochondria becomes the main source of one-carbon units for cytoplasmic OCM. This was first demonstrated in vitro by Barlowe and Appling, who showed that formate was produced from radiolabelled serine by isolated rat liver mitochondria (Barlowe and Appling 1988). The radiolabelled formate produced was released from the mitochondria, and the radiolabel was subsequently detected in purines which were synthesised in the cytoplasm. Formate is introduced into cytoplasmic OCM by the enzyme 10-formyl THF synthase (FTHFS), which catalyses the ATP-dependent conversion of formate and THF to 10-formyl THF.

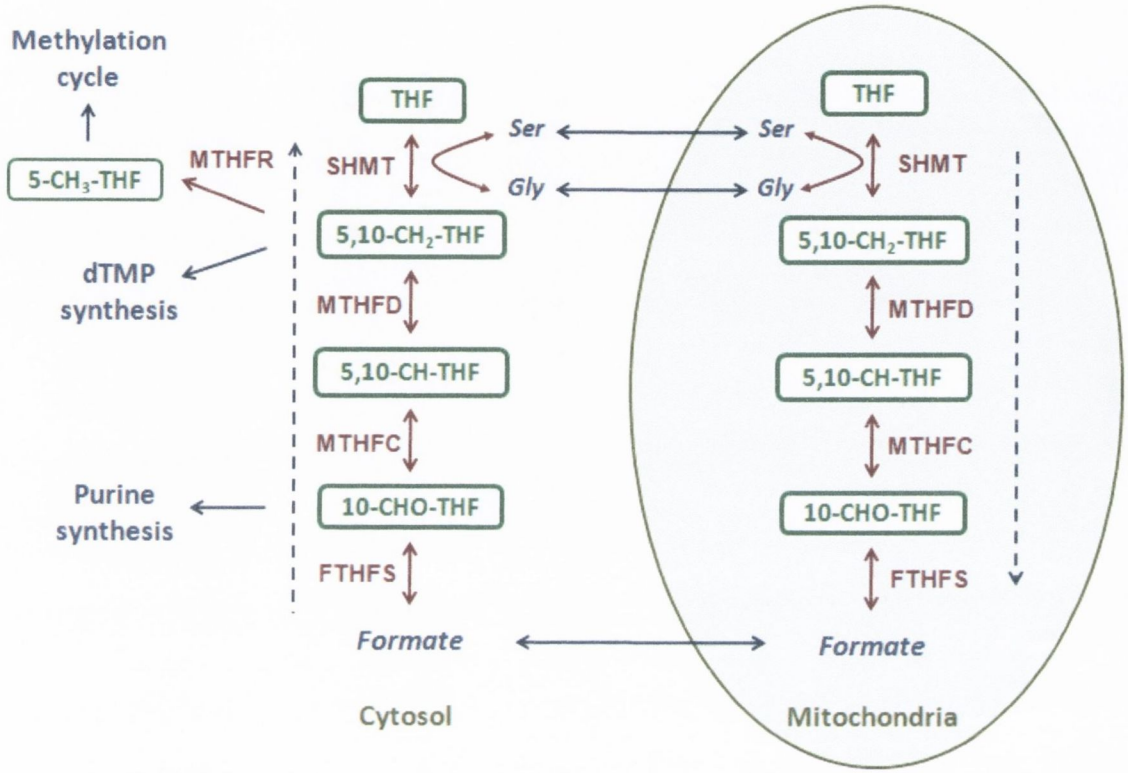

Figure 1.3 Compartmentalisation of one carbon metabolism

THF cofactors are indicated in green boxes, one-carbon donors are indicated in blue italic, and enzymes are indicated in red. The reactions catalysed by SHMT, MTHFD, MTHFC and FTHFS are reversible, the dashed arrows indicate the predominant direction under physiological conditions. **Ser**, serine; **Gly**, glycine; **THF**, tetrahydrofolate; **5,10-CH_2-THF**, 5,10-methylene THF; **5,10-CH-THF**, 5,10-methenyl THF; **10-CHO-THF**, 10-formyl THF; **5-CH_3-THF**, 5-methyl THF; **SHMT**, serine hydroxymethyltransferase; **MTHFD**, methylene THF dehydrogenase; **MTHFC**, methenyl THF cyclohydrolase; **FTHFS**, formyl THF synthase; **MTHFR**, methylene THF reductase.

Experiments using radiolabelled serine in MCF-7 cells showed that incorporation of the labelled serine into methionine and dTMP was lower than would be expected if the majority of one carbon units were obtained from cytoplasmic SHMT (Herbig et al. 2002). This indicates that mitochondrial-derived formate is the major source of one-carbon units for cytoplasmic OCM. There are also two other pathways by which one-carbon units are transferred to THF in the cytoplasm. The first is the formation of 5,10-methylene THF from serine catalysed by cSHMT, as mentioned above. The second is through catabolism of histidine and the purines adenine and guanine. During this process, the imidazole ring of these molecules is transferred to THF as a formimino group, producing 5-formimino THF. This conversion is catalysed by the bifunctional enzymes glutamate formiminotransferase/cyclodeaminase and glycine formiminotransferase/cyclodeaminase. The formiminotransferase domain of the enzyme produces 5-formimino THF which is then converted to 5,10-methenyl THF by the cyclodeaminase activity (Mackenzie and Baugh 1980).

The transfer of a one carbon unit to THF is referred to as 'activation' of the one carbon unit, because it can subsequently be used in biosynthetic reactions. The one-carbon substituents of folate cofactors can be interconverted between different levels of oxidation, in both the cytosol and mitochondria. 10-formyl THF is converted to 5,10-methenyl THF by methenyl THF cyclohydrolase (MTHFC). 5,10-methenyl THF is reduced to 5,10-methylene THF by methylenetetrahydrofolate dehydrogenase (MTHFD). The *MTHFD1* gene encodes a trifunctional enzyme containing FTHFS, MTHFC, and MTHFD activities (Hum et al. 1988). The three catalytic activities are carried out by a single protein which forms a homodimer of 100kDa subunits. Each subunit consists of two peptide domains. The methylene THF dehydrogenase/cyclohydrolase activities are located in the 30kDa N-terminal domain, and the 10-formyl THF synthetase is located in the 70 kDa C-terminal domain. There is also a mitochondrial equivalent of the trifunctional enzyme, which in fact consists of two separate gene products. *MTHFD1L* encodes a monofunctional 10-formyl-THF synthetase (Walkup and Appling 2005), and *MTHFD2L* encodes a bifunctional methylene-THF dehydrogenase/cyclohydrolase (Bolusani et al. 2011). These interconversion reactions are reversible, but the formate-producing direction is metabolically favoured in mitochondria, while the reductive direction is favoured in the cytoplasm (Tibbetts and Appling 2010).

As discussed above, production of one-carbon substituted folates in the mitochondria is a major route by which one-carbon units are provided for cellular metabolism. Mitochondrial OCM is also an essential source of glycine, as demonstrated by Chinese hamster ovary (CHO) cells with defects in mitochondrial SHMT or the mitochondrial folate transporter, which require exogenous glycine

for survival (Stover et al. 1997; Titus and Moran 2000). Folate cofactors are also required for biosynthesis within the mitochondria. One function of folate which is exclusive to mitochondria is the use of 10-formyl-THF for production of formylated tRNA, catalysed by the mitochondrial enzyme methionyl-tRNA formyltransferase (Spencer et al. 2004). This is necessary as formylated tRNA is used in the initiation step of mitochondrial protein synthesis. The human MTHFR enzyme is located in the cytoplasm, and mitochondria also cannot form SAM (Sheid and Bilik 1968). SAM is required as a source of methyl groups for mitochondrial synthesis of DNA, RNA and proteins. In order to supply methyl groups for mitochondrial reactions, SAM is transported from the cytosol into the mitochondria by the human mitochondrial SAM carrier, (SAMC) (Agrimi et al. 2004). It is thought that this transport mechanism facilitates exchange of SAM for S-adenosyl homocysteine (SAH), allowing removal of the latter compound from the mitochondria.

1.5.2 Regulation of one-carbon metabolism

The concentration of folate binding proteins in the cell is estimated to exceed the concentration of folate (Strong et al. 1990), and therefore it appears that folate does not exist freely within the cell but is largely protein bound. Many cellular proteins can bind reduced folate cofactors with high affinity, although this binding does not form part of any reaction mechanism. Non-substrate folate cofactors can also bind to enzymes which are involved in OCM, thus regulating the activity of these enzymes. The fact that folate can bind to many enzymes serves to regulate the intracellular flux of folate cofactors, and also as a mechanism to sequester folate cofactors within the cell. Some enzymes are regarded as folate binding proteins, because they are highly abundant proteins which can bind reduced folates with high affinity but do not catalyse one carbon transfer. An example of such a protein is glycine N-methyltransferase (GNMT). The catalytic function of GNMT is transmethylation of glycine to sarcosine, using SAM as a methyl donor. GNMT binds and is inhibited by 5-methyl THF, and in this context acts as a folate binding protein (Wagner et al. 1985). Other highly abundant enzymes which bind 5-methyl THF in this way are sarcosine dehydrogenase, dimethylglycine dehydrogenase (Wittwer and Wagner 1981b), and SHMT (Stover and Schirch 1991).

Several of the enzymes involved in folate metabolism are multifunctional proteins or exist in multienzyme complexes. Studies of the reaction mechanisms of these proteins have revealed that in many cases the folate cofactor is 'channelled' between different active sites rather than being released from the protein between each reaction step (Appling 1991). An example of such a mechanism is the reactions catalysed by MTHFD/MTHFC which exist as part of a trifunctional enzyme. Studies of the reaction mechanism of this enzyme show that when 5,10-methylene THF is used as a substrate, the product of the MTHFD reaction is not released from the enzyme after

this step, but is converted directly to 10-formyl THF. This channelling effect was more prevalent when a polyglutamated substrate was used in contrast to the monoglutamate form (Wasserman et al. 1983).

1.5.3 Folate in the synthesis of DNA precursors

Folates are important in the biosynthesis of DNA precursors, because the synthesis of dTMP and de novo synthesis of purines both involve folate dependent steps. The only role for dTMP is its incorporation into DNA, but the purines ATP and GTP are also active as coenzymes and regulatory factors. As well as de novo synthesis, purines can also be generated by a salvage pathway which regenerates the purine inosine monophosphate (IMP) from hypoxanthine, a product of purine catabolism.

1.5.3.1 Synthesis of dTMP

Thymidylate synthase (TS) is the enzyme required for synthesis of dTMP. This reaction involves addition of a methyl group to carbon-5 of deoxyuridine monophosphate (dUMP), using 5, 10-methylene-THF as a one-carbon donor. This reaction is unusual in that the folate cofactor is oxidized to dihydrofolate (DHF), because in this case two of the hydrogens from THF are used as reducing equivalents to reduce the one-carbon unit from 5,10-methylene-THF to a methyl group. DHF must be reduced to THF by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent action of dihydrofolate reductase (DHFR) before it can re-enter the cellular THF pool. DHFR isolated from cultured human cells is a monomer of approximately 22 kDa, with Km for DHF measured in the range 0.036-2.3μM (Blakely 1984).

The reduced folate cofactor 5,10-methylene THF which is used in dTMP synthesis can be derived from two sources; from 10-formyl THF via the MTHFD/MTHFC, or from serine via the action of cSHMT. Experiments using radiolabelled serine and formate have demonstrated that both of these routes provide one-carbon units for dTMP synthesis, but that one-carbon units derived from cSHMT are preferentially directed to dTMP synthesis over methionine synthesis (Herbig et al. 2002; Anguera et al. 2006). Increased expression of cSHMT is found to promote dTMP synthesis, possibly by increasing the availability of 5,10-methylene THF (Oppenheim et al. 2001).

TS protein levels change in a cell cycle dependent manner, with increased levels of enzyme during times of active DNA replication and cell growth (Pestalozzi et al. 1995). Activity of TS and DHFR were found to increase in the nucleus of proliferating cells, and it has been suggested that these enzymes form part of a 'replitase' enzyme complex located in the nucleus only during DNA synthesis (Prem veer Reddy and Pardee 1980). Recent work by Stover's group has demonstrated

that the three reactions of one-carbon metabolism which are required for de novo dTMP synthesis (TS, DHFR, and SHMT) also occur within the nucleus. cSHMT, TS and DHFR undergo post-translational modification by addition of a small ubiquitin-like modifier (SUMO) group (Anderson et al. 2007; Woeller et al. 2007). Modification by SUMO promotes nuclear localisation of proteins, and cSHMT, DHFR and TS were found to localise to the nucleus during S and G2/M phases of the cell cycle (Woeller et al. 2007). Intact nuclei Isolated from mouse liver were shown to produce dTMP, and this was blocked by an inhibitor of SHMT (Anderson and Stover 2009). Nuclear dTMP synthesis occurred in mice lacking expression of *SHMT1*, demonstrating that the cytoplasmic transcript of *SHMT2* can also perform this function in the nucleus (Anderson and Stover 2009). This research indicates that the complete de novo dTMP synthesis pathway occurs within the nucleus, and it is suggested that this mechanism may account for preferential incorporation of cSHMT-derived one carbon units into dTMP. Levels of cSHMT protein are increased in response to UV light, in a mechanism which involves increased translation of cSHMT mRNA and SUMOylation promoting nuclear import (Fox et al. 2009). This suggests that cSHMT and nuclear dTMP synthesis is also involved in the DNA repair response.

Recent evidence indicates that the de novo synthesis of dTMP also occurs within the mitochondrion. Isolated mitochondria from CHO and human HepG2 cells were capable of synthesising ^{3}H-dTMP when supplied with unlabelled dUMP, NADPH and [2,3 ^{3}H]-serine (Anderson et al. 2011). The localisation of SHMT2 and TS to mitochondria had previously been described (Samsonoff et al. 1997; Anderson and Stover 2009), but this study went on to identify for the first time a mitochondrial isoenzyme with DHFR activity, encoded by the *DHFRL1* gene on chromosome 3q11.2, which was previously thought to be a pseudogene (Anderson et al. 2011). Green fluorescent protein-tagged DHFRL1 was observed to localise exclusively to the mitochondria in HeLa cells (Anderson et al. 2011). McEntee et al. (2011) reported that transcripts of *DHFRL1* were present in a variety of human cell lines, indicating that the gene is widely expressed. Comparison of purified recombinant DHFRL1 and DHFR indicated that the mitochondrial form of the enzyme had a lower Km for DHF compared to DHFR (McEntee et al. 2011). The de novo dTMP synthesis pathway is thus demonstrated to occur within the nucleus and the mitochondrion, the two cellular compartments where DNA is synthesised.

1.5.3.2 Synthesis of purines

De novo purine synthesis is an 11-step process beginning with ribose-5-phosphate and ending with production of IMP. The purine nucleotides ATP and GTP are subsequently synthesized from IMP in separate two-step processes (Garrett and Grisham 1999). The folate cofactor 10-formyl

THF is used in two steps of IMP synthesis. Step three involves the conversion of glycinamide ribonucleotide (GAR) to formyl-GAR by the enzyme GAR formyltransferase (GARFT). The ninth step is the conversion of amino-imidazolecarboxamide ribosyl-5-phosphate (AICAR) to formyl-AICAR by AICAR formyltransferase (AICARFT). In these metabolic reactions the one carbon unit from 10-formyl THF is used to form the carbon-2 and carbon-8 positions of the purine ring. THF is produced by both of these reactions, and is subsequently reabsorbed into the cellular THF pool where it can be converted to another substituted form. Several of the enzymes involved in de novo purine synthesis are multifunctional enzymes, and all the enzymes required for purine synthesis have been shown to form a 'purineosome' complex in the cytoplasm of HeLa cells, as assessed by immunofluorescence staining and tagging of the enzymes with fluorescent proteins (An et al. 2008). Figure 1.4 depicts the purine synthesis and salvage pathways.

The main source of 10-formyl THF for purine synthesis is from formate synthesised in the mitochondria. As mentioned above, the enzyme which catalyses this reaction is FTHFS and forms part of a trifunctional enzyme encoded by the *MTHFD1* gene, which is ubiquitously expressed (Smith et al. 1990). Experiments using radiolabelled serine have demonstrated that one-carbon units derived from cSHMT do not make a significant contribution to purine synthesis due to the reductive environment of the cytoplasm, which does not favour synthesis of 10-formyl THF from this source (Herbig et al. 2002). Patel et al. created mouse fibroblast cells with a deletion of mitochondrial MTHFC/MTHFD activity. These cells were glycine auxotrophs, and could not produce formate by mitochondrial OCM. Radiolabelled formate was supplied to these cells, and the cells with the deletion were found to incorporate far more radiolabelled formate into DNA than wild-type cells. The mutant cells incorporated radiolabelled formate into purines but relatively little into dTMP (Patel et al. 2003). This indicates that formate is used preferentially for purine synthesis in particular.

5-formyl THF is a folate cofactor which can be formed within the cell, but is not used as a one-carbon donor in biosynthetic reactions. As well as its role in interconversion of serine and glycine, SHMT can catalyse formation of 5-formyl THF from 5,10-methenyl THF (Stover and Schirch 1990). The enzymes cSHMT and 5,10-methenyl THF synthase (MTHFS) form a futile cycle whereby 5-formyl THF is formed by cSHMT, then reconverted to 5,10-methenyl THF by MTHFS (Stover et al. 1993). MTHFS is ubiquitously expressed, and is the only enzyme which uses 5-formyl THF as a substrate. 5-formyl THF polyglutamate acts as a tight binding inhibitor of cSHMT (Stover and Schirch 1991), and 10-formyl THF is an inhibitor of MTHFS, with Ki in the nanomolar range (Field et al. 2006). These facts provide evidence for regulation of 5-formyl THF levels by feedback inhibition of cSHMT and MTHFS. It is thought that 5-formyl THF may act as a storage form of

activated one-carbon units at the formyl level, and may channel one carbon units to de novo purine synthesis. In neuroblastoma cells, 5-formyl THF was found to accumulate only in the presence of 10-formyl THF, indicating that it may be a storage form of reduced THF at the formyl level (Field et al. 2006). Increased expression of MTHFS in a neuroblastoma cell line promoted resistance to a GARFT inhibitor, but were not resistant to inhibition of TS. This suggests that MTHFS promotes de novo purine synthesis (Field et al. 2009).

Cellular supplies of 10-formyl THF can be depleted by the enzyme 10-formyl THF dehydrogenase (FDH), which catalyses the irreversible, $NADP^+$-dependent conversion of 10-formyl THF to THF and CO_2 (Min et al. 1988). The functional role of this enzyme is not precisely understood, but it is thought to regulate the supply of one carbon units available for OCM. The cytosolic form of FDH is encoded by the gene *ALDH1L1* and is expressed abundantly in the liver, and also in kidney and pancreas (Hong et al. 1999). *ALDH1L2,* a gene encoding a mitochondrial form of FDH has recently been identified, with expression in normal heart, pancreas, and brain tissue and in cancer cell lines (Krupenko et al. 2010). FDH binds THF polyglutamate with a Kd of 15nM (Kim et al. 1996), so it may sequester cellular folate in the form of THF. Mice without detectable FDH activity were found to have low levels of liver THF compared to control mice, but the viability of these mice demonstrates that the function of this enzyme is not essential (Champion et al. 1994). Recent work using neuroblastoma cells to investigate the role of FDH in OCM suggests that this protein does not alter de novo purine synthesis, but does deplete the supply of one carbon units to the methylation cycle (Anguera et al. 2006). This study found that ectopic expression of FDH in neuroblastoma cells depleted total cellular folate, casting doubt on the function of FDH in sequestration of cellular folate (Anguera et al. 2006).

1.5.4 The methylation cycle

The methylation cycle refers to the reactions of one carbon metabolism which are involved in synthesis of the essential amino acid methionine, precursor of the methyl donor SAM. These reactions also serve two other important functions; conversion of 5-methyl THF to THF allowing entry of THF to the cellular folate pool, and removal of homocysteine (Hcy) by its remethylation to methionine. Reduced folate cofactors from the cellular pool enter the methylation cycle through the enzyme MTHFR. This enzyme contains a covalently bound flavin adenine dinucleotide (FAD) cofactor, and catalyses the NADPH-dependent reduction of 5,10-methylene-THF to 5-methyl THF (Kutzbach and Stokstad 1971). The MTHFR reaction in this direction is irreversible under physiological conditions due to the high ratio of NADPH to NADP+ in the cytoplasm, and as such is an important point of regulation of intracellular folate cofactors. The only cellular enzyme reaction which uses 5-methyl THF as a cofactor is catalysed by MS, which converts Hcy and 5-

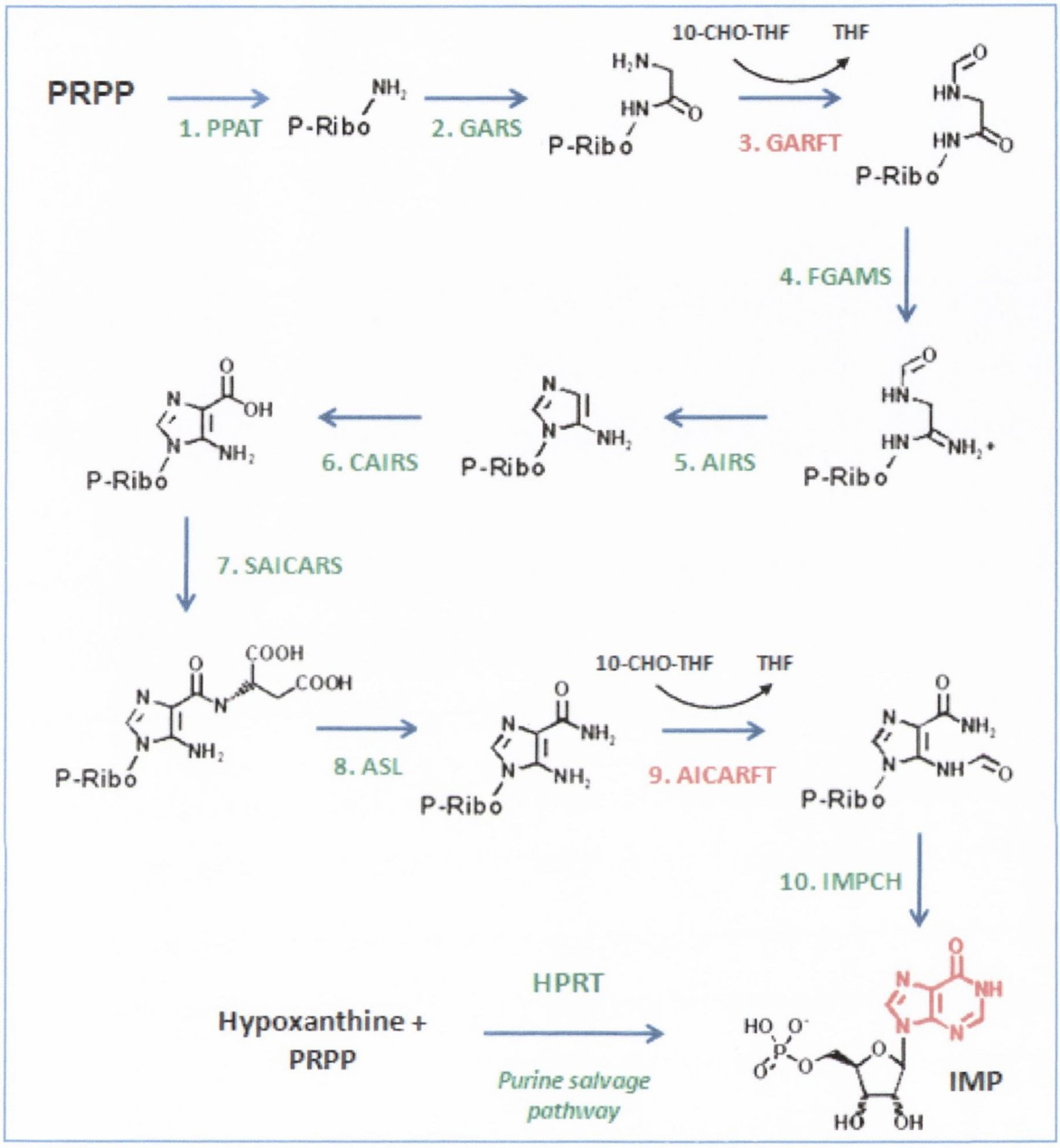

Figure 1.4 Schematic diagram of purine de novo synthesis and salvage pathways

The enzymatic reactions numbered 3 and 9 require folate in the form of 10-formyl THF (10-CHO-THF). PRPP, 5-phosphoribosyl-α-pyrophosphate; PPAT, PPRP amidotransferase; GARS, glycinamide ribonucleotide (GAR) synthase; GARFT, GAR formyltransferase; FGAMS, formyl GAR synthase; AIRS, 5-aminonimidazole ribonucleotide (AIR) synthase; CAIRS, carboxy AIR synthase; SAICARS, N-succinylo-5-aminoimidazole-4-carboxamide ribonucleotide synthase; ASL, adenylosuccinate lyase; AICARFT, 5-aminoimidazole-4-carboximide ribonucleotide formyltransferase; IMPCH, Inosine monophosphate cyclohydrolase; IMP, inosine monophosphate.

methyl THF to methionine and THF. Methionine is converted to SAM by methoinine adenosyltransferase (MAT), which transfers the adenosyl group of ATP to methionine (Mudd 1962). The methyl group of SAM can be transferred to a wide variety of substrates by a large variety of methyltransferase enzymes, resulting in production of S-adenosyl homocysteine (SAH). SAH is a product and an inhibitor of methyltransferase reactions (Ueland 1982), and is degraded to adenosine and Hcy by SAH hydrolase (SAHH) (de la Haba and Cantoni 1959). The favoured direction of the SAHH reaction is towards formation of SAH, so to keep cellular levels of Hcy and adenosine low these compounds are removed from the cell or metabolised. Adenosine is degraded by adenosine deaminase (ADA) or phosphorylated to adenosine monophosphate (AMP) by adenosine kinase (Ueland 1982). Hcy is removed either by remethylation to methionine or by degradation to cysteine via cystathionine-β synthase (CBS) (Stipanuk and Beck 1982). There is another pathway of Hcy remethylation via betaine-homocysteine methyltransferase (BHMT) which is expressed in liver and kidney (Sunden et al. 1997), whereas MTHFR is widely expressed (Blom and Smulders 2010).

Because MS is the only enzyme which uses 5-methyl THF as a cofactor, MTHFR represents a metabolic branch point where available folates are split between DNA synthesis and production of methyl group donors. The activity of MTHFR is regulated in several ways. The affinity of MTHFR for its substrate 5,10-methylene THF increases with increasing length of the polyglutamate chain, from a Km of 7.1μM for the monoglutamate form, to 0.1μM for the hexaglutamate form of THF (Matthews and Baugh 1980). MTHFR isolated from pig liver is inhibited by DHF with a Ki of 6μM, and this value decreases to 0.013μM for DHF hexaglutamate (Matthews and Haywood 1979; Matthews and Daubner 1982). DHF is a product of dTMP synthesis, so it is thought that inhibition of MTHFR activity by DHF could act as a mechanism to direct one-carbon units to biosynthesis of DNA precursors during times of increased synthesis. MTHFR is allosterically inhibited by SAM, where binding of SAM retains the enzyme in an inactive state (Jencks and Mathews 1987). SAM and SAH compete for the same binding site of MTHFR, and therefore the activity of the enzyme is regulated by the cellular SAM/SAH ratio (Matthews and Daubner 1982). When SAM is predominant MTHFR activity is inhibited, and 5-methyl THF is not produced. When SAH is abundant the enzyme is active, increasing the production of 5-methyl THF and hence SAM, restoring the SAM/SAH ratio which is regarded as representing the methylation capacity of the cell. SAM is normally present at a concentration 5-10 times that of SAH (Hoffman et al. 1979). This feedback inhibition of MTHFR by SAM prevents the formation of further 5-methyl THF when methionine and SAM are plentiful. There are also phosphorylation

sites on the MTHFR protein which can influence its activity. Dephosphorylated MTHFR is more active and less sensitive to inhibition by SAM (Yamada et al. 2005).

The activity of MTHFR is localised to the cytosol (Matthews 1984). Exogenous serine is metabolised by both cytosolic and mitochondrial SHMT, and one carbon units derived from serine are ultimately incorporated into methionine (Gregory et al. 2000). This indicates that MTHFR competes with the other enzymes which use 5,10-methylene THF as a substrate, TS and cSHMT, for supply of this folate cofactor. This competition forms one mechanism of regulation of the distribution of folate cofactors between the various one-carbon substituted forms. Herbig et al. (2002) cultured cells in the absence and presence of glycine to examine the effect of cSHMT activity on folate cofactor distribution. In the absence of glycine, cSHMT is prevented from catalysing the production of serine. Under these conditions 5-methyl THF levels were found to increase compared to when glycine was present. This indicates that competition with cSHMT regulates supply of 5,10-methylene THF to MTHFR (Herbig et al. 2002). Overexpression of MTHFS in neuroblastoma cells was found to reduce 5-methyl THF levels when the cells were cultured with excess glycine. MTHFS reduces the levels of 5-formyl THF in the cell, which is an inhibitor of cSHMT. This provides evidence that activation of cSHMT reduces the flux of one-carbon substituted folates to the methylation cycle (Girgis et al. 1997). Knockout of cSHMT in mice caused increased SAM/SAH ratio in liver cells, providing in vivo evidence for the role of cSHMT in regulating partitioning of one carbon units between DNA synthesis and methylation cycle (MacFarlane et al. 2008).

1.5.4.1 Reactions and cellular processes which require SAM

One of the major functions of one-carbon metabolism is provision of methyl one-carbon groups for the many methylation reactions that occur in the cell. The major physiological methyl donor is SAM, which is generated in the methylation cycle as described above and represented in figure 1.2. Enzymes which use SAM are present in all forms of life, and phylogenetic analysis has shown that utilisation of SAM for methyltransferase reactions has a very ancient evolutionary origin (Kozbial and Mushegian 2005). The methyl group from SAM can be transferred to specific sites on DNA and proteins by a wide variety of enzymes with methyltransferase activity. Some of the processes which require SAM are methylation of DNA and histone proteins, methylation of enzymes involved in signal transduction processes, and biosynthetic reactions.

Epigenetic modification refers to features of DNA and chromatin molecular structure which are not part of the genetic code, but which are maintained in cells through DNA replication and cell division, and which play a vital role in regulation of the subset of genes which are active in a

particular tissue or at a particular time. Methylation of DNA and chromatin proteins is one form of epigenetic modification which has an important role in the regulation of gene expression. DNA can be methylated on cytosine residues, and this modification is associated with repression of gene transcription (Klose and Bird 2006). A number of DNA-specific methyltransferases exist, which carry out de novo methylation of cytosine residues, and also maintain patterns of DNA methylation during replication. The vertebrate genome contains regions associated with gene promoters which are known as CpG islands, because they consist of many repeated CpG dinucleotides. Methylation of CpG islands is associated with repression of transcription, whereas unmethylated CpG islands are associated with actively transcribed genes. Disruption of normal DNA methylation is a feature of many cancers; this can appear as global or gene-specific DNA hypomethylation, or hypermethylation of specific gene promoter regions (Jones and Baylin 2007). Histones are the proteins which together with DNA make up chromatin. Histones can be methylated on lysine residues, and this is a dynamic process which is associated with gene regulation. There are a range of histone-specific methylases and demethylases which regulate histone methylation. The methylation state of specific Lys residues on histones is associated with changes in chromatin structure and consequent transcriptional activation or repression (Paik et al. 2007). There is a functional link between methylation of DNA and modification of chromatin, as proteins which bind to methylated DNA recruit other proteins which then alter the methylation of histones in the region, and this can lead to gene silencing (Jones et al. 1998). Methylation of DNA and histones is a highly dynamic and complex process which is still far from being completely understood, but which is known to play an important role in gene expression in health, disease and development (Ndlovu et al.).

Methylation of proteins is a post-translational modification which in many cases is essential for normal function of the modified protein. Proteins can be methylated on lysine or arginine residues, and both of these residues can have more than one methyl group attached (Paik et al. 2007). One example of such modification is myelin basic protein (MBP), which is methylated on arginine-107. There is some evidence that the severe neurological lesion of vitamin B12 sub-acute combined degeneration, which is associated with a breakdown of myelination is caused by hypomethylation of MBP (McKeever et al. 1995; Weir and Scott 1995). Reversible methylation events are associated with several proteins which are involved in signal transduction processes, and methylation may have a regulatory role in the function of these proteins (Aletta et al. 1998). The members of the family of small GTP-binding proteins including Ras, Rac, and Rho are methylated on the carboxyl group of C-terminal prenylcysteine residues. The γ subunits of heterotrimeric GTP-binding proteins are also modified in this way. These proteins must associate

with the cell membrane to perform their functions in signal transduction, and methylation is necessary for membrane association (Higgins and Casey 1996). Another intracellular signalling protein which undergoes post-translational methylation is protein phosphatase 2A (PP2A), a serine/threonine phosphatase which is involved in regulation of a variety of signal transduction processes. Specific methyltransferase and demethylase enzymes have been identified which participate in the reversible methylation of PP2A (Lee et al. 1996).

Reactions involving SAM also play an important role in biosynthesis, and examples of molecules which require SAM in their synthesis include creatine, the lipid phosphatidylcholine (PC), and polyamines. The liver is a major site of transmethylation reactions, because this organ is the site of biosynthesis of some molecules which are required in large amounts and which require methyl transfer reactions in their synthesis, such as creatine and PC (Stead et al. 2006) Creatine is synthesised in the liver by methylation of guanidinoacetate by the SAM-dependent enzyme guanidinoacetate methyltransferase. Creatine is transported from the liver to all body cells, and plays an essential role in regulation of phosphate-bound energy production (Wyss and Kaddurah-Daouk 2000). PC is a phospholipid which is abundant in cell membranes and plasma lipoproteins. There are several pathways of PC synthesis, one of which involves three sequential methylation steps beginning with phosphatidylethanolamine, using SAM as a methyl donor (Vance et al. 2007). The contribution of SAM to PC synthesis is illustrated by knockout mice lacking the gene for phosphatidylethanolamine methyltransferase (PEMT), which catalyses SAM-dependent PC synthesis. Mice lacking PEMT had 50% lower plasma Hcy than wild-type mice, suggesting that a large amount of Hcy production, and by extension consumption of SAM, was due to PC synthesis (Noga et al. 2003).

The polyamines spermidine, spermine and putrescine are small molecules consisting of a short carbon chain with an amine group at either end. Putrescine is synthesised from arginine, and spermidine and spermine are subsequently formed by addition of an aminopropyl group to putrescine from decarboxylated SAM (dcSAM) (Wallace et al. 2003). This is an unusual role of SAM, as in this case it does not function as a methyl donor but is first decarboxylated to form dcSAM before use in polyamine synthesis (Martin and McMillan 2002). Polyamines are positively charged at physiological pH, and hence can interact with negatively charged DNA and RNA. Polyamines have been associated with a range of functions in the cell and they are thought to be involved in regulation of cell growth, possibly by influencing gene transcription through alterations in chromatin structure (Morgan et al. 1987). Synthesis of polyamines changes in a cell cycle specific manner, with increases occurring during G1 and G2 phase (Bettuzzi et al. 1999). Inhibition of polyamine synthesis causes cell cycle arrest (Wallace et al. 2003), and polyamines

have also been implicated in regulation of apoptosis, most likely through changes in the expression of ornithine decarboxylase (ODC) the first enzyme in the polyamine biosynthetic pathway (Packham and Cleveland 1994). The involvement of polyamines in regulation of cell growth and apoptosis also suggests a role in cancer, and indeed increased polyamine production has been associated with carcinogenesis (Linsalata and Russo 2008). A diagram depicting the pathway of polyamine synthesis is provided in figure 1.5.

1.6 Dietary intake and deficiency of folate

1.6.1 Recommended intake of folate

As mentioned above, humans cannot synthesise folates, so they must be obtained from the diet. Foods which are naturally rich in folate include green leafy vegetables, fruits, legumes, nuts, seeds and liver (Kauwell et al. 2010). The folate present naturally in food sources consists of polyglutamated reduced folates in a variety of one-carbon substituted forms (Melse-Boonstra et al. 2002), reflecting the folate species involved in cellular metabolism. Fully oxidised folic acid is not found naturally in significant amounts. Bacteria which are resident in the gut can synthesise folate *de novo*, and bacterially synthesised folate may make a small contribution to folate absorbed from the large intestine. Asrar et al. (2005) infused (H^3)-para-aminobenzoic acid (pABA), the precursor for bacterial folate synthesis into the large intestine of piglets. Later examination showed that 0.4% of liver folate and 14% of urinary folate from the piglets was radiolabelled, indicating that the labelled molecule had been converted to folate and subsequently absorbed (Asrar and O'Connor 2005). The absorption of radiolabelled 5-formyl THF from the colon has also been demonstrated in humans (Aufreiter et al. 2009).

Considerable effort has been made to define recommended intakes for folate. A key issue in the definition of the recommended folate intake is the bioavailability of folate from foods and other sources. Bioavailability refers to the proportion of ingested folate which is absorbed and becomes available for metabolic processes or storage (McNulty and Pentieva 2004). Some evidence has indicated that synthetic folic acid is has greater bioavailability than natural food folate. Sauberlich et al. (1987) investigated the bioavailability of FA compared to natural food folate in 10 nonpregnant women in a controlled feeding study over 92 days. The subjects were placed on a folate-free diet for 28 days, after which controlled amounts of natural folate or FA were introduced to the diet, and plasma and RBC folate measured. Natural dietary folate was found have only 50% bioavailability compared with FA (Sauberlich et al. 1987). These results are supported by two subsequent controlled intervention studies which also found that FA had greater bioavailability than food folate (Hannon-Fletcher et al. 2004; Yang et al. 2005). FA

consumed as a supplement without food is thought to be almost 100% bioavailable (Gregory 1997), while FA consumed with other foods has bioavailability of approximately 85% (Pfeiffer et al. 1997). This has led to the practice in some countries of describing recommended folate intake in 'dietary folate equivalents' (DFE), where 1μg DFE is equal to 1μg natural food folate, or 1.7μg FA as a component of fortified foods (Suitor and Bailey 2000). However, other studies have found the bioavailability of natural folate to be higher, at 80% that of FA (Brouwer et al. 1999; Winkels et al. 2007), and the use of DFEs to describe folate intake is not universal. Specification of the folate available from natural food sources is also complicated by the fact that reduced folates are subject to degradation during cooking and processing. Reduced folates are prone to oxidative cleavage at the C9-N10 bond (Scott 1984), and losses of greater than 50% of folate content from foods has been reported after processing (Melse-Boonstra et al. 2002). The current recommended intake of folate by the World Health Organisation (WHO) and the Institute of Medicine of the USA is 400μg DFE per day for adults, rising to 600 μg DFE per day for pregnant women (IOM 1998; FAO/WHO 2004). The recommended intake of folate given by the Food Safety Authority of Ireland (FSAI) is 300μg/day folate for adults, 500μg/day in pregnancy (FSAI 1999).

1.6.2 Folic acid in the diet: food fortification and supplements

In 1998 the USA introduced mandatory fortification of grains with 1.4 mg FA per kg, with the aim to improve folate status in the population and thereby reduce the incidence of NTDs. Currently there is no mandatory folate fortification in the EU, but folate in the form of FA is widely available in fortified foods such as breakfast cereals, and in multivitamin supplements. This results in a situation where some individuals may regularly consume amounts of FA greater than the recommended upper intake level of 1000μg/day (Crane et al. 1995). The level of fortification in the USA was designed to provide an average intake of 100μg FA per day. However, calculations of the FA intake required to produce the observed increase in folate status post-fortification have indicated that the actual intake may be over twice that amount, at between 215-240 μg per day (Quinlivan and Gregory 2003).

The majority of folate taken orally is converted into the physiological form, 5-methyl THF during first-pass metabolism in the liver. However, there is evidence that high intake of folic acid results in the presence of unmetabolised folic acid in the blood. Kelly et al. examined the acute appearance of FA in serum after ingestion of varying amounts of FA added to foods. Consumption of food supplemented with amounts of folic acid greater than 266μg in a single bolus resulted in the appearance of folic acid in serum (Kelly et al. 1997). Circulating 5-methyl THF and FA was assessed in the Framingham Offspring Cohort before and after the introduction of folate fortification in the USA. In the post-fortification samples, the median serum FA level was increased in both supplement users and non-supplement users, indicating that folate fortification has resulted in increased exposure to plasma FA (Kalmbach et al. 2008). Sweeney et al.

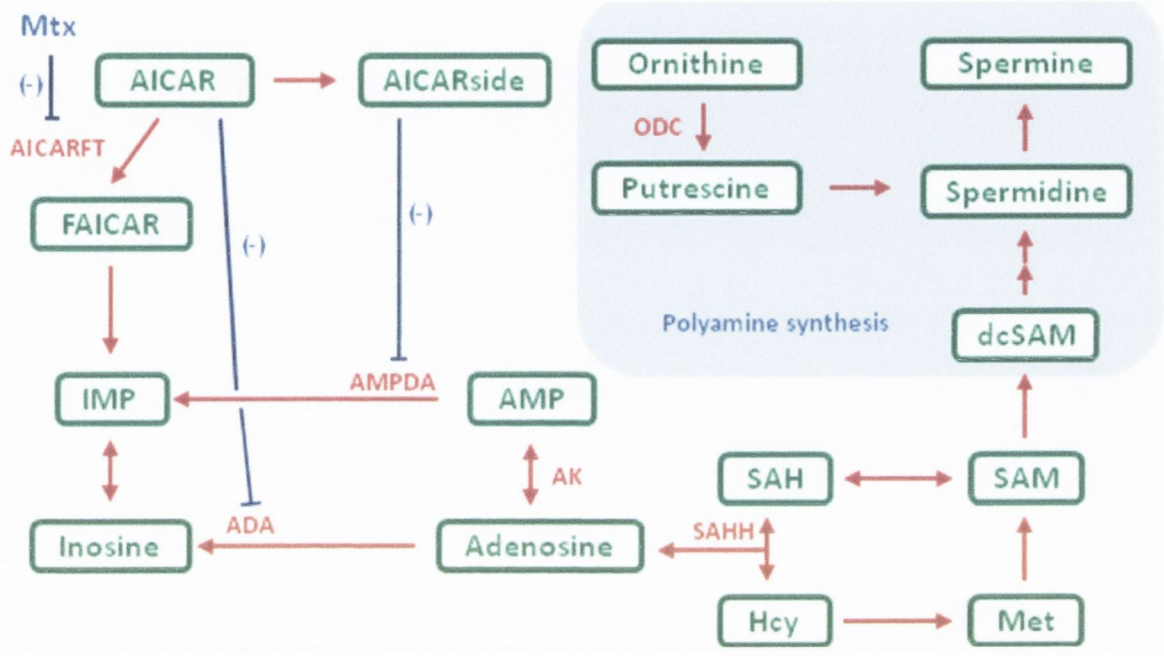

Figure 1.5: Simplified diagram of adenosine metabolism and polyamine synthesis

AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; **AICARside**, aminoimidazole carboxamidoribonucleoside; **FAICAR**, formyl AICAR; **AMP**, adenosine monophosphate; **IMP** , inosine monophosphate; **Hcy**, homocysteine; **Met**, methionine; **SAM**, S-adenosyl methionine; **SAH**, S-adenosyl homocysteine; **ADA**, adenosine deaminase; **AMPDA**, AMP deaminase; **AICARFT**, AICAR formyltransferase; **AK**, adenosine kinase; **SAHH**, SAH hydrolase; ODC, ornithine decarboxylase; **Mtx**, methotrexate.

investigated the presence of serum FA in samples taken in Ireland, a country which has voluntary FA fortification only. Levels of FA were determined from blood samples from three groups; blood donors who were not fasted prior to sampling, mothers who had not used folic acid supplements and who were fasted, and umbilical cord blood from the newborns of the mothers. Low but detectable amounts of FA were found in the majority of samples from all three groups (Sweeney et al. 2009). This study indicates that dietary intake of FA in the setting of voluntary fortification results in persistent levels of serum FA. FA does not naturally occur in serum, and it is unknown if the presence of FA in serum is harmful or benign. The ethics and long-term effects of mandatory folate fortification at population level is a subject of debate. One issue is the fact that the effect of FA in serum over a lifetime of exposure is unknown. Other problems with high intake of folic acid include the possibility of masking of vitamin B12 deficiency, and of interference with the effects of antifolate drugs, as will be discussed further in section 1.8.2.

Folic acid is a very stable form of folate, and therefore is the form used in food supplements and fortified foods. Folic acid can be absorbed into cells where it is converted to THF by the action of DHFR. 5-formyl THF (sometimes called folinic acid or leucovorin) is the only stable form of reduced folate, and is used pharmacologically. Since these compounds enter the folate cycle in a different way than natural forms of folate, they have the potential act in a different way to physiological forms of folate in regulation of the folate pools within the cell. Recent work has shown that the activity of DHFR from human liver is slow compared with rat liver, with the average activity using 7,8-DHF as a substrate of 577 nmol THF/min/g for rat liver DHFR and 16.3 nmol THF/min/g for human liver DHFR (Bailey and Ayling 2009). The activity of human DHFR was also found to vary up to 5-fold between individuals. The reduction of FA to THF by human liver was found to be 1300 times slower than the reduction of 7,8-DHF to THF (Bailey and Ayling 2009). This study is particularly useful because it improved on previous analyses of human liver DHFR by performing the measurements at physiological pH and by using fresh tissue samples. The slow rate of reduction of FA to DHF by human DHFR may limit the ability of the liver to metabolise high levels of dietary FA. In addition, the individual variation in DHFR activity may contribute to individual differences in the effectiveness of FA supplementation or treatment.

1.6.3 Clinical folate deficiency

The major symptom of clinical folate deficiency is megaloblastic anaemia, where immature erythrocytes appear in the bloodstream due to a failure of cell division during hematopoiesis. This is due to suppression of DNA synthesis, caused by a lack of folate cofactors required for synthesis of dTMP and purines. Megaloblastic anaemia can also occur as a symptom of vitamin B12 deficiency (Metz et al. 1968). MS requires vitamin B12 as a cofactor, and this enzymatic

reaction is the only route for dietary 5-methyl THF to be converted to THF and subsequently polyglutamated and retained within the cell. Therefore vitamin B12 deficiency can lead to a 'methyl trap' where folates accumulate in the form of 5-methyl THF but cannot be converted to other forms (Scott and Weir 1981). Physiologically this results in a functional deficiency of folate, although folate levels in serum may be adequate (Tisman and Herbert 1973). Chronic vitamin B12 deficiency has also been termed 'pernicious anaemia' because it can eventually progress to severe neurological symptoms (Ross et al. 1948). The precise aetiology of these symptoms is not clear, but clinically it is important to distinguish between folate and vitamin B12 deficiency because inappropriate treatment of B12 deficiency with folic acid supplementation has the potential to correct anaemia while failing to prevent neurological symptoms (Stabler 2010).

Blood folate levels can be determined by measuring serum folate or red blood cell (RBC) folate. Serum folate is sensitive to short term changes in folate intake. RBCs incorporate folate during their formation and retain these levels for the life of the cell, therefore RBC folate is regarded a better indicator of long term folate status than serum folate. There is no well defined cut-off value which indicates certain low folate status, due to individual variation and differences in assay methods (Stabler 2010). Serum folate lower than 7nmol/L is regarded as lower than normal, and this value has been used as a definition of low folate status in epidemiological studies (Clarke et al. 2004). There is an inverse relationship between folate levels and serum Hcy (Stabler et al. 1988), and measurement of serum Hcy level is also used to diagnose metabolically significant folate deficiency. In general, plasma Hcy levels above 15μmol/L have been described as indicating hyperhomocysteinemia, although plasma Hcy is responsive to folate and FA supplementation can reduce plasma Hcy levels (Tighe et al. 2011). Other substrates of folate-dependent reactions which accumulate in clinical folate deficiency are formiminoglutamic acid (FIGLU) and AICAR. FIGLU is a product of histidine catabolism and substrate for formiminotransferase enzymes which produce 5-formimino THF (Zalusky and Herbert 1962). AICAR is the substrate for AICARFT, a part of the de novo purine synthesis pathway (Herbert et al. 1964).

Traditionally, severe folate deficiency was associated with pregnancy owing to the much increased requirement for folates for foetal development. In the 1930's, Dr. Lucy Wills discovered that a yeast extract could effectively treat anaemia associated with pregnancy (Wills 1931). This compound was referred to as the 'Wills factor' was subsequently determined to be natural folate. Severe folate deficiency is also associated with chronic alcoholism, and extensive studies in animal models and humans have demonstrated that this is due to interference of dietary folate absorption by alcohol (Halsted et al. 2002).

1.6.4 Diseases associated with sub-optimal folate status

Levels of folate which are not low enough to be defined as clinical deficiency may also pose a risk to health. There is considerable evidence to show that low intake of dietary folate, or levels of folate which are in the low range of normal are associated with increased risk of cancer, cardiovascular disease and neural tube defects (NTDs).

1.6.4.1 Folate and cancer

Epidemiological studies suggest that adequate folate intake reduces risk of developing cancer, although the strongest evidence is available for certain cancer types for which large amounts of data have been accumulated. A meta-analysis of 7 cohort studies which examined the relationship between folate intake and colorectal cancer risk found that high dietary folate was significantly protective compared to low intake (OR 0.75, 95% CI 0.64-0.89) (Sanjoaquin et al. 2005). The same study found that the protective effect for folate intake which included both dietary sources and supplements was not as strong (OR 0.95, 95% CI 0.81-1.11), suggesting that natural food folates (or possibly other nutrients contained in folate-rich foods) were more protective than synthetic FA. A meta-analysis of folate intake and breast cancer risk found that increased folate intake was associated with a reduced risk in 13 case/control studies (OR 0.91, 95% CI 0.87-0.96) but did not find an association in 9 cohort studies (OR 0.99, 95% CI 0.98-1.01), and the authors concluded that overall the evidence did not support an effect of folate intake on breast cancer risk (Lewis et al. 2006). In contrast, some studies have found that high consumption of folic acid in supplement form is associated with an increased cancer risk. Women with folate intake greater than 400μg/day from supplements were found to have higher post-menopausal cancer risk than those who did not take supplements (OR 1.19, 95% CI 1.01-1.41) (Stolzenberg-Solomon et al. 2006). In a randomised trial of folate supplementation, 1021 patients with a history of colorectal adenoma were given either 1mg/day FA or a placebo, and screened by colonoscopy after 3 and 5 years. The results suggested that there was a slightly higher rate of recurrence of colorectal neoplasia in the FA treated group (Cole et al. 2007). Other studies have suggested an effect of FA supplementation on cancer development, for example a positive association of supplemental FA (but not dietary folate intake) was reported with type II endometrial cancer (Uccella et al. 2011). Cancer cells are characterised by a high proliferation rate, and therefore require folate for DNA synthesis. High levels of folate could potentially be detrimental by promoting progression of cancer once the initial lesion has become established.

Development of a cancerous phenotype is associated with genetic and epigenetic changes which alter the normal gene expression patterns and functions of a cell. As discussed above, folate

cofactors are required for key steps in DNA synthesis and methylation, and this leads to the proposal of two mechanisms by which inadequate folate might contribute to cancer progression. The first is through impaired DNA synthesis. Adequate supply of folate cofactors is required for normal DNA synthesis and replication. Decreased availability of folate is associated with increased dUMP incorporation into DNA, and this is thought to be due to reduced rate of dTMP synthesis. Blount et al. (1997) found examined the relationship between RBC folate and dUMP incorporation into human DNA isolated from blood. Folate-deficient individuals had 8-fold greater dUMP incorporation compared to individuals with normal folate status. The analysis was repeated after 8-week supplementation with a high dose of FA (5mg/day), and dUMP incorporation was dramatically decreased compared to pre-supplementation levels (Blount et al. 1997). Rats maintained on a folate-deficient diet for 8 weeks were found to have increased dUMP in lymphocyte DNA and also higher levels of DNA strand breaks compared to rats fed on a normal diet (Duthie et al. 2000a). Misincorporation of dUMP is normally corrected by DNA repair mechanisms, but the effectiveness of DNA repair could also be decreased if supply of dTMP is limited. Subsequent accumulation of defects in DNA could contribute to mutagenesis and acquisition of carcinogenic changes. Upregulation of enzymes involved in DNA repair processes has been demonstrated in response to folate deficiency in rat liver (Duthie et al. 2010) and in human colon cells *in vitro* (Duthie et al. 2008), providing further evidence that folate deficiency causes DNA damage. The second way that folate status may influence carcinogenesis is through changes in DNA or histone methylation. As discussed above, DNA methylation patterns are associated with gene expression. Alteration of DNA methylation patterns are commonly found in cancer, with both global hypomethylation of the genome and hypermethylation of specific gene promoters having been demonstrated. Folate deficiency could affect DNA methylation through decreased availability of SAM, or through increased levels of cellular SAH which is an inhibitor of methyltransferases. These effects have been demonstrated in cultured human cells. In human lymphocytes, SAH levels were positively correlated with DNA hypomethylation (Yi et al. 2000). Incubation of human colon carcinoma cells in folate-free growth medium caused DNA hypomethylation both globally and in the region of the p53 gene, compared to cells grown in medium containing 3μM FA. DNA methylation levels were subsequently normalised by supplying FA to the folate-depleted cells (Wasson et al. 2006). It is not clear how folate deficiency could lead to hypermethyation of specific sites within the genome. It has been shown that DNA methyltransferase (DNMT) enzymes bind preferentially to sites of DNA damage (James et al. 2003), and are upregulated in the livers of rats fed a folate and methyl-group depleted diet, possibly as a compensatory response to depletion of methyl groups (Ghoshal et al. 2006).

Targeting of DNMT enzymes to sites of damage could provide a mechanism whereby specific DNA sites can be preferentially methylated during times of folate deficiency.

1.6.4.2 Folate and cardiovascular disease

Cardiovascular disease includes the related conditions atherosclerosis, stroke, and coronary heart disease (CHD). These conditions represent a significant cause of mortality worldwide, as cardiovascular disease was projected to be the cause of 30% of deaths globally in 2005 (Strong et al. 2005). The earliest association of vascular disease with altered OCM was in 1969, when clinical observations of vascular plaques were made in individuals with very high levels of Hcy caused by defects in CBS and MS (McCully 1969). The observation that elevated Hcy was associated with atherosclerosis has led to extensive investigation of the role of Hcy and folate in vascular disease. A meta-analysis of 30 prospective and retrospective studies found that a 25% decrease in Hcy level was associated with decreased risk of ischemic heart disease (OR 0.89, 95% CI 0.83-0.96) and stroke (OR 0.81, 95% CI 0.69-0.95) ("Homocysteine Studies Collaboration" 2002). Low folate intake is associated with increased risk of CHD, and also with stroke and high blood pressure. The National health and nutrition examination survey (NHANES) epidemiological follow up study was a prospective study in which folate intake was determined for 9764 individuals in the US, and observed stroke and cardiovascular disease events were recorded over a 19-year follow up period. A lower relative risk (RR) of stroke (RR 0.79, 95% CI 0.63-0.99) and CHD (RR 0.86, 95% CI 0.78-0.95) was found in the group with the highest quartile of folate intake compared with the lowest quartile (Bazzano et al. 2002). A prospective study of 22,245 individuals who did not use vitamin supplements found a decreased risk of myocardial infarction in the group with folate intake above the median (adjusted hazard ratio 0.57, 95% CI 0.36-0.91) (Drogan et al. 2006). Despite the associations of plasma Hcy and low folate status with increased risk, intervention trials examining the effect of reducing plasma Hcy on cardiovascular disease have so far not found significant beneficial effects. A meta-analysis of 8 placebo-controlled trials including a total of 37,485 individuals examined the effect of folic acid-based B vitamin supplementation in disease prevention. The analysis found no influence of supplementation on risk of CHD events (OR 1.01, 95% CI 0.96–1.07) or stroke (OR 0.96, 95% CI 0.87–1.07) (Clarke et al. 2011). Seven out of the 8 trials also included vitamin B12 in the supplemented group so results for folate alone cannot be determined. A Cochrane database review of homocysteine-lowering trials in 2009 which included some overlap with the Clarke et al. (2011) meta-analysis also found no effect of B-vitamin supplementation on cardiovascular disease risk (Marti-Carvajal et al. 2009). All of the intervention trials have involved participants with previous history of disease, so the evidence presented in these meta-analyses is only applicable to secondary disease. Current thinking is that

lowering Hcy in patients with existing disease will not halt progression of symptoms, but elevated Hcy could have a role in establishment of disease.

The mechanism by which low folate increases risk of vascular disease is not well defined. A pathological effect of increased plasma Hcy is one hypothesis, although the epidemiological evidence above is not sufficient to confirm Hcy as the causal factor in these diseases. If Hcy does have a causal effect in cardiovascular disease, then in vitro experiments should provide insight on the molecular mechanism by which Hcy causes damage within the body. A number of mechanisms have been considered and there is some evidence that Hcy has direct effects on vascular tissue. Current evidence suggests that Hcy can induce apoptosis, cause production of reactive oxygen species (ROS) and increase the production of pro-inflammatory cytokines and other inflammatory mediators. Hcy has been shown to induce apoptosis by a mitochondrial pathway in rat microvascular endothelial cells (Tyagi et al. 2006), and was found to induce apoptosis by the same pathway in bone marrow stromal cells (Kim et al. 2006). In human umbilical vein endothelial cells (HUVEC), treatment with extracellular Hcy caused production of ROS, and induced expression of vascular cell adhesion molecule 1 (VCAM-1), an adhesion molecule which mediates adhesion of leukocytes to the vessel wall (Carluccio et al. 2007). Hcy exposure in vitro also induced expression of the pro-inflammatory cytokines tumour necrosis factor α (TNFα), interleukin (IL) 1β, IL-6, IL-8 and IL-12 in human monocytes (Su et al. 2005). These effects of Hcy could contribute to the development of a pro-inflammatory environment in vascular endothelium. In vitro studies have also shown that chronic folate insufficiency may promote a pro-atherosclerotic phenotype in human endothelial cells independently of Hcy. Incubation of EA.hy 926 cells in low-folate culture medium for a 3 week period resulted in morphological changes including induction of stress fibres, a disordered structure of the endothelial cell monolayer, and reduced endothelial barrier function (Brown et al. 2006). Production of the pro-inflammatory cytokine monocyte chemoattractant protein 1 (MCP-1) was also increased in the folate-deficient cells, and cells which had been grown in folate-deficient medium had a greater response to pro-inflammatory stimulus with TNF-α compared to cells maintained in medium with sufficient folate (Lu et al. 2008).

Atherosclerosis, the precursor to conditions such as ischemic heart disease, is now regarded as a chronic inflammatory condition. The in vitro evidence indicating that Hcy contributes to a pro-inflammatory state provides a mechanism by which elevated plasma Hcy could be a causal factor in cardiovascular disease. This hypothesis is further strengthened by the fact that premature atherosclerosis is common in systemic inflammatory disease (de Leeuw et al. 2005) and that rheumatoid arthritis is associated with a 50% increased risk of cardiovascular disease (Avina-

Zubieta et al. 2008). Although the in vitro evidence is incomplete, in combination with the association studies discussed above, the experimental indication of a pro-inflammatory effect of Hcy and chronic folate deficiency suggests that these biochemical circumstances may contribute to development or progression of cardiovascular disease. By extension, elevated plasma Hcy and low folate status may potentially influence the development and progression of other diseases where inflammation plays a role.

1.6.4.3 Folate and neural tube defects

Neural tube defects (NTDs) are a group of related congenital disorders including spina bifida, anencephaly and encephalocele. These conditions are the result of a failure of the neural tube to close in the developing embryo. Although the exact mechanism underlying NTDs is not understood, it is clear that both genetic and environmental factors are involved. Evidence from family studies show that NTDs have a heritable component, for example a 4.3% recurrence rate was found in families of 363 NTD-affected individuals (Partington and McLone 1995). Maternal supplementation with folic acid has been consistently shown to reduce the incidence and recurrence of NTDs. The Medical Research Council Vitamin Study carried out a randomised trial of FA or a combination of other vitamins for the prevention of recurrence of NTDs in women with a previously affected pregnancy. FA supplementation was found to have a 72% protective effect, while the other vitamins without FA had no protective effect (MRC 1991). Czeizel and Dudas (1992) carried out a randomised controlled trial where women were given either a multivitamin supplement containing 0.8mg FA or a trace element supplement only for one month previous to conception and the first two months of pregnancy. The overall incidence of congenital malformations was significantly lower in the vitamin supplemented group ($p=0.02$), and there were no NTD cases in the vitamin group compared to 6 in the trace element group (Czeizel and Dudás 1992). This study demonstrated that vitamin supplementation could prevent first occurrence of NTDs. A study of blood folate and vitamin B12 levels in 81 NTD-affected pregnancies and 247 controls found that low folate and vitamin B12 status were independently associated with the occurrence of NTDs (Kirke et al. 1993). A meta-analysis of 5 randomised trials involving a total of 6105 women confirmed that FA supplementation reduced the risk of having an NTD-affected pregnancy (RR 0.23, 95% CI 0.15-0.52) compared with placebo or multivitamins without FA (De-Regil et al. 2010). The current advice is that women who are capable of becoming pregnant should take supplemental folic acid before conception and in the first 12 weeks of pregnancy.

The clinical presentation of NTDs, in combination with the growing body of knowledge about genetic and environmental risk factors suggests that the underlying molecular mechanism could involve defects in cell migration, proliferation, or DNA synthesis. In addition to the studies described above, several other lines of evidence support a role for folate metabolism in NTD risk and aetiology. A number of folate-related polymorphisms have been associated with NTD risk in humans. The most consistent genetic association is related to a common polymorphism in the *MTHFR* gene, and which will be discussed in detail in section 1.7.3.3. Other folate-related genes which have been associated with NTD risk include *MTHFD1* (Brody et al. 2002; Parle-McDermott et al. 2006), and *MTRR* which encodes methionine synthase reductase (Wilson et al. 1999). A mouse model known as *Splotch* which has a defect in the transcription factor gene *Pax3*, exhibits an NTD phenotype which is influenced by folate. The incidence of NTDs in heterozygous *Splotch* mouse embryos could be reduced by addition of FA or thymidine, providing evidence for a gene-environment interaction (Fleming and Copp 1998). In support of the effect of altered folate metabolism in NTDs, the deoxyuridine suppression test was used to show that folate metabolism was altered in fibroblast cell lines derived from NTD-affected human embryos (Dunlevy et al. 2007). However, alterations in SAM or SAH levels, or associations with polymporphisms in folate-related genes could not be demonstrated in the fibroblast cell lines. Experiments in mouse embryos have demonstrated that alterations in methylation may be involved in NTD formation. Dunlevy et al. showed that a decrease in the SAM/SAH ratio, excess methionine and inhibitors of MAT induced failure of neural tube closure in cultured mouse embryos (Dunlevy et al. 2006a; Dunlevy et al. 2006b). Recent research using mouse models of NTD phenotypes has implicated several genes in the non-canonical Wnt signalling pathway in the development of NTDs. This signalling pathway was first identified in drosophila and has been shown to be important in establishment of cellular polarity (Boutros et al. 1998). Non-canonical Wnt signalling appears to be involved in embryonic development in a number of organisms, including gastrulation in the frog (Wallingford et al. 2000) and development of the ear in mouse (Montcouquiol et al. 2003). The presence of an NTD phenotype in mice has been linked to mutations in a number of genes which are part of, or interact with the non-canonical Wnt signalling pathway, such as *Vang2* (Kibar et al. 2001), *Scrb1* (Murdoch et al. 2003), *Dvl1* (Wang et al. 2006a), and *Fz3* (Wang et al. 2006b). These associations have not yet been translated to humans, but research in this area is ongoing.

1.7 MTHFR enzyme and its polymorphisms

As discussed above, MTHFR catalyses the reduction of 5,10-methylene THF to 5-methyl THF, using NADPH as donor of reducing equivalents. Individuals who are born with severe deficiencies in

MTHFR show serious metabolic defects, characterized by very high levels of plasma Hcy, developmental delay, and abnormalities in vascular and neurological systems. Severe deficiency of MTHFR activity is usually the result of rare autosomal recessive mutations in the MTHFR gene that severely disrupt protein function (Goyette et al. 1995; Urreizti et al. 2010). The presence of this condition demonstrates the importance of MTHFR for normal cellular function.

1.7.1 Gene and protein structure

The human MTHFR gene is located on chromosome 1, at position 36.3 (Goyette et al. 1994). The gene contains 11 exons, and exon 1 is subject to alternative splicing resulting in two isoforms of the enzyme, of 70 and 77 kDa (Tran et al. 2002). Examination of purified porcine MTHFR by scanning electron microscopy showed that the mammalian enzyme is a dimer of identical subunits of 70-77kDa. Limited proteolysis showed that each subunit consists of two protein domains of roughly equal size (Matthews et al. 1984). MTHFR isolated from *E.coli* contains a single subunit type, and exists as a homotetramer (Sheppard et al. 1999). The prokaryotic form of MTHFR has 30% homology to the N-terminal domain of the mammalian enzyme (Guenther et al. 1999). Much of the early work on the catalytic activity of MTHFR was carried out on the *E.coli* enzyme due to relative ease of purification. The N-terminal domain contains the binding sites for FAD, NADPH and folate, and is the catalytic part of the protein. The C-terminal domain found only in mammalian MTHFR is regarded as a regulatory domain because it contains a binding site for the allosteric inhibitor SAM (Sumner et al. 1986). Northern blot analysis of human tissue samples indicates that MTHFR is ubiquitously expressed (Gaughan et al. 2000).

1.7.2 Polymorphisms of the MTHFR gene

In a 1991 study of patients with coronary artery disease, Kang et al. (1991) reported that a thermolabile form of MTHFR was associated with increased plasma homocystiene and also was a risk factor for development of coronary artery disease (Kang et al. 1991). The existence of a thermolabile form of MTHFR had been known for some time, but the underlying biochemical defect was as yet unknown. Kang et al. (1991) studied the inheritance pattern of the thermolabile MTHFR phenotype, and found that it was inherited in an autosomal recessive manner (Kang et al. 1991). In 1995, Frosst et al. reported the discovery of a mutation in the MTHFR enzyme which was associated with thermolability of the enzyme and reduced enzyme activity (Frosst et al. 1995). The mutation responsible is a change from cytosine to thymine at position 677 in the nucleotide sequence of the MTHFR gene, and results in conversion of alanine to valine at position 222 in the protein, a conserved site. Frosst et al. (1995) examined enzyme activity in a group of vascular disease patients and controls, finding that MTHFR from individuals homozygous for the

mutation had only 30% of the wild-type enzyme activity, and heterozygotes had intermediate activity at 65%. Homozygosity for the mutation was also associated with increased homocysteine level in this group (Frosst et al. 1995).

MTHFR has a non-covalently bound FAD cofactor, which participates in the transfer of reducing equivalents from NADPH to folate (Kutzbach and Stokstad 1971). Experiments using E. Coli MTHFR with a mutation analogous to the human Ala→Val mutation showed that the mutation resulted in increased thermolability, as observed in the human enzyme. The kinetic properties of the mutant enzyme were similar to the wild-type, but observation of the rate of dissociation of the FAD cofactor on dilution of the purified enzyme showed that the mutation reduced the affinity of the enzyme for its FAD cofactor (Guenther et al. 1999). Following production of recombinant human MTHFR, Yamada et al. (2001) showed that similar effects of the mutation were observed in the human enzyme. The mutant enzyme was thermolabile, and lost the FAD cofactor on dilution approximately three times faster than the wild-type enzyme. Loss of the FAD cofactor caused dissociation of the enzyme into inactive monomers. Both the substrate 5-methyl THF and the allosteric inhibitor SAM were found to protect from loss of FAD on dilution (Yamada et al. 2001). The crystal structure of E.coli MTHFR shows that each subunit forms a β8α8 barrel structure. The folate cofactor binds to the enzyme in a conformation where the pteridine ring is stacked on top of the FAD moiety (Guenther et al. 1999). The mutated residue is located far from the FAD binding site, however a recent crystal structure of the mutant E. coli enzyme indicates that the Ala→Val substitution causes distortion of α-helix 5 which contains several residues which interact with the FAD cofactor (Pejchal et al. 2006).

Since these early discoveries, the MTHFR 677C→T polymorphism (rs 1801133) has been studied extensively, and a clearer picture of its influence on health and disease has gradually emerged. Frosst et al. found a distribution of 12% TT individuals in their original identification of the polymorphism (Frosst et al. 1995). Subsequent studies have shown that the MTHFR 677C→T polymorphism is found in populations all over the world, with considerable geographic variation in allele frequency. The T allele is more prevalent in European and Asian populations, and rarer in populations of African origin. Schneider et al. found a T allele frequency of 18.6% in a European population, 20.8 % in an Asian population, and 6.6% in an African population (Schneider et al. 1998). A different analysis observed T allele frequencies of 35.4% in Britain, 43.8% in Italy, 35.2% in Japan, 41% in US Hispanics, 6.3% in Africa and 14% in African-Americans (Botto and Yang 2000). In a large study of an Irish population, the frequency of individuals with TT genotype was found to be 9.8% (Mills et al. 1999). There are also reports of high frequency of the TT allele in small

isolated populations, for example 44.9% in a population of Brazilian Amerindians (Schneider et al. 1998).

Two other common polymorphisms which result in changes in the protein sequence have been found in the MTHFR gene, 1298A→C (rs1801131) and 1793G→A (rs2274976). The 1298A→C polymorphism was first described by two different groups in 1998, and is the better characterized of the two polymorphisms. van der Put et al. identified the 1298A→C polymorphism as part of a study on the role of MTHFR in birth defects (van der Put et al. 1998). This polymorphism is located in exon 7 of the MTHFR gene, and results in change of a glutamate residue to alanine at position 429 in the protein sequence. In this study the 1298C allele was associated with a reduction in MTHFR activity, but to a lesser extent than the 677T allele, and was not associated with thermolability of the enzyme. Weisberg et al. found the same 1298A→C polymorphism in a study of a Canadian population, observing that 10% of Canadian individuals were homozygous for the 1298C allele. They found that MTHFR from 1298CC individuals had 60% enzyme activity compared to controls with 1298AA genotype. There was an interaction with the 677C→T polymorphism, with compound heterozygotes having lower MTHFR activity than individuals with heterozygosity at the 677C→T position alone (Weisberg et al. 1998). The allele frequency of the 1298C form was 33% in a sample of 1962 Caucasians in Germany (Meisel et al. 2001). The biochemical effects of the 1298A→C polymorphism were investigated by Yamada et al. in their experiments on recombinant human MTHFR, along with the 677C→T mutation. They found that the 1298A→C mutant protein was indistinguishable biochemically from the wild type protein, and that a double mutant with both the 1298A→C mutation and the 677C→T substitution had the same effects as 677C→T alone (Yamada et al. 2001). Their work suggests that the 1298A→C does not have a significant effect on MTHFR activity.

The 1793G→A polymorphism was first described by Rady in 2002 (Rady et al. 2002). This polymorphism results in an Arg to Glu substitution at position 594 of the protein. Frequency of the A allele varied between 1.3% and 6.9% depending on the population studied, and was much lower than the frequency of the other two polymorphisms. A study of 923 Chinese individuals found an allele frequency of 9.3% for the 1793A allele (Mao et al. 2008). This polymorphism has been included in several recent association studies along with 677C→T and 1298A→C, however no significant associations have been found.

The 677C→T and 1298A→C polymorphisms are in strong linkage disequilibrium. Rosenberg et al. (2002) identified a common haplotype encompassing both 677T and 1298A alleles along with two flanking intronic polymorphisms, which was very strongly associated with the 677T allele

(Rosenberg et al. 2002). Their evidence supports the idea that the 677T mutation was ancestral, and has been inherited as part of this haplotype ever since. A meta-analysis of the haplotype distributions of these polymorphisms, which included a total of 5389 individuals found no instances of the 677TT/1298CC genotype (Ogino and Wilson 2003). Alleles containing 677T and 1298C in *cis* conformation have been described, but the occurrence is very rare (Weisberg et al. 1998; Isotalo et al. 2000). Although the 677TT/1298CC genotype has been found in foetal tissue (Isotalo et al. 2000), there is only one report in the literature of a living person with compound homozygosity for 677TT/1298CC genotype – a healthy mother of a child with spina bifida (Volcik et al. 2001). The implication of the haplotype structure of the MTHFR gene is that analysis of effects of the 1298A→C polymorphism without also accounting for 677C→T genotype is likely to lead to inaccurate findings. The 677C→T polymorphism is more important to MTHFR function than the 1298A→C polymorphism as it is has confirmed effects on enzyme activity and folate metabolism. The MTHFR 677C→T polymorphism also has strong associations with a number of diseases as will be discussed in the following sections. It has been suggested that the 677C→T polymorphism may have a balancing effect, whereby the T allele may confer a survival advantage in populations where folate status is adequate, but be deleterious when folate is inadequate. Such an effect would explain the variation in prevalence of the T allele in different geographic populations (Rosenberg et al. 2002).

1.7.3 Metabolic effects and disease associations of altered MTHFR function

The understanding of allosteric regulation of MTHFR by SAM, with competition for binding by SAH, and the importance of folate and FAD for stability of the functional enzyme dimer builds up a picture of the complex network of regulation of MTHFR activity within the cell. Since enzyme activity and stability are closely related, the overall activity of MTHFR depends on folate and riboflavin status, along with SAM:SAH ratio within the cell. These effects are further modified by the MTHFR 677C→T polymorphism. The mutated form of the enzyme has lower activity, which leads to altered distribution of the folate species within the cell. Bagley and Selhub (1998) used high-performance liquid chomatography (HPLC) to measure the levels of different reduced folate cofactors in RBCs from individuals of 677 CC and TT genotype. In CC individuals, only 5-methyl THF was found, while in TT individuals both 5-methyl THF and formylated THF were detected (Bagley and Selhub 1998). This suggests that the polymorphism reduces flux of folates through the MTHFR enzyme, and supports the idea that the mutation reduces MTHFR activity in vivo. A study by another group found similar effects of the polymorphism on folate cofactor distribution in cultured immortalised lymphocytes. Cells of TT genotype had higher levels of formylated folates and lower levels of methylated folates than cells of CC genotype (Lathrop Stern et al.

2003). Davis et al. (2005) carried out an intervention study where 9 healthy women homozygous for each allele of the MTHFR 677C→T polymorphism (i.e. CC or TT genotype) were maintained on a folate-restricted diet for 7 weeks. Folate cofactor distribution in RBCs was analysed at baseline and after the intervention. Formylated folates were found in RBCs from TT individuals but not from CC individuals both before and after the intervention (Davis et al. 2005).

Low folate status is associated with increased levels of plasma Hcy, which itself is associated with a range of diseases as discussed previously. The MTHFR 677C→T polymorphism is also associated with elevated plasma Hcy, and the effect of the polymorphism varies with folate status. A study of 365 individuals found that TT genotype was associated with higher fasting plasma Hcy (Jacques et al. 1996). Division of the cohort based on folate status found that the genotype effect was significant in the sub-group who had plasma folate levels below the median value of 15.4 nmol/L, but not in those who had higher folate status (Jacques et al. 1996). A study of 625 healthy male volunteers showed that TT genotype was strongly predictive of elevated Hcy, and that heterozygosity for the polymorphism was also associated with a small but significant increase in plasma Hcy in cases of low folate status (Harmon et al. 1996). An interaction between folate status and MTHFR 677C→T genotype was further observed in a study of 1042 healthy elderly individuals. In this group, TT genotype was associated with higher plasma Hcy compared to CC genotype, and the genotype effect was more marked in cases of lower folate status (Devlin et al. 2006). A large population-based study including 10,601 participants examined the effects of 13 polymorphisms in enzymes of OCM on biomarkers of folate status. The results confirmed that the MTHFR 677C→T polymorphism had the strongest effect on plasma Hcy of all polymorphisms tested. TT genotype was associated with a 32% higher plasma Hcy level, and a 29% lower serum folate level (Fredriksen et al. 2007). A recent genome-wide association study (GWAS) which examined 336,469 single nucleotide polymorphisms (SNPs) in 13,974 healthy women for associations with plasma Hcy confirmed the association of the MTHFR 677C→T mutation with high levels of plasma Hcy (Pare et al. 2009).

MTHFR also requires FAD, which is obtained from dietary intake of riboflavin. Three groups examined the interaction between folate status, riboflavin status and the MTHFR 677C→T polymorphism on plasma homocysteine. A cross-sectional study of 423 blood donors from a Norwegian population found that low riboflavin status was associated with high plasma Hcy, and that this association was driven by the individuals with the C667T allele (Hustad et al. 2000). Jacques et al. examined this relationship in a large sample from the Framingham Offspring Cohort which included approximately 150 individuals of each genotype (i.e. CC, CT or TT). Plasma Hcy level was higher in the TT group than in the CC or CT groups. There was an inverse relationship

between riboflavin and plasma Hcy, which was only observed in the group with folate status below the median. A statistical interaction between the effects of folate and riboflavin status on plasma Hcy was only significant in the TT group (Jacques et al. 2002). A study of 286 healthy volunteers found that mean plasma Hcy level was higher in TT individuals than in those with CT or CC genotype. The subset of TT individuals with low riboflavin status made the greatest contribution to the elevated Hcy level observed in this genotype group (McNulty et al. 2002). These studies indicate that for individuals with MTHFR 677TT genotype, both folate and riboflavin status have an influence on plasma Hcy due to effects on MTHFR activity. This relationship is supported by the results of an intervention study, which showed that supplementation with riboflavin increased levels of the vitamin in all genotypes, but caused a reduction in plasma Hcy levels only in individuals with TT genotype. When the TT group from this study were stratified based on riboflavin status at baseline, the reduction in Hcy was found to be driven by the individuals who had the lowest riboflavin levels before the intervention (McNulty et al. 2006). An *in vitro* study showed that in extracts of MTHFR from cultured lymphocytes enzyme activity was lower in cells of TT genotype compared to cells of CC genotype. Low riboflavin availability further decreased MTHFR activity in cells of TT genotype, but did not significantly affect activity in cells of CC genotype (Lathrop Stern et al. 2003).

Through its effects on Hcy level and folate metabolism, the MTHFR 677C→T polymorphism has potential to influence a large number of disease processes. A very large number of studies have investigated its role in a range of disorders, including cardiovascular disease, cancer, and neural tube defects.

1.7.3.1 Cardiovascular disease

The rationale for an impact of the MTHFR 677C→T polymorphism in cardiovascular disease is based on its effect on plasma Hcy levels. While there is a very clear association between elevated plasma Hcy and cardiovascular disease, it is still not certain if Hcy plays a causal role, or if it is a consequence of the disease process. Two meta-analyses published in 2002 investigated the role of Hcy and the MTHFR 677C→T polymorphism in cardiovascular disease. Wald et al. examined the results of 72 genetic studies which investigated the presence of the polymorphism in case-control groups, and also 20 prospective studies which tested the relationship between serum Hcy level and disease risk. They found that both TT genotype and increased plasma Hcy significantly increased risk for ischemic heart disease (IHD), deep vein thrombosis (DVT) and stroke (Wald et al. 2002). Summary ORs for TT genotype compared with CC genotype were 1.21 (95% CI 1.06-1.39) for IHD, 1.29 (95% CI1.08 to 1.54) for DVT, and 1.65 (95% CI 0.66-4.13) for stroke. Klerk et al.

(2002) carried out a meta-analysis investigating the association of the MTHFR 677C→T polymorphism with coronary heart disease (CHD). They found that TT genotype was associated with a 16% increased risk of CHD (OR 1.16, 95% CI 1.05-1.28). When the data was stratified by folate status where it was available, increased risk of CHD in all genotype groups (CC, CT, and TT) was only apparent when low folate status was also present. The most significant risk under low-folate status was for TT genotype (OR 1.44, 95% CI 1.12-1.83) (Klerk et al. 2002). The principle of Mendelian randomisation indicates that the MTHFR 677T allele will be randomly distributed in a population, and will therefore randomly associate with lifestyle factors which influence folate status, Hcy level and disease risk. The observed associations of TT genotype with increased Hcy are therefore unlikely to be due to chance. The evidence from these studies pointed towards a causal role for Hcy in CHD, particularly for the Wald et al. study which obtained similar results by analysing two different types of study design independently. However a subsequent meta-analysis including 26,000 cases and 31,183 controls suggests the evidence for a causal role of the MTHFR 677C→T polymorphism in CHD is not as strong as suggested in the Wald et al. study. This work found a reduced effect of TT genotype on CHD risk, with an OR of 1.14 (95 % CI 1.05-1.24). When the data were split by geographic region the effect was significant in populations from the Middle East and Asia, but not significant in populations from Europe, North America, or Australia (Lewis et al. 2005).

1.7.3.2 Cancer

As described in section 1.6.4.1, folate deficiency is associated with risk of developing several types of cancer, and has been mechanistically linked to DNA damage, impaired DNA repair, and also epigenetic changes in DNA methylation. Alteration of cellular function through acquired changes in DNA sequence, or altered gene expression due to changes in DNA methylation patterns are two mechanisms which can lead to development of cancer. Since the MTHFR enzyme lies at a branch point between supply of folate cofactors for DNA synthesis and repair, and also for provision of methyl donors, it is clear that the polymorphism could influence both of these processes. A large number of studies have investigated the association of the MTHFR 677C→T polymorphism with cancer risk.

There is a strong association between the MTHFR 677C→T polymorphism and reduced risk of colorectal cancer (CRC). Chen et al. (1996) carried out the first case-control study in this area, with 144 cases of CRC and 627 controls. They found that TT genotype was associated with a reduced risk of CRC (OR 0.57, 95% CI 0.30-1.06) (Chen et al. 1996a). Since then, many more studies have been carried out investigating the relationship of MTHFR 677C→T genotype with

CRC. Two meta-analyses published in 2007 were in agreement with the findings of Chen et al. (1996), reporting a reduced risk of CRC associated with MTHFR 677 TT genotype. Hubner et al. (2007) reported a reduced risk for TT genotype compared to CC (OR 0.83, 95% CI 0.75-0.93), and found that heterozygosity (CT) did not affect risk (OR 0.99, 95% CI: 0.94-1.04) (Hubner and Houlston 2007). Huang et al. (2007) reported an OR based on the presence of the 677T allele, reporting a modest protective effect (OR 0.93, 95% CI 0.89-0.98). This study found no association of the polymorphism with colorectal adenoma, suggesting that the reduced risk associated with the T allele may be effected by inhibiting progression of pre-cancerous lesions (Huang et al. 2007). A 2009 meta-analysis of 29 studies including 11,963 cases and 18,714 controls found an overall reduced risk of CRC associated with the MTHFR TT genotype (OR 0.83, 95% CI 0.77 to 0.90) (Taioli et al. 2009). When the data was stratified by ethnicity this association was found to be true for Asians and Caucasians only. This study also included a pooled analysis to investigate the effect of smoking, alcohol consumption and body mass index (BMI) on CRC risk, and found no statistically significant interactions between these factors and MTHFR 677C→T genotype.

A recent meta-analysis of 21 publications on the effect of the MTHFR 677C→T polymorphism on childhood acute lymphoblastic leukaemia (ALL) found that the TT genotype was associated with a reduced risk of the disease. The study included 4,706 cases and 7,414 controls, and used several different models to analyse the data, with similar results by all types of analysis. For example, using a fixed-effects model, they calculated OR 0.80 (95% CI = 0.70-0.93) for TT genotype compared to CC (Yan et al. 2011). The association with childhood ALL is interesting as the aetiology of this disease is likely to be somewhat different from adult cancer. Since it occurs early in life, the cumulative effects of environmental influences on cancer risk (for example smoking) are less likely to have an impact. It might be expected that any genetic predispositions would be more readily apparent in studies of this condition. This meta-analysis is an improvement over previous studies, as it exclusively included studies on childhood ALL, and showed an effect by several different types of analysis.

There are studies of other types of cancer which show an increased risk associated with MTHFR 677C→T polymorphism, although the evidence is not as clear as fewer studies have been undertaken in these cases. In some cancer types, presence of the MTHFR 677C→T polymorphism has been found to be protective, and in others it has been found to be a risk factor. For example, recent study by a group in Iran have found associations between TT genotype and increased risk of clear cell renal carcinoma (OR 1.58, 95% CI 1.21-2.44) (Safarinejad et al. 2011), and decreased risk of TT genotype with prostate cancer (OR 0.59, 95% CI 0.41-0.94) (Safarinejad et al.). A 2006 meta-analysis found no association of breast cancer risk with folate intake, or with the MTHFR

677C→T polymorphism (OR 1.05, 95% CI 0.88-1.25) (Lewis et al. 2006). A meta-analysis which investigated the role of folate status (but not the MTHFR polymorphism) in breast cancer risk showed no significant associations (Larsson et al. 2007). A case-control study reported an increased risk of sporadic breast cancer associated with MTHFR C667T in a study of 222 cases and 235 controls (OR 1.84, 95% CI 1.14-3.00) (Mohammad et al. 2010). A recent meta-analysis which examined the association of MTHFR 677C→T with several cancer types found that the 677 TT genotype was a risk factor for oesophageal cancer (OR 1.77, 95% CI 1.17-2.68) and for gastric cancer (OR 1.40, 95% CI 1.19-1.66), but was protective against colorectal cancer (OR 0.85, 95% CI 0.77-0.94) (Zacho et al. 2010). Based on the diversity of findings in different cancer types, it is likely that the effect of the MTHFR 677C→T polymorphism on cancer risk varies depending on tumour site.

A disadvantage of many studies of the MTHFR 677C→T polymorphism and cancer is that they do not all take folate status into account. Folate deficiency has been associated with risk of many cancer types, with the strongest evidence for CRC as discussed previously. Some studies have reported conflicting results, for example a study of prostate cancer reported no association between of folate status and disease in a case-control study, but in a meta-analysis of 7 studies found a positive association between higher folate levels and cancer risk (OR 1.11, 95% CI 0.96-1.28) (Collin et al. 2010). It is clear that the effect of the MTHFR 677C→T polymorphism on cancer is likely to be influenced by folate status. The interaction of MTHFR 677C→T polymorphism and folate status was investigated in 471 colorectal adenoma cases and 510 matched controls. TT genotype was associated with increased risk of colorectal adenoma in individuals in the lowest quartile of folate status, but decreased risk in the group in the highest quartile of folate status (Levine et al. 2000). A recent study has shown a similar interaction of TT genotype and folate status in a study of CRC. TT genotype conferred an increased risk in the group with the lowest folate status (RR 1.39, 95% CI 0.87-2.21), and in contrast a decreased risk in the group with the highest folate status (RR 0.74, 95% CI 0.39-1.37), although the effect was non-significant in this instance (Eussen et al. 2010).

1.7.3.3 Neural tube defects

As discussed previously, maternal peri-conceptional folic acid supplementation reduces the risk of an NTD-affected pregnancy. However, it is not an overt folate deficiency that leads to NTD risk, as it has been shown that mothers of NTD cases have folate levels in the normal range (Molloy et al. 1985; Kirke et al. 1993). The association of the T allele of the MTHFR 677C→T polymorphism with increased plasma Hcy led to the suggestion that this polymorphism might also influence NTD risk.

Two papers published in 1995 investigated the prevalence of the MTHFR 677C→T polymorphism in NTD cases. A study of 82 NTD-affected children in Ireland found that the frequency of TT genotype in 82 NTD cases was 18.3%, significantly higher than in healthy unrelated controls where the TT frequency was 6.1% (Whitehead et al. 1995). Similar results were reported in a Dutch population, where 13% of NTD cases had TT genotype, compared to 5% of controls (Van der Put et al. 1995). Many other studies in different population groups followed these early investigations. A further study of MTHFR 677C→T genotype in NTD cases and their families in the Irish population determined that TT genotype in the affected child was more influential in disease risk than maternal MTHFR 677 genotype (Shields et al. 1999). In 2000, a meta-analysis of 15 studies on the effect of the MTHFR 677C→T polymorphism and NTDs found that TT genotype in the mother or in the affected child (OR = 1.8 95% CI 1.4 to 2.2) was associated with a significantly increased risk of NTDs, although some individual studies had not demonstrated this association (Botto and Yang 2000). In an Irish population of 395 cases of spina bifida and 848 controls, it was found that an increased risk of NTDs was associated with heterozygosity for the MTHFR 677C→T polymorphism as well as TT genotype. Both CT (OR 1.52, 95% CI 1.16 to 2.00) and TT (OR 2.56, 95% CI 1.75 to 3.74) genotype were associated with increased risk compared to CC genotype (Kirke et al. 2004). In a recent study which investigated a number of candidate SNPs as NTD risk factors, the association of the MTHFR 677T allele was confirmed in a in an expanded case-control group from the same Irish population (Carter et al. 2011). Further evidence supporting this association comes from a recent meta-analysis which reported an increased risk for risk of NTDs in TT genotype compared to CT+CC genotype, using 3,530 cases and 6,296 controls from 32 separate studies (OR 1.41, 95% CI 1.24–1.59) (Amorim et al. 2007).

1.8 Methotrexate

1.8.1 History of methotrexate

The potential use of folate antagonists in cancer treatment came from the clinical observation that administration of folic acid to a patient with leukaemia resulted in acceleration of the disease (Farber and Diamond 1948). This observation led to the trial of structurally related compounds which were folic acid antagonists in cancer treatment. Aminopterin (4-amino pteroylglutamic acid) was the first antifolate drug shown to be effective in treatment of paediatric leukaemia, however the remission produced was temporary and the drug was associated with significant toxicity (Farber and Diamond 1948). Methotrexate (Mtx) is structurally similar to aminopterin, and was found to have a superior therapeutic index compared to aminopterin (Goldin et al. 1955). The molecular structure of Mtx is shown in Figure 1.1. Mtx was the agent used for the first

medical cure of a solid cancer, in a case of choriocarcinoma (Li et al. 1958). This early research paved the way for the chemotherapeutic treatment of cancer, and today Mtx is still commonly used to treat several types of cancer. These include acute lymphocytic leukaemia, non-Hodgkin's lymphoma, carcinomas of the breast, tongue, pharynx and testes, choriocarcinoma, osteogenic sarcoma, urinary bladder tumours, brain tumours and cutaneous neoplasms (Fleisher 1993).

The observation that aminopterin could suppress proliferation of connective tissue led to the first trial of an antifolate drug in treatment rheumatoid arthritis (RA) and psoriasis by Gubner et al. in 1951 (Gubner et al. 1951). An improvement in symptoms of disease was observed, but the doses used were high enough to cause toxic side effects, and Gubner concluded that this would "impose serious limitations on clinical applicability" of the drug. However, randomised trials conducted in the 1980's explored the usage of the antifolate drug Mtx in RA and found it to be effective and well tolerated (Weinblatt et al. 1985). The dosage regimens have been much refined over the years, and today low dose Mtx is used in treatment of a range of autoimmune and inflammatory conditions. These include RA, psoriasis, Crohn's disease, cutaneous lupus erythematosus, systemic lupus erythematosus, polymyositis, scleroderma, graft-versus-host disease and organ allograft rejection (Grim et al. 2003). Mtx is recommended as the first line treatment for RA (Combe et al. 2007) and for moderate to severe psoriasis (Roenigk et al. 1998).

1.8.2 Clinical use of methotrexate

There are two different clinical dose regimens of Mtx use. Higher doses of Mtx are used for the treatment of cancers, and within this regimen the amount and route of administration of the drug varies depending on the type of malignancy being treated. Doses can range from 50mg to upwards of 5000mg per m^2 of body area (Cronstein 2005). The highest doses are administered intravenously over a period of 24h, but lower doses in this range can be given orally (Walling 2006). The dosage regimen used for treatment of diseases such as RA and psoriasis involves much smaller amounts of the drug, ranging from 15- 30 mg in a single weekly dose, administered orally (Visser and van der Heijde 2009). Methotrexate is also used as part of combination therapy with non-steroidal anti-inflammatory agents and newer biological drugs such as anti-TNFα agents in treatment of autoimmune disease (Emery et al. 2010).

Mtx is transported by the same transporter proteins as for physiological folates, RFC and PCFT. The affinity of the RFC for Mtx is similar to that for the physiological substrate 5-methyl THF (Henderson et al. 1980). Experiments in the human colon cell line Caco-2, which express the RFC reported a Km of 1μM for Mtx, and that transport of the L-Mtx stereoisomer was preferred over D-Mtx (Narawa et al. 2007). Mtx resistance due to decreased expression of RFC is a common

feature in cancer. In one study, 70% of relapsed ALL patients were found to have reduced Mtx transport compared to only 13% of untreated ALL patients. Expression of RFC was measured by northern blot and reverse-transcriptase polymerase chain reaction (RT-PCR), and reduced expression was found in cells which had demonstrated lower Mtx transport activity (Gorlick et al. 1997). Studies in murine leukemia cells have shown that mutations in the RFC protein can result in decreased Mtx transport (Brigle et al. 1995). The PCFT transports Mtx with a Km of 5μM, and as for physiological folates, transport is optimal at pH 5.5 and declines as pH is increased. Transport of Mtx via the PCFT is highly stereospecific for L-Mtx (Narawa and Itoh 2010). A study of leukaemia cells over-expressing FRα demonstrated that Mtx can also be transported into cells by receptor-mediated routes (Spinella et al. 1995). As discussed above, FRs have an affinity for FA in the low nanomolar range, and for some FRs there is a slightly lower affinity for reduced folates. The affinity of FRs for Mtx is much lower than for FA, with Mtx having a relative affinity less than 1% of FA (Westerhof et al. 1995). However, receptor mediated transport of Mtx could be important in disease, because expression of folate receptors can become upregulated in some types of cancer cells. High levels of FR expression has been demonstrated cancer of the ovary, uterus, brain, colon, breast and kidney (Campbell et al. 1991; Weitman et al. 1992a; Ross et al. 1994; Wu et al. 1999) At very high concentrations, Mtx can enter cells by passive diffusion, which may have a role where cells have become resistant to Mtx due to acquired defects in carrier mediated transport (Hill et al. 1986).

Once Mtx has been transported into cells, it is polyglutamated by FPGS in the same manner as natural folates (Baugh et al. 1973). As was described above for tetrahydrofolates, FPGS catalyses the sequential addition of glutamate groups to the molecule, and this modification causes Mtx to be retained within the cell. Incubation of human breast cancer cells with Mtx resulted in formation of Mtx polyglutamates containing 2-5 glutamate residues, and when cells were subsequently placed in medium without Mtx the retention time of the drug was greater with increased polyglutamate chain length (Jolivet and Chabner 1983). Addition of glutamate residues to Mtx also increases the affinity of the drug for several enzymes, as will be discussed further below. The fact that Mtx is a substrate for FPGS is therefore an important aspect of its efficacy. Experiments using lysosomes from murine S180 cells have demonstrated that Mtx polyglutamates can be transported into lysosomes, where glutamate groups are removed by γ-glutamyl hydrolase (Barrueco et al. 1992). The extent of Mtx polyglutamation may depend on the balance between the action of FPGS in the cytosol and γ-glutamyl hydrolase in the lysosomes. Pharmacokinetic studies have shown that after administration of Mtx in the low dose regimen, serum concentrations of the drug fall rapidly (Tishler et al. 1989), however Mtx polyglutamates are

retained within body cells for several weeks after cessation of treatment (Dalrymple et al. 2008). A study of the accumulation of Mtx after low-dose therapy of RA indicated that the intracellular concentration of Mtx is also dependent on other factors such as age, Mtx dosage and renal function (Stamp et al. 2009).

There is a significant risk of toxicity associated with Mtx treatment, which can manifest as effects on rapidly proliferating tissue such as bone marrow suppression or gastro-intestinal mucositis. Adverse effects of Mtx can also occur as acute or chronic neurological toxicity, hepatic toxicity, or impaired renal function due to the high demand for metabolism and excretion of Mtx and its breakdown products (Attar 2010). 5-formyl THF supplementation is often given alongside Mtx treatment in an attempt to reduce toxicity. This approach was first shown to be effective in animal models of leukaemia and sarcoma, where treatment with high dose Mtx followed by rescue therapy with 5-formyl THF was found to produce an anti-tumour effect without toxicity compared to Mtx alone (Sirotnak et al. 1978). For very high dose Mtx used in cancer chemotherapy, high doses of folate rescue may be used, along with hydration and alkalinisation of the urine to ensure sufficient excretion of Mtx (Sand and Jacobsen 1981). Folate rescue is also a feature of low-dose Mtx therapy for inflammatory disease, where folic acid or 5-formyl THF is administered orally the day following the Mtx dose to avoid interference with absorption of the drug. Folate rescue has been shown to reduce side effects of Mtx treatment without compromising efficacy in the low-dose regimen (Morgan et al. 1994; Ortiz et al. 2000; Cronstein 2005). However other studies have shown that folate therapy may reduce the effectiveness of Mtx treatment, and that the form of folate used may have different effects. A randomised controlled trial which examined the potential of FA and 5-formyl THF to reduce toxicity of Mtx found that both folate compounds were effective in reducing toxicity. However, higher doses of Mtx were required to achieve the same clinical effect in the groups who were given folate supplementation (Van Ede et al. 2001b). Another study found that administration of folic acid in combination with low-dose Mtx reduced the efficacy of the drug for treatment of psoriasis, compared with Mtx alone (Chládek et al. 2008). A study which examined the effects of folic acid or 5-formyl THF in combination with Mtx in treatment of RA measured urinary AICAR level as a measurement of inhibition of purine synthesis. 5-formyl THF but not folic acid was found to normalise the urinary AICAR levels of patients taking Mtx for treatment of rheumatoid arthritis (Morgan et al. 2004). Increased urinary AICAR was correlated with an improvement in disease symptoms. This result suggests that 5-formyl THF may interfere with the efficacy of low-dose Mtx.

1.8.3 Enzyme inhibition by methotrexate

Mtx was originally synthesised as an antagonist of folic acid, and the mechanism of this antagonism was later shown to be due to inhibition of DHFR and several other folate-dependent enzymes. The inhibition of DHFR by Mtx is extremely potent, and this is regarded as the primary mechanism by which Mtx has its mode of action. Methotrexate has much higher affinity for DHFR than the physiological substrate DHF does. The K_m for methotrexate is in the order of 10^{-7} M compared to 10^{-5} M for DHF (Williams et al. 1979). A Ki value of 3.4pM has been reported for Mtx in a ternary complex with human DHFR and NADPH, and the Ki for Mtx tetraglutamate was slightly lower at 1.4pM (Appleman et al. 1988). The normal function of DHFR is to reduce the DHF produced during the synthesis of dTMP by TS, regenerating THF, the fully reduced form of folate. Inhibition of DHFR enzyme activity reduces the availability of THF in the intracellular environment, and hence decreases the supply of reduced folate cofactors for synthesis of purines and dTMP and other cellular processes (see figure 1.2). This blockade of the normal folate cycle causes inhibition of DNA synthesis and results in a halt of cell proliferation. DNA synthesis is particularly important in rapidly proliferating cells, and this is the rationale behind the use of Mtx in treatment of malignancy. The inhibition of DHFR also leads to accumulation of DHF polyglutamate, which is an inhibitor of several folate-dependent enzymes. Allegra et al. demonstrated in MCF-7 cells that treatment with 1μM Mtx led to accumulation of DHF to greater than 30% of total cellular folate content, compared to less than 1% in untreated cells (Allegra et al. 1986). Mtx is catabolised to 7-hydroxy methotrexate (7-OH-Mtx) by the enzyme aldehyde oxidase (Fabre et al. 1986). 7-OH-Mtx is a less effective inhibitor of DHFR than Mtx itself. The extent of this catabolism varies from person to person and may contribute to individual variations in response to Mtx. In a study of low-dose Mtx used in treatment of RA, supplementation with folic acid was found to reduce the formation of 7-OH-Mtx in vivo, while 5-formyl-THF supplementation did not. This could contribute to differences of effect between the two supplements (Baggott and Morgan 2009).

Mtx has also been found to directly inhibit other enzymes which use reduced folate cofactors, which may contribute to the potent effects of Mtx. As well as indirect inhibition of the TS reaction by depletion of THF, Mtx has also been shown to directly inhibit TS. Polyglutamation of Mtx does not markedly increase its affinity for DHFR, but it does result in considerable increases in inhibitory potential for TS. The monogluatamated form of Mtx has a K_i of 13μM for TS, but for the pentaglutamate form the K_i is 0.047μM (Allegra et al. 1985a). Mtx can also inhibit AICARFT, an enzyme involved in the *de novo* synthesis of purine nucleotides (see figure 1.4). AICARFT catalyses the transfer of a one carbon unit from 10-formyl-THF to AICAR, producing formyl AICAR. Mtx tetra- and penta- glutamates can directly inhibit AICARFT, and are 2,500 times more potent inhibitors than the monglutamate form of the drug (Allegra et al. 1985b). AICARFT is also

inhibited by DHF (Baggott et al. 1986), which accumulates due to the primary effect of Mtx on DHFR. The inhibition of AICARFT blocks the de novo purine synthesis pathway, and results in accumulation of AICAR.

Fairbanks et al. suggested that Mtx can inhibit amidophosphoribosyl transferase (APRT), which catalyses the very first step of de novo purine synthesis (Fairbanks et al. 1999). This study involved measurement of the levels of purine and pyrimidine nucleotide pools in PHA-stimulated T-cells treated with Mtx. This study was carried out in T-cells, and does not concur with other studies that have shown an accumulation of AICAR in response to Mtx. The authors suggested that inhibition of APRT by Mtx leads to an accumulation of 5-phosphoribosyl-1-pyrophosphate and a consequent stimulation of de novo pyrimidine synthesis. APRT is not a folate dependent enzyme, so inhibition must be due to alteration in the amounts of another metabolic intermediate, rather than a folate species. Kinetic studies of Mtx inhibition of APRT have not been carried out, so it is not known if Mtx has a direct inhibitory effect on this enzyme. Others have shown that APRT is inhibited non-competitively by DHF pentaglutamate with a Kd of 3.4μM (Sant et al. 1992), and that inhibition of purine synthesis by Mtx is related to the accumulation of DHF which has an inhibitory effect on enzymes in this pathway (Allegra et al. 1987). It is likely that the inhibition of APRT observed by Fairbanks et al. is due to accumulation of DHF caused by Mtx inhibition of DHFR.

The rationale for the mode of action of Mtx in cancer treatment is inhibition of DNA synthesis secondary to inhibition of DHFR. Mtx is also effective in treatment of RA and other inflammatory diseases, but the mechanism of action in these cases is not certain due to the low doses used. One possibility is that inhibition of DNA synthesis leads to inhibition of proliferation of aberrantly activated immune cells that were potentiating symptoms of these diseases. However, THF synthesis can continue up until 95% of DHFR in the cell has been inhibited (Fleisher 1993), and concentrations of Mtx higher than needed to saturate DHFR are required to completely inhibit nucleotide synthesis (White et al. 1975). Since the doses of Mtx used to treat auto-immune disease are low, and some studies indicate that folic acid can be given without apparently reducing the efficacy of the drug, suppression of proliferation due to inhibition of DNA synthesis does not fully explain the effectiveness of low-dose Mtx treatment. Mtx has also been shown to cause apoptosis in a variety of cell types, and this property of Mtx may be important in both high and low-dose regimens. In addition to the anti-proliferative and apoptotic effects of Mtx, there are several alternative mechanisms which have been proposed to account for the anti-inflammatory activity of low-dose Mtx treatment. These mechanisms include release of adenosine, apoptosis of activated T-cells or other cell types, effects on cytokine levels and

expression of adhesion molecules, release of adenosine or reduced formation of polyamines. These aspects of Mtx activity will be discussed further in later sections of this chapter.

1.9 Apoptosis

A large part of this thesis consists of the investigation of the mechanism of Mtx-induced apoptosis in transformed B-lymphocyte cell lines. In order to provide a full understanding of the importance of this process, this section describes the mechanisms and physiological relevance of this process, as well as an overview of experimental methods used to investigate apoptosis.

1.9.1 The cellular process of apoptosis

Apoptosis is a term used to describe a coordinated, energy-dependent process of cell death. The characteristic features of apoptosis are cell shrinkage and chromatin condensation, followed by nuclear fragmentation, and formation of protrusions of the plasma membrane known as 'blebbing'. Ultimately the cell breaks up into membrane-bound apoptotic bodies which are then engulfed by phagocytotic cells. Apoptosis occurs as a normal part of embryonic development, and is also important in immune responses to infection, homeostasis of immune cell populations, and in removal of cells irreparably damaged by disease or cytotoxic agents. The physiological benefit of this process is that it allows for removal of cells without release of the intracellular contents, which would induce an inflammatory response. There are specific mechanisms and signalling pathways which are involved in causing cells to undergo apoptosis, and the regulation of apoptotic signalling is highly complex.

The caspases are a group of cysteine proteases, certain members of which are centrally involved in the apoptotic process. Caspases are normally present in the cytoplasm as inactive pro-caspases. Apoptotic stimuli causes initiation of a proteolytic cascade, beginning with cleavage and activation of initiator caspases, which then go on to cleave and activate effector caspases, followed by activation of various proteases and endonucleases which result in the ultimate destruction of the cell. The initiation of this cascade is tightly regulated by a large number of other proteins. There are two main pathways by which the initial apoptotic stimulus can originate. A schematic diagram of the pathways of apoptosis is presented in figure 1.6.

The death-receptor or 'extrinsic' pathway of apoptosis involves ligation of membrane bound death receptors, resulting in activation of Caspase-8. These receptors are part of the TNF-receptor superfamily, and include CD95 (also known as Fas). Death receptors have intracellular protein domains called death domains (DD). When the receptor is activated by binding its specific ligand, the cytosolic adaptor protein FADD is recruited via binding to the receptor DD. FADD in

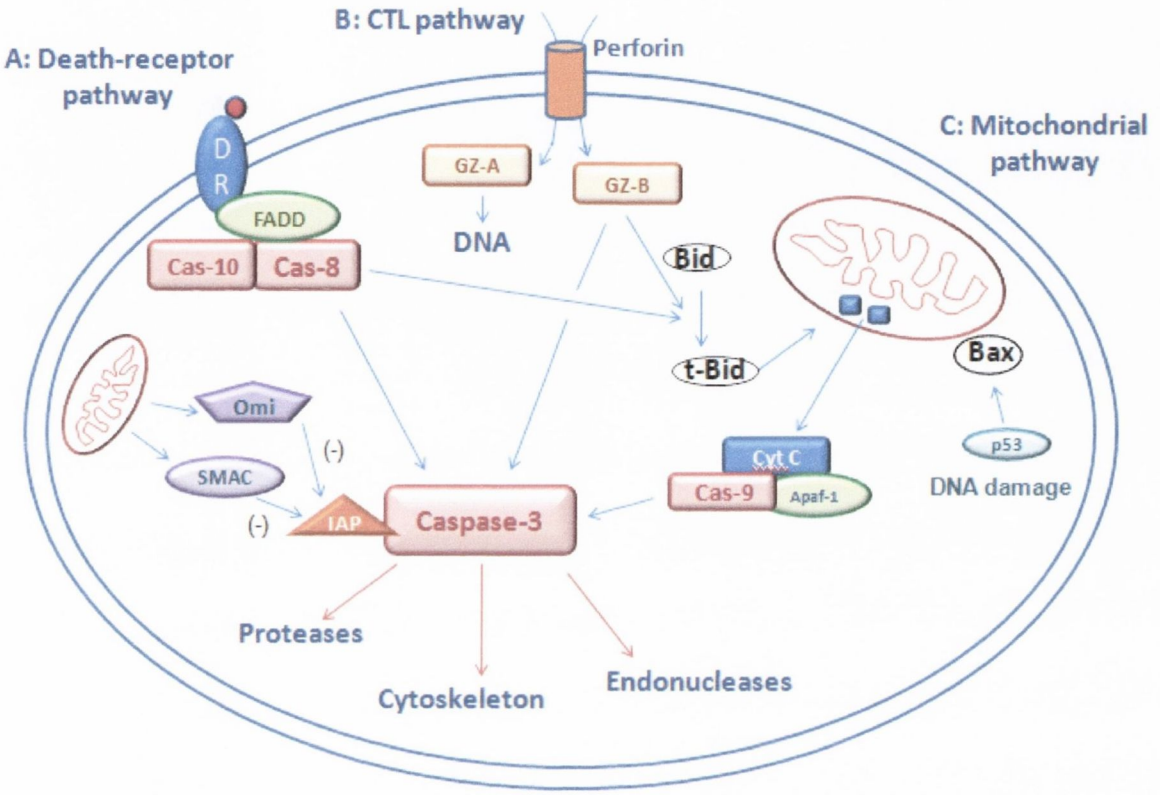

Figure 1.6 Schematic diagram of apoptotic pathways

A: Death receptor pathway. **DR**, death receptor; **FADD**, adaptor protein; **Cas-8**, caspase 8; **Cas-10**, caspase 10; **IAP**, inhibitor of apoptosis protein. B: Ctyotoxic T-lymphocyte (**CTL**) pathway. **GZ-A**, granzyme A; **GZ-B**, granzyme B. C: Mitochondrial pathway. **Cyt C**, cytochrome C; **Cas-9**, caspase 9; **Bax, Bid** are members of the Bcl-2 protein family; **t-Bid**, truncated Bid.

turn recruits procaspase-8 and procaspase-10, along with other proteins to form a complex called the death-inducing signalling complex (DISC). The formation of this complex allows proteolytic cleavage of the procaspases, resulting in activation of caspase-8 and caspase-10 (Kantari and Walczak 2011).

The mitochondrial or 'intrinsic' pathway of apoptosis is mediated through changes in the mitochondria, resulting in activation of Caspase-9. Activation of this pathway occurs in response to DNA damage or oxidative stress. Cytochrome C is released from the mitochondrion, where it associates with pro-caspase 9 and APAF-1 to form a protein complex known as the apoptosome (Cain et al. 1999). Formation of this structure allows autoproteolytic activation of Caspase-9. The release of cytochrome C is regulated by a group of proteins known as the Bcl-2 family. The family is sub-divided into three groups based on function and structural similarity. One group are pro-survival proteins (e.g. Bcl-2 and Bcl-XL), a second group known as BH3-only proteins are pro-apoptotic (e.g. Bid, Puma), and a third group have pore-forming properties (e.g. Bax, Bak) (Westphal et al. 2011). It is thought that the pore-forming factors can associate with the mitochondrial membrane to facilitate cytochrome C release, and that this process is regulated by the BH3-only proteins which can also be bound and sequestered by the pro-survival proteins (Vaux 2011). The permeability of the mitochondrial membrane for cytochrome C is therefore determined by the relative levels of pro- and anti- apoptotic Bcl-2 proteins. Apoptotic stimuli result in an alteration of this balance, either by causing activation of BH3-only proteins which are present in the cytosol in inactive forms, or by up-regulating transcription of BH3-only proteins. For example, transcription of the BH3-only protein Puma is up-regulated in response to DNA damage via transcription factor p53 (Nakano and Vousden 2001).

Further regulation of apoptosis is achieved downstream of release of cytochrome C from the mitochondria. Heat shock proteins can inhibit assembly of the apoptosome, and inhibitor of apoptosis proteins (IAPs) act by inhibiting caspase activity. There are also other proteins released from the mitochondria which promote cell death by apoptosis, for example Smac/DIABLO, which can bind to IAPs and interfere with their activity (Vaux 2011).

The initiator caspases (caspase-8, caspase-9) have the ability to cleave and activate caspase-3, the central 'effector' caspase (Slee et al. 2001). There is also cross-talk between the different activation pathways, for example caspase-8 has been shown to become active downstream of caspase-9 (Slee et al. 1999). One of the substrates of caspase-8 is Bid, which is cleaved to form tBid, a pro-apoptotic protein which stimulates the mitochondrial apoptosis pathway. Signalling through death receptors is sufficient to cause apoptosis in some cell types, but in others

activation of the mitochondrial pathway is also required (Barnhart et al. 2003). The numerous levels of regulation of apoptotic initiation allow integration of different signals and pathways, so that cell death will only occur when the initiating stimulus is sufficiently strong.

Cytotoxic T-lymphocytes can stimulate infected cells to undergo apoptosis by stimulation through the death receptor pathway. They can also cause apoptosis by release of a pore-forming molecule called perforin and granules containing the enzyme granzyme B. This enzyme enters the target cell , where it can directly activate caspases and also cause activation of the mitochondrial pathway through cleavage of Bid (Elmore 2007). Granzyme A is another enzyme produced by cytotoxic T-lymphocytes. It is a trypsin-like protease and can cause apoptosis in a caspase-independent manner by direct targeting to the nucleus causing DNA degradation (O'Connell and Stenson-Cox 2007).

Once the central effector caspase, caspase-3, is converted to its active form, it acts on a large number of protein substrates resulting in destruction of the cellular structure and ultimately cell death. During apoptosis chromosomal DNA is degraded by a nuclease called caspase-activated deoxyribonuclease (CAD). CAD is normally present in the cytosol as a complex with its inhibitory protein, ICAD. Caspase 3 causes cleavage of ICAD, freeing CAD which then translocates to the nucleus and catlyses inter-nucleosomal hydrolysis of DNA (Sakahira et al. 1998). Cytoskeletal proteins such as the actin-binding protein gelsolin, are also cleaved by caspase-3 (Kothakota et al. 1997). This results in disassembly of the cytoskeleton and ultimately cell fragmentation into apoptotic bodies. During apoptosis, the lipid phosphatidylserine is translocated to the outer leaflet of the plasma membrane, targeting the cell for recognition by phagocytes (Fadok et al. 2001). The exact mechanism of this lipid re-arrangement is not understood, but it is known to occur downstream of caspase activation.

As well as the caspases, there are other types of protein which are involved in apoptosis, although, these are not as well understood at the present time. These proteins act by promoting caspase activation, and there is some evidence of pathways that may be independent of caspase activation (Stenson-Cox et al. 2003). Granzyme A, an enzyme produced by cytotoxic T-lymphocytes has already been mentioned above. Other types of protein thought to be involved in apoptosis include calpains, cathepsins and serine proteases. Calpains are cysteine proteases which are activated in response to increases in intracellular calcium, and result in activation of procaspase-12 (Tan et al. 2006). Cathepsins are a group of proteases which can be released from lysosomes during apoptosis and are thought to promote caspase activation through activation of the mitochondrial pathway (Stoka et al. 2007). Omi (also known as HtrA2) is a trypsin-like serine

protease released from the mitochondria during apoptosis. Part of its mechanism of action is by binding to and inhibiting IAPs, thereby promoting caspase activity. The serine protease activity of Omi may also be able to cause apoptosis independently of caspases, as over expression of Omi induced apoptosis when caspases are inhibited (Suzuki et al. 2001). AP24 is a chymotrypsin-like protease which is activated by several cytotoxic stimuli, and indirectly causes DNA fragmentation by activating a nuclease called L-DNAse II (Altairac et al. 2003).

1.9.2 Experimental methods used to detect apoptosis

The methods used to study apoptosis in the laboratory rely on detection of characteristic morphological and biochemical changes that occur in cells during the process of cell death. There are other pathways of controlled cell death which are recognized, e.g. autophagy, a process involving degradation of organelles and which can lead to cell death. It is recognized that cell death does not occur in exactly the same way in every cell type and for every type of stimulus (Golstein and Kroemer 2007). In an experimental setting it is also important to distinguish apoptosis from necrosis, an unregulated form of cell death where cells swell and lyse, releasing their cytosolic contents. There are certain techniques and molecular markers which currently make up the 'gold standard' for experimental detection of apoptosis. As there is no single method for definitive detection of apoptosis, it is common practice to use several different types of assay to demonstrate that cell death occurs by apoptosis in a particular in vitro system. In cell culture there are no phagoctyes to engulf apoptotic cells, so these cells ultimately enter a stage of secondary necrosis where membrane permeability is lost. To reflect this, it is also good practice to observe the cell death process at several time points in order to ensure detection of apoptotic markers at the optimum point (Krysko et al. 2008b).

The major difference between apoptosis and necrosis is the morphological change within the cell. During apoptosis the cell shrinks, and the nucleus breaks up into fragments. These changes can be detected by electron or light microscopy, or by flow cytometry. As cells pass through a flow cytometer, one of the parameters detected is the direction of light scatter caused by each cell, in terms of forward scatter (FSC) or side scatter (SSC). During apoptosis, cells shrink in size and increase in granularity due to nuclear fragmentation. This can be detected as a decrease in FSC and an increase in SSC compared to healthy cells (Krysko et al. 2008a).

Degradation of chromosomal DNA is another characteristic feature of apoptosis. This can be detected by extraction of DNA and separation on an agarose gel. In some cases DNA from apoptotic cells forms a DNA 'ladder' of fragments differing in size by increments of approximately 200bp, and in others DNA fragments ranging in size from 50-300 kilobases are found

(Oberhammer et al. 1993). Another way to measure DNA degradation is by fixation of cells with 70% ethanol followed by staining with the DNA dye propidium iodide (PI). Subsequent analysis by flow cytometry allows detection of apoptotic cells, which present as cells with lower than normal DNA content (Krysko et al. 2008a). DNA strand breaks can also be detected in fixed cells by labelling the breaks with fluorescently conjugated molecules, which can be quantified by flow cytometric analysis (Darzynkiewicz et al. 2008).

Phosphatidyl serine (PS) exposure on the outer surface of cells is a marker which is commonly used to detect apoptosis by flow cytometry. This process is regarded as an early event in the apoptotic mechanism (Martin et al. 1995). To detect PS, a fluorescently labelled conjugate of the PS binding protein Annexin V is used to label live unfixed cells. The intensity of fluorescent labelling is proportional to the amount of PS exposure, and this is quantified by flow cytometry. The cells are usually stained with PI simultaneously to allow detection of secondary necrosis. PI cannot cross the plasma membrane and so does not stain the DNA of live cells, but will stain DNA of necrotic cells which have lost membrane permeability (Krysko et al. 2008a).

Caspase activation is also regarded as a central feature of apoptosis. Caspase activity in cell extracts can be quantified by enzyme-linked immunosorbent assay (ELISA), or can be detected by Western blot using antibodies specific for the active form of the caspase. Cleavage of caspase substrates such as poly-ADP-ribose polymerase (PARP) can also be detected by Western blot (Huerta et al. 2007). General caspase inhibitors and inhibitors of specific caspases are available which allow investigation of the caspase-dependence of cell death following a particular stimulus.

Mitochondrial changes during apoptosis can be detected by using dyes which are sensitive to changes in mitochondrial membrane potential, or by detection of cytochrome C release. These parameters can be detected by flow cytometry of live cells, or in isolated mitochondria (Arnoult 2008). Western blotting can also be used to detect the presence and relative abundance of Bcl-2 family proteins, which have a central role in regulating the mitochondrial pathway of apoptosis, as described above.

1.9.3 Apoptosis in response to DNA damage

Of particular interest in the context of methotrexate are the signalling events that lead to activation of apoptosis in response to DNA damage. This process is not yet fully understood, but there is one key protein which has received much attention. This is the transcription factor protein p53, nicknamed 'guardian of the genome' due to its central role in cellular responses to DNA damage (Lane 1992). In response to DNA damage, p53 is capable of causing cell cycle arrest, activating DNA repair mechanisms or initiating apoptosis if the DNA damage is sufficiently severe.

The p53 protein is constitutively present in the cell, and under normal conditions levels of p53 are regulated by binding to MDM2, which targets p53 for proteasomal degradation. Expression of the MDM2 gene is induced by p53 itself, so this feedback loop preserves p53 at a constant level under normal conditions. In response to DNA damage, specific residues on the p53 protein become phosphorylated, blocking its interaction with MDM2 and hence allowing accumulation of p53 (Oren 1999). P53 is then capable of inducing expression of genes involved in apoptosis, such as Puma and Noxa, or in cell cycle arrest such as p21 and cyclin-G1. It is thought that post-translational modifications involving acetylation of specific lysine residues of p53 may act as a 'sensor' mechanism, influencing which genes are activated by p53 at a particular time (Yoshida and Miki 2010). The regulation of p53 function is complex, and includes regulation of its DNA binding properties by post-translational modifications, regulation of transcription of the p53 gene by other transcription factors, and regulation of p53 protein levels by targeting for proteasomal degradation.

The mechanisms by which p53 is activated in response to DNA damage are not fully understood, although some elements of this undoubtedly complex process have been elucidated. Transcription of p53 is upregulated by protein kinase C δ (PKCδ) in response to DNA damage, in collaboration with the transcription factor Btf (Liu et al. 2007). The transcription factor Ras responsive element-binding protein 1 (RREB-1) has also been shown to bind to the p53 promoter in response to DNA damage and induce expression of p53 (Liu et al. 2009). There are also some kinases which have been shown to phosphorylate and activate p53. Homeodomain-interacting protein kinase-2 (HIPK2) is a nuclear kinase that activates p53 by phosphorylating serine 46 of the protein, and is involved in the apoptotic response to UV-irradiation (D'Orazi et al. 2002). Serine 46 of p53 is also phosphorylated by dual-specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2), which translocates to the nucleus in response to DNA damage and induces p53 dependent expression of pro-apoptotic genes (Taira et al. 2007).

The effects of p53 on apoptosis are mainly exerted through the mitochondrial pathway, as it is involved in regulating expression of pro-apoptotic Bcl-2 family proteins. There is a second function of p53 in mitochondrial promotion of apoptosis, as it has also been shown to interact with these proteins at the mitochondrial membrane and facilitate release of pro-apoptotic factors (Wolff et al. 2008). However p53 also induces expression of certain death receptors such as Fas and DR5, and can therefore sensitise cells to apoptosis via the death receptor pathway (Müller et al. 1998).

1.9.4 Methotrexate and apoptosis

Mtx has a general anti proliferative effect due to its ability to inhibit DNA synthesis. In 1990, Barry et al. demonstrated that exposure of CHO cells to Mtx caused fragmentation of cellular DNA followed by cell shrinkage and loss of membrane integrity, features associated with apoptotic cell death (Barry et al. 1990). There is now evidence that Mtx causes apoptosis in a variety of cell types, and that this may be an important aspect of the therapeutic effect of Mtx.

There are many reports of Mtx-induced apoptosis in cancer cells and cell lines, including human leukaemia cell lines (da Silva et al. 1996; Gazzanelli et al. 2000), hepatoma (Müller et al. 1997), cervical cancer (Mazur et al. 2009), lung cancer (Huang et al. 2010) and head and neck cancer (Kraljevic Pavelic et al. 2008). The exact mechanism by which apoptosis proceeds downstream of Mtx exposure may vary depending on cell type. Several reports have indicated involvement of the extrinsic apoptotic pathway. Expression of the Fas (also termed CD95) receptor was upregulated in response to Mtx in CEM and Jurkat leukemic T-cell lines (Friesen et al. 1996) and also in hepatoma cells (Müller et al. 1997). Contrasting effects were observed in a different human leukemia cell line (HL-60), where Mtx was found to induce apoptosis and was associated with downregulation of Fas and BCL2 which is involved in the mitochondrial apoptotic pathway (Floros et al. 2006). In normal rat intestinal epithelial cells, Mtx was found to induce apoptosis with involvement of caspase-9 which is associated with the mitochondrial pathway, in contrast to caspase-8 which was not activated (Papaconstantinou et al. 2001).

There is considerable evidence that intracellular signalling events associated with the DNA-damage response are involved in activation of apoptosis in response to Mtx. Fritsche et al. (1993) demonstrated that p53 accumulated in nuclei of fibroblasts which were exposed to Mtx (Fritsche et al. 1993). In a study of a non-small cell lung cancer (NSCLC) cell line, Mtx was found to inhibit cell growth and induce apoptosis in a NSCLC cell line, and also to inhibit tumour formation in a mouse xenograft model. In this case apoptosis was p53-dependent and induction of p53 target molecules including Puma, Noxa, DR5 and p21 was observed (Huang et al. 2010). Kraljevic et al. (2008) examined Mtx-induced apoptosis in human laryngeal squamous carcinoma cells (HEp-2), which lack p53 protein expression. Mtx was found to induce apoptosis in a large proportion of the cultured cell population. Overexpression of the cyclin-dependent kinase (CDK) inhibitior p21 reduced the apoptotic effect of Mtx (Kraljevic Pavelic et al. 2008). P21 is a transcriptional target of p53, so this result suggests that apoptosis in response to Mtx is altered by downstream targets of the p53 pathway.

Frouin et al. examined the effects of Mtx in cell lines (colon cancer and HeLa) which had either normal DNA mismatch repair (MMR) function, or were deficient in mismatch repair. Mtx-induced apoptosis was detected in both types of cell and surprisingly, higher levels of apoptosis were detected in the cells with intact DNA repair function (Frouin et al. 2001). This suggests that activation of DNA repair mechanisms may be a part of the intracellular process which leads to induction of apoptosis in response to Mtx. In contrast to these results, another study showed that human endometrial adenocarcinoma cells lacking MSH2, a protein involved in the MMR process were more sensitive to Mtx than isogenic cells with functional MSH2 (Martin et al. 2009). This difference was attributed to accumulation of 8-hydroxy-2'-deoxyguanosine lesions in DNA, which were removed in the cells with functional MSH2, but not in the repair-deficient cells. The authors suggested that Mtx may be particularly beneficial in treatment of tumours with acquired MSH2 defects.

As well as its effects on DNA synthesis, a second cellular effect of Mtx is alteration of the distribution of folate cofactors within the cell. Allegra et al. demonstrated in the human breast cancer cell line MCF-7 that 3hr exposure to 1μM Mtx resulted in accumulation of DHF, and reduced levels of 5-methyl THF compared to untreated cells (Allegra et al. 1986). Increased plasma methionine and decreased plasma Hcy following Mtx treatment have been observed, indicating that Mtx reduces the normal flux through the methylation cycle (Broxson Jr et al. 1989). Accumulation of Hcy within the cell drives SAHH in the reverse direction, leading to increased levels of SAH which is a potent inhibitor of methyltransferase enzymes. The influence of Mtx on the methylation cycle could have subsequent effects on many levels of cellular function which are governed by methylation. In particular, this effect of Mtx has been linked to impairment of signalling through Ras, a G-protein which is involved in signalling pathways of cell growth and differentiation. Methylation by Isoprenylcysteine carboxymethyltransferase (ICMT) is a key step in post-translational processing of Ras, and is necessary for its membrane localisation and therefore its correct function in signal transduction. Winter-Vann et al. (2003) examined the effects of Mtx on Ras methylation and function in mouse embryonic fibroblasts (MEF) and DKOB8 cells, a human colon cancer cell line engineered to express inductible Ras. Mtx treatment increased levels of intracellular SAH, and caused 70% reduction in Ras methylation in DKOB8 cells. In MEFs, Mtx treatment caused mislocalisation of Ras to the cytosol and prevented signalling via the mitogen-activated protein kinase (MAPK) pathway which is downstream of Ras (Winter-Vann et al. 2003). This study suggests that Mtx interferes with Ras signalling due to inhibition of ICMT. Ras is one of many proteins involved in signalling which require methylation to ensure correct subcellular location and function, as described in section 1.5.4.1. As well as apoptosis, these

proteins are involved in signalling in response to growth factors, and processes such as cell adhesion and migration (Cushman and Casey 2011). The influence of Mtx on these methylation events could provide another mechanism whereby cell signalling is altered due to Mtx treatment.

1.10 Mechanisms of methotrexate action in inflammatory disease

Low-dose Mtx is used commonly to treat RA and psoriasis. Although these diseases are both described as autoimmune disorders, there are differences in pathology between the two. RA is primarily a disease of the joints, although other organs can also be affected. It is characterised by symmetrical joint inflammation, which progresses to cartilage and bone erosion in later stages of the disease. The underlying cause of RA is not completely clear, although it involves many arms of the immune system including aberrant activation of both T and B cells, and production of autoantibodies (McInnes and Schett 2007). Chronic inflammation of the joint in RA causes tissue damage, which is likely to contribute to sustained activation of the inflammatory response. Psoriasis is a disease characterised by the major symptom of scaly red lesions of the skin, although 10-30% of people with psoriasis also suffer from joint inflammation which is known as psoriatic arthritis. Psoriasis is regarded as a disorder driven by activated T cells, which stimulate hyperplasia of keratinocytes. Unlike RA, psoriasis is not associated with production of autoantibodies (Guttman-Yassky and Krueger 2007). Despite these differences in pathology, Mtx is an effective treatment for both diseases. It is recommended as the first line treatment for RA (Combe et al. 2007) and for moderate to severe psoriasis (Chládek et al. 2008).

1.10.1 Methotrexate-induced apoptosis of immune cells

Cells of the immune system can be divided into two broad categories, myeloid cells and lymphoid cells. These classifications are based on the pathways of differentiation during early development of immune cells. Cells of the myeloid lineage include granulocytes (basophils, neutrophils and eosinophils) and monocytes (macrophages). Cells of lymphoid lineage include B-lymphocytes, T-lymphocytes and natural killer (NK) cells. A diagram of immune cell lineage is provided in figure 1.7.

The immune system can also be divided functionally into the innate and adaptive immune response. Cells which are involved in the innate immune response include the granulocytes, macrophages, and NK cells. Monocytes and neutrophils can recognise and engulf extracellular pathogens such as bacteria. Monocytes further differentiate into macrophages, which as well as phagocytosis play an important role in activation of other immune cells via cytokine production, and can act as antigen-presenting cells resulting in T-cell activation. NK cells can recognise and kill virus-infected cells, and are also involved in the immune response to tumour cells.

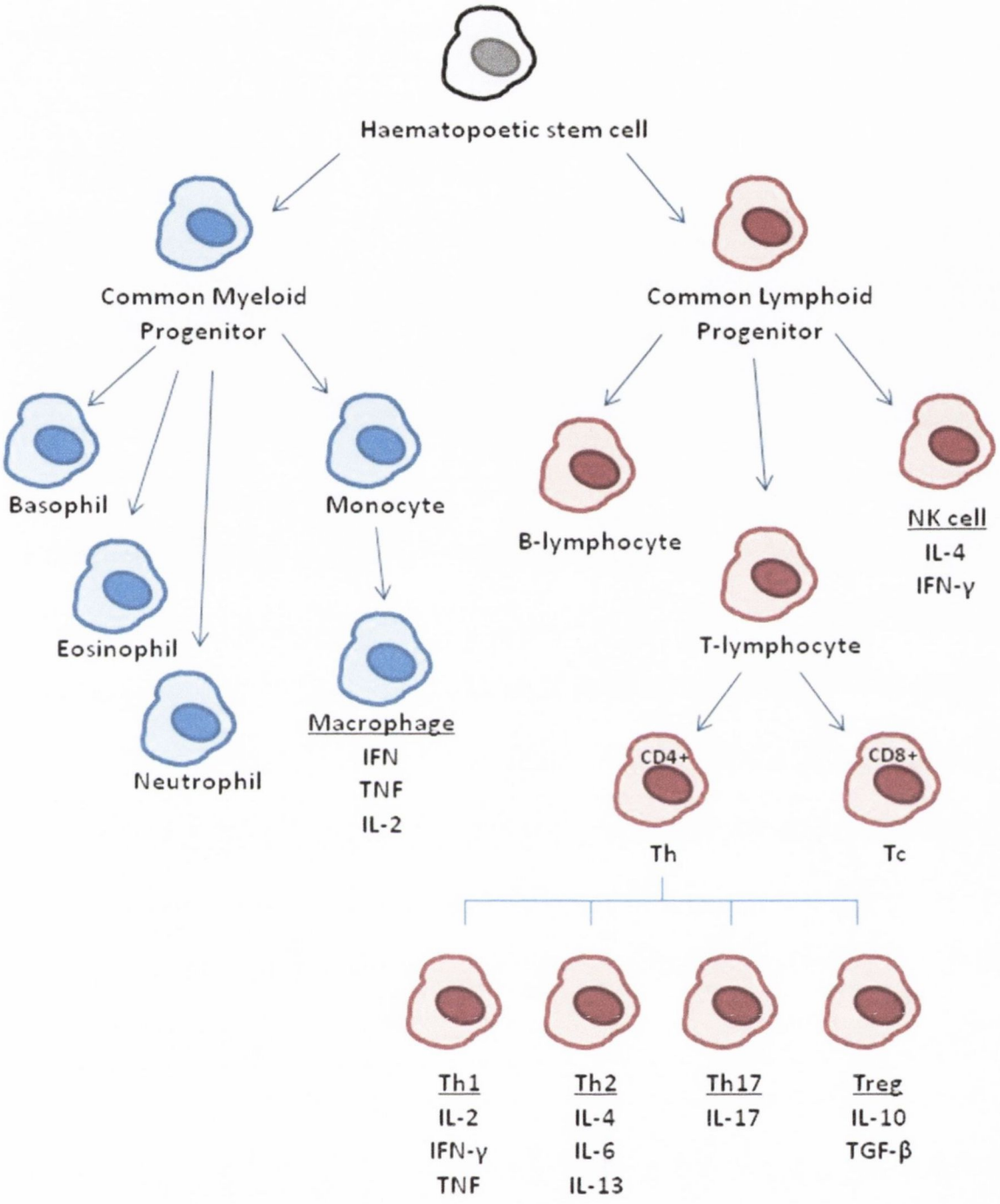

Figure 1.7 Diagram of immune cell lineage

This diagram presents a schematic description of the differentiation process giving rise to the various cells of the immune system. Selected cytokines which are produced by particular cell types are indicated underneath the cell name label. Th = helper T-lymphocyte, Tc = cytotoxic T-lymphocyte.

The primary cell types involved in the adaptive immune response are T and B lymphocytes, which are responsible for immune recognition of specific pathogens. B-lymphocytes mature in the bone marrow, and are capable of producing soluble antibodies. After activation by recognition of a specific pathogen, B-lymphocytes differentiate into antibody-producing plasma cells, or memory B-cells which become re-activated in the event of a second exposure to their specific pathogen. T-lymphocytes mature in the thymus, and function as antigen presenting cells. T-lymphocytes are also major regulators of the B-cell response and the immune system as a whole. T-lymphocytes are sub-divided into several categories, which are classified by the presence of cell surface molecules. The broadest division is between CD4+ T-helper cells and CD8+ cytotoxic T cells. Cytotoxic T-lymphocyes (Tc) are capable of directly killing cells which express specific antigens on the cell surface. CD4+ T-helper (Th) lymphocytes have a major role in regulation of other immune cells. Th cells and are further divided into Th1, Th2, Treg, and Th17 subsets on the basis of particular cytokines produced. The different subsets of Th cell have functionally distinct roles in regulation of the immune response. Th1 cells are considered to be pro-inflammatory, and produce Il-2, IFN-γ and TNF. Th2 cells produce IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13, and stimulate antibody production by B-cells as well as downregulate other arms of the immune response. Treg cells are also considered to be anti-inflammatory. Th17 cells are distinguished by production of IL-17 and are regarded as activators of immune responses.

Genestier et al. (1998) showed that Mtx can induce apoptosis of activated T cells. *In vitro* activation of T cells with phytohaemagglutinin (PHA) followed by Mtx treatment resulted in apoptosis. T cells isolated from patients who had undergone Mtx treatment also underwent apoptosis when subsequently activated by PHA *in vitro*. Apoptosis was found to be independent of Fas ligation, and could not be attributed to adenosine release (Genestier et al. 1998). Another study found that Mtx was accumulated by dividing CD4+ T cells, and caused an increase in the proportion of apoptotic cells. However, these effects were not seen in resting (non-dividing) T-lymphocytes (Nielsen et al. 2007). The same group showed that the effect of Mtx on activated T-lymphocytes differed depending on the mitogen used to stimulate the cells in vitro. PHA stimulation induced apoptosis in both dividing and non-dividing T cells, while stimulation with the antigens tetanus toxoid and *Candida albicans* only caused apoptosis in dividing T cells (Nielsen et al. 2007). It could be difficult to translate these results to the in vivo situation, as the method of activation in vitro affects outcome of apoptosis. However it is suggested that stimulation with antigens may better reflect the type of stimulation that occurs in the body during autoimmune disease.

Some experiments have suggested that Mtx-induced apoptosis of immune cells may be due to generation of reactive oxygen species (ROS). Mtx treatment resulted in increased intracellular peroxide levels and growth arrest in a monocyte cell line, and a more pronounced increase in peroxide and induced apoptosis in a Jurkat T-lymphocyte cell line (Phillips et al. 2003). Herman et al. compared the effects of Mtx on cell lines of lymphocytic and monocytic lineage. Apoptosis associated with Mtx treatment was accompanied by generation of ROS, and was partially inhibited by the anti-oxidant N-acetyl-L-cysteine (NAC) (Herman et al. 2005). A second observation of this study was that lymphocyte cell lines were more susceptible to Mtx-induced apoptosis than monocytic cell lines. However an in vivo study has demonstrated an effect of Mtx on apoptosis in neutrophils (which are of monocytic lineage) in patients with very early RA. Neutrophils isolated from untreated RA patients showed delayed apoptosis compared with the same cell type from healthy controls. Treatment with low dose Mtx restored the kinetics of apoptosis in RA patients to a level comparable with the control group (Weinmann et al. 2007).

The reports described above indicate that Mtx induces apoptosis in several types of immune cell, including T cells, neutrophils and monocytes. There is evidence that lymphocytes are more susceptible to apoptosis than monocytes, and that activated T cells may be more susceptible than resting cells. This property of Mtx could be beneficial in autoimmune diseases if it promotes removal of inappropriately activated immune cells. Since there is substantial networking between different types of immune cell during inflammation, an effect on one group of cells could have ensuing effects on other cell types, ultimately leading to a halt in the cycle of inflammation in autoimmune diseases. Apoptosis of one or more types of immune cell would also play a part in alteration of the cytokine environment present at sites of inflammatory disease.

1.10.2 Cytokine responses

Cytokines are a group of signalling molecules which act as regulators of immune cell growth and function. There is a very large number of cytokines which are divided into several sub-groups including interleukins (IL), tumour necrosis factors (TNF), interferons (IFN), transforming growth factors (TGF), colony stimulating factors, and chemokines. Cytokines are produced by and act on immune cells and other cell types such as endothelial cells via binding to specific cell-surface receptors. The effect of a particular cytokine can vary depending on the type of cell to which it is bound. In general, certain cytokines are associated with activation of immune responses and inflammation, for example IL-1, IL-2, IL-6, TNF and IFN-γ. There are also cytokines which function to dampen the immune response, for example IL-4, IL-10 and TGF-β.

Inflammatory disease is characterised by infiltration of immune cells into the affected areas. The initiating events causing this inappropriate activation of the immune system are not clear, and are likely to vary depending on the specific disease. However it is understood that the accumulated immune cells produce cytokines at the site of inflammation, leading to sustained activation of the immune and inflammatory response in the area. Due to its effectiveness in treating inflammatory diseases, many studies have investigated the effects of low-dose Mtx on cytokine levels.

In several cases, low-dose Mtx treatment has been shown to reduce the production of pro-inflammatory cytokines. Constantin et al. investigated cytokine levels in vitro in peripheral blood mononuclear cells (PBMCs) isolated from healthy controls, and from RA patients who were as yet untreated or who had undergone treatment with low-dose Mtx. Basal levels of IL-4 were lower in the untreated group than in the healthy controls. In vitro exposure of the cells to Mtx increased levels of anti-inflammatory IL-10 in all groups. In cells from the untreated patient group, IL-4 was increased and IL-2 and IFN-γ were decreased following Mtx exposure, changes which are associated with reduction of inflammation (Constantin et al. 1998). Similar results were found in a study which investigated the effect of Mtx on T cells in vitro. T cells were isolated from RA patients and healthy controls. Mtx treatment during in vitro stimulation suppressed TNF and IFN-γ production by T cells from both groups. IL-4 levels were increased by Mtx in naive but not memory T-cells (Hildner et al. 1999). This suggests that the effects of low dose Mtx on cytokine production could vary depending on the activation state of immune cells. A further study on PBMCs from active RA patients found that Mtx treatment induced secretion of IL-10, and promotes a Th2 type response (Herman et al. 2008). A recent study showed that increased production of IL-10 by PBMCs *in vitro* was correlated with improved clinical response of RA to Mtx treatment (Seitz et al. 2001).

In a mouse model of collagen-induced arthritis, Mtx injection before administration of collagen completely prevented disease symptoms. Mtx reduced the production of TNF by T cells, but not by macrophages, in a dose-dependent manner. In this case there was a lesser reduction of IFN-γ production, and no difference in Il-4 caused by Mtx. The same study also reported that the effects of Mtx were abolished in a mouse with an inactivated TNF-α gene, underlining the importance of TNF-α as a mediator of Mtx anti-inflammatory action (Neurath et al. 1999). Further immunosuppressive effects of Mtx were demonstrated in a rat model of collagen-induced arthritis. Low-dose Mtx treatment was found to promote the expansion of Treg cells and to induce a Th1 to Th2 shift in the cytokine profile (Xinqiang et al. 2010).

1.10.3 Adhesion molecules

Adhesion molecules are cell surface molecules which mediate cell-cell interactions between systemic cells and cells of the immune system. Adhesion molecules are expressed by endothelial cells and also by circulating immune cells. Selectins are one class of adhesion molecules, including E-selectin which is expressed on endothelial cells, L-selectin which is expressed on leukocytes and P-selectin which is expressed on platelets. Intercellular adhesion molecule 1 (ICAM-1) and VCAM-1 are members of the immunoglobulin superfamily, and are expressed in endothelial cells and leukocytes. Some adhesion molecules are expressed constitutively, for example L-selectin on leucocytes. Expression of others is induced by signalling events, for example E-selectin is expressed on endothelial cells in response to pro-inflammatory cytokines. One function of adhesion molecule interactions is to mediate migration of lymphocytes into tissues, and altered expression of adhesion molecules is known to play a role in psoriasis and RA. Efalizumab is a monoclonal antibody which blocks the interaction of lymphocytes with the adhesion molecule ICAM-1. This drug was effective in treatment of psoriasis (Berger and Gottlieb 2007), but has since been withdrawn due to side effects associated with immunosuppression. Several studies have shown that low-dose Mtx is also associated with changes in expression of adhesion molecules, and this may be another facet of its anti-inflammatory action.

Much of the work in this area has used histological staining of skin biopsies from patients with psoriasis to examine the immune cells, cytokines and adhesion molecules involved in the disease. Mtx treatment has been shown to reduce hyperplasia, lymphocyte infiltration, markers of proliferation such as Ki67 and proliferating cell nuclear antigen (PCNA), along with expression of adhesion molecules CD31 and ICAM-3 in psoriatic skin lesions (Yazici et al. 2005). Another study involving histopathological analysis of psoriatic skin lesions showed reduced levels of the adhesion molecules ICAM-1, VCAM-1 and E-Selectin after Mtx treatment (Torres-Alvarez et al. 2007). This group also reported a reduction in leukocyte infiltration by Mtx treatment, but noted the persistence of cytotoxic CD8+ T cells which may contribute to reactivation of disease after treatment is discontinued. Kane et al. (2004) investigated the effects of Mtx treatment in synovial biopsies of psoriatic arthritis. There was reduced lymphocyte infiltration after treatment, along with reduced levels of ICAM-1 and E-selectin. Matrix metalloproteinase -3 (MMP-3) and IL-8 were also reduced (Kane et al. 2004).

T-cells which express the skin-homing marker cutaneous lymphocyte-associated antigen (CLA) are associated with psoriatic plaques and have been demonstrated to appear in the early stages of disease (Davison et al. 2001). An interesting study monitored the effect of Mtx treatment on expansion of CLA+ positive T cells by taking daily blood samples from a psoriasis patient who

received weekly Mtx doses over a 5 week period. Levels of CLA+ T cells decreased in the days immediately after the Mtx dose, then increased again until the next dose was administered. When treatment was stopped, disease symptoms reoccurred after 9 days. Reccurrence of disease was associated with an increase in CLA+ cells in blood, and increased E-selectin and leukocyte infiltration in skin biopsy (Sigmundsdottir et al. 2004). The effects of Mtx on CLA were confirmed in a study by Johnson et al, who found that Mtx suppressed T cell activation and reduced expression of CLA and ICAM-1 in PBMCs from healthy donors (Johnston et al. 2005). In this case it was found that the expression of CLA and decrease in ICAM-1 levels was reversed by addition of 5-formyl THF, demonstrating that these effects of Mtx are downstream of its influence of folate metabolism. The reduction in CLA expression was enhanced by adenosine receptor agonists (Johnston et al. 2005). This provides evidence of a link between adenosine release as discussed in the section 1.10.4, and the effect of Mtx on expression of adhesion molecules.

Klimiuk et al. (2002) investigated the levels of soluble adhesion molecules in serum from patients with early signs of RA, before and after Mtx treatment. After 6 months of treatment, levels of soluble ICAM-1, VCAM-1 and E-selectin were significantly decreased, and this was correlated with clinical improvement (Klimiuk et al. 2002). There is increasing focus on early treatment of RA, to prevent later effects of the disease which cause devastating loss of function due to joint erosion. This study suggests that Mtx can be a particularly effective therapy if administered early in the course of disease. Further evidence of the effect of Mtx on adhesion molecules came from a study carried out in HUVEC cells. Low-dose Mtx treatment reduced TNF-α-induced expression of ICAM-1 and to a lesser extent VCAM-1 (Yamasaki et al. 2003).

The above studies demonstrate that treatment with low-dose Mtx is associated with a reduction in expression of adhesion molecules, with most significant effect on ICAM-1, and also E-selectin and on CLA in treatment of psoriasis. It is suggested by several of these authors that effects of Mtx on adhesion molecules may inhibit vascularisation. This is an important component of disease pathology in both psoriasis and rheumatoid arthritis, and may represent another way in which Mtx could improve clinical symptoms.

1.10.4 Adenosine release

Extracellular adenosine functions as a signalling molecule, which can cause vasodilation and also has important functions in regulation of immune responses. Adenosine can be directly released from cells through nucleotide transporters (Dagnino et al. 1991), or adenine nucleotides can be exported from the cell and subsequently converted to adenosine by ectonucleotidases (Lennon et al. 1998). Adenosine concentrations in the extracellular environment are normally less than 1μM,

but can reach much higher levels in cases of cellular injury or inflammation. Plasma levels of adenosine were found to be 8.4μM in cases of systemic sepsis, higher than in healthy controls which had a mean plasma adenosine level of 0.8μM (Martin et al. 2000). HPLC analysis of synovial fluid from joints of RA patients found adenosine concentrations of 10-100μM (Sottofattori et al. 2001). Adenosine acts by binding to specific adenosine receptors on the surface of target cells. All of the adenosine receptors are G-protein coupled receptors (GPCRs), and there are four known subtypes of adenosine receptor; A_1, A_{2A}, A_{2B} and A_3. Adenosine receptors are expressed on many cell types including endothelial cells and immune cells (Haskó and Cronstein 2004) (Review). Specificity of adenosine action is restricted to the localised area where it is released, as it has a short half-life in plasma. The response of a specific cell type to adenosine may also depend on the concentration of extracellular adenosine in the local environment. In general, adenosine has an inhibitory effect on cells of the immune system. Extracellular adenosine inhibits superoxide production by neutrophils in vitro (Cronstein et al. 1983) and subsequent work has shown that inhibition of neutrophil function by adenosine is mediated through A_{2A} and A_3 receptors (Bouma et al. 1997). Adenosine has also been shown to mediate immunosupression by reducing production of the pro-inflammatory cytokine IL-12 and TNF-α by monocytes and macrophages (Hasko et al. 2000; Link et al. 2000).

There is a large body of work which suggests that Mtx mediates its anti-inflammatory action partially through increase of adenosine concentrations in the extracellular environment. Within the cell, Mtx and DHF inhibit the enzyme AICARFT, resulting in accumulation of the substrate AICAR. AICAR can be metabolised to adenosine or AMP, both of which can be transported out of the cell by nucleoside transporters. Adenosine is metabolised by ADA, but this enzyme is inhibited by AICAR (Baggott et al. 1986). Therefore the downstream effects of Mtx enzyme inhibition lead to accumulation of adenosine and promote its efflux from the cell. Cronstein et al. were the first to demonstrate *in vitro* that Mtx can result in production of extracellular adenosine from human fibroblasts and umbilical vein endothelial cells (Cronstein et al. 1991). This study also showed that extracellular adenosine reduced adherence of neutrophils to fibroblasts, because this interaction was promoted by addition of exogenous ADA to the culture medium. It has been shown that Mtx treatment is ineffective in mice lacking ecto-5'-nucleotidase, an enzyme which converts AMP to adenosine (Montesinos et al. 2007). This suggests that methotrexate increases intracellular AMP concentration which is subsequently converted to adenosine by extracellular processes. A diagram of adenosine metabolism is presented in figure 1.5.

Further evidence for the involvement of adenosine in the anti-inflammatory action of Mtx has come from experiments carried out using mouse models of inflammation. Montesinos et al.

induced inflammation in mice using an air-pouch model, and measured inflammatory mediators in the inflammatory exudates. Compared to untreated mice, low-dose Mtx treatment for 5-weeks before induction of inflammation increased adenosine concentration in inflammatory exudates. In wild-type mice, infiltration of lymphocytes and TNF-α were reduced by Mtx treatment, but these effects were not observed in mice which were knockouts for the A_{2A} or A_3 receptor (Montesinos et al. 2003). This suggested that both A_{2A} receptors and A_3 receptors were involved in the anti-inflammatory response. Using a different model of inflammation, the same group reported that A_{2A} adenosine receptors were preferentially involved in response to Mtx. When acute peritoneal inflammation was induced by injection of thioglycollate, Mtx-treated mice had greater adenosine concentrations than untreated animals, similar to the previous study (Montesinos et al. 2006). However, Mtx treatment was associated with improvement of inflammatory markers in wild type and A_3 knockout mice, but not A_{2A} knockouts. Reduced leukocyte infiltration and TNF-α, and increased levels of the anti-inflammatory cytokine IL-10 were observed in wild-type and A_3 knockout mice, but not in A_{2A} knockouts. A study by a different group found that A_3 receptors were increased in PBMCs from RA patients, and that in rats with adjuvant-induced arthritis, treatment with Mtx plus A_3 receptor agonist produced an additive reduction in disease (Ochaion et al. 2006). Clearly adenosine released due to Mtx treatment can act on any of the adenosine receptor subtypes, but the type of receptor present depends on what cell types are involved and the site of inflammation.

A study by Riksen et al. (2006) used the vasodilation response to adenosine or dipyridimole, (an inhibitor of the nucleoside transporter) as a read out of adenosine receptor stimulation. This method was used to compensate for the fact that it is difficult to directly measure adenosine concentrations in plasma due to its short half-life (Moser et al. 1989). They showed that in patients who were undergoing Mtx treatment for RA, vasodilation response to both agents was enhanced (Riksen et al. 2006). This provides evidence that Mtx increases response to adenosine in humans, and suggests that both intracellular and extracellular generation of adenosine is involved. In support of the above, it has been shown that genetic polymorphisms in enzymes involved in adenosine release are predictive of clinical response to Mtx (Wessels et al. 2006).

Some studies have investigated the downstream effects of adenosine, and how the signal from adenosine receptors might be transmitted and go on to modulate inflammation. NURR1 is an orphan nuclear receptor, which is involved in transduction of multiple types of inflammatory signal. Both Mtx and adenosine suppress expression of NURR1, and it has been shown that the effect of Mtx on NURR1 is mediated through the A_{2A} receptor (Ralph et al. 2005). NF-κB is another central signalling pathway which is involved in immune signalling. Mtx reduces NF-κB

activation through an adenosine-dependant mechanism (Majumdar and Aggarwal 2001). A_3 receptor agonists are also known to signal through the NF-κB pathway (Bar-Yehuda et al. 2007). NURR1 and NF-κB represent two pathways by which adenosine may mediate anti-inflammatory signalling, and which may therefore be influenced by Mtx treatment.

1.10.5 Polyamines

Polyamines are a group of molecules derived from amino acids, including spermine, spermidine and putrescine. They have a role in cell growth and development, and have also been associated with cancer (Gerner and Meyskens 2004). Polyamines also act as scavengers of ROS. Increased polyamine levels are found in synovial fluid of patients with RA (Hawkes et al. 1994). dcSAM is required for the synthesis of polyamines, as described in section 1.5.4.1. Treatment with Mtx may reduce polyamine levels if it acts by reducing the availability of SAM for formation of dcSAM. Nesher et al. (1996) examined this possibility in cultured lymphocytes isolated from RA patients. Treatment of the cells with Mtx reduced the concentration of spermine and spermidine, but did not affect levels of putrescine. Addition of either 5-formyl THF or SAM to the culture medium prevented the Mtx-associated decrease in polyamine levels, indicating that the effect was due to depletion of SAM (Nesher et al. 1996). These results reflect the function of dcSAM in polyamine synthesis as it is not required for synthesis of putrescine but is used in subsequent synthesis of spermidine from putrescine (See figure 1.5). The role of polyamines as ROS scavengers could also influence Mtx-induced apoptosis in immune cells. Increased expression of ornithine decarboxylase (ODC) can reduce Mtx-induced apoptosis. ODC is the first enzyme in the polyamine pathway, and so overexpression of ODC would promote increased synthesis of putrescine. Huang et al. demonstrated that exogenous putrescine as well as over-expression of ODC could decrease Mtx-induced ROS production and apoptosis in T-lymphocyte cell lines (Huang et al. 2005).

Despite the many studies which have been carried out, and the evidence to support the several mechanisms described above, there is still no consensus on exactly how low-dose Mtx exerts its anti-inflammatory action. It is likely that more than one mechanism is involved. The several mechanisms discussed here need not be exclusive as there are ways in which they could be inter-related. For example, the expression of adhesion molecules is influenced by signalling from cytokines. Apoptosis or reduced proliferation of immune cells would alter cytokine production at the site of inflammation, thereby altering expression of adhesion molecules. Signalling via adenosine receptors may also ultimately result in apoptotic or anti-proliferative effects. It seems likely that the anti-proliferative and apoptotic effects of Mtx are most important, as this will dampen the hyper-proliferative and inflammatory tendencies in auto-immune disease. This will

have a subsequent effect in reducing production of pro-inflammatory cytokines from immune cells.

1.11 Interaction of methotrexate with the MTHFR 677C→T polymorphism

The MTHFR 677C→T polymorphism can influence folate levels, distribution of folate cofactors, and cellular methylation capacity. Due to these effects, the MTHFR 677C→T polymorphism has been investigated for potential influence on the efficacy or toxicity of Mtx therapy. Most of the work in this area to date has centred on the use of Mtx in treatment of ALL, or RA in the low-dose context.

There are several reports of MTHFR 677 increasing risk of Mtx toxicity in treatment of ALL. The 677 T allele was associated with risk of relapse after Mtx therapy in a study of 520 childhood ALL cases (Aplenc et al. 2005). In a study of 122 cases of adult ALL, the 677 T allele was associated with increased risk of gastrointestinal or hepatic toxicity of Mtx treatment and reduced 2-year survival after treatment (Ongaro et al. 2009). Another study of childhood ALL found that the 677 TT genotype was associated with increased risk of both toxicity of Mtx and disease relapse (D'Angelo et al. 2011). MTHFR 677 TT genotype was also associated with risk of mucosal toxicity in Mtx treatment of childhood ALL and lymphoma, although the number of cases studied was small (n=64) (Faganel Kotnik et al. 2011).

RA patients treated with Mtx had higher plasma Hcy concentrations than patients treated with another drug, sulfasalazine. Homozygosity for the 677C→T polymorphism was associated with a higher level of plasma Hcy in response to Mtx treatment than in wild type individuals (Haagsma et al. 1999). RA patients who were heterozygous or homozygous for the 677T allele had an increased risk of discontinuation of Mtx treatment due to elevated liver enzyme levels, a marker of hepatic toxicity, compared to individuals of CC genotype. There was no association with clinical efficacy of Mtx (van Ede et al. 2001a). An association between 677TT genotype and liver toxicity, but not efficacy of Mtx treatment, was also found in a study of 281 psoriatic arthritis patients (Chandran et al. 2010). A higher risk of adverse reactions to Mtx was associated with 677 CT or TT genotype in a study of 110 RA patients in China (Xiao et al. 2010). Others have found negative results for associations of the MTHFR polymorphism with efficacy or toxicity of low-dose Mtx (Wessels et al. 2006; Owen et al. 2010). A disadvantage of these studies, both positive and negative is that they have quite small sample size, and the Mtx treatment regimens used vary between studies. A recent meta-analysis attempted to address this problem, and found no association between MTHFR 677 C→T genotype and Mtx toxicity or efficacy in 1514 RA patients

(Lee and Song 2010). However the studies used to conduct the meta-analysis varied widely in the treatment protocols and methods of reporting adverse reactions, and not all studies reported toxicity due to Mtx. Further investigation is required to define the effects of the MTHFR 677 C→T polymorphism on low-dose Mtx treatment.

Altered response to Mtx treatment has also been demonstrated in mouse models with deficient or over expressed levels of MTHFR (Celtikci et al. 2008). Mice which were heterozygous or homozygous knockouts for MTHFR had increased haematological toxicity in response to Mtx compared to wild-type mice. In addition, MTHFR-null mice showed increased kidney and liver toxicity compared to wild-type. Mice which had increased MTHFR also had increased haematological toxicity due to Mtx, and showed decreased levels of thymidine synthesis.

Overall, the evidence above suggests that reduced MTHFR activity can result in increased risk of toxicity of Mtx treatment, although more studies are required to fully investigate the relevance of this in a clinical setting.

1.12 Aims

As discussed above, there is a body of work which suggests that MTHFR 677C→T genotype may influence the efficacy or toxicity of Mtx. However, there is insufficient evidence from clinical studies to draw firm conclusions, and there is also a lack of experimental studies to test this hypothesis. The current study aims to investigate the influence of MTHFR 677C→T genotype in a cell culture model involving transformed B-lymphocytes of CC or TT genotype. Evidence from a mouse model of altered MTHFR expression indicates that such alteration of MTHFR protein levels may alter the level of apoptosis in response to Mtx (Celtikci et al. 2008). Mtx is also understood to induce apoptosis in a wide variety of cell types, however the potential influence of MTHFR 677C→T genotype on apoptosis induced by Mtx has not previously been examined. Additionally, the extent of apoptotic induction by Mtx and the mechanism of such apoptosis have been observed to vary depending on cell type. Mtx-induced apoptosis has not been previously investigated in transformed B-lymphocytes, therefore the mechanism of apoptosis induced by Mtx in this cell type is also of interest. Mtx is known to inhibit the synthesis of DNA precursor molecules, but there is also some evidence that Mtx may partially act via alteration of intracellular transmethylation reactions. Winter-Vann et al. (2003) suggested that Mtx may mediate its effect through inhibition of ICMT and subsequent alterations of intracellular signalling (Winter-Vann et al. 2003). The potential contribution of such a mechanism of Mtx action has not been investigated in other cell models. It is possible that inhibition of methylation-dependent cell

signalling by Mtx may promote apoptosis, although this was not investigated by Winter-Vann et al.

The aims of the current study are threefold:

- To determine if MTHFR 677C→T genotype influences the cellular response to Mtx by examining the effect of Mtx exposure on transformed B-lymphocyte cell lines in terms of cell proliferation and apoptosis.
- To investigate the mechanism of Mtx-induced apoptotic cell death in transformed B-lymphocyte cell lines.
- To examine the possibility that inhibition of ICMT is a feature of the cellular response to Mtx in transformed B-lymphocyte cell lines by chemical inhibition of ICMT.

2 Chapter 2: Materials and methods

2.1 Materials

2.1.1 Equipment

Pipettes:

Anachem, UK	Gilson Automatic pipette, 0.2-2µl, 2-20µl, 20-200µl, and 200-1000µl
Cruinn, Ireland	Micropipette tips 0.1-10µl, 2-200µl and 100-1000µl
Fisher Scientific, Ireland	Gel loading pipette tips

Consumables:

Fisher Scientific, Ireland	20ml sterile universal centrifuge tubes
	50ml sterile centrifuge tubes
	$25cm^3$, $75cm^3$, $125cm^3$ tissue culture flask for suspension cells
	96 well and 12-well tissue culture plates
Sarstedt, Ireland	1.5ml and 0.5ml Eppendorf microcentrifuge tubes

Equipment for cell culture:

AGB, Ireland	Cover slips 22 x 22mm
Fisher Scientific, Ireland	Haemocytometer
Nikon	Eclipe TS100 microscope
Atlas Clean Air Ltd.	Laminar flow hood / Class II Microbiologial Cabinet
NuAire, USA	CO_2 water-jacketed incubator
Sorvall Products, USA	RT7 Centrifuge81

Milipore, USA	Vacuum filter flasks, 0.22µM pore size

Equipment for flow cytometry:

BD Biosciences, Canada	FACSCalibur flow cytometer
	Cell Quest analysis software
	Polystyrene tubes, 5ml
Tree Star Inc., USA	FlowJo™ analysis software

Equipment for Western blot assay:

Atto Corporation, Japan	Electrophoresis gel chamber
	Semi-dry transfer system
Life Technologies GibcoBRL, UK	Power suppy, Model 400L

Equipment for cell proliferation assay:

Molecular devices, CA, USA	Microplate reader, VersaMax

Equipment for DNA extraction & MTHFR genotyping:

Life Technologies GibcoBRL, UK	Horizon 11.14 Gel Box
UVP, USA	Gel-Doc imaging system
MJ Research Inc., USA	PCR machine, PTC-100

Equipment for homocystiene assay

Abbott Diagnostics, USA	IMx analyser

Other equipment:

Sigma-Aldrich Ireland Ltd	Syringe filter unit, aqueous solutions (0.22µM, 25mm diameter)
	Luer lock disposable syringes, 20ml

2.1.2 Reagents

Cell culture media and sera:

Sigma-Aldrich Ireland Ltd	Roswell Park Memorial Institute (RPMI) 1640 Medium
	10X RPMI 1640 media without L-glutamine, folic acid and $NaHCO_3$
	Foetal bovine serum (FBS)
Invitrogen, USA	RPMI 1640 medium without folate or riboflavin
JRH Biosciences, USA	Dialysed foetal bovine serum (dFBS)

Reagents used in cell culture:

Sigma-Aldrich Ireland Ltd	Trypan blue solution (0.4%)
	Dimethyl sulfoxide, sterile (DMSO)
	Tissue culture water
	L-glutamine (200mM)
	Sodium bicarbonate
	1N Hydrochloric acid, sterile

	1N Sodium hydroxide
	Methotrexate (Mtx)
Schirks, Switzerland	(6S)-5-Methyl-5,6,7,8-tetrahydrofolic acid, calcium salt
	(6S)-5-Formyl-5,6,7,8-tetrahydrofolic acid calcium salt
BOC Gases, Ireland	CO_2 gas cylinder

Reagents used in flow cytometry:

Sigma-Aldrich Ireland Ltd	Phosphate buffered saline (PBS) tablets
	Ethanol
	RNAse A
	Propidium iodide (PI)
	Paraformaldehyde (PFA)
BD Biosciences, Canada	APO-DIRECT™ Kit
Roche Diagnostics Ltd., UK	Annexin V FLUOS Staining Kit

Reagents used in Western blot assay:

Sigma Aldrich Ireland Ltd	Trizma Base
	Trizma hydrochloride
	Sodium dodecyl sulphate (SDS)
	Glycerol
	Bromophenol blue
	β-mercaptoethanol
	Protease inhibitor cocktail

	Triton X-100
	Sodium chloride (NaCl)
	Sodium fluoride (NaF)
	Ethylenediaminetetraacetic acid (EDTA)
	30% Acrylamide solution
	Ammonium persulfate (APS)
	N,N,N',N'-Tetramethylethylenediamine (TEMED)
	Glycine
	Tween-20
	Monoclonal anti β-Actin antibody, mouse
	Polyvinylidene difluoride (PVDF) membrane
Thermo Scientific, USA	Pierce bicinchoninic acid (BCA) Protein Assay Kit
Licor Biosciences, USA	IRDye 800CW Goat anti-Mouse IgG
	IRDye-680 Goat anti-rabbit IgG
	Protein markers
	NewBlot PVDF stripping buffer
Invitrogen, USA	SeeBlue Plus2 Prestained Protein Standard
Chivers Ireland Ltd	Dried skimmed milk powder
Calbiochem, USA	Anti-PARP-1 Mouse monoclonal antibody
Cell Signaling Technology, USA	Cleaved Caspase-3 Rabbit monoclonal antibody

Reagents used in MTS cell proliferation assay:

Promega, USA	CellTiter 96 AQueous One Solution Cell Proliferation Assay

Reagents used in DNA extraction:

Qiagen, UK	QIAmp®DNA Blood Mini Kit
Sigma Aldrich Ireland Ltd	Disodium hydrogen phosphate, Na_2HPO_4
	Citric acid

Reagents used in MTHFR genotyping:

Sigma Aldrich Ireland Ltd	PCR Buffer (10x)
	Magnesium Chloride (25mM)
	Taq polymerase (5U/µl)
	DNAse free-water
Roche Ltd., Switzerland	Deoxy Trinucleotidephosphates (dNTPs) 100mM
Gibco, Biosciences, Tallaght	Primer 1 (Forward) 5'-TGAAGGAGAAGGTGTCTGCGGGA-3'
	Primer 2 (Reverse) 5'-AGGACGGTGCGGTGAGAGTGG-3'
Isis, Bray	HinF1 restriction enzyme
	Digestion buffer

Reagents used in homocysteine assay:

Abbott Diagnostics, USA	IMx Homocystiene Reagent Pack

Other reagents:

R&D systems, UK	Caspase 9 inhibitor Z-LEHD-fluoromethylketone
	Caspase 8 inhibitor Z-IETD-fluoromethylketone
	General caspase inhibitor (Z-VAD-fluoromethylketone)
Enzo Life Sciences, UK	N-Acetyl-S-geranylgeranyl-L-cysteine (AGGC)
Fisher Scientific, Ireland	N-Acetyl-L-cysteine (NAC) 98%
	Hypoxanthine (Hx)
Sigma Aldrich Ireland	Thymidine (Thy)
	Electrophoresis grade agarose
	Orange G dye
	Staurosporine
	N-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK)
	N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK)
Schircks Laboratories, Switzerland	(6S)-5-Methyl-5,6,7,8-tetrahydrofolic acid, calcium salt

2.1.3 Cell lines

Coriell Institute for Medical Research, USA	Lymphoblast cell lines: 17226, 17235, 17241, 17257, 17234, 17240, 17274, 17287

2.1.4 Composition of stock solutions

Tris-Borate-EDTA (TBE) buffer (10X)

1.0M Tris, 0.9M Boric Acid, 0.01M EDTA. Made up to 1X with dH_2O

Cell lysis buffer for Western blot

1% Triton X-100, 20mM Tris-HCl ph 7.4, 150mM NaCl, 5mM NaF, 5mM EDTA

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Sample Buffer (5X)

250mM Tris, 10% SDS, 50% glycerol, 0.2% bromophenol blue, 10% β-mercaptoethanol

SDS-PAGE running buffer

25mM Trizma, 20mM glycine, 0.1% w/v SDS

Western blot transfer buffer

48mM Trizma base, 39mM Glycine, 0.04 5 w/v SDS, 20% methanol

(6S)-5-Formyl-5,6,7,8-THF - 400μM stock solution

0.0041g of (6S)-5-Formyl-5,6,7,8-THF was weighed out and made up to 20ml with tissue culture water in a volumetric flask then filtered through a syringe filter with 0.22μM pore size. The solution was aliquoted in a laminar flow hood and stored at -40^{o}C.

(6S)-5-Methyl-5,6,7,8-THF - 500μM stock solution

0.0049g of (6S)-5-Methyl-5,6,7,8-THF was weighed out and dissolved in tissue culture water. The volume was made up to 20ml in a volumetric flask. The solution was filtered through a 0.22μm syringe filter. This solution was prepared immediately before use.

Folic acid - 400μM stock solution

0.0035g folic acid was weighed out and dissolved in 5ml tissue culture water. This was made up to 20ml with tissue culture water in a volumetric flask, then filtered through a syringe filter with 0.22μM pore size. The solution was aliquoted in a laminar flow hood and stored at -40^{o}C.

Thymidine - 10mM stock solution

0.121g thymidine was weighed out and dissolved in 1ml of tissue culture water with one drop of 0.1M NaOH. This was made up to 50 ml with tissue culture water in a volumetric flask, then filtered through a syringe filter with 0.22μM pore size. The solution was aliquoted in a laminar flow hood and stored at -40^{o}C.

Hypoxanthine - 20mM stock solution

0.136g hypoxanthine was weighed out and dissolved in a few drops of 0.1M NaOH. This was made up to 50 ml with tissue culture water in a volumetric flask, then filtered through a syringe

filter with 0.22µM pore size. The solution was aliquoted in a laminar flow hood and stored at -40°C.

Methotrexate - 500µM stock solution

0.00222g of Methotrexate was weighed out and dissolved in 5ml tissue culture water with 1 drop 1M NaOH. This was made up to 10ml with tissue culture water in a volumetric flask. The solution was filtered through a 0.22µM filter in the laminar flow hood. The solution was aliquoted and stored at -20°C.

N-acetylcysteine – 100mM stock solution

1.632g of N-acetylcysteine was weighed out and dissolved in 4ml tissue culture water. This was made up to 10ml with tissue culture water in a volumetric flask. The solution was filtered through a 0.22µM filter in the laminar flow hood. The solution was aliquoted and stored at -40°C.

Phosphate citrate buffer

192 parts 0.2M Na_2HPO_4, 8 parts 0.1M citric acid (pH 7.8)

Orange G loading buffer

0.25% (v/v) Orange G Dye, 30% (v/v) glycerol, dH_2O

TPCK- 40mM stock solution

0.07037g TPCK was dissolved in 5ml sterile DMSO.

TLCK- 40mM stock solution

0.0738g TLCK was dissolved in 5ml sterile DMSO.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Cell lines

Human lymphoblast cell lines were obtained from Coriell Cell Repositories (CCR), at the Coriell Institute for Medical Research in New Jersey, USA. These cell lines are established by transformation of peripheral blood mononuclear cells by Epstein - Barr virus using

phytohemagluttinin as a mitogen. This produces a transformed cell line of B-lymphocyte lineage. The CCR provides cell lines from a variety of ethnic backgrounds. The cell lines used in the current study were chosen from a Caucasian variation panel, primarily on the basis of their MTHFR genotype. The cell lines were also chosen because they all require 15% uninactivated FBS for growth and have similar split ratios. The MTHFR C677T genotype of a number of cell lines had previously been determined by Dr. Larry Brody. On receipt of the stock cultures from the CCR, the MTHFR C677T genotype was confirmed by polymerase chain reaction (PCR) genotyping as described in Section 2.2.7.

2.2.1.2 Maintenance of cell lines

Cell lines were maintained under sterile conditions in a Class II biological safety cabinet. The cells grow in suspension and form loose clumps. Cell cultures were maintained in RPMI 1640 medium supplemented with 15% FBS. They were stored in a humidified incubator at 37°C, with a CO_2 concentration of 5%.

Cell cultures were fed every 2-3 days with fresh medium. Cells were harvested by centrifugation at 1100 rpm, 5 min. The exhausted medium was discarded and the pellet resuspended in 1ml pre-warmed medium per 10ml original culture volume. Three 5µl aliquots of this suspension were taken to determine cell counts. Each aliquot was diluted 1:10 using trypan blue solution. 10µl of this mixture was applied to a haemocytometer and live and dead cells were counted using a light microscope. Live cells appear white because they exclude the dye, while dead cells appear dark blue. The cell count was defined as:

$$\text{Number of cells/ml} = \text{average cell count} \times \text{dilution factor} \times 10^4$$

Cells were resuspended in pre-warmed medium at a density of 2-4 x 10^5 cells/ml. Cell viability was calculated as the percentage of total cells counted which were live. Cells were used for experiments only if viability was greater than 80%.

2.2.1.3 Freezing cells for long-term storage of stocks

Cells were harvested by centrifugation at 1100 rpm for 5 min. The supernatant was discarded and the cell pellet resuspended in 1ml medium per 10ml original culture volume. Cells were counted using a haemocytometer as described in section 2.2.1.2 and viability assessed by trypan blue staining. Cells were not used to make frozen stocks if viability was less than 80%. Once cells had been counted they were centrifuged again at 1100 rpm for 5 min. The supernatant was discarded and the pellet resuspended in RPMI containing 6% DMSO, at a density of 5 x 10^6 cells/ml. One ml aliquots of this suspension were placed in cryovials and the cryovials placed in a polystyrene box.

The box was placed in a freezer at -20°C for one hour, then at -40°C for one hour, then at -80°C overnight. The following day the cryovials were transferred to a dewar containing liquid nitrogen for long term storage.

2.2.1.4 Revival of cells from frozen stocks

Medium was pre-warmed to 37°C in a water bath. A 10ml volume of pre-warmed medium was pipetted into a sterile container. A cryovial containing cells from the desired line was removed from liquid nitrogen storage and thawed in the hand. As soon as the cell suspension had thawed, it was pipetted into the previously aliquoted medium. The suspension was centrifuged at 1100rpm for 5 min. Supernatant was removed and the cells resuspended in 1ml medium. At this point a 5µl aliquot was removed to use for counting and viability assessment. A further 9ml medium was added to the remaining cell suspension, and this was centrifuged again at 1100 rpm for 5 min. The supernatant was removed, and pellet resuspended in 1ml medium. The cells were seeded in a sterile tissue culture flask at 2 x 10^5 cells/ml, and placed a water jacketed incubator at 37°C, 5% CO_2.

2.2.1.5 Preparation of cell culture media

Cells were maintained in RPMI 1640 medium supplemented with 15% Fetal Bovine Serum.

Folate-free RPMI medium was required for some experiments. Concentrated 10X RPMI 1640 without sodium bicarbonate ($NaHCO_3$), L-glutamine and folic acid was obtained from Sigma. A one in ten dilution of this 10X RPMI was prepared using sterile tissue culture water as a diluent. This was supplemented with 2g/L $NaHCO_3$ and 0.3g/L L-glutamine. The pH was adjusted to 7.4 at ~18°C with either 1N NaOH or 1N HCl. The medium was then filtered using a Milipore vacuum filter flask with 0.2µm pore size. Dialysed FBS was then added to 15%.

For experimental setup, cells were harvested by centrifugation (1100rpm, 5 min), washed three times in sterile PBS and resuspended in folate-free RPMI which was then supplemented with methotrexate or folate sources. Solutions of Mtx or folates were made up in sterile tissue culture water, sterile filtered using a syringe-filter and stored in aliquots at -80°C.

2.2.1.6 Cell viability assessment

Assessment of cell viability was carried out routinely and for experimental results by staining with trypan blue as described in section 2.2.1.2.

2.2.2 Flow cytometry

2.2.2.1 Cell cycle analysis

Cells were seeded at $2x10^5$ cells/ml, with addition of methotrexate as required, and harvested by centrifugation (1100rpm, 5min) at appropriate time points. Pellets were resuspended in ice-cold PBS and ice-cold 70% ethanol was added to each sample. The samples were stored at 4^oC overnight. The following day, samples were centrifuged at 2400rpm for 10 mins. The supernatant was discarded and pellet resuspended in PBS. RNAse A and Propidium Iodide (PI) were added to each sample and incubated for 30min. Samples were analysed for DNA content using a BD FACSCalibur flow cytometer and CellQuest software. A two-parameter dot plot of side-scatter (SSC) vs forward scatter (FSC) was used to exclude debris. PI staining was detected in channel FL2. A two-parameter dot-plot of FL2 pulse-width (FL2-W) vs FL2 pulse-area (FL2-A) was used to define the single-cell region. The cell-cycle profile of the single-cell population was displayed as a histogram of FL2-A. A sample of untreated cells was used to select the regions for each phase of the cell cycle, and these markers were used to analyse the experimental samples. Figure 2.1 shows an example of flow cytometry output from the cell cycle analysis method.

2.2.2.2 Annexin V/PI staining

This staining method is used to detect the proportion of cells in a population which are undergoing apoptosis. During the early stages of apoptosis, phosphatidylserine (PS) translocates from the inner leaflet to the outer leaflet of the plasma membrane. As apoptosis progresses further, the cell membrane becomes permeable. In this assay, fluorescein isothiocyanate-labelled Annexin V (FITC-AnnV) and the DNA stain propidium iodide (PI) are added to samples of live cells. FITC-AnnV binds to any exposed PS on the surface of early apoptotic cells. PI can cross the plasma membrane of late apoptotic cells and stain the DNA. The intensity of FITC-AnnV and PI staining is detected using a flow cytometer. Healthy cells do not stain with either compound, in contrast to to early apoptotic cells which stain positively for FITC-AnnV only, and late apoptotic or necrotic cells which stain positively for both FITC-AnnV and PI.

Cells were processed for Annexin V/PI staining using a kit from Roche. To perform the assay, cells which had previously been incubated under desired experimental conditions were harvested by centrifugation at 1100rpm for 5min in a bench centrifuge. Each sample was resuspended in 1ml PBS and centrifuged again at 1100rpm for 5min. Each sample was then resuspended in 50µl incubation buffer containing FITC-AnnV and PI (1µl of each stock solution per 50µl incubation buffer). Cells were incubated in staining buffer for 10 mins at room temperature in the dark. Samples of cells which were left unstained, or single-stained with either FITC-AnnV or PI were also

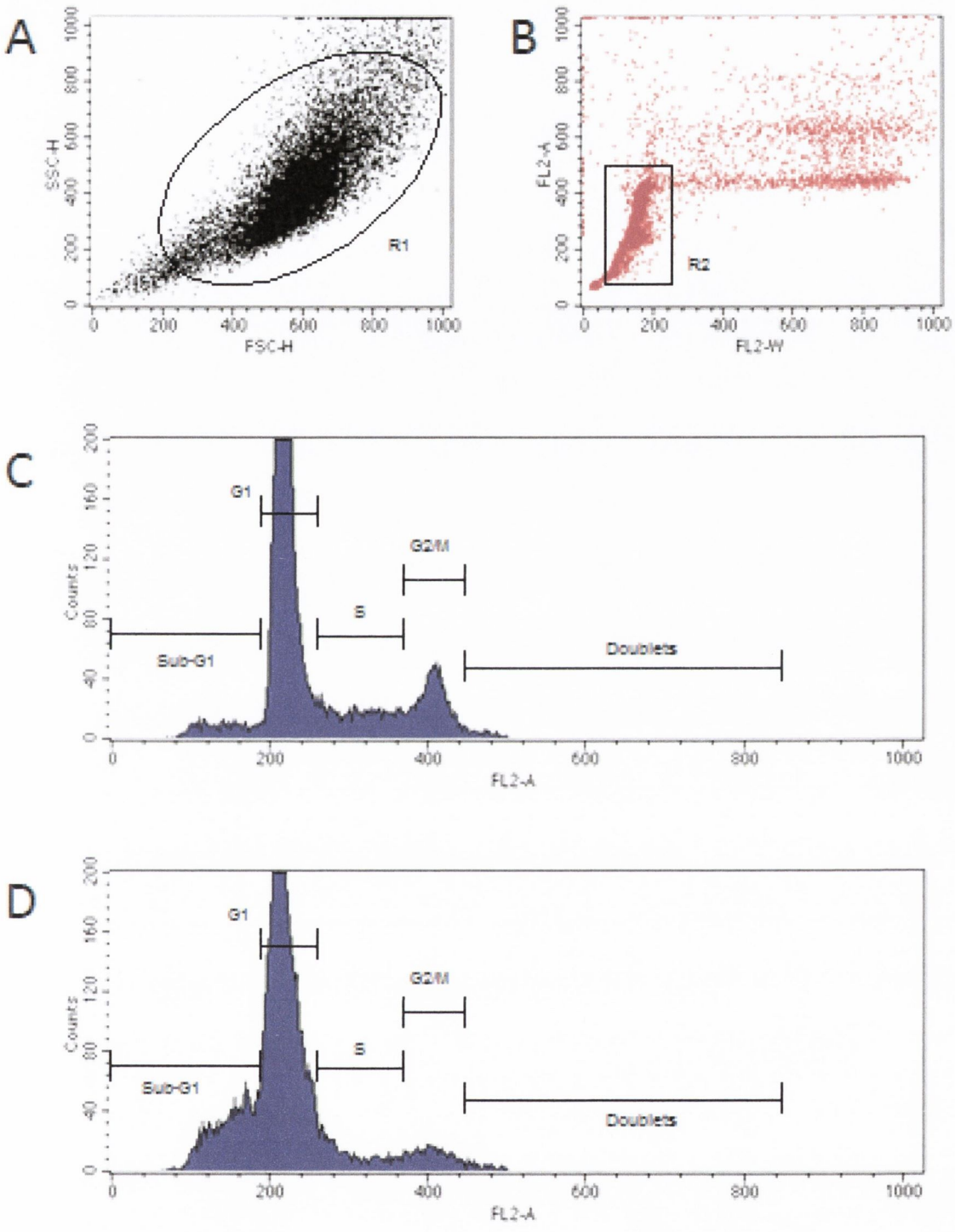

Figure 2.1 Example of flow cytometry output from the cell cycle analysis method

(A) Dot-plot of side scatter (SSC-H) vs. Forward scatter (FSC-H). This plot is used to exclude debris, by enclosing single cells in the gate labelled R1. (B) Dot plot of Fluorescence channel 2 pulse area (FL2-A) vs pulse width (FL2-W) showing events gated on R1. This plot is used to exclude doublets, triplets etc., by creating gate R2. (C) Histogram of FL2-A for a sample of control cells. The regions representing different stages of the cell cycle are shown. (D) Histogram of FL2-A for a sample of cells treated with 100nM Mtx for 48h. The increase in Sub-G1 cells compared to control can be seen.

prepared for use as controls. The samples were immediately analysed using a BD FACSCalibur flow cytometer. PI was detected in channel FL3, and FITC-Annexin V was detected in channel FL1.

Further analysis of data was carried out after data acquisition using FloJo software. A two-parameter dot plot of Forward scatter (FSC) vs. Side scatter (SSC) was used to gate out debris and define the single-cell population. The single-stained samples were used to compensate for interference between the two fluorescent stains. After compensation, a two-parameter dot plot of FL3 vs FL1 was used to display the cells with different levels of staining for both markers. The single-stained samples were also used to define the quadrants which allow analysis of the percentage of cells in the sample which are positive for each type of stain, or both. An example of flow cytometry output for the Annexin V/PI method is shown in figure 2.2.

2.2.2.3 Modified TUNEL assay with Uracil-DNA glycosylase treatment

The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay is a method used to detect DNA strand breaks in cells. Cells are first fixed in paraformaldehyde (PFA) and permeablised by treating with 70% ethanol. The enzyme terminal deoxynucleotidyl transferase (TdT) is used to catalyse the linkage of fluorescein isothiocyanate-labelled dUTP (FITC-dUTP) to the 3'-hydroxyl terminal of double or single stranded breaks in cellular DNA. The extent of labelling can then be measured using a BD FACSCalibur flow cytometer and CellQuest software.

Cells which had been previously incubated under desired experimental conditions were harvested by centrifugation at 1100rpm for 5min. The pellets were resuspended in Iml 1% w/v PFA in PBS (ph7.4), and incubated on ice for 30min. Samples were then centrifuged at 300g for 5min and the supernatant discarded. Samples were washed twice in 1ml PBS and pelleted by centrifugation. Cells were then resupended in 50µl ice-cold PBS, and 1ml ice-cold 70% ethanol was added. Samples were stored at -20^{o}C until further analysis.

Half of each sample was treated with the enzyme uracil DNA glycosylase (UDG) in an attempt to quantify uracil incorporation into cellular DNA. This occurs when the thymidylate synthase (TS) pathway is disrupted due to folate deficiency or inhibition of dihydrofolate reductase (DHFR). The samples were pelleted by centrifugation at 300g for 5min, then rinsed with 1ml PBS to remove any residual ethanol. The pellets were then resuspended in 1ml UDG buffer (20mM Tris-HCl, 1mM EDTA, 1mM dithiothreitol, pH 8) and each sample was split into two 500µl aliquots. A 2µl volume of UDG enzyme (corresponding to 10U active enzyme) was added to one part of each sample. All tubes were placed in a water bath at 37^{o}C for 60min.

The APO-DIRECT™ Kit from BD was used to carry out TUNEL staining. The samples were pelleted by centrifugation at 300g for 5min and the supernatant was removed. Samples were washed twice in 1ml Wash Buffer. Each pellet was then resuspended in 50µl DNA labelling solution, which contained Reaction Buffer, TdT enzyme, FITC-dUTP and distilled water in quantities according to manufacturer's directions. The cells were incubated in this solution for 60min in a water bath at 37°C. A 1ml volume of Rinse Buffer was added to each tube after incubation, and then the cells were pelleted by centrifugation at 300g for 5min. The rinse step was repeated with a further 1ml of Rinse Buffer. The cell pellets were then resuspended in 0.5ml PI/RNAse staining buffer. The samples were incubated in the dark at room temperature for 30min, and then analysed using a BD FACSCalibur flow cytometer and CellQuest software. A two-parameter dot plot of forward scatter (FSC) vs. side scatter (SSC) was used to exclude debris and define the single-cell population. FITC-dUTP staining was detected in channel FL1, and PI staining was detected in channel FL3. A two-parameter plot of FL1 vs FL3 and a histogram plot of FL1 was used to display the data and identify cells which were positive and negative for TUNEL staining (i.e. FITC-dUTP positive cells). An example of output from the TUNEL assay is shown in figure 2.3.

2.2.3 Western blot assay

2.2.3.1 Cell lysis and sample preparation

After the desired treatment and incubation time, cell pellets were harvested by centrifugation (1100rpm, 5min). Cells were lysed in cell lysis buffer (1% Triton X-100, 20mM Tris-HCl ph 7.4, 150mM NaCl, 5mM NaF, 5mM EDTA), on ice for 30min, with occasional agitation using a vortex mixer. A protease inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin) was added to the lysis buffer immediately before use. Samples were then centrifuged at 1000g, 4°C for 5min. Aliquots of each supernatant were kept for protein assay (section 2.2.3.2) and the remainder was mixed with 5x SDS-PAGE Sample Buffer (250mM Tris, 10% SDS, 50% glycerol, 0.2% bromophenol blue, 10% β-mercaptoethanol) and boiled for 5mins. Samples were stored at -20°C until they were loaded onto gels.

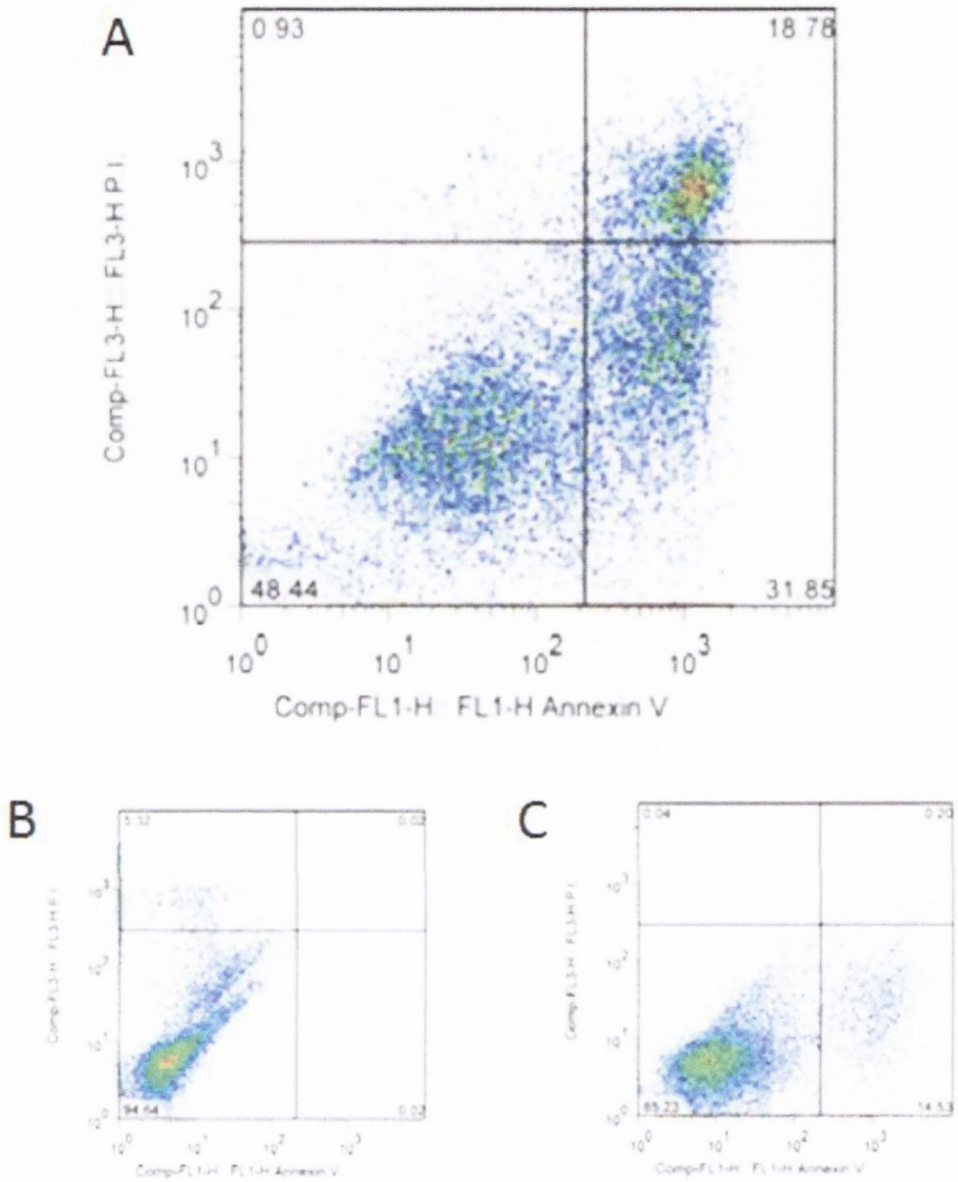

Figure 2.2 Example of flow cytometry output for Annexin V/PI staining method

Cells were treated with 100nM Mtx for 48h, then processed for Annexin V/PI staining as described in section 2.2.2.2. Samples were run on a BD FACScalibur flow cytometer, and subsequent data analysis was carried out using FloJo software. (A) Two-parameter dot plot, with intensity of Annexin V staining on the X-axis and PI staining on the Y-axis. Samples of cells which were single stained with PI (B) and Annexin V (C) were used to define the quadrants. Lower left quadrant- cells negative for both. Lower right quadrant– cells positive for Annexin V, negative for PI. Upper right quadrant – cells positive for both Annexin V and PI. Upper left quadrant – cells positive for PI only. The numbers in each quadrant represent the percentage of the cell population in that quadrant.

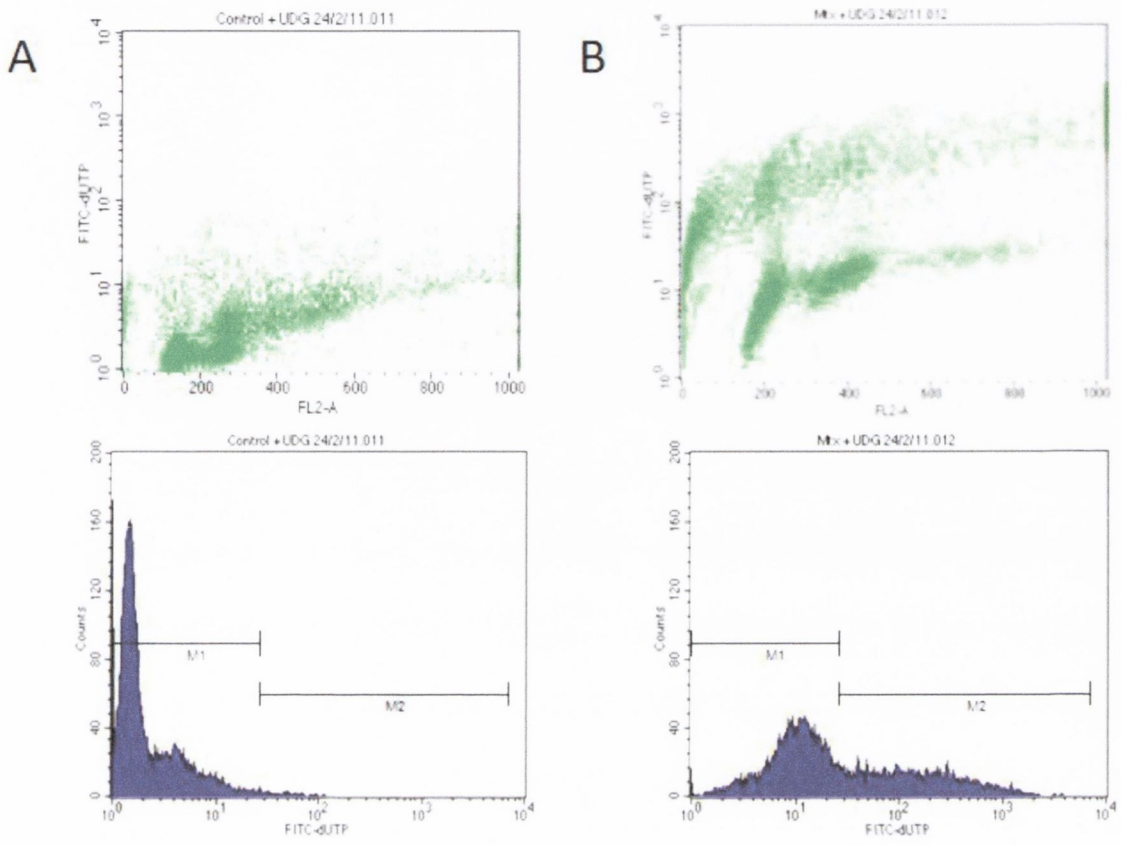

Figure 2.3 Example of flow cytometry output for TUNEL staining.

Cells were incubated for 48h without (A) or with 100nM Mtx (B), then fixed and processed for TUNEL staining as described in section 2.2.2.3. The upper panels show a two-parameter dot plot of FITC-dUTP vs PI staining. The lower panels show a histogram of FITC-dUTP staining only, with markers indicating TUNEL negative (M1) and TUNEL positive cells (M2).

2.2.3.3 Protein assay

The BCA protein assay was used to determine the total protein content of samples to ensure equal loading on the SDS-PAGE gel. This is a colorimetric assay which couples reduction of Cu^{2+} to Cu^{1+} by protein to chelation of bicinchoninic acid, forming a purple product with a strong absorbance at 562nm. The assay is linear over a concentration range of 20-2000 µg/ml protein.

To carry out the assay, BCA reagent A and BCA reagent B were mixed in a 50:1 ratio to produce the working reagent. 10µl of each sample to be tested was pipetted into wells of a microplate, along with a range of bovine serum albumin (BSA) protein standards (0-2000µg/ml). 200µl of working reagent was added to each well, the plate was covered with parafilm and incubated at 37°C for 30mins. Absorbance at 562nm was then determined using a microplate reader. The protein concentration of each sample was calculated from a standard curve of the absorbance values of the BSA standards.

2.2.3.4 Preparation of an SDS-PAGE gel

The glass plates of the Atto gel rig were rinsed with ethanol and assembled to allow pouring of the gel. A 10% acrylamide resolving gel was made up according to the scheme provided in table 2.1, adding reagents in the order given in the table:

Component	Volume
dH_2O	7.9 ml
30% Acrylamide	6.7 ml
1.5M Tris-HCl pH 8.8	5 ml
10% w/v SDS	200 µl
10% w/v APS	200 µl
TEMED	8 µl

Table 2.1: Reagents used in preparation of an SDS-PAGE resolving gel

The gel was mixed quickly and poured in between the glass plates, and overlaid with 1ml of isopropanol. It was allowed to set for 40mins at room temperature. The isopropanol was poured off and the top of the gel rinsed with dH^2O. A 5% acrylamide stacking gel was then prepared by combining the reagents in the order given in table 2.2:

Component	Volume
dH_2O	3.4 ml
30% Acrylamide	0.83 ml
1.5M Tris-HCl pH 8.8	0.63 ml
10% w/v SDS	50 µl
10% w/v APS	50 µl
TEMED	5 µl

Table 2.2: Reagents used in preparation of an SDS-PAGE stacking gel

The stacking gel was pipetted between the plates on top of the set resolving gel, and the comb was added to form wells. The stacking gel was allowed to set for 40mins at room temperature.

2.2.3.5 Running an SDS-PAGE gel

The set gels were placed into the electrophoresis rig and secured using the supplied components. SDS-PAGE running buffer (Section 2.1.4) was poured in up to the top level of the gel plates. The combs were removed and protein samples were loaded into the wells. The SDS-PAGE gels were run at 100V and 40mA for approximately 1.5 hours.

2.2.3.6 Transfer to PVDF membrane

Separated proteins were transferred to a PVDF membrane using the ATTO Semi-Dry transfer system. The gel was placed on top of the PVDF membrane within a 'sandwich' of filter paper soaked in Western blot transfer buffer (48mM Trizma base, 39mM Glycine, 0.04 5 w/v SDS, 20% methanol). Transfer was carried out for 1hr at 100V 100mA.

2.2.3.7 Antibody detection

Membranes were then blocked in 5% w/v non-fat dry milk powder in TBST, rinsed three times in tris buffered saline containing 0.1% v/v TWEEN-20 (TBST), then incubated with primary antibody against either PARP, caspase 2 or β-actin. The antibodies were diluted in 5% w/v non-fat dry milk powder overnight at $4^{o}C$. Membranes were rinsed three times in TBST and incubated for 40 – 60 mins with fluorescently labelled secondary antibodies (Licor). The secondary antibodies were diluted in 5% w/v non-fat dry milk powder. The protein bands were visualised using the Licor Odyssey system. This system uses a fluorescence scanner to scan the PVDF membrane and detect the presence of the fluorescent-labelled secondary antibodies.

2.2.4 MTS cell proliferation assay

The CellTiter 96 Aqueous one solution cell proliferation Assay (Promega) was used for this purpose.

The CellTiter reagent contains the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) and an electron coupling reagent phenazine ethosulfate (PES). MTS is reduced to a coloured formazan product by live cells, and so the number of live cells in a culture can be quantified by the amount of formazan produced. The concentration of formazan is determined by measuring absorbance at 490nm.

Experimental media were prepared in advance by adding appropriate amounts of stock solutions of methotrexate (or other compounds) to cell culture medium supplemented with 15% FBS. Solutions of compounds of interest in cell culture medium were prepared at twice the final assay concentration. 50µl of prepared these stock solutions were pipetted into the wells of a 96-well tissue culture plate, using triplicate wells for each experimental condition. Cells were counted as described in section 2.2.1.2, and a cell suspension at twice the final cell concentration was prepared. 50µl of this cells suspension was added to each prepared well of the 96-well plate. The final cell density was $2.5x10^4$ cells/well in a 96 well plate, in a total volume of 100µl. The plates were placed in an incubator at 37°C, 5% CO_2. After the desired treatment time (0-72 hours), 20µl of CellTiter reagent was added to each well, and the plate returned to the incubator for 4 hours. Absorbance at 490nm was then measured using a microplate reader.

2.2.5 Homocysteine assay

Total homocystiene (Hcy) in samples of cell culture supernatants was determined by the Abbott IMx homocysteine assay. This is an automated fluorescence polarization immunoassay, and can detect the presence of Hcy at concentrations greater than 0.8µmol/L.

2.2.6 DNA extraction

Two methods were used to extract DNA from cells for different subsequent analysis. The Qiagen method was used to purify genomic DNA for MTHFR genotyping, and the phosphate-citrate method was used to extract DNA for analysis of fragmentation caused by experimental treatments.

2.2.6.1 Qiagen method

A 20µl volume of Qiagen Proteinase was pipetted into a 1.5ml microcentrifuge tube. $3x10^6$ cells suspended in 200µl of PBS and were added to the tube. 200µl of buffer AL (Qiagen lysis buffer) was added and the contents of the tube were mixed by vortexing for 15 sec. The tube was incubated at 56°C for 10min, then briefly centrifuged. 200µl of 100% ethanol was added and mixed by vortexing for 15 seconds. The tube was centrifuged briefly and then the contents were applied to a QIAmp spin column in a collecting tube. The column was centrifuged at 8000 rpm for

1 min, then placed in a clean collecting tube. The column was washed by addition of 500µl buffer AW1 (Qiagen wash buffer 1), and centrifuged at 8000 rpm for 1 min, then placed in a clean collecting tube. A 500µl volume of buffer AW2 (Qiagen wash buffer 2) was added and the column centrifuged at 15000 rpm for 3 minutes. The column was placed in a clean collecting tube and centrifuged again at 15000 rpm for 1 min to ensure removal of all wash buffer. The column was placed in a labelled 1.5ml microcentrifuge tube, and 200µl buffer AE was carefully added to the top of the column. After incubation at room temperature for 5 min, the column was centrifuged at 8000 rpm for 1 min. A further 200µl of buffer AE (Qiagen elution buffer) was added to the column, and allowed to stand for 5 min at room temperature. This was collected into the same microcentrifuge tube by centrifugation at 8000 rpm for 1 min. DNA extracts were stored at -20°C until further analysis.

2.2.6.2 Phosphate-citrate method

Cells were harvested by centrifugation at 200g for 5min. The growth medium was discarded, and pellets were washed by resuspending in 1ml ice-cold PBS followed by a further centrifugation. The pellets were resuspended in 100µl ice cold PBS, and 1.2ml ice-cold 70% ethanol was added to each sample. Samples were stored at -20°C for at least 24h. The fixed cells were pelleted by centrifugation at 800g for 5min and the ethanol removed. The pellets were suspended in 40µl phosphate citrate buffer (192 parts 0.2M Na_2PO_4 and 8 parts 0.1M ctiric acid (pH 7.8). Samples were incubated at room temperature for 30minutes. The cells were pelleted by centrifugation at 1000g for 5min. The supernatant was transferred to fresh microcentrifuge tubes and pellets were discarded. 3µl of Triton X-100 (0.25% in water) and 3µl of RNAse A (1mg/ml) was added to each tube, and all tubes were incubated at 37°C for 30min. 3µl of proteinase K (1mg/ml) was then added to each tube and incubation at 37°C was continued for a further 30min. After incubation the DNA was run on a 1.5% agarose gel as described in section 2.2.7.2.

2.2.7 MTHFR genotyping

2.2.7.1 Polymerase chain reaction

DNA extracts which had been prepared using the Qiagen method were thawed to room temperature. PCR master mix was prepared by mixing the components described in table 2.3 in the order listed. The amounts stated in table 2.3 are sufficient for one reaction and can be scaled up according to need.

Reagent	Amount (µl)
Water (RNAse/DNAse Free)	34.8
dNTPs (2.5mM)	4
Forward primer P1 (1/40)	1
Reverse primer P2 (1/40)	1
$MgCl_2$ (25mM)	4
10x PCR buffer	5
Taq polymerase	0.2

Table 2.3: Reagents used in preparation of PCR master mix

A 49µl volume of PCR master mix was aliquoted into labelled PCR tubes, one for each DNA extract to be analysed. A 1µl aliquot of the appropriate DNA extract was added to each tube and the lid of the tube closed. A positive control containing DNA from a known heterozygote for the MTHFR C677T mutation was included, and also a negative control containing PCR master mix only. The tubes were placed in the PCR machine and run according to the MTHFR programme, as follows:

1. 94°C for 5 min
2. 94°C for 1 min
3. 62°C for 1 min
4. 72°C for 1 min

Stages 2 through 4 were repeated for 30 cycles. The programme took approximately 3 hours to complete. After amplification, the presence PCR of products in each reaction was checked by running all samples on a 1.5% agarose gel (section 2.2.7.2). A 5µl volume of each product was mixed with 2µl orange G and applied to the wells of the agarose gel. The gel was run at 100V for 20 min.

2.2.7.2 Preparation of an agarose gel

For a 1.5% agarose gel, 1.5g of electrophoresis grade agarose was added to 100ml TBE buffer in a 500ml pyrex bottle. The solution was boiled in a microwave to dissolve the agarose and allowed to cool slightly. A 1.5µl volume of ethidium bromide solution (10mg/ml) was added to the gel and mixed by swirling the bottle. A gel frame was prepared by taping the ends with masking tape and placing a comb in position. When the gel was cool enough to handle, it was poured into the gel frame and allowed to solidify.

2.2.7.3 Restriction enzyme digest

Restriction digest master mix was prepared as in table 2.4. Amounts stated are sufficient for one reaction and can be scaled up according to need:

Reagent	**Amount (µl)**
Digestion buffer	3
HinF1 enzyme	1.5
DNAse-free water	6

Table 2.4: Reagents used in preparation of restriction digest master mix

A 10.5µl volume of digestion master mix was added to labelled 0.2ml tubes, one for each reaction required. 20µl of the appropriate PCR product was added to each tube, including the positive and negative controls. The tubes were covered and incubated at 37^{o}C overnight. After digestion, 3µl Orange G was added to each tube and the samples were loaded on a 2.5% agarose gel (2.5g/100ml 1x TBE buffer). The gel was run for 1hour at 100V, 250mA. Bands were visualized and photographed using the UVP Gel Doc system. The wild type (CC genotype) is represented by an uncut band of 198 base pairs. Heterozygote DNA (CT genotype) produces two bands of 198 and 175 base pairs. DNA from homozygotes for the mutation (TT genotype) produce one band of 175 base pairs.

2.3 Statistical analysis

Statistical analysis was carried out using Prism Version 5.01, GraphPad Software Inc. Student's t-test was used to make comparisons between two groups. One-way ANOVA with Tukey's post-tests was used to compare three or more groups. Two-Way ANOVA was used to analyse data

where there were two factors affecting the outcome, which might interact. Bonferroni correction for multiple testing was used in conjunction with TWO-way ANOVA.

3 Chapter 3: Investigation of the effect of methotrexate on transformed B-lymphocyte cell lines

3.1 Introduction

It is well established that the 677C→T mutation in MTHFR decreases enzyme activity by approximately 70% compared to the wild type. This was first shown by Frosst et al. (1995) in human enzyme isolates from patients with vascular disease (Frosst et al. 1995). The detrimental effect of this polymorphism on activity of MTHFR has been extensively investigated and confirmed by others. In vitro experiments using purified enzyme have shown that the mutation does not directly influence catalytic parameters, but its influence on enzyme activity is due to decreased stability of the mutant holoenzyme. Structural studies of MTHFR from *E. Coli* which had a mutation homologous to the 677C→T mutation in the human enzyme suggested that a defect in binding of the FAD cofactor was possible due to the location of the altered amino acid residue relative to the FAD binding site (Guenther et al. 1999). In vitro experiments confirmed this supposition, as the mutant form lost the essential FAD cofactor 11 times more rapidly on dilution (Guenther et al. 1999). Similar results were obtained in experiments using recombinant human MTHFR. The 677C→T form of the enzyme lost its essential FAD cofactor on dilution three times more rapidly than the wild type enzyme, and this was accompanied by dissociation of the active dimeric enzyme into inactive monomers (Yamada et al. 2001). The reduced activity of the 677C→T form of human MTHFR has also been confirmed in experiments using enzyme isolated from human cells (Lathrop Stern et al. 2003). Lesser activity of the mutant enzyme relative to the wild-type was also noted in protein extracts from human cancer cell lines which had been transfected with wild-type or mutant MTHFR (Sohn et al. 2004).

In addition to these demonstrations of reduced enzyme stability and activity associated with the MTHFR 677C→T mutation, the presence of the 677C→T mutation is also functionally significant in vivo. Differences in the distribution of intracellular folate species have been observed based on MTHFR 677C→T genotype, with TT cells having higher levels of formylated folates and lower methylated folate than cells of CC genotype (Bagley and Selhub 1998; Lathrop Stern et al. 2003; Davis et al. 2005). Homozygosity for the T allele is an independent risk factor for elevated plasma Hcy (Guttormsen et al. 1996), a biochemical change associated with cardiovascular disease. As discussed in Chapter 1, the T allele is also associated with increased risk of certain diseases

including NTDs (Kirke et al. 2004) and cardiovascular disease (Lewis et al. 2005) and with altered risk of some types of cancer.

A further feature of the MTHFR 677C→T polymorphism which is functionally relevant is its interaction with folate and riboflavin nutritional status. In vitro experiments show that the stability of the mutant form of MTHFR is increased in the presence of higher concentrations of folate or FAD (derived from riboflavin) (Guenther et al. 1999; Yamada et al. 2001). Elevated plasma Hcy associated with TT genotype is exacerbated by low folate and riboflavin status (Jacques et al. 2002). In some cases it has been observed that the effects of the T allele on disease risk may be more potent in individuals with low folate status. For example, a study of MTHFR genotype and folate status and colorectal adenoma risk showed that individuals with TT genotype had increased risk if their folate status was also low, but decreased risk if they were in the highest plasma folate quartile of the group studied (Levine et al. 2000).

Methotrexate is a folate analogue used in chemotherapy of cancer and autoimmune disease. Although Mtx does not directly affect MTHFR activity, because this enzyme plays central role in regulation of folate metabolism there have been several investigations of the possible influence of the 677C→T polymorphism on Mtx efficacy or toxicity. Most of the studies in this area have been observational studies of patient cohorts, and there is evidence that the MTHFR 677C→T mutation is associated with increased risk of toxicity during Mtx treatment. Several studies have shown that TT genotype is associated with higher risk of Mtx toxicity during treatment of ALL (Aplenc et al. 2005; Ongaro et al. 2009; D'Angelo et al. 2011; Faganel Kotnik et al. 2011). Treatment of inflammatory disease with Mtx uses far lower doses of the drug than those used for cancer therapy, however the presence of the T allele has also been associated with increased risk of Mtx toxicity during this type of treatment (Van Ede et al. 2001b; Chandran et al. 2010). A study carried out in mice with both over and under expression of MTHFR showed that both of these alterations led to increased toxicity of Mtx treatment. Both under and over expression of MTHFR was associated with reduced haematopoiesis compared to wild-type mice (Celtikci et al. 2008). Mice with reduced MTHFR expression showed increased plasma Hcy and toxicity of the liver and kidney after administration of Mtx. This suggests that decreased MTHFR activity renders the organism more susceptible to toxic effects of Mtx. Overexpression of MTHFR in mice was associated with a decrease in thymidine synthesis compared to wild-type animals, but did not alter Hcy levels.

Less is known about how the MTHFR 677C→T polymorphism affects the response to Mtx on a cellular level. Sohn et al. investigated the response to Mtx in human colon and breast cancer cell lines overexpressing wild-type or 677C→T MTHFR (Sohn et al. 2004). Compared to cells

overexpressing wild-type MTHFR, expression of the 677 C→T form of the enzyme resulted in a lower proportion of 5-methyl THF, increased thymidylate synthase (TS) activity and increased cellular growth rate. Using an in vitro chemosensitivity assay, breast cancer cells overexpressing MTHFR with the 677T mutation were less sensitive to Mtx than the same cells over expressing wild-type MTHFR, but there was no difference in chemosensitivity in the colon cancer cell line (Sohn et al. 2004). The conclusion from this study was that overexpression of the 677T form of MTHFR opposed the action of Mtx by increasing levels of 5,10-methyl THF available for dTMP and purine synthesis. This is biologically plausible given the role of MTHFR in regulation of the distribution of folate cofactors between those necessary for DNA precursor synthesis and for the methylation cycle. However it does not explain the observed associations of TT genotype with increased risk of Mtx toxicity, which suggests that such individuals may be more sensitive to Mtx. A possible explanation for this discrepancy could be due to differences between the effect of overexpression of the 677T enzyme and the implications of the altered enzyme in vivo.

The initial area of interest for the current study was to investigate if cells with unaltered MTHFR expression had different sensitivity to Mtx based on MTHFR 677C→T genotype. EBV-transformed B-lymphocyte cell lines were chosen as a cell model because this cell type has previously been successfully used in this laboratory and therefore protocols for cell culture were well established. This type of cell line has been previously used in studies of the folate-dependent influence of the MTHFR 677C→T polymorphism (Lathrop Stern et al. 2003; Chiang et al. 2007), and separately in experiments investigating the growth inhibitory effects of Mtx (Dudman 1982). In addition, the effect of Mtx on cells of B-lymphocyte lineage is relevant because this cell type is inappropriately activated in RA (Edwards and Cambridge 2001), one of the conditions for which Mtx is the first-line treatment. The influence of the MTHFR 677C→T polymorphism on Mtx sensitivity has not been previously investigated in this cell type.

Mtx inhibits enzymes involved in de novo synthesis of both dTMP and purines. A blockade of de novo dTMP synthesis can be overcome by addition of thymidine (Thy) to cell culture medium. Thy is converted to dTMP by thymidine kinase, and can therefore act as a source of dTTP without the requirement for active TS (Lee and Cheng 1976). The consequences of inhibition of de novo purine synthesis can be rescued by using hypoxanthine (Hx), a substrate for hypoxanthine-guanine phosphoribosyl transferase (HGPRT). This enzyme is part of the purine salvage pathway and converts Hx to inosine monophosphate (IMP), circumventing the de novo purine synthesis pathway. Cell culture studies with Mtx have shown that the relative effect of the drug on dTMP and purine synthesis can vary depending on cell type. A study comparing normal cells of T-lymphocyte and B-lymphocyte lineage with tumour-derived cell lines showed that T-lymphocytes

required lower concentrations of Thy and Hx to overcome the growth inhibitory effect of Mtx than tumour-derived cells, and normal B-lymphocytes required higher concentrations again (Howell et al. 1981). A synergistic effect of Thy and Hx in blocking the effects of Mtx has been shown for many cell lines including murine lymphoma cells and normal fibroblasts (Refsum et al. 1991) and human T-lymphocyctes (Budzik et al. 2000). The response to Mtx in terms of growth inhibition or cell death can also differ by cell type. In many in vitro investigations of Mtx, treatment with the drug is cytotoxic, resulting in cell death, while in other cases the drug has a cytostatic effect, preventing proliferation but without killing cells. Cell types for which a cytotoxic effect of Mtx has been observed include endothelial cells (Merkle et al. 2000), human T-lymphocytes (Herman et al. 2005), and a number of tumour-derived cell lines including human leukemia (da Silva et al. 1996), hepatoma (Müller et al. 1997) and lung cancer (Huang et al. 2010). A cytotoxic effect of Mtx was observed in cultured human THP-1 cells, but the same study did not observe a cytotoxic effect of the drug in macrophages isolated from the joints of RA patients (Cutolo et al. 2000). In human T-lymphocytes, Mtx was observed to have a cytostatic effect without evidence of cell death (Fairbanks et al. 1999).

The influence of different folate cofactors on the actions of Mtx is a further question of interest. Previous cell culture studies have shown that 5-formyl THF and 5-methyl THF can have different effects on Mtx rescue in cell culture. Dudman et al. (1982) showed that both 5-methyl THF and 5-formyl THF could rescue lymphocytes from growth inhibition by Mtx equally well at low drug concentrations, but that 5-formyl THF was a more effective rescue agent at higher Mtx concentrations which inhibited greater than 85% of cell growth (Dudman 1982). This result suggests that increasing concentrations of Mtx inhibit cellular metabolism of 5-methyl THF to a greater extent than 5-formyl THF. Supplementation with folate is often a feature of clinical Mtx treatment, in an effort to reduce side effects of the drug. FA and 5-formyl THF are the folate forms commonly in pharmacological use, due to their high stability compared to 5-methyl THF. There is some evidence that the two forms of folate supplement can act differently in a clinical context. Morgan et al. showed that 5-formyl THF was able to normalise increased urinary 5-aminoimidazole-4-carboxamide (AICAR), a marker of inhibited purine synthesis caused by low-dose Mtx treatment; folic acid (FA) did not have this effect (Morgan et al. 2004). Recently there have been concerns about the appearance of unmetabolised FA in serum due to high intake of FA from supplements and fortified foods (Kalmbach et al. 2008). The potential of unmetabolised FA to interfere with antifolate therapies such as Mtx is one of the potential problems associated with this observation. A study of the mean annual Mtx dose for RA patients in the US observed that the mean dose was stable during the period 1998-1996, then increased in the period 1997-1999

(Arabelovic et al. 2007), coinciding with the introduction of mandatory fortification of grain products with FA. This result suggests that the increase in FA consumption post-fortification may have influenced Mtx treatment, although the study has several limitations including small sample size and the fact that the study design was a retrospective examination of patient data. Further information is necessary to establish if serum FA can interfere with Mtx treatment, and therefore it is of interest to establish how FA may affect Mtx treatment on a cellular level.

In this chapter, transformed B-lymphocyte cell lines of MTHFR 677 CC and TT genotype were used to determine if this polymorphism affects cellular response to Mtx. Experiments involving supplementation of the cell culture medium with either FA, 5-methyl THF, or 5-formyl THF were carried out to investigate how Mtx interferes with folate metabolism in these cell lines. The effect of Mtx on dTMP and purine synthesis was examined using hypoxanthine (Hx) and thymidine (Thy). In addition, Hcy release from cells was measured to determine if differences in Hcy release due to MTHFR 677C→T genotype can be detected in this cell culture system.

3.1.1 Aims

- To confirm the MTHFR 677C→T genotype of transformed B-lymphocyte cell lines.
- To investigate the functional effect of the MTHFR 677C→T polymorphism in human transformed B-lymphocyte cell lines by measuring cell growth and viability after methotrexate treatment.
- To determine the ability of various folate forms and sources of purines and dTMP to block the effect of Mtx in these cell lines.
- Investigate if Hcy efflux is affected by folate and riboflavin status in these cell lines.

3.2 MTHFR genotyping of transformed B-lymphocyte cell lines and establishment of cell stocks

Eight cell lines were purchased from the Coriell Cell Repository (CCR) collection. A number of cell lines from the CCR collection had previously been genotyped for the MTHFR C677→T polymorphism by Dr Larry Brody, and four cell lines of CC genotype and four cell lines of TT genotype were chosen. On receipt of the stock cultures from the CCR collection, each cell line was expanded and stocks were frozen down in liquid nitrogen as described in section 2.2.1.3, providing a bank of cells for use throughout this project.

DNA was extracted from a sample of cells from each cell line to confirm the MTHFR 677C→T genotype of each cell line. MTHFR genotyping was carried out as described in Chapter 2. An agarose gel showing the results of genotyping is presented in figure 3.1. Lane 2 is a positive control using DNA of known MTHFR 677 CT genotype, and shows two bands of 198 and 175 base pairs. Lane 3 is a negative control containing PCR reagents without any DNA, and does not show any band. The genotype of samples being tested can be determined by comparing the sizes of bands produced to the positive control sample. The wild type (MTHFR 677 CC) is represented by a single band of 198 base pairs. Lanes 4-7 show cell lines of CC genotype. Homozygotes for the mutation (MTHFR 677 TT) produce one band of 175 base pairs. Lanes 8-11 show cell lines of TT genotype. A list of each cell line by identification number with confirmed genotype is shown in table 3.1.

Cell line	MTHFR 677C→T genotype
17226	CC
17235	CC
17241	CC
17257	CC
17234	TT
17240	TT
17274	TT
17287	TT

Table 3.1 Identification number and MTHFR 677C→T genotype of transformed B-lymphocyte cell lines

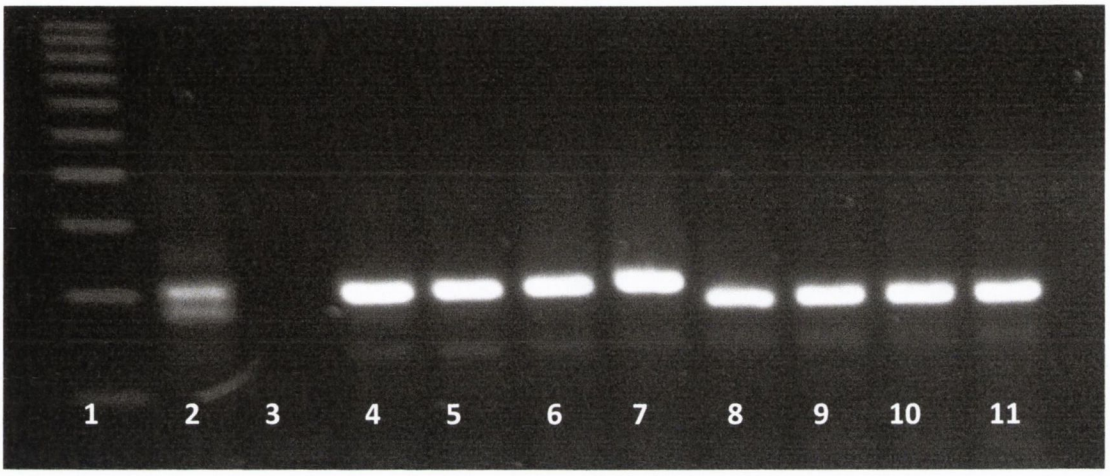

Figure 3.1 Agarose gel showing the results of MTHFR genotyping of transformed B-lymphocyte cell lines

DNA was extracted from $3x10^6$ cells from each cell line using Qiagen DNA Blood Mini kit. The MTHFR C677T region was amplified by PCR. The resulting product was digested with the restriction enzyme *Hin*F1 overnight at 37°C and samples were run on a 2.5% agarose gel.

Lane No:

1: 100bp DNA ladder
2: Positive control- 198 and 175 base pair bands - CT genotype
3: Negative control
4: Cell line 17241, 198 bp band – CC genotype
5: Cell line 17235, 198 bp band – CC genotype
6: Cell line 17257, 198 bp band – CC genotype
7: Cell line 17226, 198 bp band – CC genotype
8: Cell line 17287, 175 bp band – TT genotype
9: Cell line 17240, 175 bp band – TT genotype
10: Cell line 17274, 175 bp band – TT genotype
11: Cell line 17234, 175 bp band – TT genotype

3.3 Initial experiment to establish the effect of a range of methotrexate concentrations on proliferation of cell line 17241

In order to establish a suitable working concentration of Mtx to use with transformed B-lymphocyte cell lines, an initial experiment was carried out to investigate the effect of a wide range of Mtx concentrations on proliferation of cell line 17241. In order to investigate if the presence of FA in the growth medium would influence the effect of Mtx, the experiment was carried out in both standard RPMI growth medium which contains 2μM FA, and in folate-free RPMI.

The Mtx cell proliferation assay was used for this initial experiment. Several dilutions of Mtx stock in standard and folate-free RPMI were prepared, at twice the final assay concentration required (10,000/1000/100/10/1nM). 50μl of each of these Mtx solutions were pipetted into the wells of a 96-well plate. Triplicate wells were used for each condition, and one plate was prepared for each time point to be analysed. Cells (17241 CC) were washed three times in folate-free RPMI, and then counted as described in section 2.2.1.2. Two cell suspensions at $5x10^5$ cells/ml were prepared, one in standard RPMI and one in folate-free RPMI. 50μl of these cell suspensions was added to the appropriate wells containing growth medium with Mtx. This resulted in a final cell density of 0.25 $x10^5$ cells/well, at a range of Mtx at concentrations from 0-5000nM. A well containing 100μl RPMI medium without cells was included on each plate as a blank. At the time of setting up the experiment and 24h intervals thereafter, 20μl MTS assay reagent was added to each well of one 96-well plate. The plate was returned to the incubator for 4 hours, and then absorbance at 490nm was determined.

The results from this experiment are shown in figure 3.2. A log scale has been used in order to fit all Mtx concentrations on to the graph, and so the zero Mtx data point is not shown. Mtx has a dose-dependent cytotoxic effect in both folate-free RPMI medium and in standard RPMI containing 2μM FA. At Mtx concentrations below 5nM there is very little effect of Mtx on cell proliferation. At concentrations below this level proliferation was within 5% of the control level, with the exception of the folate-free condition after 48h incubation. In this case cell proliferation in the presence of 0.5nM Mtx was only 80% of the control value. At concentrations above 500nM the effect of Mtx is maximal. The presence of FA in the growth medium does not block the anti-proliferative effect of Mtx, because after 48h the curves for cell proliferation under both folate conditions are very similar. However, from the 24h time point, it can be seen that Mtx exerts its cytotoxic effect at an earlier stage in folate-free conditions than in the presence of 2μM FA. At 24h the effect of Mtx is more pronounced in folate-free condition than in standard RPMI which

contains 2μM FA. This result establishes the dose-dependent inhibition of cell proliferation by Mtx, and indicates that while FA does not block the effect of Mtx, it can reduce the effectiveness of the drug after short incubation times or at very low doses.

3.4 The influence of MTHFR 677C→T genotype on cell growth and viability in response to methotrexate

3.4.1 Comparison of the effect of methotrexate on growth and viability between cell lines of MTHFR 677 CC or TT genotype, by trypan blue staining and manual counting

The aim of this experiment was to further examine the effect of Mtx on cell growth and viability in these cell lines, and to determine if MTHFR 677C→T genotype has an influence on the cellular response to Mtx. The effect of several concentrations of Mtx on cell growth and viability was assessed in three cell lines of CC genotype and three cell lines of TT genotype using trypan blue staining and manual counting as described in chapter 2. This method of analysis is useful as it quantifies both live and dead cells, allowing discrimination between a cytostatic effect of Mtx and a truly cytotoxic effect. This experiment was carried out in folate-free RPMI because differences between CC and TT genotype are more likely to be observed under conditions of folate deficiency.

Cells which had previously been cultured in standard RPMI were harvested and rinsed three times in folate-free RPMI. The cells were counted as described in section 2.2.1.2 and then seeded in $25cm^3$ culture flasks at $2x10^5$ cells/ml in folate-free RPMI containing concentrations of Mtx from 0-1000nM. At 24h intervals, each flask was mixed with a sterile pipette to distribute cells evenly. A 1ml aliquot was taken from each flask, and cell count and percentage viability was determined by manual counting with trypan blue (Section 2.2.1.2.). This experiment was carried out for three cell lines of CC genotype (17235, 17257 and 17241), and three cell lines of TT genotype (17240, 17274 and 17287). The experiment was carried out three separate times for each cell line. The results were pooled together by genotype and are presented in Figure 3.3 (cell growth) and Figure 3.4 (percentage viability).

From figure 3.3 it can be seen that in folate-free conditions without Mtx, the number of live cells increases with time. This shows that cell proliferation continues during incubation without folate in the growth medium for 72h. At all concentrations of Mtx tested, the number of live cells decreases compared to initial cell density. This shows that the effect of Mtx is not simply a block of cell proliferation, and that cell death does occur due to the effect of Mtx.

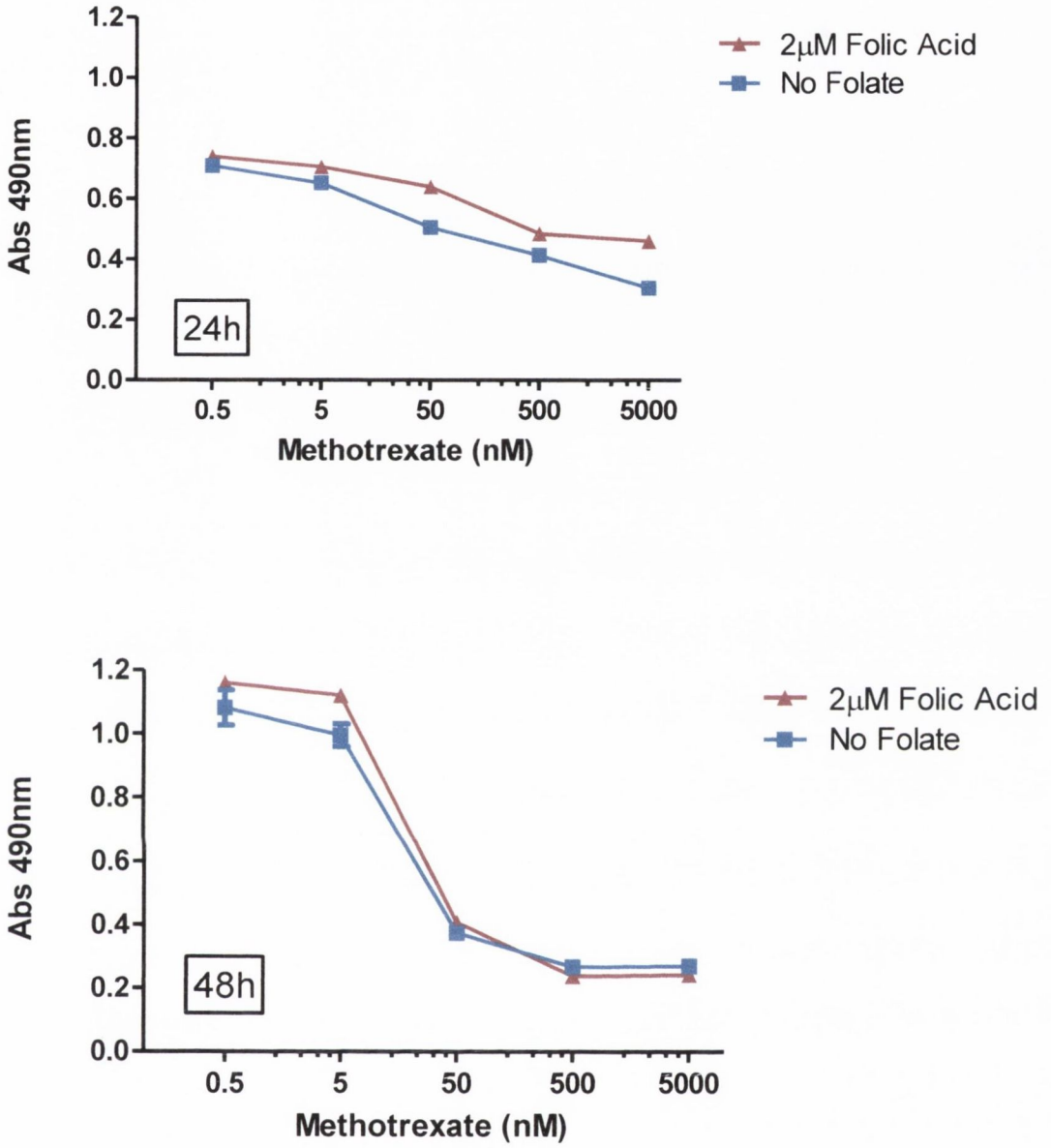

Figure 3.2 Initial experiment to establish the effect of a range of Mtx concentrations on proliferation of cell line 17241

Cells (17241 CC) were seeded at $2x10^5$ cells/ml at a range of Mtx concentrations from 0-5000nM, in both standard and folate-free RPMI. Proliferation was assessed by the MTS assay after 24h (upper panel) and 48h (lower panel). Data shown are the means +/- SD for 3 replicate wells for each data point.

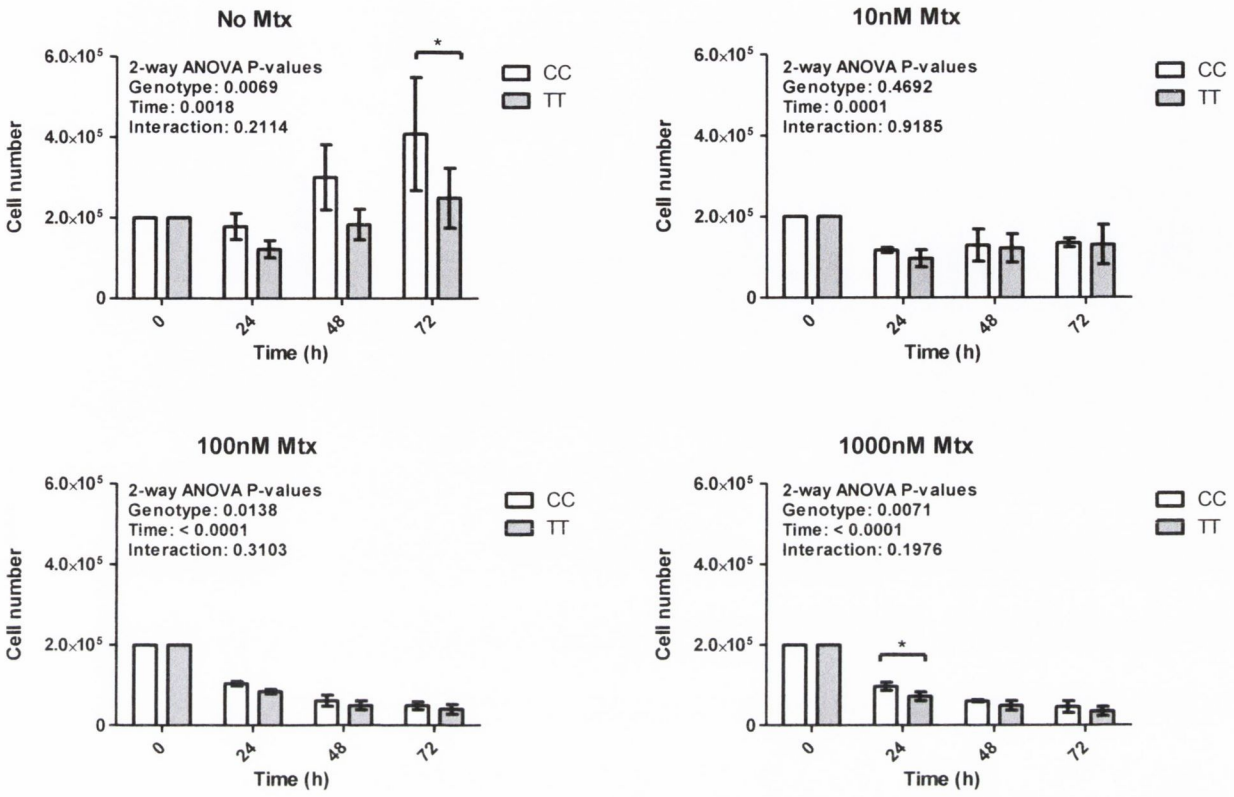

Figure 3.3 The effect of methotrexate on cell growth of transformed B-lymphocyte cell lines as assessed by trypan blue staining and manual counting.

Cells were seeded at $2x10^5$ cells/ml in folate-free medium, with Mtx concentrations from 0-1000nM. At 24h intervals, a sample was removed from each flask, and live cell counts determined by trypan blue staining and manual counting.

Data shown are the means +/- SD for 3 cell lines of CC genotype and 3 cell lines of TT genotype. The experiment was carried out two or three times with each individual cell line.

Two-way ANOVA for genotype and time point was used to test for differences due to genotype under each treatment condition. P-values for genotype, time and the interaction of these factors are shown on the graphs. Data points which remain significant after Bonferroni correction for multiple testing ($P < 0.05$) are marked with an asterisk.

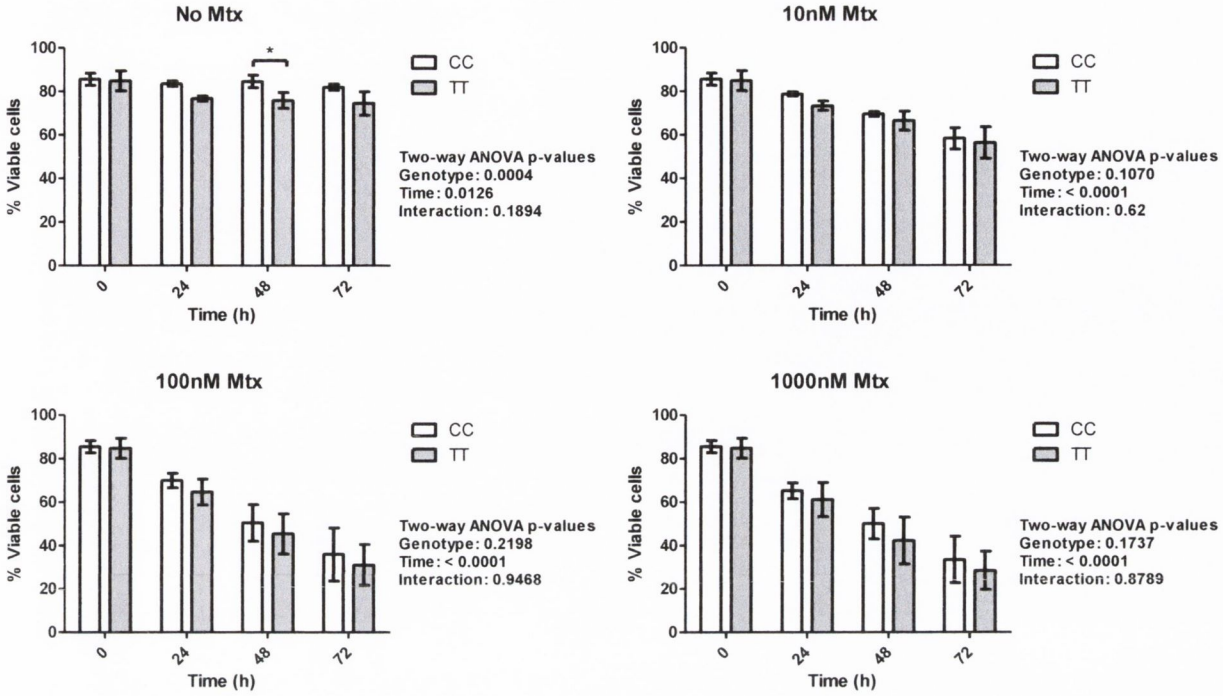

Figure 3.4 The effect of methotrexate on cell viability of transformed B-lymphocyte cell lines as assessed by trypan blue staining and manual counting

Cells were seeded at $2x10^5$ cells/ml in folate free RPMI with varying concentrations of methotrexate. At 24h intervals a sample was taken from each flask and cell viability assessed by trypan blue exclusion method.

Data are the means +/- SD for 3 cell lines of CC genotype and 3 cell lines of TT genotype. The experiment was carried out two or three times with each individual cell line.

Two-way ANOVA for genotype and time point was used to test for differences due to genotype under each treatment condition. P-values for genotype, time and the interaction of these factors are shown on the graphs. Data points which remain significant after Bonferroni correction for multiple testing ($P < 0.05$) are marked with an asterisk.

Two-way ANOVA was used to test the effects of genotype and time on cell growth under each treatment condition. Under folate-free conditions without Mtx there is a statistically significant difference in cell growth between genotypes, with TT cell lines having lower growth rate than CC cell lines ($P = 0.0069$, two-way ANOVA). Only the 72h time point remains significant after correction for multiple testing ($P<0.05$, Bonferroni), but the low sample size of $n = 3$ for each genotype is likely to contribute to lack of significance after Bonferroni correction at the other time points. For the Mtx-treated conditions, there is a significant difference in viable cell number by genotype in the presence of Mtx at 100nM ($P = 0.0138$, two-way ANOVA) and 1000nM ($P = 0.0071$, two-way ANOVA), with CC cell lines maintaining slightly higher viable cell numbers than TT cell lines. There is no difference according to genotype at 10nM Mtx ($P = 0.4692$, two-way ANOVA). The effect of time is significant under all treatment conditions, and there is no interaction between genotype and time, which is as expected. These results indicate that MTHFR 677C→T genotype does influence the effect of Mtx on cell proliferation at the higher concentrations of Mtx tested.

Percentage cell viability compares the number of live cells at a given time point with the total number of cells counted, both live and dead. When percentage cell viability is considered (figure 3.4) it can be seen that percentage viability remains largely stable in folate-free conditions over 72h, but at all concentrations of Mtx tested there is a decrease in cell viability with time over this period.

Two-way ANOVA was used to test the effects of genotype and time on percentage viability under each treatment condition. There is a significant difference by genotype in folate-free RPMI when Mtx is not present, with CC cell lines having higher cell viability than TT cell lines ($P = 0.0004$, two-way ANOVA). Only the 48h time point remains significant after Bonferroni correction. The magnitude of this difference in cell viability is small, for example at 24h cell viability for CC cell lines is 83.5% compared to 76.7% for TT cells, but it is statistically significant. This indicates that TT cell lines are more sensitive to low folate conditions than CC cell lines. This correlates with the results of the viable cell counts, which showed higher proliferation of CC cell lines under these conditions. This indicates that in conditions of low folate availability, TT cell lines have lower proliferation and a higher rate of cell death than CC cell lines.

The presence of Mtx causes a reduction in cell viability with time in cell lines of both genotypes, at all Mtx concentrations tested ($P < 0.0001$ for time, 2-way ANOVA). There is no difference in percentage viability by genotype at any of the Mtx concentrations tested. There is no interaction between time and genotype, which is as expected.

These results show that CC cell lines have higher proliferation and percentage viability under folate-free conditions, although the magnitude of this effect is small. Cell lines of CC genotype also had a higher proliferation rate than TT cell lines in the presence of 100nM and 1000nM Mtx. There were no statistically significant differences in percentage cell viability by MTHFR 677C→T genotype when Mtx was present. Mtx has a very strong effect on cell viability overall, which may obscure any smaller difference due to MTHFR 677C→T genotype. These results also demonstrate that Mtx causes cell death in transformed B-lymphocyte cell lines, rather than simply growth arrest.

3.4.2 Comparison of the effect of methotrexate on the proliferation rate of cell lines of MTHFR 677 CC or TT genotype by the MTS cell proliferation assay

In order to verify the results of the above experiment, the MTS cell proliferation assay was used as an alternative method for measurement of cell proliferation. This method was chosen as it is quicker and more objective than counting cells manually with a haemocytometer. The MTS assay is carried out in a 96-well format, and the output measured is absorbance at 490nm. The intensity of absorbance is proportional to the number of metabolically active cells in that well.

The assay was set up as described in section 3.3.. Briefly, 96-well plates were prepared with folate-free medium containing Mtx to result in final concentrations of 0, 10, 100 and 1000nM. Triplicate wells were used for each condition, and one plate was prepared for each time point to be analysed. Cells were washed three times in folate-free RPMI, then seeded at a final cell density of 0.25 $x10^5$ cells/well. A well containing RPMI medium without cells was included on each plate as a blank. At the time of setting up the experiment and 24h intervals thereafter, MTS reagent was added to one plate and absorbance at 490nm determined after a 4h incubation time. In order to compare genotypes, proliferation was calculated by expressing the absorbance for a particular condition as a percentage of the control (0nM Mtx) absorbance value for that time point. This was carried out for three cell lines of MTHFR 677 CC genotype (17235, 17241, 17257) and three cell lines of MTHFR 677 TT genotype (17240, 17274, 17287).

The results from this experiment are presented in Figure 3.5. Using 2-way ANOVA, there is a statistically significant difference in cell proliferation by genotype in the presence of 10nM Mtx ($P < 0.0001$), 100nM Mtx ($P = 0.014$) and 1000nM Mtx ($P = 0.023$) with TT cells having a lower proliferation rate when compared to CC cells. This difference remains significant after Bonferroni correction at all time points for 10nM Mtx, but was not significant after Bonferroni correction for the higher Mtx concentrations used. From the experiment carried out in section 3.3, it can be seen that Mtx at 10nM is very much at the lower end of the effective concentration range. It is

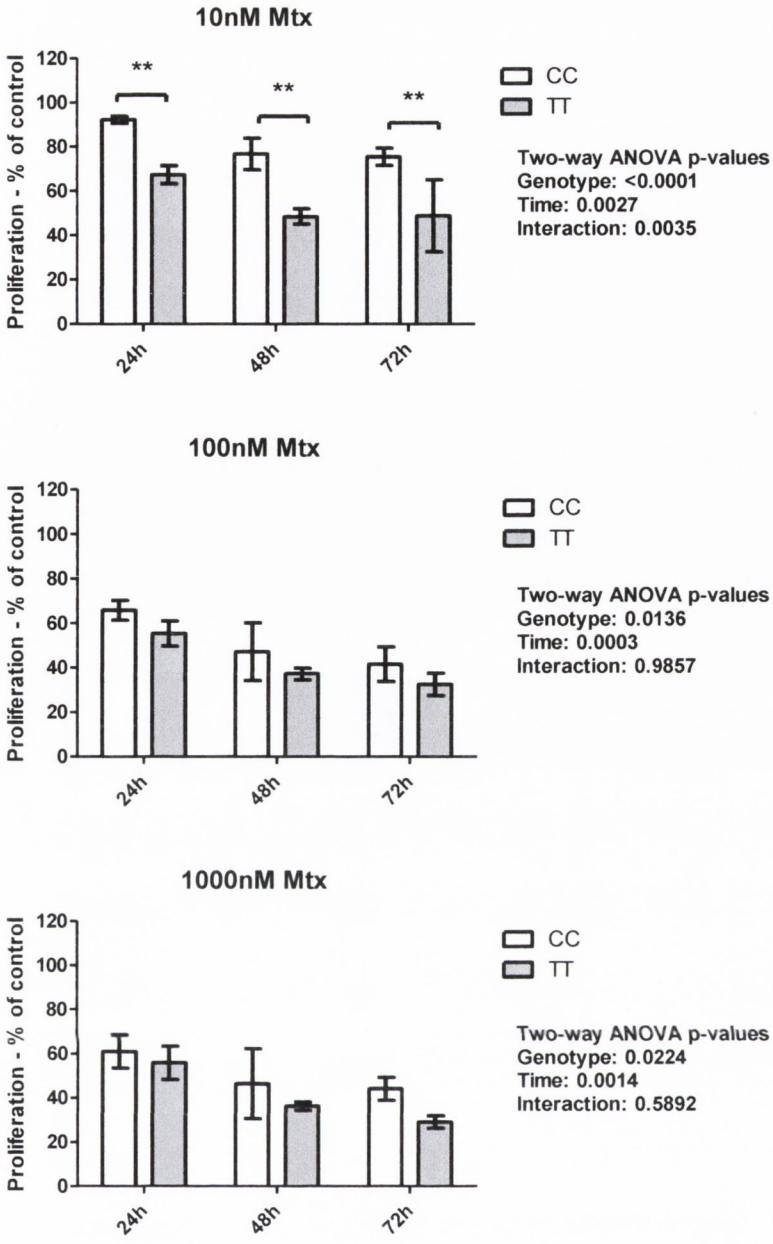

Figure 3.5 Comparison the effect of methotrexate on the proliferation rate of cell lines of MTHFR CC or TT genotype by the MTS cell proliferation assay

Cells were seeded in 96-well plates at a density of $0.25x10^5$ cells/well, in folate-free media containing Mtx at concentrations from 0-1000nM. MTS assay reagent was added at 24h intervals and absorbance at 490nm determined. Absorbances are expressed as a percentage of the control (0nM Mtx) value at that time point.

Data are the means +/- SD for 3 cell lines of CC genotype, or 3 cell lines of TT genotype. The experiment was carried out in triplicate for each individual cell line.

Two-way ANOVA for genotype and time point was used to test for differences due to genotype under each treatment condition. P-values for genotype, time and the interaction of these factors are shown on the graphs. ** = significant after Bonferroni correction for multiple testing ($P < 0.01$).

interesting to note that an effect of genotype is extremely significant at 10nM Mtx, but not at higher concentrations of the drug which have a much stronger effect on cell proliferation. This is in agreement with the results in section 3.4.1, where a genotype effect on cell viability was observed without Mtx but was obscured by the presence of the drug. This experiment also confirms the result presented in section 3.4.1, where TT cells had a lower proliferation rate than CC cells in the presence of Mtx.

3.4.3 Investigation of the effect of methotrexate concentrations below 10nm on transformed B-lymphocyte cell lines

In the preceding section, it was noted that TT cell lines had lower cell viability and a lower proliferation rate than CC cell lines in folate free conditions. There was also a statistically significant difference in cell proliferation by genotype in the presence of 10nM Mtx. The MTS assay was repeated with even lower concentrations of Mtx in order to investigate if the same difference by genotype was observed in very low concentrations of the drug. The experiment was set up as detailed in section 3.4.2, with a range of Mtx concentrations of 0, 1, 5 and 10nM. Figure 3.6 shows results of this experiment which was carried out twice in cell line 17274. As expected based on the results in section 3.4.2, there was a significant reduction in cell proliferation in the presence of 10nM Mtx after 48h incubation ($p = 0.024$, Student's t-test). However, at concentrations of Mtx lower than 10nM proliferation was not significantly different compared to untreated cells. This analysis was also carried out once each in cell lines 17240, 17235 and 17226, and there was no effect of Mtx concentrations of 5nM or lower observed in any cell line. For this reason further repeats of this experiment in a greater number of cell lines were not carried out.

3.5 Investigation of the ability of various folate forms to block the effect of methotrexate on transformed B-lymphocyte cell lines

Initial results presented in section 3.3 suggest that the presence of FA does not block the effect of Mtx, although it does appear to reduce the effect of the drug after 24h incubation time. This is consistent with the fact that FA requires reduction via DHFR before it can enter the cellular THF pool, and is thus in direct competition with Mtx for its main site of action. Other forms of folate such as 5-formyl THF or the physiological form 5-methyl THF do not require DHFR for their entry to the folate pool. In order to investigate if certain forms of folate are more effective at protecting against the effects of Mtx in transformed B-lymphocyte cell lines, the MTS assay was used to assess proliferation of cells exposed to 100nM Mtx with supplementation of either FA, 5-formyl THF or 5-methyl THF.

Folate-free RPMI containing Mtx and either 5-methyl THF, 5-formyl-THF or FA at twice the required concentrations was prepared. 50µl aliquots of the prepared medium were pipetted into 96-well plates, using three wells for each experimental condition. Cells of cell line 17274 were washed three times in folate-free RPMI to remove residual folate, then counted as described in section 2.2.1.2. A cell suspension was prepared at $5x10^5$ cells/ml in folate-free RPMI. 50µl of this cell suspension was added to each well in the plates. Control wells containing folate-free medium without Mtx, and one blank well containing medium without cells were also included. The final cell concentration was $2.5x\ 10^5$ cells/ml, and final concentrations in the medium were 100nM Mtx, and ranges from 0-25µM 5-methyl THF, 0-5µM 5-formyl THF, and 0-50µM FA. These ranges were chosen on the basis of values previously used by others (Dudman 1982). 5-formyl THF is known to be a very stable form of folate, so the maximum concentration used was 5µM. 5-methyl-THF is much less stable in aqueous solution, so a higher maximum concentration of 25µM was used. The standard concentration of FA in RPMI 1640 medium is 2µM, and it has already been determined in this study that this concentration of FA does not block the effect of Mtx. Higher concentrations of FA were thus used to investigate the possibility that addition of sufficient FA to the culture medium might overcome the effects of Mtx. The experiment was repeated on two separate occasions.

The results of this experiment are presented in figure 3.7. FA did not allow cell proliferation in the presence of Mtx at any of the concentrations tested. The effect of 5-formyl-THF and 5-methyl THF was very different, in that both forms of folate allowed cell proliferation in the presence of 100nM Mtx, with a dose-dependent increase in cell proliferation with increased folate concentration. Cell proliferation in the presence of 100nM Mtx was restored to control levels by 5-methyl THF at 15µM, and the highest concentration of 5-formyl THF tested (5µM) restored proliferation to 90% of the control value.

These results are interesting because rather than looking at direct inhibition of an enzyme by different folate forms, they show how different forms of folate oppose the effect of Mtx on the cell as a whole. Although Mtx is a competitive inhibitor of DHFR, even very high supra-physiological concentrations of FA have no effect on the action of Mtx. However 5-Methyl THF and 5-formyl THF interfere with the action of Mtx within the cell, as they show a dose-dependent opposing effect which at sufficient concentration can completely block the antiproliferative effect of Mtx on the cell.

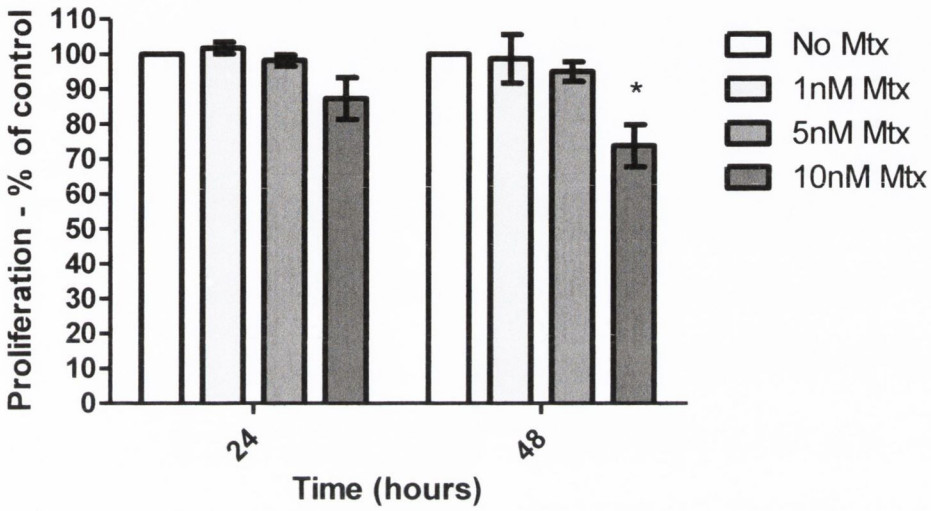

Figure 3.6 Investigation of the effect of Mtx concentrations below 10nM on cell proliferation of transformed B-lymphocyte cell lines

Cells (17274) were seeded in 96-well plates at a density of 0.25 x 10^5 cells/well, in folate-free media containing Mtx at concentrations from 0-10nM. At 24h intervals, MTS assay reagent was added and absorbance at 490nm determined after a 4h incubation at 37°C.

Absorbances are expressed as a percentage of the control (No Mtx) value at that time point. Data are the means +/- SD of two independent experiments with cell line 17274. * = different from respective control, $p < 0.05$, Student's t-test.

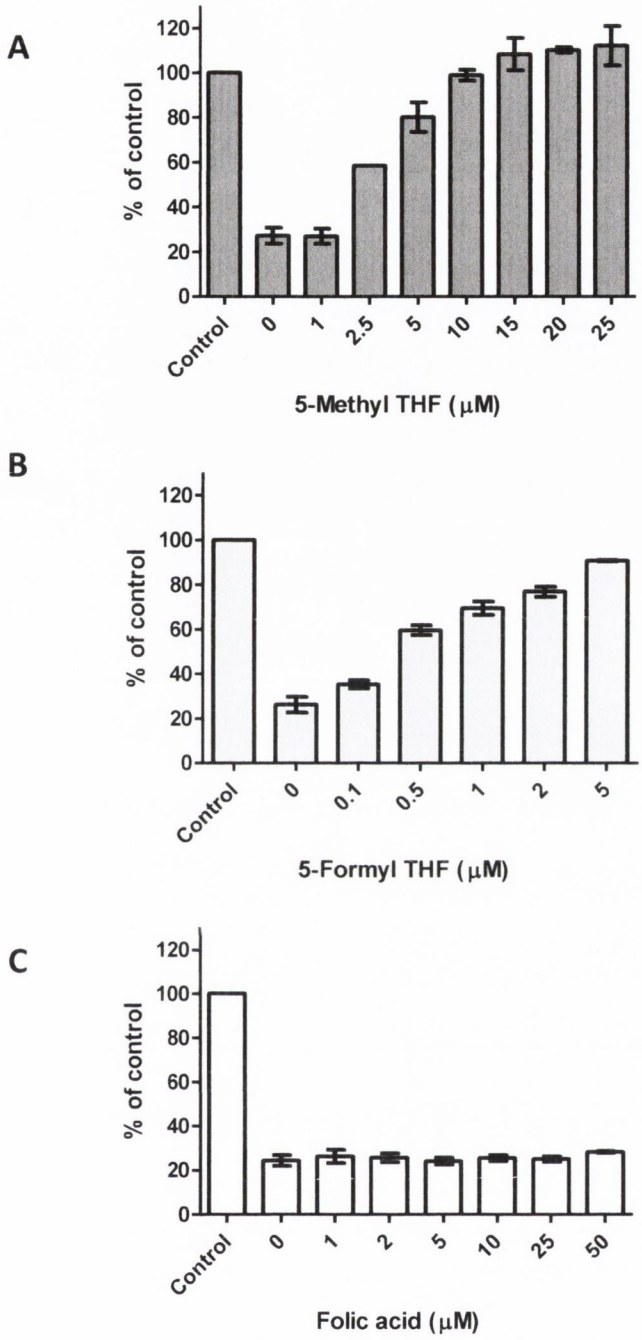

Figure 3.7 Investigation of the ability of various folate forms to block the effect of methotrexate on transformed B-lymphocyte cell lines

Cells (17274) were seeded at $2x10^5$ cells/ml in folate-free RPMI medium containing 100nM Mtx and a range of concentrations of 5-methyl THF (A), 5-formyl THF (B) or folic acid (C). After 48h incubation, proliferation was measured by the MTS assay. Data are the means +/- SD of two independent experiments. Cell proliferation is expressed as a percentage of the control value (without Mtx).

3.6 Evaluation of the ability of thymidine and hypoxanthine to protect against the cytotoxic effect of methotrexate

The major enzyme target of methotrexate is DHFR. Inhibition of this enzyme prevents regeneration of THF from DHF produced in synthesis of dTMP, and this ultimately blocks DNA synthesis. However Mtx also inhibits folate-dependent steps in the purine synthesis pathway. In order to investigate the relative importance of these biosynthetic pathways in transformed B-lymphocyte cell lines, the effect of supplementation with thymidine (Thy) and hypoxanthine (Hx) on the cytotoxic effect of Mtx was determined. Thy will overcome Mtx-induced inhibition of dTMP synthesis, and Hx will overcome the inhibition of purine synthesis.

3.6.1 Initial experiment to establish the effect of a range of thymidine and hypoxanthine concentrations in protection against methotrexate cytotoxicity

The first step was to investigate the effects of Hx and Thy separately, in order to assess suitable concentrations of each compound for use in this system. Cells of cell line 17241 were set up for the MTS cell proliferation assay as described in section 3.3, in standard RPMI containing 100nM Mtx and increasing concentrations of hypoxanthine (0-1000 μM) or thymidine (0-1000 μM). A condition of standard RPMI medium without Mtx was also included as a control. Absorbance was measured at initial time of setup, and after 24, 48 and 72 hours incubation time. The results from this experiment are presented in figure 3.8.

Figure 3.8 (A) shows cell proliferation in the presence of 100nM Mtx and increasing concentrations of Hx. The presence of 100nM Mtx caused a decrease in absorbance at 490nm over the time of the experiment, indicating that the cells are not proliferating and are in fact dying off. The addition of Hx at concentrations from 1-1000μM did not prevent this decrease in viable cells at any of the concentrations tested. This indicates that provision of a substrate for the purine salvage pathway does not block the cytotoxic effects of Mtx in this cell line. Figure 3.8 (B) shows cell proliferation in the presence of 100nM Mtx and increasing concentrations of Thy. In contrast to Hx, Thy prevents the drop in cell numbers associated with Mtx treatment, and even supports a small amount of cell proliferation in the presence of Mtx. This effect of Thy is maximal at concentrations of 10μM or greater. However, even at the highest concentration tested, Thy does not restore cell growth to the level of the control without Mtx. Provision of dTMP alone can partially prevent the effect of Mtx by inhibiting the cytotoxicity of the drug, but cannot support normal levels of proliferation. This result indicates that inhibition of dTMP synthesis is not the sole consequence of Mtx treatment in this transformed B-lymphocyte cell line.

Investigation of the ability of thymidine or hypoxanthine or a combination of the two to prevent the cytotoxic effects of methotrexate on transformed B-lymphocyte cell lines

A combination of hypoxanthine (Hx) and thymidine (Thy) together have previously been shown to rescue cells from the effects of Mtx more effectively than thymidine alone in human T-lymphocytes (Budzik et al. 2000), and in murine fibroblasts and lymphoma cells (Refsum et al. 1991). In order to investigate if this is effective in transformed B-lymphocyte cell lines, MTS assays were carried out using a combination of Hx and Thy in the presence of 100nM Mtx. Two cell lines, 17241 CC and 17274 TT, with differing MTHFR 677 genotype but with similar growth rates were used to investigate if MTHFR 677 genotype might influence the ability of Hx and Thy to prevent the cytotoxic effect of Mtx.

Standard RPMI containing Mtx and either Hx, Thy or both at twice the required final concentrations was prepared. 50µl aliquots of the prepared medium was pipetted into 96-well plates, using three wells for each experimental condition. Cells were counted as described in section 2.2.1.2. A cell suspension was prepared at $5x10^5$ cells/ml in standard RPMI. 50µl of this cell suspension was added to each well in the prepared 96-well plates. The final cell concentration was $2.5x\ 10^5$ cells/ml, and final concentrations in the wells were 100nM Mtx, 100µM Thy and 40µM Hx. The thymidine concentration was chosen based on section 3.6.1 above, as it is above the level which produces maximal rescue of proliferation from Mtx. The same experiment did not show any effect for Hx alone, so the concentration of Hx used here was chosen based on a level that had previously been used for Mtx rescue in the literature (Refsum et al. 1991).

Proliferation was measured at 24, 48 and 72 hours after setting up the assay. Differences between the treatment conditions were not apparent after 24h, so the 48h and 72h time points were used for analysis, as shown in figure 3.9. Methotrexate treatment reduced proliferation to 30% of the control level after a 48h incubation, and to 15% of the control level after a 72h incubation. One-way ANOVA was used to compare the groups for each cell line at each time point. At both time points, Thy was found to partially protect from the effects of Mtx, restoring proliferation to 50% of the control level after a 48h incubation, and to 35 % of the control level after a 72h incubation. Hx alone was not protective, because proliferation remained at the same level as for Mtx treatment. However, a combination of Hx plus Thy had a synergistic effect, and restored proliferation to control levels. The same effects of Thy and Hx were observed in both cell lines tested.

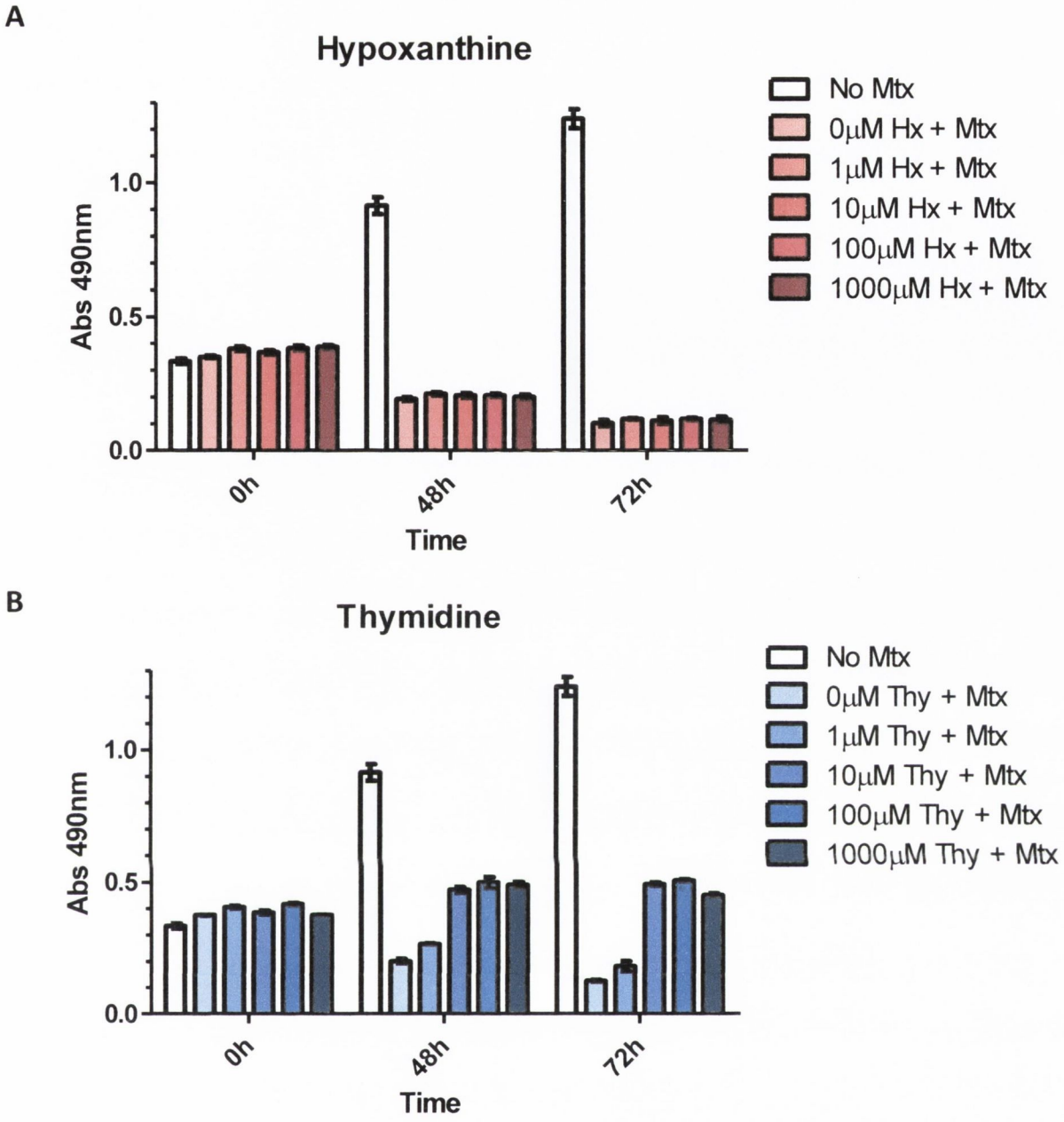

Figure 3.8 Initial experiment to establish the effect of a range of thymidine and hypoxanthine concentrations in protection against Mtx-induced cytotoxicity

Cells (17241 CC) were seeded at $2x10^5$ cells/ml in standard RPMI containing 100nM Mtx, and a range of concentrations of either (A) hypoxanthine (Hx) or (B) thymidine (Thy). Cell proliferation was assessed by the MTS assay after 48h and 72h incubation time.

Data are the means +/- SD of an experiment carried out in triplicate with cell line 17241 CC.

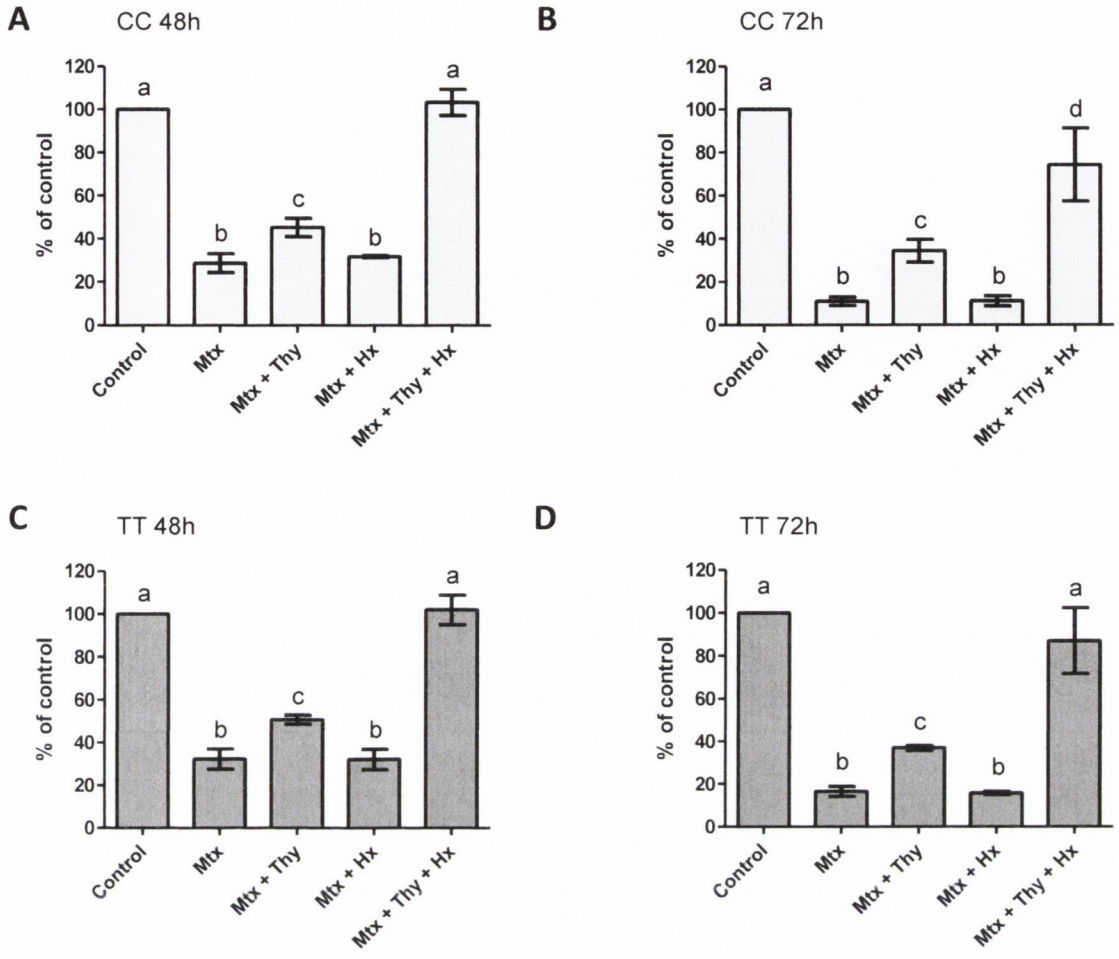

Figure 3.9 Investigation of the ability of hypoxanthine and thymidine or a combination of the two to prevent the cytotoxic effects of methotrexate on transformed B-lymphocyte cell lines

Cells were seeded in 96-well plates at a density of $0.25x10^5$ cells/well, in folate-free media containing Mtx (100nM) and hypoxanthine (40μM) or thymidine (100μM) as indicated. Proliferation was determined at 48h and 72h by MTS assay. Panels A (48h) and B (72h) show results for cell line 17241 CC, panels C (48h) and D (72h) shown results for cell line 17274 TT. Absorbances are expressed as a percentage of the control (0nM Mtx) value at that time point.

Data are the means +/- SD of 3 separate experiments for each cell line. Within each graph, the groups were compared by one-way ANOVA with Tukey's post-tests. Bars without a common letter are statistically different ($p < 0.05$).

A Student's t-test was used to compare the values at each data point between CC and TT cell lines. There was only one instance where there was a significant difference by genotype. At 72h, the CC cell line had lower proliferation in the presence of Mtx than the TT cell line, but this barely reached statistical significance. The difference between means was 5.5% ± 1.754 (p=0.0351, 95% CI -10.36 to -0.625).

Overall, this experiment shows that supplementation with Thy partially blocks the effects of Mtx, but Hx alone does not. However, these two compounds together have a synergistic effect and completely block the cytotoxicity of Mtx. This demonstrates that the effect of Mtx occurs by blockade of both dTMP and purine synthesis in transformed B-lymphocyte cell lines. This experiment does not provide evidence that the MTHFR C677→T genotype modifies the blockade of Mtx-induced cytotoxicity by Hx, Thy or a combination of the two compounds.

3.7 Investigation of the influence of folate, riboflavin and MTHFR 677C→T genotype on homocysteine efflux from cultured B-lymphocyte cell lines

In order to investigate whether cells of MTHFR CC and TT genotype were functionally different in terms of homocysteine (Hcy) efflux, experiments were carried out to measure homocysteine (Hcy) levels in the supernatant from cell cultures in standard RPMI, folate-free RPMI (FF) and riboflavin and folate-free RPMI (RFF).

Cells were seeded at $2x10^5$ cells/ml in a 96-well plate, in a final volume 200µl of culture per well. At the time of setting up the experiment, and at 24h intervals thereafter, the contents of triplicate wells were transferred to separate 0.5ml microcentrifuge tubes. The tubes were centrifuged briefly in a microcentrifuge to pellet the cells. The supernatant growth medium was transferred to a clean labelled microcentrifuge tube, and stored at -40°C for later determination of Hcy content. The cell pellet was resuspended in 0.4% Trypan Blue and the cells counted as described in section 2.2.1.2. The volume of Trypan Blue used was adjusted to provide a suitable cell density for accurate counting, and this volume was recorded to subsequently allow calculation of the total number of cells in each well.

Initially this experiment was carried out with cell line 17241 CC, in order to determine a time point when Hcy becomes detectable in the supernatant growth medium under these experimental conditions. Figure 3.10 shows the relationship between Hcy efflux and cell growth for cell line 17241 CC. There is a strong correlation between Hcy efflux and cell growth. Hcy is present above the limit of detection for the assay at all time points, however Hcy levels remain very low up to 8h

of incubation time. For further analysis, Hcy concentration in the growth medium was measured at time of experimental setup, then after later time points of 24 and 48h, and measurements were extended to include a 72h time point.

This experiment was repeated three times with a TT cell line (17274 TT) in standard RPMI. The experiment was also carried out in FF medium and RFF medium for both cell lines (17241 CC and 17274 TT). Three independent repeats of the experiment were carried out for each cell line in each type of medium. The aim of these experiments was to determine how the supply of folate and riboflavin would affect cell growth, cell viability or export of Hcy in these cell lines, and if any such effects are modified by MTHFR 677C→T genotype.

The results for cell growth are shown in figure 3.11. The number of viable cells per well increased over time in both standard and FF medium, although higher maximum cell numbers were achieved in standard medium than in FF medium. In contrast, cells in the RFF condition did not proliferate at all, as cell numbers decreased from initial levels. Although these cells appear to have sufficient folate reserves to maintain a certain level of proliferation over 72 hours in folate-free conditions, starvation of both folate and riboflavin is a much more damaging condition, causing significant decline in live cell numbers within this timeframe. Two-way ANOVA for genotype and time was used to test for differences by genotype. There was no effect of genotype on total cell number in standard ($p= 0.18$) or FF media ($p = 0.12$), but in RFF medium the TT cell line had lower total cell number compared to the CC cell line ($p=0.0016$, 48h and 72h time points remain significant after Bonferroni correction $p < 0.05$). There was a difference in cell number with time in all cases ($p < 0.001$), but there was no interaction between genotype and time, which is as expected.

Results for percentage cell viability are shown in figure 3.12. In standard RPMI medium, there was no decrease in cell viability over time, because for both cell lines viability remained above 80% throughout the experiment. Two-way ANOVA showed no significant difference in viability over time in standard RPMI ($p = 0.54$ for time). From the two-way ANOVA analysis, there was a difference in viability between CC and TT in standard medium, with TT cells having slightly higher viability. However the actual difference was very small (viability ranged between 85 and 92%) and this was not significant after Bonferroni correction. There was no interaction between genotype and time ($p = 0.97$).

When cells were incubated in FF medium, the mean cell viability for the CC cell line was 80% after 72h, and mean viability for the TT cell line was 62% after 72h. Two-way ANOVA showed that there was a significant difference in cell viability between genotypes under folate-free conditions,

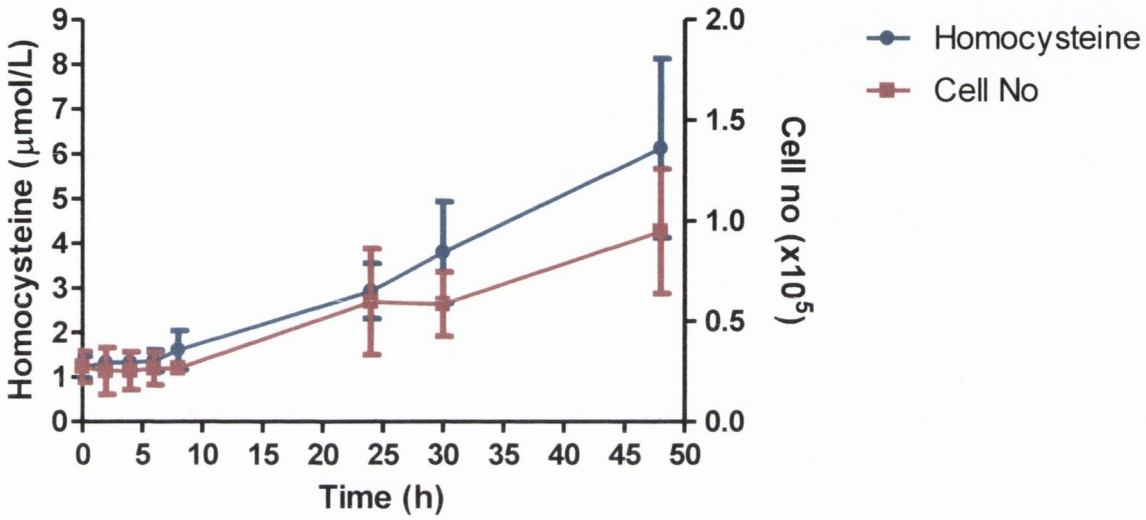

Figure 3.10 The relationship between homocysteine efflux and cell growth in cell line 17241

Cells (17241 CC) were seeded at $2x10^5$ cells/ml in standard RPMI in a 96-well plate. At each time point, cell number was determined for triplicate wells, and Hcy concentration of the growth medium was determined. Data are the means +/- SD of three separate experiments.

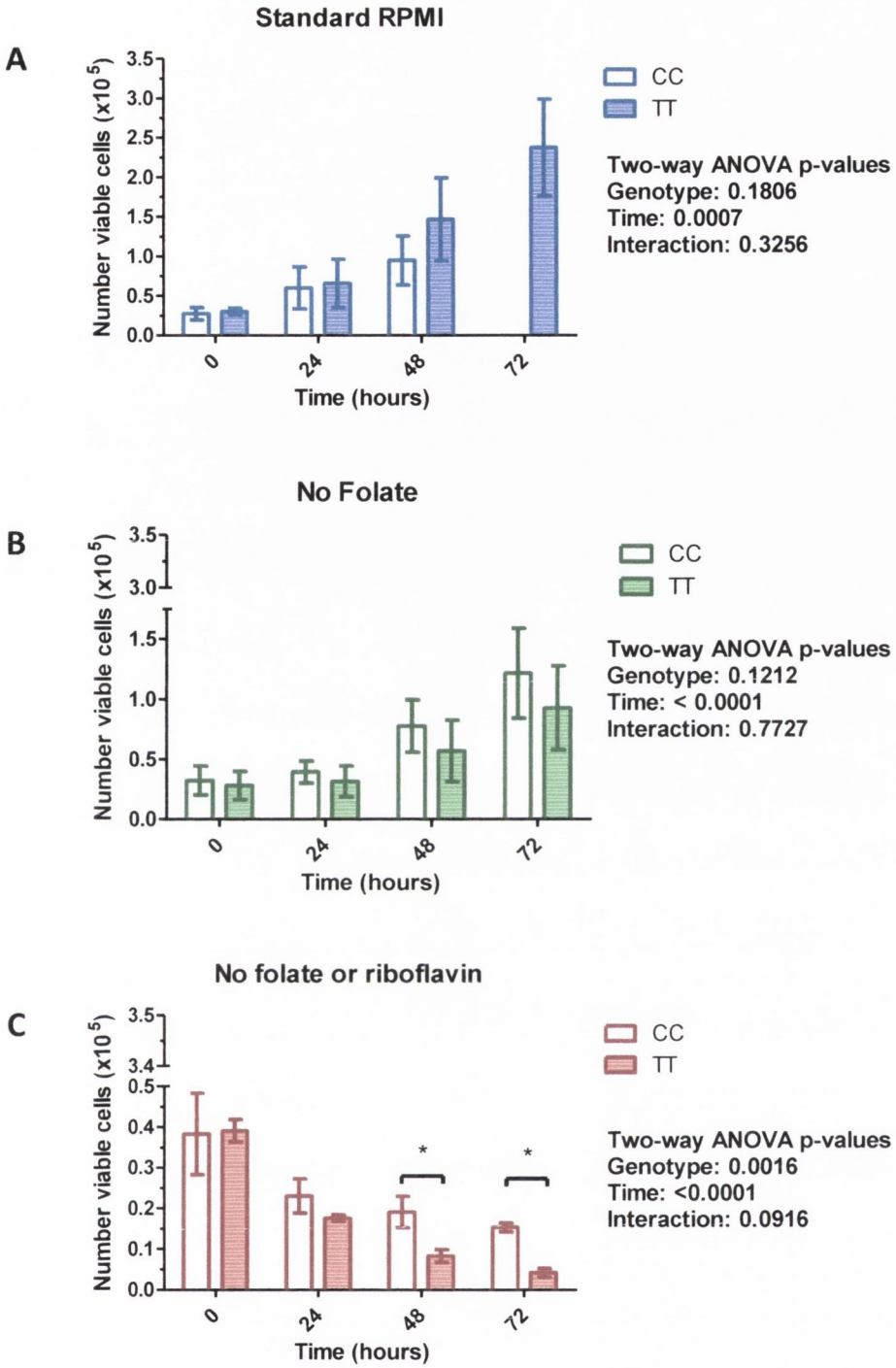

Figure 3.11 The effect of folate and riboflavin availability in culture medium on cell growth of cell lines 17241 CC and 17274 TT

Cell lines 17241 CC and 17274 TT were seeded at $2x10^5$ cells/ml in standard RPMI (A), RPMI without folate (B), or RPMI without folate or riboflavin (C). The number of viable cells/well was determined by manual counting at the indicated time points. Data are the means +/- SD of 3 independent repeats with each cell line. Two-way ANOVA was used to assess the effects of genotype and time for each condition. Marked bars are significantly different after Bonferroni correction, * = $p < 0.05$, *** = $p < 0.0001$.

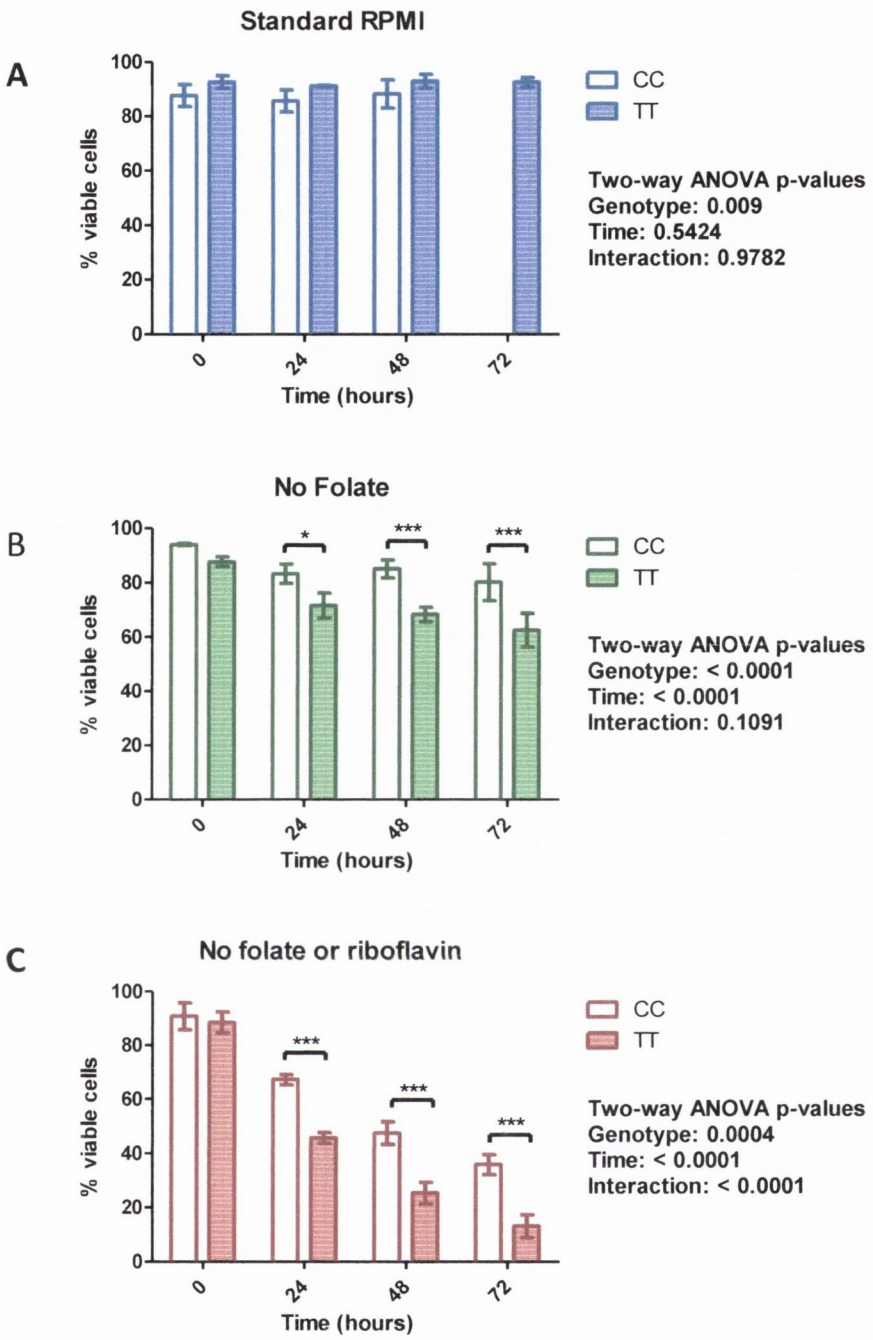

Figure 3.12 The effect of folate and riboflavin availability in culture medium on cell viability of cell lines 17241 CC and 17274 TT

Cell lines 17241 CC and 17274 TT were seeded at 2x105 cells/ml in standard RPMI (A), RPMI without folate (B), or RPMI without folate or riboflavin (C). Cell viability was determined by trypan blue staining and cell counting at the indicated time points. Data are the means +/- SD of 3 independent repeats with each cell line. Two-way ANOVA was used to assess the effects of genotype and time for each condition. Marked bars are different after Bonferroni correction, * = $p < 0.05$, *** = $p < 0.001$

with the TT cell line having lower viability ($p = < 0.0001$). This difference remained significant after Bonferroni correction at all time points (excepting the 0h control time point). There was no interaction between genotype and time ($p = 0.1$). This result confirms the effect of genotype on cell viability in folate-free conditions observed in section 3.4.1.

There was a decrease in cell viability with time in RFF medium, of greater magnitude than that in FF conditions. After 72h incubation in RFF conditions, mean cell viability of the CC cell line was 35% and for the TT cell line mean cell viability was 13%. This is as expected considering the decline in cell number in RFF medium which was noted above. Two-way ANOVA showed a difference in viability by genotype under this condition, with the TT cell line again having the lower viability ($p = 0.0004$). This difference remained significant after Bonferroni correction for multiple testing. In this case there was an interaction between the factors genotype and time, indicating that the difference by genotype is not the same at all time points. This suggests that while both cell lines were severely hindered by folate and riboflavin deficiency, the effect was more severe in the TT cell line.

The concentration of Hcy in the supernatant growth medium was also determined for each sample. In all cases, the concentration of Hcy measured in the supernatant medium increases with time, reflecting continued release from cells into the culture medium. To allow comparison between different cell lines, Hcy levels were corrected for number of viable cells and this data is presented in figure 3.13. In standard RPMI medium, there is no statistical increase in Hcy/10^5 cells over time ($p = 0.28$ for time, two-way ANOVA). In contrast, the level of Hcy in supernatant medium increases over time in both FF ($p = 0.0004$ for time, two-way ANOVA) and RFF free conditions ($p = < 0.0001$ for time, two-way ANOVA). In standard RPMI, levels of Hcy do not exceed 6.5 μmol/L/10^5 cells. Under FF conditions, the level of Hcy is slightly higher, reaching 9.0 μmol/L/10^5 cells in the CC cell line and 7.8 μmol/L/10^5 cells in the TT cell line after 72h. Hcy levels are much higher in RFF conditions, at 17.5 μmol/L/10^5 cells for CC and 46.0 μmol/L/10^5 cells for TT cells after 72h. By two-way ANOVA, there is no effect of genotype detected in FF culture medium ($p = 0.41$), but in RFF medium Hcy concentration was higher in the TT cell line than the CC cell line, and this remains significant after Bonferroni correction (72h time point). There is a significant effect of genotype on Hcy level in standard RPMI ($p = 0.02$), but in this case the overall level of Hcy does not increase with time.

Overall these results indicate that transformed B-lymphocyte cell lines do not produce large amounts of Hcy under standard cell culture conditions where folate is plentiful. If folate is deficient, the level of Hcy produced by the cells is increased, and cell viability decreases although

a proportion of cell proliferation is maintained. Complete deficiency of folate plus riboflavin is a serious insult to the cells, resulting in a sharp decline in cell numbers and cell viability. This is associated with an increase in the Hcy output per cell. In agreement with results observed above, there is evidence that cells with MTHFR 677 TT genotype are more sensitive to deficiency of folate and the combination of folate and riboflavin than cells of MTHFR 677 CC genotype.

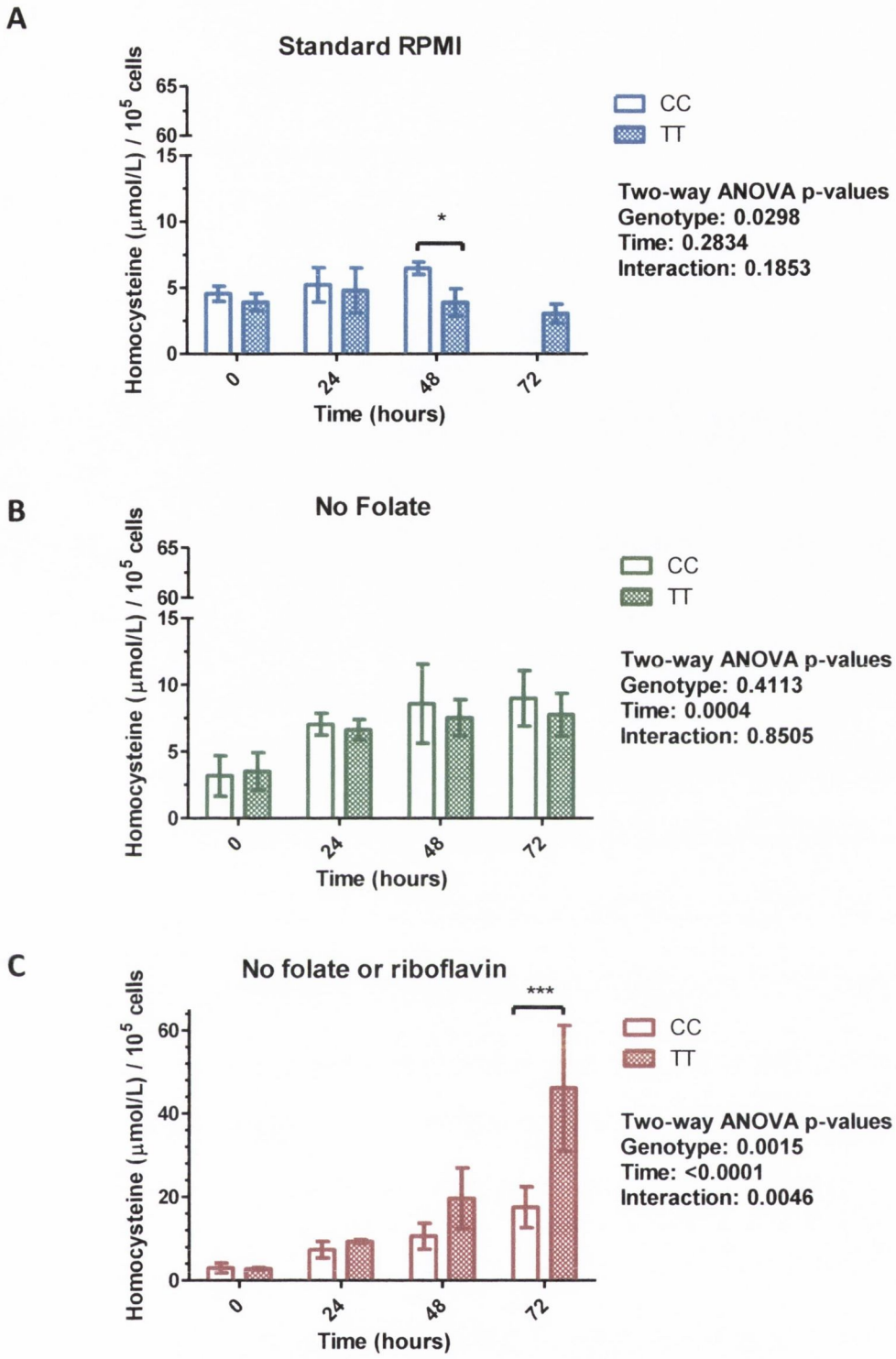

Figure 3.13 Homocysteine efflux from cell lines 17241 CC and 17274 TT under varying availability of folate and riboflavin

Cell lines 17241 CC and 17274 TT were seeded at $2x10^5$ cells/ml in standard RPMI (A), RPMI without folate (B), or RPMI without folate or riboflavin (C). Homocysteine concentration per 10^5 viable cells was determined at the indicated time points. Data are the means +/- SD of 3 independent experiments with each cell line. Two-way ANOVA was used to assess the effect of genotype and time for each condition. Marked bars are different after Bonferroni correction, * = $p < 0.05$, *** = $p < 0.001$.

3.8 Discussion

In this chapter, transformed B-lymphocyte cell lines which were homozygous for either allele of the MTHFR 677C→T polymorphism were used to investigate the hypothesis that this polymorphism would influence cell proliferation and viability in response to Mtx. The MTHFR 677 genotype of eight transformed B-lymphocyte cell lines was confirmed by PCR genotyping. An initial experiment was carried out to establish a suitable concentration of Mtx to use with these cell lines. The effect of Mtx was dose dependent. The lowest concentration of the drug which reduced cell proliferation relative to controls was 5nM, and the effect of Mtx was maximal at 500nM. The presence of FA in cell culture medium was observed to delay the effect of Mtx at a shorter incubation time of 24h, but after 48h there was no difference in the effect of Mtx in the presence or absence of FA. The effective range of Mtx concentrations determined in this work is similar to that of other cell culture studies, for example Huang et al. (2010) who reported a dose-dependent effect of Mtx on cell proliferation human lung carcinoma cells, with at a maximum effect at 1μM Mtx (Huang et al. 2010). It is difficult to directly relate the concentration of Mtx used in cell culture studies to the plasma concentration of Mtx achieved during drug treatment, due to the large variation in Mtx doses used for various conditions, and the variable effects of absorption and clearance between different individuals. However, the effective Mtx concentration range reported here is comparable to the steady state serum Mtx concentration range of 40.8-190nM reported during treatment of psoriasis (Chladek et al. 1998), and the intracellular Mtx polyglutamate concentration of 85.8 +/- 48.4 nM reported in treatment of juvenile idiopathic arthritis (Becker et al. 2010).

The effect of Mtx on cell proliferation and viability of three cell lines of MTHFR 677 CC genotype and three cell lines of MTHFR 677 TT genotype was assessed by trypan blue staining and manual counting. Three concentrations of Mtx were used which spanned the effective range of the drug (10nM, 100nM and 1000nM) as determined for these cell lines (see fig.3.2). This experiment was carried out in folate-free culture medium to avoid potential interference from extracellular folate sources. In the control condition of folate-free culture medium, cells of MTHFR 677 TT genotype were found to have a lower proliferation rate compared to cells of CC genotype. This difference in proliferation rate was also observed in the presence of Mtx at concentrations of 100nM and 1000nM, but was not apparent at 10nM Mtx. It is notable that the lower proliferation rate in cell lines of TT genotype was observed in the control condition, where cell proliferation continued over the time frame of the experiment, and also in the presence of Mtx which caused a decline in the number of live cells. When cell viability was examined, TT cell lines had lower percentage viability than CC cell lines in the control condition of folate-free RPMI, but there was no difference

by genotype detected for any of the Mtx concentrations tested. A second assessment of the effect of Mtx on cell proliferation was carried out using a complementary method for quantification of cell proliferation, the MTS assay. This assay also showed that TT cells had lower levels of proliferation compared to CC cells in the presence of all concentrations of Mtx tested. The difference by genotype was most significant at 10nM Mtx, the lowest concentration tested, and was less so at higher concentrations of the drug. Taken together, these results indicate that MTHFR TT genotype confers increased sensitivity to low-folate conditions, both in the presence and absence of Mtx. The difference in cell proliferation or viability due to genotype is small, in the order of 10-15% and becomes less apparent at higher concentrations of Mtx, which have a potent cytotoxic effect, reducing cell proliferation to less than 50% of the control level after a 48h incubation. Overall, the magnitude of the cytotoxic effect of Mtx is larger compared to the influence of MTHFR genotype on cell proliferation in these cell lines. However, this does not exclude the possibility that MTHFR 677C→T genotype may be relevant in the response to Mtx in a physiological setting, particularly in the case of low-dose Mtx where treatment is long term and the effects of the drug will be cumulative over time.

Many of the symptoms of Mtx toxicity are observed in tissues that undergo cell proliferation, such as bone marrow and gastrointestinal tract (McKendry and Dale 1993; Attar 2010), and it is likely that such toxicity is related to suppression of normal cell proliferation by the drug. There are several reports that the MTHFR 677C→ T polymorphism is associated with increased risk of Mtx toxicity. The presence of the T allele was associated with increased risk of gastrointestinal or hepatic toxicity of Mtx (Ongaro et al. 2009), and other studies have reported increased risk of relapse after Mtx treatment of ALL (Aplenc et al. 2005; D'Angelo et al. 2011). However, this association is not universally observed because other studies have not found such an effect (Wessels et al. 2006; Owen et al. 2010). The results presented here show that cells of TT genotype have a lower proliferation rate than CC cells in the presence of Mtx, and also under control conditions without Mtx. This supports the results of the clinical studies which have observed increased Mtx toxicity associated with the TT genotype, but because the underlying effect of genotype was reduced by the higher concentrations of Mtx used, the results of the current study suggest that an effect of genotype is more likely to be influential in cases of low-dose Mtx treatment. Experiments where cellular methionine synthase (MS) activity was inhibited by nitrous oxide showed that such inhibition reduced the effectiveness of 5-methyl THF to rescue lymphocytes from Mtx-induced cytotoxicity (Dudman 1982). Based on this observation, along with earlier work which showed that tumour cells with low MS activity were not rescued from the effect of Mtx by 5-methyl THF (Halpern et al. 1975), Dudman et al. suggested that cells with lower

MS activity may be more sensitive to cytotoxic effects of Mtx. The reduction in enzyme activity associated with MTHFR TT genotype has been shown to reduce levels of cellular 5-methyl THF (Davis et al. 2005), and this could also have the effect of reducing flux through MS because 5-methyl THF is required as a substrate for MS. This would be particularly relevant in a situation where folate supply is reduced, because this is associated with lower activity of the 677 TT form of MTHFR (Yamada et al. 2001). Chiang et al. (2007) have shown that TT genotype is associated with decreased methionine synthesis under low folate conditions, using transformed B-lymphocytes similar to the cell lines used in this project (Chiang et al. 2007). These findings support the suggestions that TT genotype is associated with Mtx toxicity, and also correlate with the results presented here which show that cells of TT genotype have a lower proliferation rate than CC cell lines in folate-free culture medium.

The ability of FA, 5-formyl THF and 5-methyl THF to prevent the cytotoxic effect of Mtx were investigated using the MTS cell proliferation assay. FA is commonly used in fortified foods and vitamin supplements, and all three forms of folate can be used pharmacologically and are often prescribed as an adjunct to Mtx therapy in order to reduce the risk of side effects. However, it is not clear if such supplements can interfere with the efficacy of the drug. A recent analysis of randomised controlled trials found that supplementation with either FA or 5-formyl THF reduced hepatic toxicity of Mtx, and there was a non-significant benefit in terms of gastrointestinal toxicity. However, there was insufficient evidence to assess the influence of folate supplementation on Mtx efficacy (Prey and Paul 2009). In recent years it has become apparent that consumption of large amounts of FA in the diet can result in the appearance of unmetabolised FA in the serum, and there is some concern that this may interfere with antifolate therapies such as Mtx. In the present study, it was found that FA did not block the effect of Mtx on cell proliferation in a B-lymphocyte cell line, even at very high supra-physiological concentrations. In contrast, 5-methyl THF and 5-formyl THF inhibited the cytotoxic effect of Mtx in a dose dependent manner. A higher concentration of 5-methyl THF was required to produce maximum inhibition of Mtx-induced cytotoxicity than for 5-formyl THF, and this may be due to the inferior stability of 5-methyl THF in aqueous solutions. FA must be reduced to THF by the action of DHFR before it can become a functional part of cellular folate metabolism. Mtx inhibits DHFR, and so blocks the entry of FA to the folate cycle. In contrast, 5-methyl THF and 5-formyl do not require the action of DHFR for entry to the folate cycle. It may be suggested that 5-formyl and 5-methyl THF allow normal one-carbon metabolism to continue in the presence of Mtx, supplying sufficient THF to allow continued synthesis of purines and dTMP in the face of DHFR inhibition. The result presented here suggests that the presence of unmetabolised FA in serum would be

unlikely to interfere with pharmacological effects of Mtx, because the serum levels of FA which have been measured are in the nanomolar range (Obeid et al. 2010), very much lower than the concentrations of FA used in the current study which did not interfere with the effect of Mtx. However, most folate consumed orally is converted to 5-methyl THF during absorption. Since 5-methyl THF did block the effects of Mtx in this investigation, it is possible that a general elevation of plasma folate levels due to supplementation with any form of folate could influence the efficacy of Mtx treatment. This could be beneficial in that it may reduce symptoms of Mtx toxicity, but by contrast could reduce efficacy of the drug if the desired effect of Mtx is a reduction in cell proliferation. Achieving an appropriate balance between Mtx concentration and folate status should be a topic of concern in a clinical setting, because the correct balance should allow maximal efficacy of the drug while reducing the risk of side effects.

Experiments using Thy and Hx were carried out to investigate the effect of Mtx on purine and dTMP synthesis in these transformed B-lymphocytes. Thy provides a source of dTMP therefore overcoming the inhibition of dTMP synthesis by Mtx. Hx is a substrate for the purine salvage pathway, thereby supplying an alternative source of purine nucleotides. Addition of Thy was found to partially protect cells from the effect of Mtx. Thy prevented cell death and supported a small amount of cell proliferation in the presence of Mtx, but did not restore proliferation to control levels. Hx alone had no influence on the effect of Mtx. The two compounds together had a synergistic effect, restoring cell proliferation completely to control levels. These results indicate that the mechanism of Mtx action in these cells involves inhibition of both dTMP and purine synthesis. Provision of dTMP appears to be sufficient to prevent cell death caused by Mtx, but supply of purine nucleotides in addition to Thy is required to completely block the effect of Mtx and restore normal cell proliferation.

In order to investigate if differences in Hcy production could be detected in cells of MTHFR CC and TT genotype, two cell lines were maintained in either standard RPMI, folate free RPMI (FF) or RPMI without folate or riboflavin (RFF). The results of this analysis confirmed the observations in earlier experiments in this chapter that cells of MTHFR TT genotype are more sensitive to low-folate conditions than cells of CC genotype. As observed in previous experiments in this chapter, the TT cell line had a lower proliferation rate and lower viability than the CC cell line in folate-free medium, but not in standard RPMI medium. The concentration of Hcy in cell culture supernatants was measured, and the amount of Hcy released per 10^5 viable cells was calculated for each cell ine in each type of culture medium. Hcy efflux was correlated with cell growth, suggesting that cells continually release Hcy into the culture medium. The same relationship between Hcy efflux and cell growth was observed by Refsum et al. in murine cells (Refsum et al. 1991). In the current

study, the amount of Hcy produced by cells was greater in folate-free conditions than in standard RPMI which contains 2μM FA. This is expected, because low levels of folate are likely to reduce the remethylation of Hcy to methionine. There was no difference in the level of Hcy released by the CC and TT cell line in standard or FF medium. Hcy release was greatest in RFF medium, and the production of Hcy was higher in the TT cell line in this case, however deficiency of both nutrients was highly toxic to these cells.

Lathrop Stern et al. (2003) have previously investigated the effect of folate and riboflavin status on Hcy release from transformed B-lymphocytes (Lathrop Stern et al. 2003). They observed that Hcy efflux was increased under reduced folate conditions, but not when riboflavin only was reduced. They found no difference in Hcy efflux between MTHFR 677 CC and TT cells under any culture condition. There are some differences between the current study and the work of Lathrop Stern et al. (2003). The current study investigated complete deficiency of folate or riboflavin over a 72h period, while Lathrop Stern et al. used reduced levels of these nutrients for an undefined period of time. Lathrop Stern et al. also used 5-formyl THF as a folate source, in contrast to FA which was used in the current study. The concentration of Hcy in culture medium measured by the Lathrop Stern study was lower than that reported here, but because the length of culture time is not reported it is difficult to compare these measurements directly. However, the results presented here are in agreement with Lathrop Stern et al. in that Hcy concentration in the culture supernatant increased over time in FF and RFF conditions, but not in standard RPMI where supply of folate was adequate. There are strong associations between MTHFR 677 TT genotype and increased plasma Hcy levels in clinical studies (Harmon et al. 1996; Jacques et al. 1996), however, this relationship was not observed in the cellular experiments of the current study or of Lathrop Stern et al (2003). This discrepancy may be due to the fact that the liver is a major source of Hcy production in vivo, due to the high rates of folate metabolism in this organ. Plasma Hcy is a reflection of whole-body metabolism, and could therefore be influenced by MTHFR 677 genotype to a far greater extent than release of Hcy from cultured lymphocytes.

In conclusion, the results presented in this chapter demonstrate that transformed B-lymphocyte cell lines of MTHFR 677 TT genotype have a lower proliferation rate than cells of CC genotype, both in the absence and presence of Mtx. The cytotoxic effect of methotrexate could be blocked by reduced folates, but not by FA, and was due to blockade of both purine and dTMP synthesis. In the next chapter, the cytotoxic effect of Mtx is investigated further by examining the ability of Mtx to induce apoptosis in these cell lines.

4 Chapter 4: Analysis of methotrexate-induced apoptosis in transformed B-lymphocyte cell lines

4.1 Introduction

Induction of apoptosis is one of the mechanisms of action proposed for the clinical effect of Mtx treatment. In treatment of cancer, promotion of apoptosis is desirable because many tumour cells lose their normal regulation of apoptosis during establishment of the malignant phenotype. A pro-apoptotic effect of Mtx would also be of benefit in treatment of autoimmune diseases, which are characterised by inappropriate activation of the immune response, involving proliferation of several types of immune cells. A further consequence of Mtx-induced apoptosis which is not desirable is the potential contribution to toxicity of Mtx treatment. Symptoms of toxicity often occur in the gastrointestinal tract, lungs, and mucosal tissues, and it is conceivable that apoptosis of epithelial cells in such tissues plays a role in development of symptoms.

There are conflicting opinions about the importance of apoptosis in the mechanistic effect of Mtx, particularly in the low-dose treatment regimen. Clinical studies have demonstrated that Mtx treatment causes apoptosis in neutrophils isolated from the blood of rheumatoid arthritis (RA) patients (Weinmann et al.), and in eosinophils during treatment of the auto-immune skin disease bullous pemphigoid (Dahlman-Ghozlan et al. 2008). Several in vitro studies have demonstrated that Mtx causes apoptosis in activated, but not resting primary lymphocytes. Mtx was found to cause apoptosis in human T-lymphocytes which were activated with the mitogen phytohemagluttinin (PHA) in vitro, but not in resting cells (Genestier et al. 1998). Apoptosis was also observed after PHA-activation of lymphocytes isolated from RA patients who had been treated with Mtx (Genestier et al. 1998). A second group also reported that Mtx caused apoptosis in PHA-activated human mononuclear cells (Pawlik et al. 2005). Nielsen et al. (2007) examined the ability of Mtx to induce apoptosis in CD4+ T-lymphocytes stimulated in vitro with either PHA or the physiological antigens *Candida albicans* (CA) and tetanus toxoid (TeT). In this case, Mtx was found to cause apoptosis in CD4+ T-lymphocytes activated by CA or TeT, while PHA activation caused inherent apoptosis which was not increased by Mtx. When the cells were separated into subsets of cells which had or had not undergone division, Mtx-induced apoptosis was apparent only in the divided cells (Nielsen et al. 2007). Johnston et al. (2005) also observed different effects of Mtx on apoptosis of lymphocytes depending on the nature of in vitro stimulation. Mtx increased apoptosis in PHA-stimulated cells but not in cells stimulated by a variety of bacterially derived antigens (Johnston et al. 2005). It was observed that Mtx down-regulated the expression

of several adhesion molecules, and the authors suggested that this may be the mode of Mtx action in inflammatory disease (Johnston et al. 2005). Although most studies demonstrate Mtx-induced apoptosis in stimulated cells or cell lines, there is also at least one report of Mtx causing apoptosis in cells which are not actively undergoing proliferation, for example confluent bovine pulmonary artery endothelial cells (Merkle et al. 2000).

In studies which have demonstrated apoptosis in response to Mtx, there is heterogeneity in the mechanisms of apoptosis that have been ascribed to the drug. There are a number of studies which demonstrate the activation of caspase-3 in response to Mtx. Mazur et al. (2009) demonstrated the activation of caspase-3 in human cervical cancer cells and normal rat kidney fibroblasts in response to Mtx (Mazur et al. 2009). Mtx-induced activation of caspase-3 was also observed in lung cancer cells (Huang et al. 2010). In contrast, apoptosis of CD4+ T-lymphocytes in response to Mtx was observed to be independent of caspase-3 (Nielsen et al. 2007). Caspase-3 is regarded as the central effector caspase of the apoptotic process, and the upstream events that lead to caspase-3 activation in response to Mtx are not clear. As described in Chapter 1, caspase-3 can be activated via caspase-9 in the mitochondrial activation pathway, or via caspase-8 in the extrinsic activation pathway. In rat intestinal epithelial cells, Mtx-induced apoptosis was found to be caspase-dependent and involved activation of caspase-9, but not caspase-8 (Papaconstantinou et al. 2001). The mitochondrial pathway of apoptosis has also been implicated in the response of choriocarcinoma cells to Mtx, where decreased mitochondrial membrane potential and activation of caspase-9 but not caspase-8 was detected (Chen et al. 2009). However, in other cell types, caspase-8 has been implicated in Mtx-induced apoptosis. In a range of tumour cell lines and primary leukaemia cells, Mtx exposure led to upregulation of caspase-8 and increased sensitivity of the cells to apoptosis through the extrinsic activation pathway (Ehrhardt et al. 2007).

Generation of intracellular reactive oxygen species (ROS) is also associated with the induction of apoptosis (Fleury et al. 2002). Mtx was shown to cause an increase in intracellular peroxide, which correlated with cytostasis in a monocyte cell line, and with apoptosis in a T-lymphocyte cell line (Phillips et al. 2003). Herman et al. (2005) showed that generation of ROS was correlated with Mtx-induced apoptosis, and that apoptosis was partially reversed by the anti-oxidant N-acetylcysteine. However the contribution of ROS to Mtx-induced apoptosis varied depending on cell type, with T-cell lines being more susceptible than cells of moncyte lineage (Herman et al. 2005).

The role of caspases in apoptotic mechanisms is well established; however apoptotic cell death can also occur independently of caspases. For example, staurosporine was observed to induce cell death in HL-60 cells with characteristic features of apoptosis under conditions where caspase activity was inhibited (O'Connell et al. 2006). Other types of protease activity are involved in apoptotic cell death, particularly serine proteases. Using a fluorescent substrate of serine proteases, Grabarek et al. (2002) demonstrated that serine protease activity was enhanced in HL-60 cells in response to two different apoptotic agents, TNF-α and camptothecin (Grabarek et al. 2002a). Two examples of serine proteases for which a role in apoptosis has been described are HtrA2/Omi and AP24, although it is thought that other serine proteases which have not yet been fully identified may also be involved (O'Connell et al. 2006). HtrA2/Omi is a trypsin-like serine protease which promotes caspase activation by disrupting the interaction of caspases with inhibitory proteins, and can also cause apoptosis by a caspase-independent mechanism (Hegde et al. 2002). AP24 is an elastase-like serine protease which leads to nuclear DNA fragmentation through activation of L-DNase II (Altairac et al. 2003). Serine-protease activation may occur upstream or downstream of, or in parallel to caspase activation, and the contributions of these mechanisms to cell death may vary depending on cell type or apoptotic stimulus. Grabarek et al. (2002) suggested that different types of serine protease might act upstream and downstream of caspases. They observed that a pan-caspase inhibitor reduced the activation of serine proteases in response to apoptotic stimuli, and an inhibitor of serine proteases also partially reduced caspase activation (Grabarek et al. 2002b). In a prostate cancer cell line, inhibition of serine proteases was found to prevent caspase activation in response to the death-receptor ligand TRAIL, but promoted caspase activation in response to wortmannin (Rokhlin et al. 2004). The role of serine proteases in Mtx-induced apoptosis has not previously been investigated, but because caspase-independent cell death in response to Mtx has been reported (Nielsen et al. 2007) it is possible that other factors such as serine protease-related mechanisms may also be involved. Inhibitors of serine proteases which include a reactive fluoromethylketone group which binds to the His 57 residue in the active site are used to interrogate the role of serine proteases in vitro. Substrate specificity of such inhibitors is achieved by structural similarity to the normal substrate of the enzyme. TPCK is an inhibitor of chymotrypsin-like serine proteases, which preferentially cleave at the carboxyl side of aromatic amino acid residues. TLCK is an inhibitor of trypsin-like serine proteases which have specificity for proteolytic cleavage at basic residues such as lysine.

The experiments in chapter 3 demonstrated that Mtx caused cell death in transformed B-lymphocytes, and postulated that cell death was a consequence of folate antagonism and inhibition of dTMP and purine synthesis. From the studies mentioned above, it is likely that this

cell death involves apoptosis, but Mtx-induced apoptosis has not been previously examined in transformed-B lymphocyte cell lines. It was also noted in chapter 3 that cells of MTHFR 677 TT genotype were more sensitive to low-folate conditions than cells of CC genotype. Examination of apoptosis in these cell lines may also reveal a role for apoptosis in this effect of genotype. In a mouse model of altered MTHFR expression, both decreased and increased expression of the enzyme resulted in increased apoptosis in spleen cells after Mtx treatment, when compared to wild-type controls with normal MTHFR expression (Celtikci et al. 2009). Celtikci et al. (2009) suggested that the increase in apoptosis in mice with decreased MTHFR expression was due to an increased level of plasma Hcy, and in the case of MTHFR over expression it was due to imbalances in the dUTP/dTTP ratio. It is feasible that increased Hcy promotes apoptosis, because there are several reports of apoptosis caused by exogenous Hcy in cell culture. Hcy has been shown to cause apoptosis in vascular endothelial cells from rat (Tyagi et al. 2006) and human (Lee et al. 2005), in human primary bone marrow stromal cells (Kim et al. 2006) and in mesangial cells from rat kidney (Shastry et al. 2007). Mtx has been shown to increase intracellular dUTP levels and misincorporation of uracil into DNA (Goulian et al. 1980), although it is not known if this can lead to apoptosis. The observations from the mouse model mentioned above provide evidence that alteration of MTHFR activity can influence apoptosis in response to Mtx. The MTHFR 677C→T polymorphism is also known to decrease MTHFR activity in vitro (Yamada et al. 2001), and is associated with altered distribution of intracellular folate species (Bagley and Selhub 1998), and increased Hcy levels in vivo (Jacques et al. 1996). It is possible that MTHFR 677C→T genotype may influence Mtx-induced apoptosis on a cellular level. MTHFR 677 TT genotype has also been associated with increased risk of Mtx toxicity. Since the toxic effects of Mtx treatment are observed in rapidly proliferating tissues such as bone marrow or the gastro-intestinal tract, it is of interest to define whether TT genotype increases sensitivity to Mtx-induced apoptosis.

An issue associated with the study of apoptosis in cell culture systems is differentiation of apoptosis from other types of cell death. In the absence of other cell types such as macrophages, apoptotic bodies cannot be engulfed by phagocytes as would be their normal physiological fate. Cultured cells undergoing apoptosis instead eventually reach a state of secondary necrosis, which displays features associated with necrosis. There is no positive biochemical marker for necrosis, so it is difficult to distinguish true necrosis from apoptosis in cell culture systems (Krysko et al. 2008b). For this reason, when studying apoptosis in vitro, it is recommended to use a variety of different methods to observe several aspects of the process, thus confirming that cell death occurs by apoptosis and not by necrosis. It is also advisable to observe the process at several time points during the response. The ultimate definition of apoptotic death is by detection of changes

in cell morphology, including cell shrinkage, 'blebbing' of the cell membrane and nuclear condensation and fragmentation. These can be detected by microscopy or by changes in light scatter properties in flow cytometry. Biochemical markers such as externalisation of phosphatidylserine (PS) on the cell membrane, and activation of caspases or proteolytic cleavage of their substrates are used to support these observations. In this chapter, the occurrence of apoptosis was examined using Western blot to detect activated caspase-3 and cleavage of the caspase substrate PARP. Flow cytometry was used to quantify apoptosis by detecting DNA fragmentation and Annexin V exposure on the cell membrane. Inhibitors of caspases and serine protease inhibitors were used to investigate some of the mechanistic features of Mtx-induced apoptosis in transformed B-lymphocyte cell lines.

4.1.1 Aims

- To determine if the cell death caused by Mtx in transformed B-lymphocytes is apoptotic in nature, and if such apoptosis can be prevented by the interventions used to restore cell proliferation in chapter 3.
- To investigate if Mtx-induced apoptosis is influenced by MTHFR 677C→T genotype.
- To explore the mechanisms involved in Mtx-induced apoptosis in transformed B-lymphocytes, investigating the roles of caspase and serine protease-mediated mechanisms of cell death.

4.2 Methotrexate causes apoptotic cell death in transformed B-lymphocyte cell lines

4.2.1 Methotrexate causes activation of caspase-3 and cleavage of PARP in transformed B-lymphocyte cell lines

Western blot analysis was carried out to demonstrate that apoptosis occurs in transformed B-lymphocyte cell lines in response to treatment with 100nM Mtx. Cells which had previously been cultured in standard RPMI were harvested by centrifugation and counted as described in section 2.2.1.2. Cells were rinsed three times in folate free RPMI to remove residual folate, then were seeded at $2x10^5$ cells/ml in folate-free RPMI containing 100nM Mtx, and also in medium without Mtx as an untreated control. Two $25cm^3$ tissue culture flasks were prepared for treated and untreated cells, with a total volume of 10ml in each flask. After 24h and 48h incubation at 37^oC, 5% CO_2, cells from one flask of control cells and one flask of treated cells were harvested by centrifugation and cell lysates were prepared as described in section 2.2.3.1. Cell lysates were also prepared from a flask of cells which had been treated with 1μM staurosporine for 4h as a

positive control for apoptosis. The protein concentration of each lysate was determined by the BCA assay (section 2.2.3.2). Aliquots of cell lysates containing equal amounts of protein were separated on an SDS-PAGE gel. The separated proteins were transferred to a PVDF membrane, which was then incubated with antibodies against PARP and activated Caspase 3, and β-actin as a loading control.

Figure 4.1 shows the results of Western blot analysis for cleaved Poly-ADP-ribose polymerase (PARP) and activated caspase-3 in two different cell lines. Caspase-3 becomes active during apoptosis and is regarded as the central enzyme in the apoptotic cascade. PARP is a caspase substrate which is characteristically cleaved during apoptosis. Bands corresponding to intact PARP (116kDa) are visible in all samples, but bands corresponding to cleaved PARP (89 kDa) are only visible in samples which have been treated with Mtx for 24 and 48 hours, and in the staurosporine-treated positive control. Bands for activated caspase-3 (17 kDa) can be seen in samples which have been treated with Mtx and in the staurosporine-treated positive control, but are not present in untreated control samples. This provides evidence that Mtx causes cell death by apoptosis in these cell lines. Since all treatments and controls were maintained in folate-free RPMI medium, this result also demonstrates that deprivation of folate for this period of time does not cause apoptosis in these cell lines.

4.2.2 The use of the Annnexin V/PI staining and cell cycle analysis methods to quantify apoptosis in transformed B-lymphocyte cell lines

Western blots are an excellent and well regarded method to determine the presence or absence of a particular protein, and are thus extremely useful for confirmation of the presence of apoptotic markers in response to Mtx in these cell lines. However, because it is a semi-quantitative method, Western blots are not ideal for quantifying protein levels with a great degree of accuracy. It is hypothesized that the presence of the MTHFR 677C→T polymorphism may result in higher levels of apoptosis in response to Mtx. Therefore quantitative measurements of apoptosis are necessary for investigation of the influence of MTHFR 677C→T genotype on Mtx-induced apoptosis. The methods used to achieve this are cell cycle analysis and Annexin V/PI staining. Both of these methods quantify levels of apoptosis in a cell population by flow cytometry.

In order to determine a suitable time point after Mtx exposure for measurement of apoptosis using these methods, a time course exposure of cells to 100nM Mtx was carried out. Cells of cell line 17241 which had previously been cultured in standard RPMI medium were harvested by centrifugation and counted as described in section 2.2.1.2. The cells were washed three times in

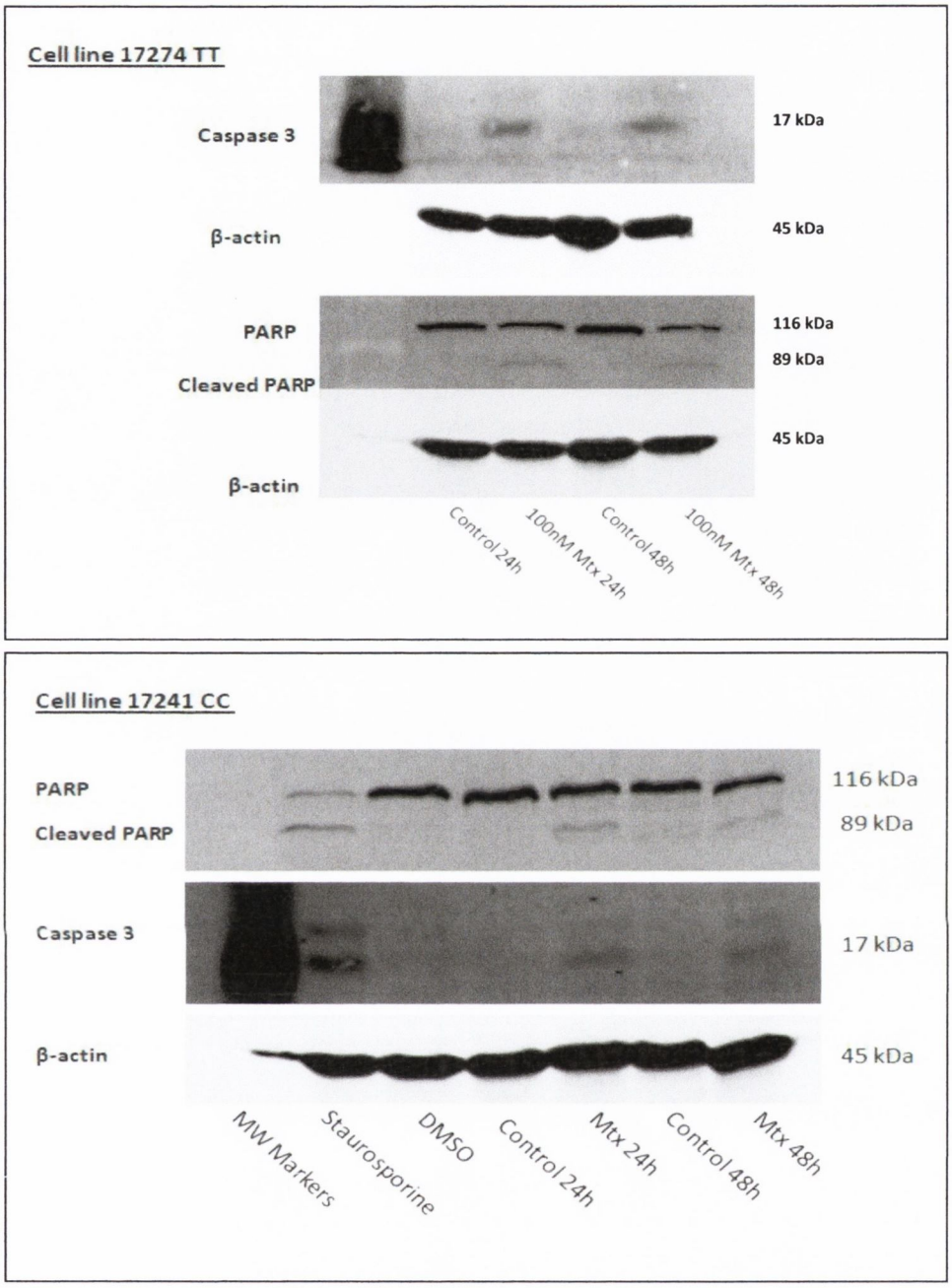

Figure 4.1 Mtx causes activation of caspase-3 and PARP in transformed B-lymphocyte cell lines

Two cell lines, 17274 TT (upper panel) and 17241 CC (lower panel), were treated with 100nM Mtx for the indicated times, then processed for Western blot analysis as described in chapter 2. Western blots were treated with antibodies which recognise activated Caspase 3, and the whole and cleaved forms of the caspase substrate, PARP. An antibody against β-actin was used as a loading control.

folate-free RPMI to remove residual folate, then seeded at $2x10^5$ cells/ml in folate-free RPMI containing 100nM Mtx, in a total volume of 20ml. One flask was prepared for each time point, and a control flask of cells in folate-free RPMI without Mtx was also prepared. After incubation for 8-72 hours at 37°C, 5% CO_2, one flask was taken at each time point and cells were harvested by centrifugation. The sample was split in two, and one half was processed for the cell cycle analysis method (section 2.2.2.1) and the other for Annexin V/PI staining (section 2.2.2.2). This experiment was carried out on three separate occasions with cell line 17241.

The results of this experiment are shown in Figure 4.2. Apoptosis becomes apparent after 24h incubation with 100nM Mtx. The level of apoptosis reach statistical significance compared to control cells after 48h using both the cell cycle analysis method and the Annexin V/PI method ($P < 0.01$, One-way ANOVA with Tukey's post-tests). For subsequent experiments to quantify apoptosis, an end point of 40h or longer was used. In some cases apoptosis was quantified at more than one time point because this gives a more complete picture of the response of a cell population to an apoptotic stimulus.

Flow cytometry also allows detection of morphological changes in cells which are characteristic of apoptosis. During apoptotic cell death, cells shrink in size and the nucleus fragments. This decrease in cell size and increase in granularity translates to a change in light scatter properties during flow cytometric analysis (Krysko et al. 2008b). Figure 4.3 shows the occurrence of these characteristic changes in transformed B-lymphocyte cells during the time course exposure to 100nM Mtx. As incubation time increases, the forward scatter (FSC, x-axis) decreases and side scatter (SSC, y-axis) increases. This change is indicative of a decrease in cell size, and an increase in granularity, indicating a progressive increase in apoptotic cells over the time of incubation with Mtx.

As well as allowing quantification of the proportion of apoptotic cells, the cell cycle analysis method provides information about the proportion of cells in each phase of the cell cycle. This can provide useful information about the cell cycle stage at which Mtx has its action. Figure 4.4 shows the proportion of cells in each phase of the cell cycle during the time course exposure to 100nM Mtx. After treatment with 100nM Mtx, the proportion of cells in G1 phase initially increases, and then decreases with the decrease becoming statistically significant compared to untreated cells after 48h ($P < 0.01$, repeated measures ANOVA). The decrease in G1 cells coincides with the increased proportion of cells in the sub-G1, or apoptotic phase. The proportion of cells in S phase does not change significantly at any time point. The proportion of cells in G2/M phase decreases over time, and this decrease is statistically significant after only 8h incubation

($P < 0.05$, repeated measures ANOVA). These results suggest that Mtx treatment causes an arrest of the cell cycle during the transition from G1 to S-phase. Cells which are prevented from progressing through the cell cycle subsequently die by apoptosis, as demonstrated by the increase in cells in the sub-G1 region over time.

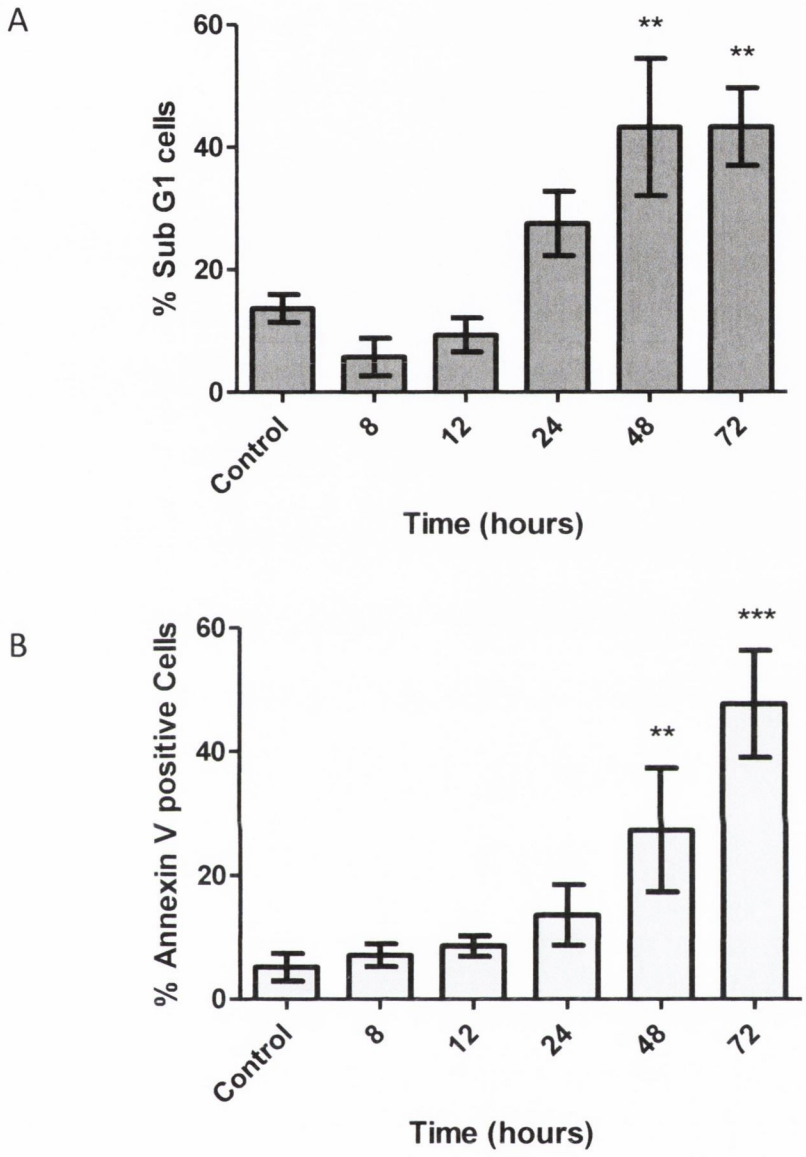

Figure 4.2 The use of Annexin V and cell cycle analysis assays to quantify Mtx-induced apoptosis in transformed B-lymphocyte cell lines

Cells (17241) were seeded at 2.5 x 10^5 cells/ml in standard RPMI media containing 100nM Mtx. At the indicated timepoints, cells were processed for (A) cell cycle analysis and (B) Annexin V/PI staining. Apoptotic cells were quantified by flow cytometry. Data are the means +/- SD of 3 separate experiments. Results were analysed using repeated-measures ANOVA with Tukey's post-tests. * = significantly different from control, ** = $P < 0.01$, *** = $P < 0.001$.

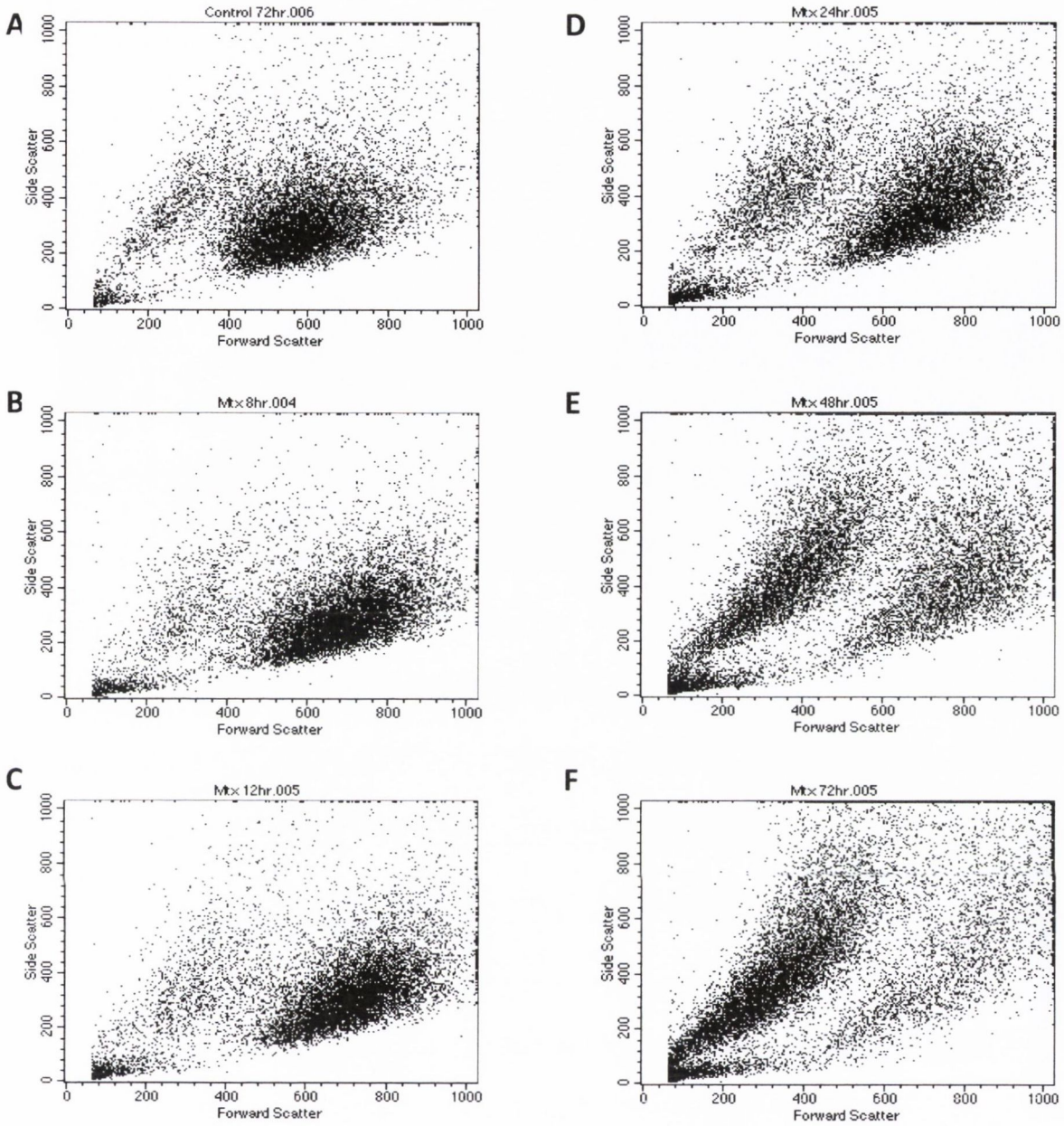

Figure 4.3 Transformed B-lymphocytes undergo morphological changes associated with apoptosis during exposure to 100nM Mtx

Cells (17241) were seeded at 2 x 10^5 cells/ml in folate-free medium with 100nM Mtx. At intervals of 8h (B), 12h (C), 24h (D), 48h (E) and 72h (F) samples of cells were analysed by flow cytometry for morphological changes characteristic of apoptosis, which are detected as changes in light scatter properties. Panel (A) shows untreated cells after 72h incubation. Forward scatter decreases and side scatter increases over time when cells are exposed to Mtx.

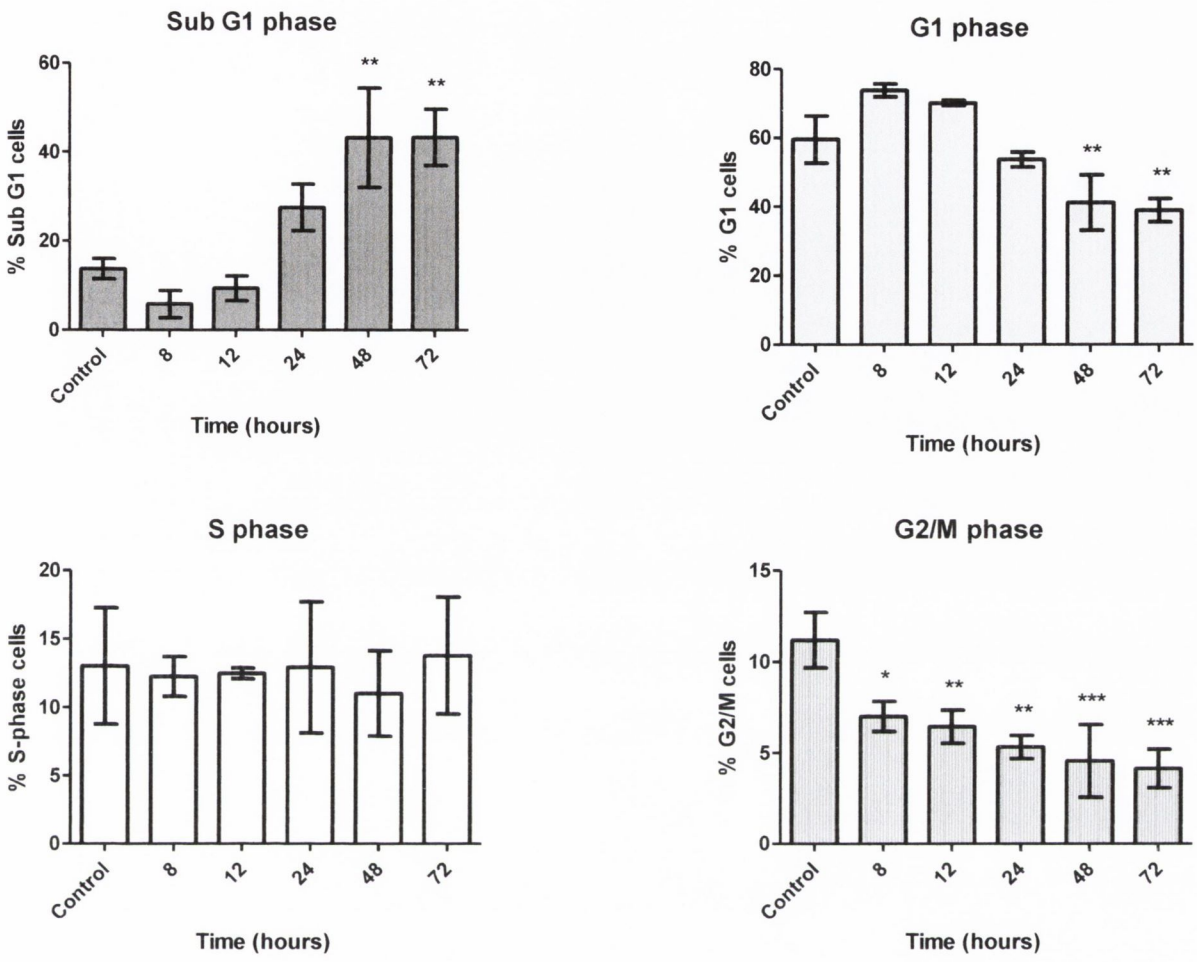

Figure 4.4 The effect of methotrexate on cell cycle stage distribution in transformed B-lymphocytes

Cells (17241) were seeded at 2.5 x 10^5 cells/ml in standard RPMI medium containing 100nM Mtx. At the indicated time points, a sample of cells was processed for cell cycle analysis. The proportion of cells in each phase of the cell cycle over a 72h exposure to 100nM Mtx is shown. Data are the means +/- SD of 3 separate experiments. Repeated-measures ANOVA was used to analyse the data. * = different from respective control. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

4.3 Investigation of the influence of MTHFR 677C→T genotype on methotrexate-induced apoptosis in transformed B-lymphocyte cell lines

In chapter 3, it was observed that transformed B-lymphocytes of MTHFR 677 TT genotype have lower levels of proliferation than cells of CC genotype in low folate conditions and in the presence of Mtx. In order to address the question of whether MTHFR 677C→T genotype may affect the level of apoptosis induced by Mtx, three cell lines of MTHFR 677 CC genotype and three cell lines of MTHFR 677 TT genotype were incubated in varying concentrations of Mtx and the level of apoptosis measured over time. Cells which had previously been cultured in standard RPMI medium were harvested by centrifugation and counted as described in section 2.2.1.2. The cells were washed three times in folate-free RPMI to remove residual folate, then seeded at $2x10^5$ cells/ml in folate-free RPMI containing Mtx at concentrations of 0 (control), 10, 100 and 1000nM. After 24 and 48h incubation at 37°C, 5% CO_2, one flask of cells from each condition were taken and harvested by centrifugation. The cells were processed for Annexin V/PI analysis (section 2.2.2.2), and the proportion of apoptotic cells in each sample was determined by flow cytometry. This experiment was carried out in three cell lines of CC genotype (17257, 17241, 17226) and three cell lines of TT genotype (17234, 17274, 17240).

The results were pooled by genotype to produce figure 4.5. Two-way ANOVA was used to analyse these data, to test for effects of genotype and Mtx, and also for interaction of these two factors. After 24h incubation, the effect of Mtx on levels of apoptosis was not significantly different from untreated controls ($p = 0.16$, two-way ANOVA for Mtx). The fact that the effect of Mtx is not statistically significant after 24h incubation is as expected based on the results in section 4.2.2, where the level of apoptosis due to Mtx was not significantly different from the control until 48h incubation time. However, although there was no effect of Mtx at 24h, there was a highly significant difference by genotype, with TT cell lines having higher levels of apoptosis compared to CC cell lines ($P = 0.003$). There was also no interaction between the two factors.

After 48h incubation time the effects of genotype and Mtx were quite different. There was a statistically significant increase in apoptosis due to Mtx ($P= 0.0009$, two-way ANOVA for Mtx), but there was no longer an effect of genotype observed ($P = 0.93$, two-way ANOVA for genotype).

Again, there was no interaction between the two factors after 48h. To exclude the possibility that the exclusion of cells which were positive for PI staining skewed this result, data for total cell

death (including cells positive for Annexin V only, and cells positive for both Annexin V and PI) was also analysed by two-way ANOVA, and this analysis is presented in figure 4.6. The same pattern of statistical significance is observed when total cell death is taken into account. After 24h there was a significant effect of genotype ($P = 0.014$) but not of Mtx, and after 48h there was a significant effect of Mtx ($P < 0.0001$) but no effect of genotype.

These data illustrate two different effects. TT cell lines exhibit higher basal levels of apoptosis than CC cell lines under folate-free conditions. Exposure to Mtx causes an increase in apoptosis in cells of both genotypes, and the effect of Mtx is sufficient to obscure any smaller differences in apoptosis that are due to genotype.

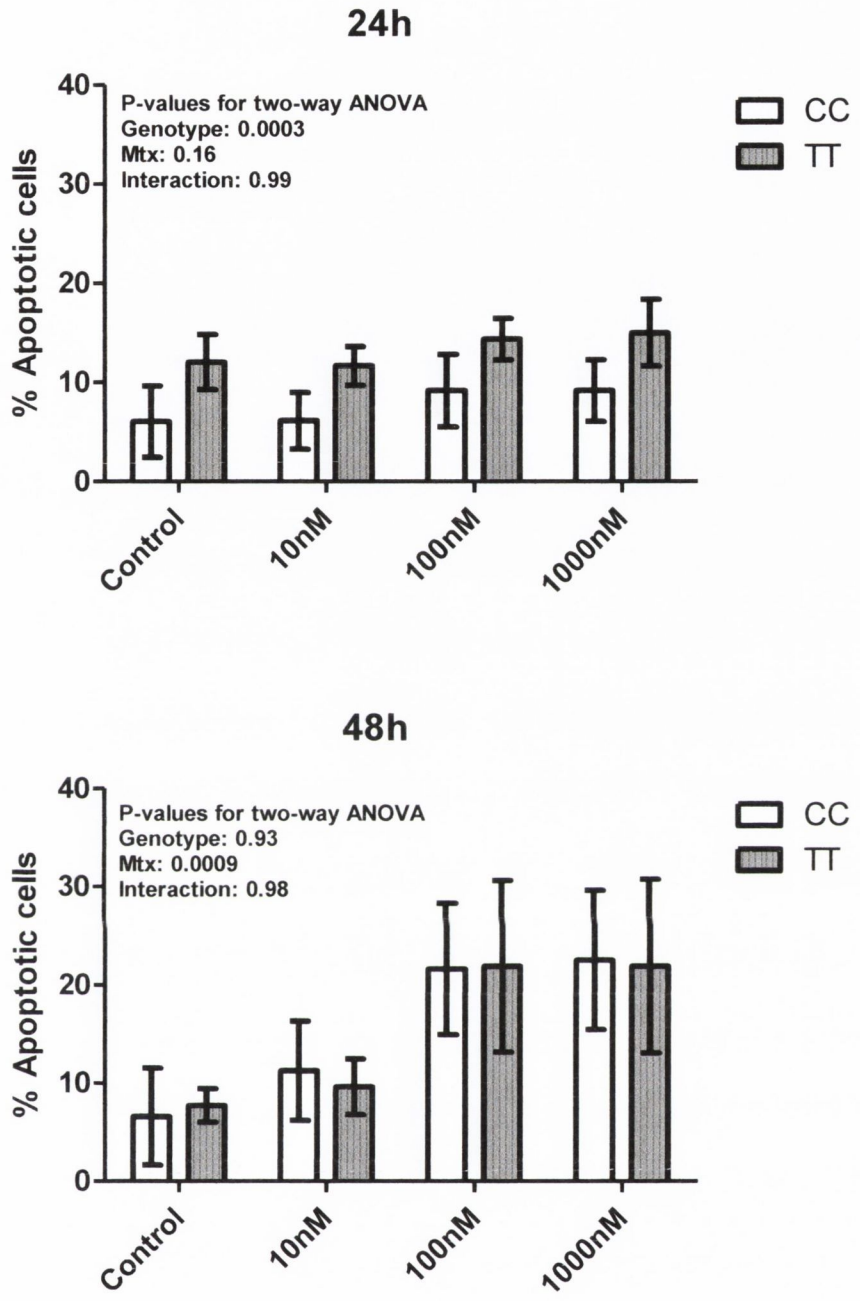

Figure 4.5 Early apoptosis in cell lines of MTHFR 677 CC and TT genotype during methotrexate exposure.

Cells were seeded at $2x10^5$ cells/ml in folate free medium containing Mtx at the concentrations indicated (Control = no Mtx). After 24h (upper panel) and 48h (lower panel), samples from each condition were processed for Annexin V/PI analysis. The proportion of early apoptotic (Annexin V +/PI –) cells was quantified by flow cytometry. Data are the means +/- SD for 3 cell lines of CC genotype and 3 cell lines of TT genotype. The effects of genotype and Mtx were analysed by two-way ANOVA at each time point.

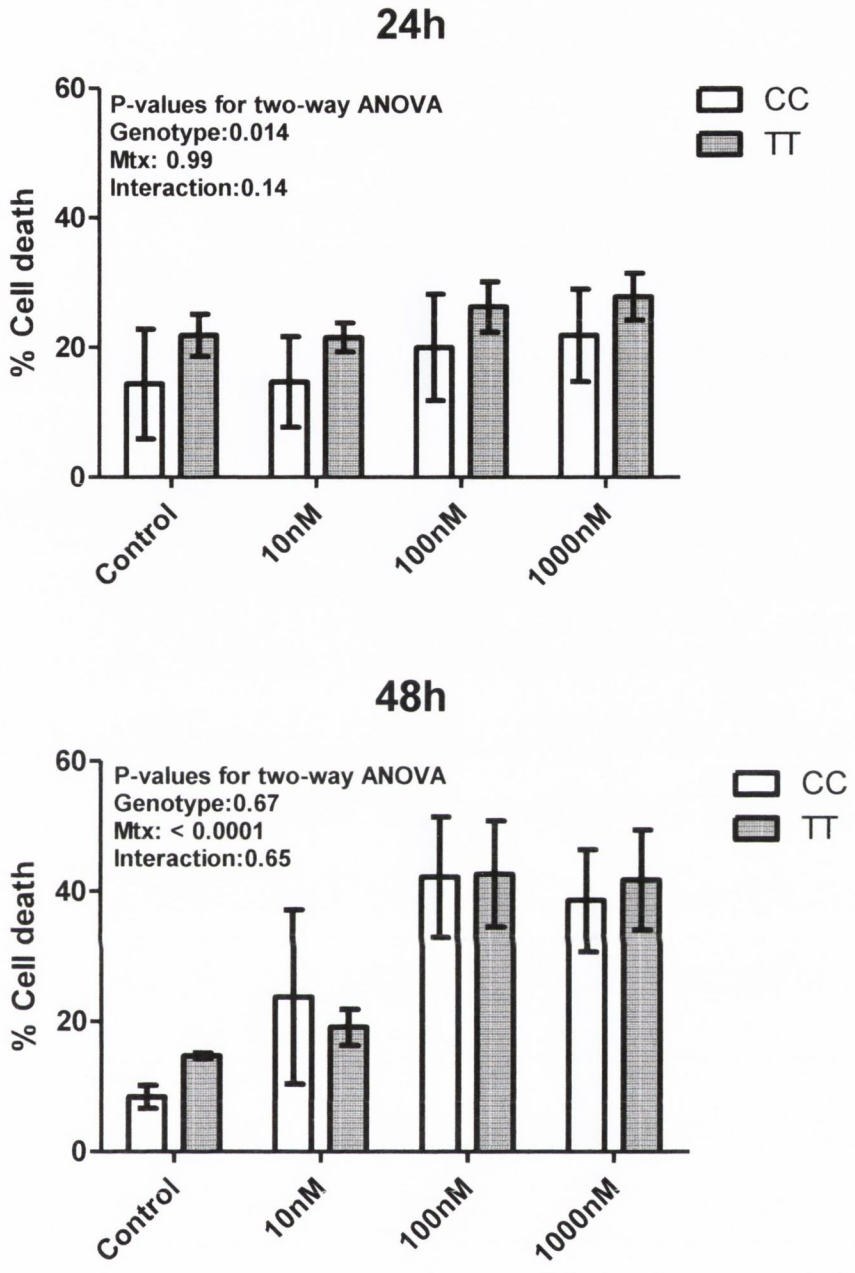

Figure 4.6 Total apoptosis in cell lines of MTHFR 677 CC and TT genotype during methotrexate exposure

Cells were seeded at $2x10^5$ cells/ml in folate free medium containing Mtx at concentrations indicated (Control = no Mtx). After 24 (upper panel) and 48h (lower panel), samples from each condition were processed for Annexin V/PI analysis. The proportion of cells in both early and late stages of apoptosis (total Annexin V positive cells) was quantified by flow cytometry. Data are the means +/- SD for 3 cell lines of CC genotype and 3 cell lines of TT genotype. The effects of genotype and Mtx were analysed by two-way ANOVA at each time point.

4.4 Investigation of the ability of various folate cofactors to prevent methotrexate-induced apoptosis in transformed B-lymphocyte cell lines

In chapter 3 it was shown that 5-formyl THF and 5-methyl THF were more effective at preventing the antiproliferative effect of Mtx than folic acid (FA), using the MTS assay. In order to investigate if the same interactions with folate could be detected at the level of apoptosis, cells were incubated in folate-free RPMI with 100nM Mtx and either 5-methyl THF, 5-formyl-THF or FA for a 72h period, and apoptosis was then quantified using Annexin V/PI staining and cell cycle analysis.

Folate-free medium containing either no added folate (control), 20μM 5-methyl THF, 5μM 5-formyl-THF or 2μM FA was prepared. 2.5ml of each type of medium was pipetted into wells of a 12-well tissue culture plate. Two wells were prepared for each folate condition. Cells of cell line 17241 were rinsed three times in folate-free RPMI to remove residual folate. The cells were counted as described in section 2.2.1.2. A cell suspension at $125x10^6$ cells/ml was made up in folate-free RPMI, and 50μl of this cell suspension was added to each well giving a final cell density of $2.5x10^5$ cells/ml. Medium containing 5μM Mtx was prepared by diluting 5μl of 500μM Mtx stock with 495μl folate-free RPMI. 50μl of this Mtx medium was added to one well of each folate condition, giving a final Mtx concentration of 100nM. After 48h incubation at 37°C, 5% CO_2, the cells were harvested by centrifugation. Each sample was split in two and processed for cell cycle analysis and Annexin V/PI staining as described in Chapter 2. This experiment was repeated on three separate occasions.

The results of this experiment are presented in figure 4.7. One-way ANOVA with Tukey's post-tests was used to compare the groups for each type of apoptosis assay. In the absence of Mtx, the level of apoptosis in all of the folate-containing conditions was not different from the folate-free control, demonstrating that folate deficiency for 48h does not affect cellular apoptosis. As observed previously, 100nM Mtx caused apoptosis significantly above the background level ($p < 0.001$, one-way ANOVA). Supplementation of the culture medium with either 20μM 5-methyl THF or 5μM 5-formyl THF reduced levels of apoptosis in the presence of Mtx back to control levels. Supplementation with 2μM FA did not reduce apoptosis due to Mtx, because levels of apoptosis in this condition were indistinguishable from the Mtx-only sample. The same pattern of results was observed in the cell cycle analysis assay and Annexin V/PI staining assay.

4.5 Investigation of the ability of thymidine and hypoxanthine to prevent methotrexate-induced apoptosis in transformed B-lymphocyte cell lines

In chapter 3, it was also observed that a combination of thymidine (Thy) plus hypoxanthine (Hx) could restore proliferation of transformed B-lymphocytes to control levels in the presence of Mtx. An experiment was carried out to investigate if the protective effect of Thy plus Hx could be detected at the level of apoptosis, and if this protection is modified by MTHFR 677C→T genotype. Cells of one cell line of MTHFR 677 CC genotype (17241) and one cell line of MTHFR 677 TT genotype (17274) were seeded at a density of $2x10^5$ cells/ml in folate-free RPMI containing 100nM Mtx, and either 40μM Hx, 100μM Thy or a combination of the two. After 48h and 72h incubation time, one flask of cells from each condition was processed for analysis of apoptosis by cell cycle analysis and Annexin V staining. This experiment was carried out three separate times for each cell line. The results of this experiment are presented in figure 4.8 for cell line 17241 CC and figure 4.9 for cell line17274 TT.

One-way ANOVA with Tukey's post-tests was used to compare differences between treatments at each time point. There was no increase in apoptosis compared to controls where cells were incubated in the presence of either Thy or Hx alone, demonstrating that neither of these compounds have a pro-apoptotic effect. In the CC cell line (fig 4.8), Thy partially reduced Mtx-induced apoptosis, but did not fully reduce apoptosis to control levels ($p < 0.001$ for Mtx vs. Mtx+Thy after 72h). Hx alone did not reduce Mtx-induced apoptosis, because the level of apoptosis was indistinguishable from the Mtx treated condition. A combination of Thy plus Hx restored the level of apoptosis completely to control levels. This pattern of effect of Thy and Hx was observed at both time points and with both types of apoptosis assay. The same overall

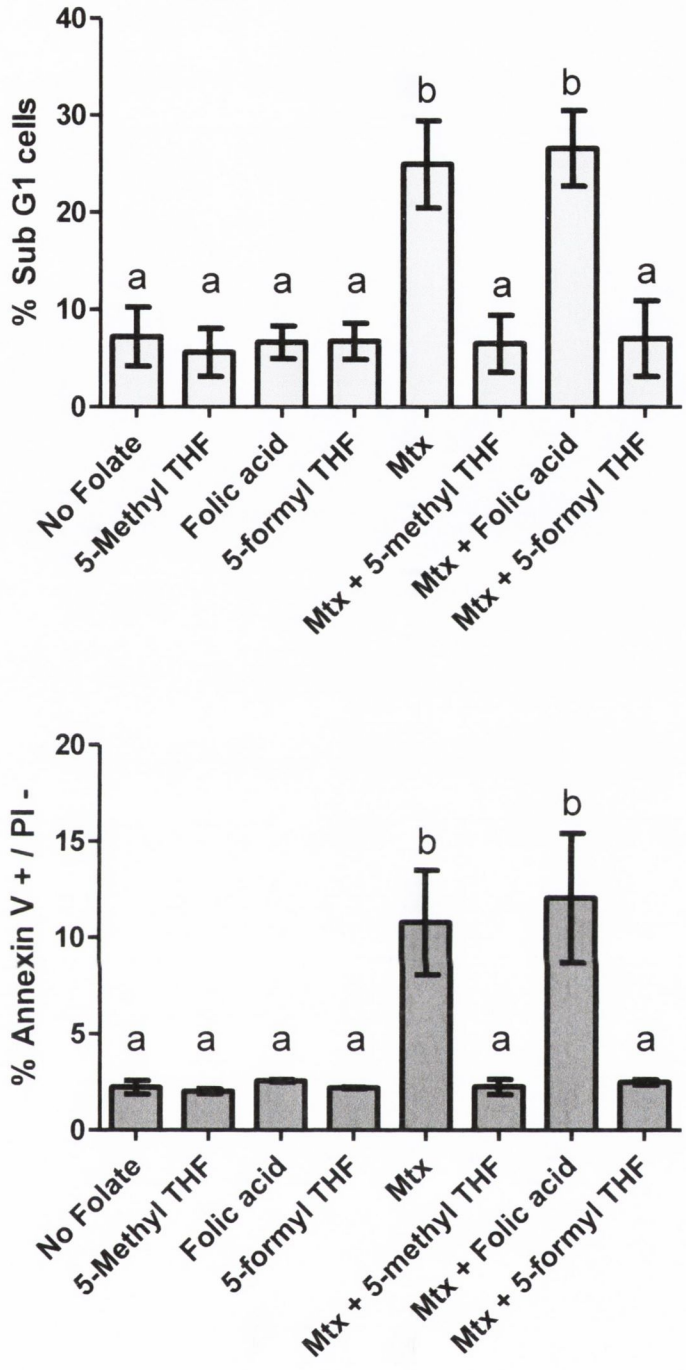

Figure 4.7 Investigation of the ability of various folate cofactors to prevent Mtx-induced apoptosis in transformed B-lymphocyte cell lines

Cells (17241) were seeded at 2.5 x 10^5 cells/ml in folate-free RPMI medium containing 100nM Mtx and supplemented with 5-methyl THF, 5-formyl THF or folic acid as indicated. After 48h, cells were processed for cell cycle analysis (upper panel) and Annexin V/PI staining (lower panel). Data are the means +/- SD of three independent experiments. One-way ANOVA with Tukey's post-tests was used to compare groups within each graph. Bars without a common letter are significantly different, $p < 0.001$.

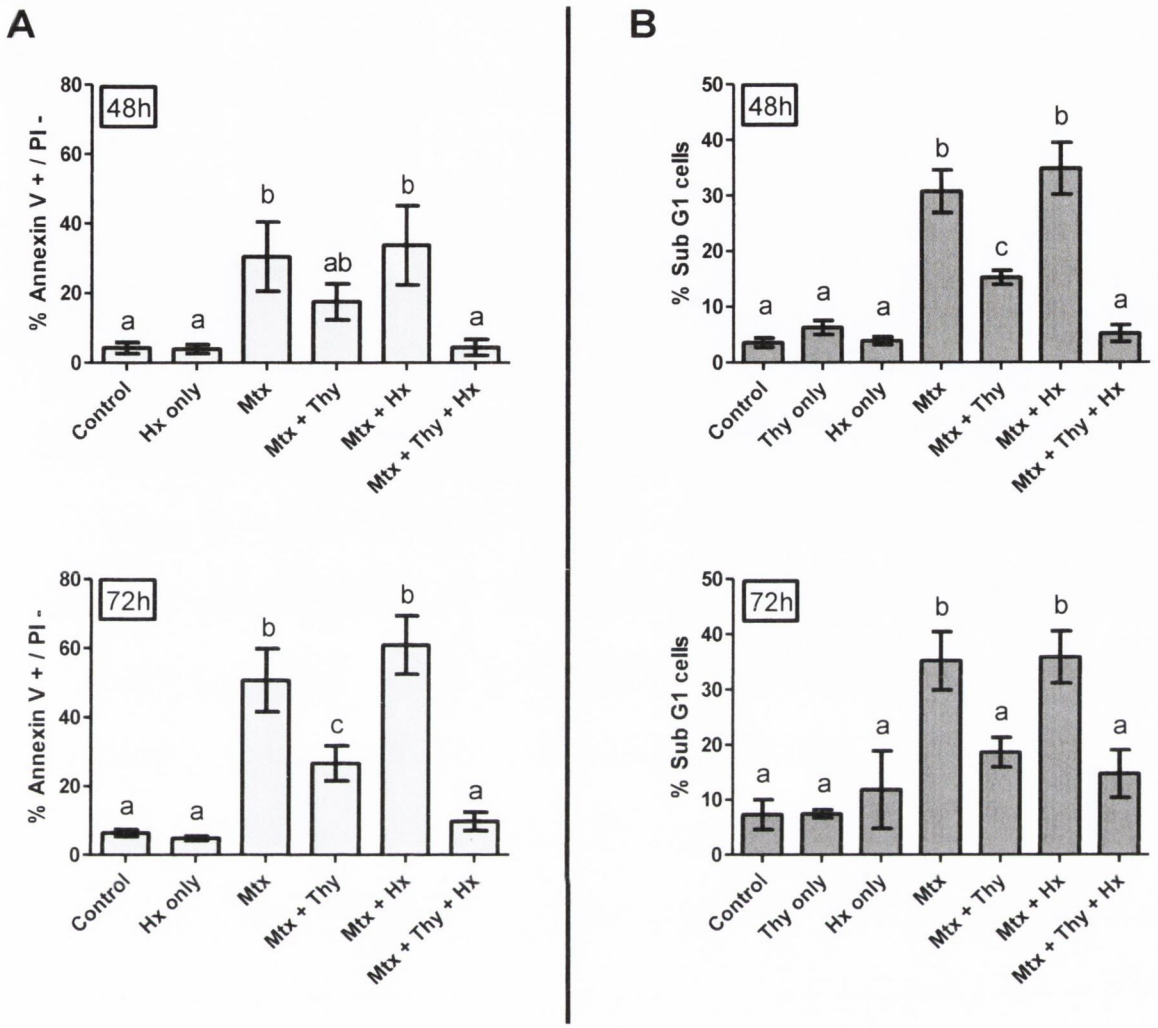

Figure 4.8 Investigation of the ability of hypoxanthine and thymidine to prevent Mtx-induced apoptosis in cell line 17241 CC

Cells (17241) were seeded at 2.5 x 10^5 cells/ml in standard RPMI medium containing 100nM Mtx and Hx or Thy as indicated. After 48h and 72h, cells were processed for Annexin V staining (A) and cell cycle analysis (B). Data are the means +/- SD of 3 independent experiments. One-way ANOVA with Tukey's post-tests was used to compare groups within each graph. Bars without a common letter are significantly different, $p < 0.01$.

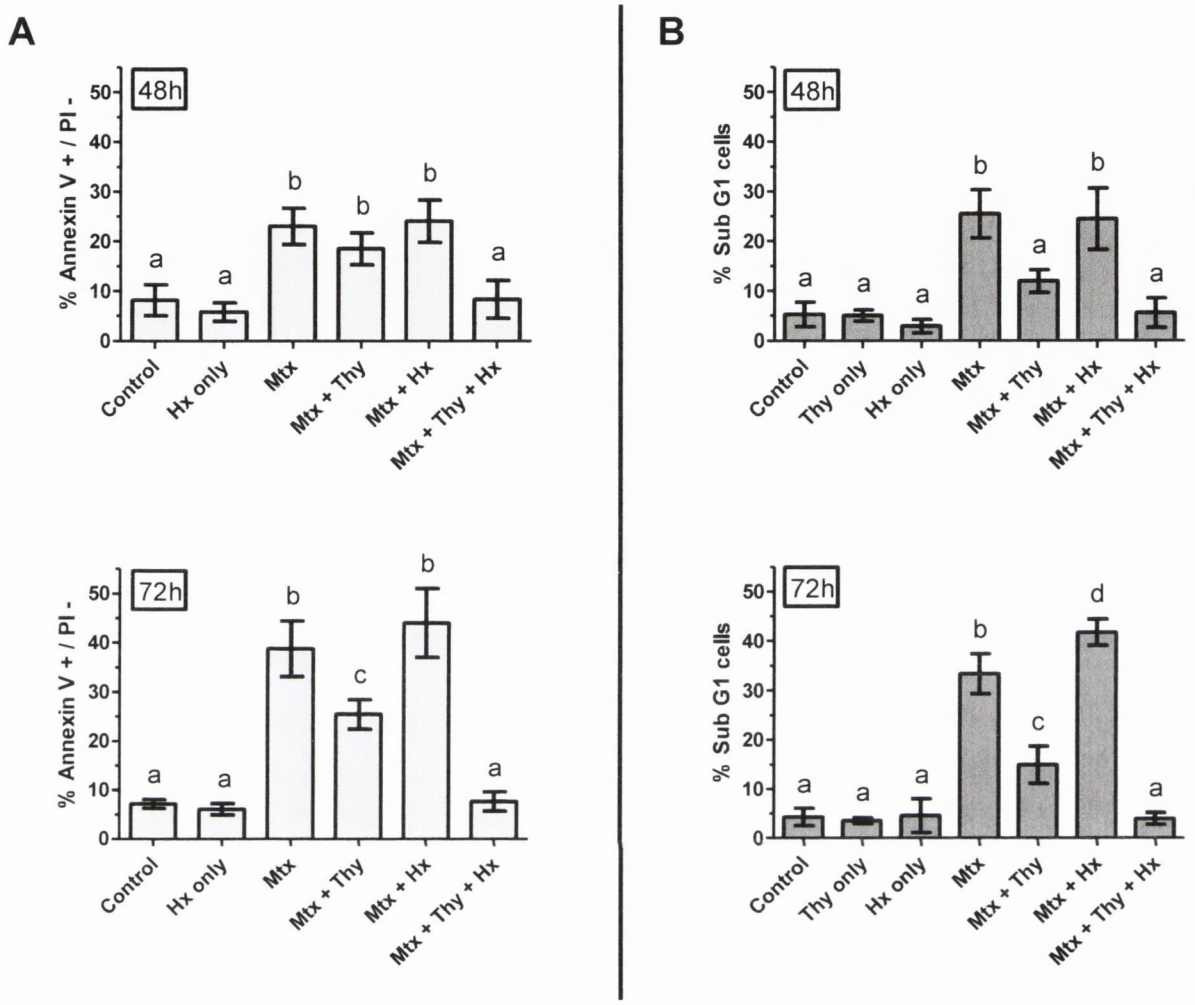

Figure 4.9 Investigation of the ability of hypoxanthine and thymidine to prevent Mtx-induced apoptosis in cell line 17274 TT

Cells (17274) were seeded at 2.5 x 10^5 cells/ml in standard RPMI medium containing 100nM Mtx and Hx or Thy as indicated. After 48h and 72h, cells were processed for Annexin V staining (A) and cell cycle analysis (B). Data are the means +/- SD of 3 independent experiments. One-way ANOVA with Tukey's post-tests was used to compare groups within each graph. Bars without a common letter are significantly different, $p < 0.01$.

effects of Hx and Thy were observed in the TT cell line (fig 4.9). There is one difference between the two cell lines; for the TT cell line, the combination of Hx plus Mtx appeared to cause a greater degree of apoptosis compared to Mtx alone, although this is significant only by the cell cycle analysis method and only after 72h incubation time ($p < 0.01$, one-way ANOVA).

4.5.1 A modification of the TUNEL assay to investigate incorporation of uracil into DNA

The results in section 4.5 showed that addition of Thy could partially prevent Mtx-induced apoptosis, in contrast to Hx which did not reduce apoptosis and in one case appeared to potentiate the apoptotic effect of Mtx. Following on from this observation, it was hypothesized that the presence of Hx during exposure to Mtx in deficient folate conditions might allow DNA synthesis to occur, but may result in increased uracil incorporation into DNA due to the lack of dTMP availability. This could account for the slight increase in apoptosis observed when Mtx and Hx are present compared to Mtx alone. In order to investigate this possibility, a method to quantify uracil incorporation and DNA breaks is necessary. It was attempted to use a modification of the TUNEL assay to investigate both of these events. The TUNEL assay is a well established method that is used to measure the extent of apoptotic DNA breaks by flow cytometry or other imaging methods. The proposed modification of the standard TUNEL assay protocol involves splitting each sample in half and treating one half with the enzyme uracil DNA glycosylase (UDG) prior to TUNEL staining. UDG treatment should digest any uracil residues incorporated into the DNA, leaving a break which would be detected as an increased level of TUNEL staining compared to the untreated half of the sample. The use of UDG to quantify uracil incorporation in combination with the TUNEL assay has not been previously attempted, although UDG treatment has been used to assess uracil incorporation in the qualitative COMET assay (Duthie and McMillan 1997).

Cells of cell line 17241 were seeded at $2x10^5$ cells/ml in folate-free RPMI containing 100nM Mtx and 40μM Hx or 100μM Thy. After incubation for 40h, the cells were harvested and fixed with 1% PFA. The TUNEL assay was carried out as described in Chapter 2. The experiment was repeated three times with cell line 17241, and results are presented in Table 4.1. The results from the samples which were not treated with UDG confirm the observations of section 4.5. In the control sample and Mtx+Thy+Hx sample, there is very little DNA damage detected. Mtx-treatment causes DNA a greater extent of DNA damage than in the control sample, and in some cases this is reduced by the presence of Thy, and exacerbated by Hx alone. However, the values obtained for each sample in the three experiments were not very repeatable. Additionally, UDG-treatment did not have a consistent effect; in experiments 1 and 3 UDG-treatment decreased the

amount of TUNEL staining compared to the untreated portion of the sample, but in experiment 2 the amount of TUNEL staining was increased in the UDG-treated samples. Although extensive wash steps were carried out, there is a possibility that residual UDG could interfere with the TUNEL staining reaction. Another problem encountered in the development of this assay was extensive loss of cell pellet during the TUNEL staining process, even though all procedures were carried out as recommended. In conclusion, while the results from standard TUNEL staining do confirm the general result that a combination of Hx plus Thy protects cells from Mtx-induced DNA damage, it is not suitable to provide evidence of increased uracil incorporation into DNA of Mtx-treated cells. Further optimization of this method may improve results, but this was not possible within the scope of this project.

Experiment 1		
% TUNEL positive cells		
	UDG -	UDG +
Control	1.24	0.73
Mtx	47.85	38.87
Mtx + Hx	68.02	58.79
Mtx + Thy	35.04	24.07
Mtx + Thy+ Hx	0.58	0.38

Experiment 2		
% TUNEL positive cells		
	UDG -	UDG +
Control	0.34	0.1
Mtx	13.74	41.69
Mtx + Hx	30.74	39.9
Mtx + Thy	18.3	18.25
Mtx + Thy+ Hx	0.29	0.37

Experiment 3		
% TUNEL positive cells		
	UDG -	UDG +
Control	2.74	1.69
Mtx	15.54	10.15
Mtx + Hx	10.34	4.17
Mtx + Thy	23.31	19.26
Mtx + Thy+ Hx	1.7	1.92

Table 4.1 Results of modified TUNEL assay with UDG treatment

Cells (17241) were seeded at 2.5x105 cells/ml in folate-free RPMI containing 100nM Mtx, Hx, and Thy as indicated. After 40h incubation, the samples were processed for TUNEL staining as described in Chapter 2. The experiment was repeated 3 times and results from each experiment are presented separately.

4.6 Investigation of the mechanism of methotrexate-induced apoptosis in transformed B-lymphocytes

4.6.1 Caspase-dependence of methotrexate induced apoptosis in transformed B-lymphocyte cell lines

To explore the mechanism of Mtx-induced apoptosis in transformed B-lymphocyte cell lines, the general caspase inhibitor Z-VAD-fmk was used to investigate if the process is caspase dependent. Cells which had previously been cultured in standard RPMI medium were harvested by centrifugation and counted as described in section 2.2.1.2. Cells were washed three times in folate-free RPMI to remove residual folate. Cells were then seeded at $2x10^5$ cells/ml in a 12-well tissue culture plate, at a total volume of 2.5ml per well. Each plate contained two wells of folate-free RPMI (control), and two wells of folate-free RPMI containing 100nM Mtx. 12.5µl of Z-VAD-fmk was added to one control well and one well containing Mtx in each plate, giving a final concentration of 50µM. After 48h and 72h incubation at 37^oC, 5% CO_2, samples from each condition were taken and processed for cell cycle analysis as described in section 2.2.2.1. The percentage of apoptotic cells in each sample was measured by flow cytometry. This experiment was repeated three separate times with cell line 17241 (CC genotype).

The results of this experiment are shown in figure 4.10. The level of apoptosis measured in the presence of Mtx + Z-VAD-fmk were significantly lower than levels of apoptosis with Mtx alone at both time points investigated ($p = 0.014$ 24h, $p = 0.0127$ 72h, Student's t-test). As Z-VAD-fmk is a general caspase inhibitor, this indicates that Mtx-induced apoptosis is caspase dependent. Levels of apoptosis in the presence of Mtx plus Z-VAD-fmk remain higher than controls. After 48h controls have 4.6% apoptosis compared to 10.05% for Mtx + Z-VAD-fmk ($p = 0.036$, Student's t-test), and after 72h the levels are 5.4% for controls and 17.84% for Mtx + Z-VAD-fmk ($p = 0.011$, Student's t-test). These results suggest that while Mtx-induced apoptosis in transformed B-lymphocyte cell lines is largely caspase-dependent, other mechanisms may also be involved.

To further investigate the caspase-dependence of Mtx-induced apoptosis, specific inhibitors of caspase-8 and caspase-9 were used. These enzymes are 'initiator caspases', and are involved in the two main pathways of activation of apoptosis, caspase-9 in the mitochondrial activation pathway, and caspase-8 in the extrinsic activation pathway. Cells of cell line 17241 which had previously been cultured in standard RPMI medium were harvested by centrifugation and counted as described in section 2.2.1.2. Cells were washed three times in folate-free RPMI to

remove residual folate. Cells were then seeded at $2x10^5$ cells/ml in a 12-well tissue culture plate, at a total volume of 2.5ml per well. The wells contained folate-free RPMI with either Z-VAD-fmk (50μM), caspase-8 inhibitor (Z-IETD-fmk, 40μM), or caspase-9 inhibitor (Z-LEHD-fmk, 40μM). Control wells were also included with folate-free RPMI alone and with 0.0026% DMSO as a vehicle control, because the caspase inhibitors are supplied dissolved in DMSO. After pre-incubation at 37°C 5% CO_2 for 1 hour, Mtx was added to one well of each condition to a final concentration of 100nM Mtx. After 40h and 72h incubation at 37°C, 5% CO_2, samples from each condition were harvested by centrifugation. The samples were split in half, and processed for cell cycle analysis and Annexin V/PI staining as described in chapter 2. The percentage of apoptotic cells in each sample was measured by flow cytometry. This experiment was carried out on four separate occasions with cell line 17241.

In this experiment analysis of apoptosis was carried out at two time points after Mtx exposure, 40h and 72h. The results are shown in Figure 4.11. One-way ANOVA with Tukey's post-tests was used to compare the treatments at each time point. After incubation with Mtx for 40h, the overall levels of apoptosis detected were lower than at 72h, which was as expected. As observed previously, the general caspase inhibitor Z-VAD-fmk reduced the level of Mtx-induced apoptosis at both time points and by both types of assay ($P< 0.01$). After 40h incubation, samples treated with Mtx + Caspase-8 inhibitor had lower levels of apoptosis than samples treated with Mtx alone by both types of assay ($P < 0.01$), but apoptosis was not restored to control levels by caspase-8 inhibition. The caspase-9 inhibitor had no statistically significant effect at this time point. After 72h incubation, the samples with Mtx plus either the caspase-8 and caspase-9 inhibitor had decreased levels of apoptosis compared to the Mtx condition when analysed by Annexin V/PI staining ($P< 0.001$), but neither caspase inhibitor had a significant effect by the cell cycle analysis assay. These results suggest that caspase-8 may be activated earlier than caspase-9 in Mtx-induced apoptosis, but ultimately both caspases are involved in the process.

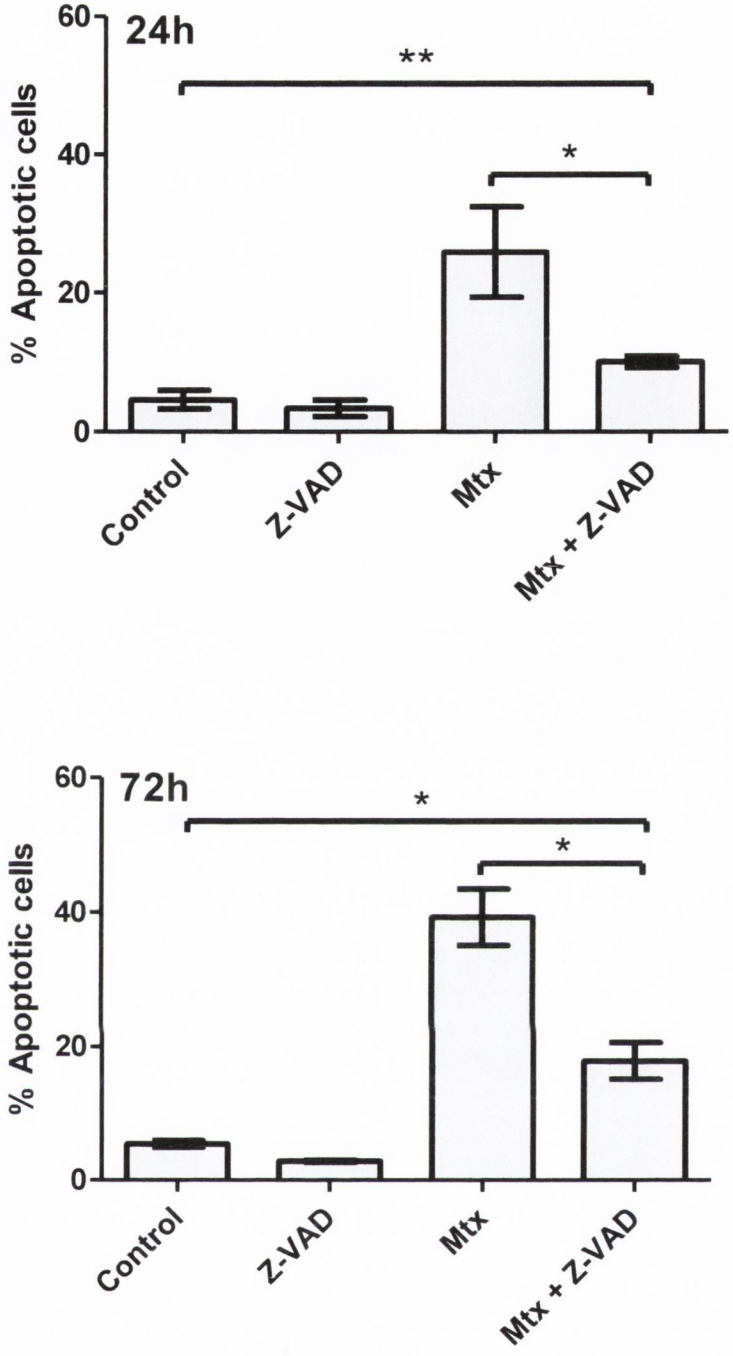

Figure 4.10 Mtx-induced apoptosis in B-lymphocyte cell lines is prevented by the general caspase inhibitor Z-VAD-fmk

Cells (17241) were seeded in folate-free RPMI medium. Mtx (100nM) or Z-VAD-fmk (50µM) were added as indicated. After 48h (upper panel) and 72h (lower panel) samples were processed for cell cycle analysis, and the percentage of apoptotic cells measured by flow cytometry. Data are the means +/- SD of 3 independent experiments. The data were analysed by Student's t-test, * = $p < 0.02$ ** = $p < 0.01$.

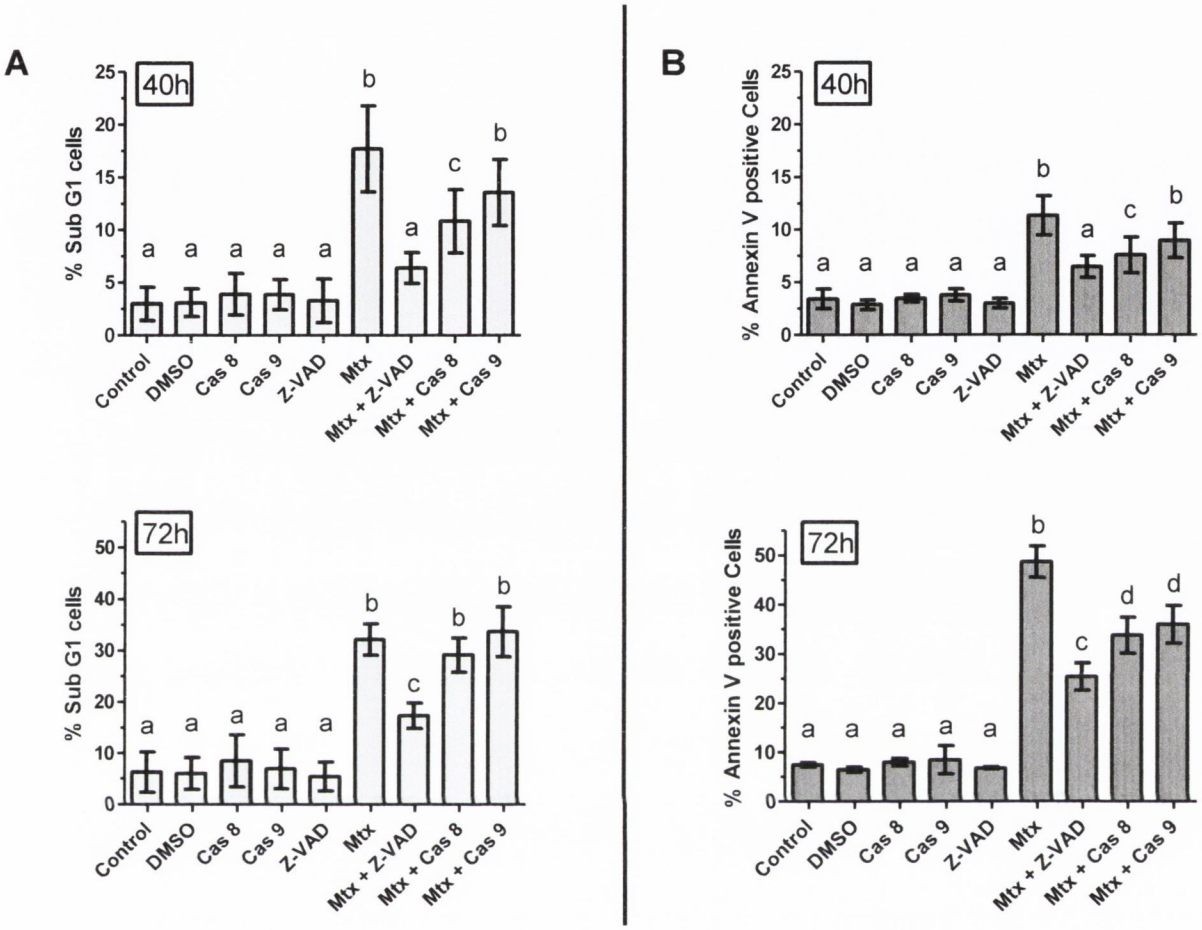

Figure 4.11 The effect of general and specific caspase inhibitors on Mtx-induced apoptosis in transformed B-lymphocytes

Cells (17241) were incubated with Mtx (100nM) and either the general caspase inhibitor Z-VAD-fmk (Z-VAD) or inhibitors of Caspase-8 (Cas-8) or Caspase-9 (Cas-9) as indicated. After incubation for 40h and 72h, samples processed for cell cycle analysis (A) and annexin V staining (B) and the percentage of apoptotic cells were determined by flow cytometry. Data are the means +/- SD of 4 independent experiments. One-way ANOVA with Tukey's post-tests was used to compare groups within each graph. Bars without a common letter are significantly different, $p > 0.05$.

4.6.2 Investigation of the effect of the antioxidant compound N-acetylcysteine on methotrexate-induced apoptosis in transformed B-lymphocyte cell lines

The aim of this experiment was to determine if generation of ROS plays a role in Mtx-induced apoptosis in transformed B-lymphocyte cell lines. Cells of cell line 17241 which had previously been cultured in standard RPMI medium were harvested by centrifugation and counted as described in section 2.2.1.2. Cells were washed three times in folate-free RPMI to remove residual folate. Cells were then seeded at $2x10^5$ cells/ml in a 12-well tissue culture plate, at a total volume of 2.5ml per well. N-acetylcysteine (NAC) at a final concentration of 5 or 10mM and Mtx to a final concentration of 100nM were added to wells as required. After 48h and 72h incubation at 37°C, 5% CO_2, samples of cells from each condition were harvested by centrifugation and processed for cell cycle analysis as described in chapter 2. The percentage of apoptotic cells was measured by flow cytometry. Three independent repeats of this experiment were carried out with cell line 17241.

The results of this experiment are presented in figure 4.12. One-way ANOVA was used to compare the level of apoptosis between groups at each time point. Samples treated with NAC alone did not have an increased level of apoptosis compared to untreated controls, demonstrating that NAC on its own does not cause apoptosis. Samples treated with Mtx + 10mM NAC had significantly lower levels of apoptosis than Mtx alone after 48h incubation ($P < 0.05$, one-way ANOVA). The effect of NAC did not remain significant after 72h incubation at either of the concentrations tested. This indicates that reducing oxidative stress by using NAC is not an effective method of blocking the apoptotic effect of Mtx treatment in this cell culture model. For this reason the experiment was not repeated using Annexin V staining.

4.6.3 The effect of the serine protease inhibitors TPCK and TLCK on methotrexate-induced apoptosis in transformed B-lymphocyte cell lines

In section 4.6.1 it was observed that the general caspase inhibitor Z-VAD-fmk reduced but did not completely prevent Mtx-induced apoptosis in a transformed B-lymphocyte cell line. The aim of this experiment was to investigate if serine proteases are involved in Mtx-induced apoptosis. Cells of cell line 17241 which had previously been cultured in standard RPMI medium were harvested by centrifugation and counted as described in section 2.2.1.2. Cells were washed three times in folate-free RPMI to remove residual folate. Cells were then seeded at $2x10^5$ cells/ml in a 12-well tissue culture plate, at a total volume of 2.5ml per well. The wells contained folate-free RPMI with either the chymotrypsin-like serine protease inhibitor TPCK (100μM) or the trypsin-like serine protease inhibitor TLCK (200μM). These concentrations were chosen based on a preliminary experiment carried out using a range of concentrations of these compounds. Control

wells were also included with folate-free RPMI alone and with 0.0026% DMSO as a vehicle control, because the stock solutions of TPCK and TLCK were prepared using DMSO as a solvent. After pre-incubation at 37°C 5% CO_2 for 1 hour, Mtx was added to one well of each condition to a final concentration of 100nM Mtx. After incubation at 37°C, 5% CO_2 for 72h, samples from each condition were harvested by centrifugation. The samples were split in half, and processed for cell cycle analysis and Annexin V/PI staining as described in chapter 2. The percentage of apoptotic cells in each sample was measured by flow cytometry.

The first experiment with these compounds was conducted for a 72h incubation time, after which samples were split in two and analysed by cell cycle analysis and Annexin V staining. This experiment was repeated on three separate occasions and the results combined to produce figure 4.13. One-way ANOVA with Tukey's post tests was used to compare the groups for each type of apoptosis assay.

TLCK caused significant amounts of apoptosis by itself, as the sample treated with TLCK alone had a significantly greater level of apoptosis compared to untreated cells as assessed by Annexin V staining ($P < 0.001$), and also by cell cycle analysis although this was not significant by one-way ANOVA. TLCK also did not reduce the level of apoptosis caused by Mtx by either type of apoptosis assay. The effects of TPCK were quite different to those of TLCK. TPCK did not cause significant apoptosis by itself, although there was a non-significant increase in apoptosis caused by TPCK by Annexin V staining only. TPCK reduced the level of Mtx-induced apoptosis when analysed by the cell cycle analysis method ($P < 0.05$), however TPCK did not significantly reduce Mtx-induced apoptosis as measured by Annexin V staining.

To attempt to resolve this conflicting result for the effect of TPCK between the two apoptosis assays, the experiment was repeated using a shorter incubation time of 40h. Although the overall levels of apoptosis measured at this time point are lower than at 72h, it is possible that the effects might be clearer at an earlier time point where apoptosis had not progressed as extensively in the cell population. The experiment was not repeated with TLCK as it was clear from the result above that this compound does not have any protective effect against Mtx-induced apoptosis. The experiment was set up exactly as described above, with the omission of the TLCK conditions. This was repeated on six occasions with cell line 17241. The results of this second analysis of TPCK are presented in figure 4.14.

After 40h incubation time, TPCK did not cause significant levels of apoptosis by itself, as measured by either assay method. However, again there was a non-significant increase in apoptosis by TPCK alone when measured by Annexin V staining. The same pattern of protective effect of TPCK

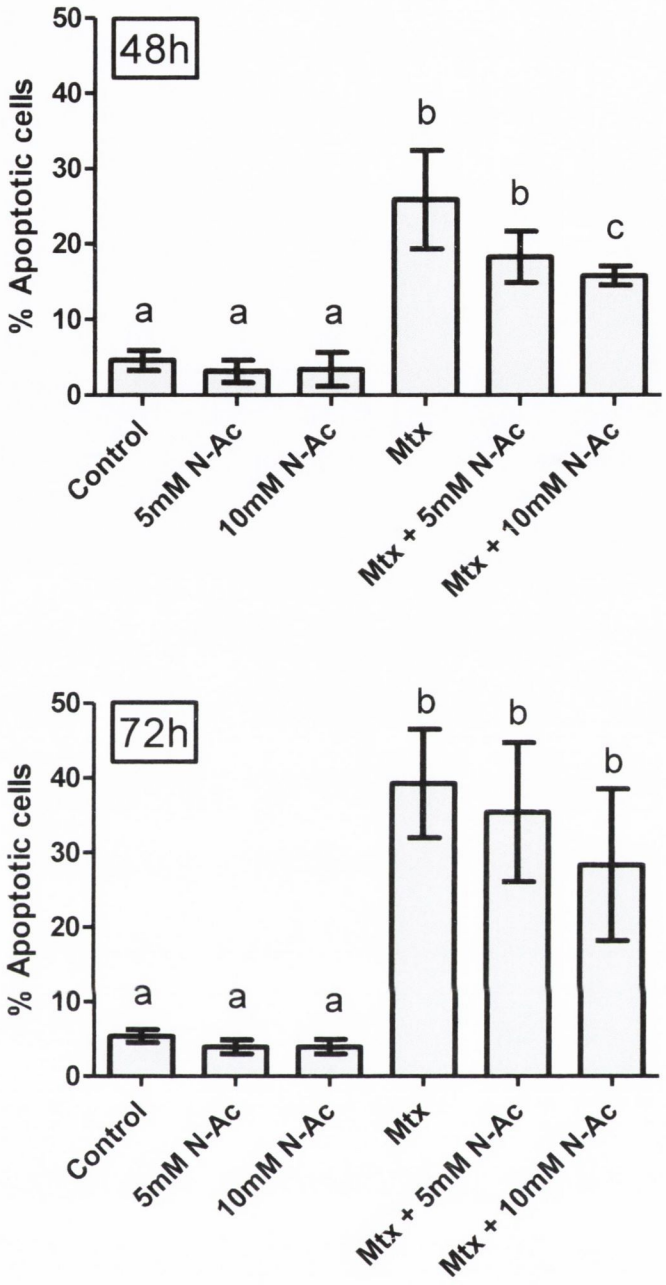

Figure 4.12 The effect of the antioxidant N-acetyl cysteine on Mtx-induced apoptosis in transformed B-lymphocytes

Cells (17241) were seeded in folate-free RPMI. Mtx (100nM) or N-acetyl cysteine (N-Ac) were added as indicated. After 48h and 72h samples were processed for cell cycle analysis and the percentage of apoptotic cells measured by flow cytometry. Data are the means +/- SD of 3 independent experiments. Data were analysed by one-way ANOVA with Tukey's post-tests. Bars without a common letter are significantly different, $p < 0.05$.

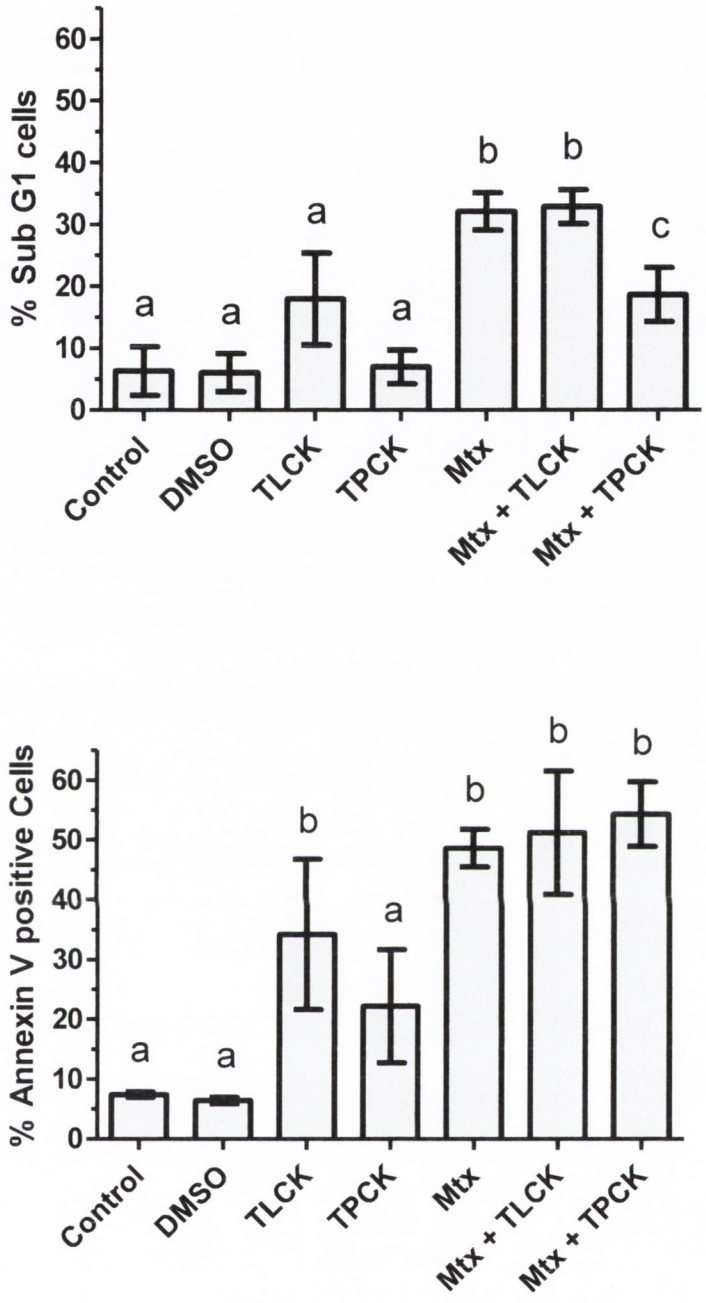

Figure 4.13 The effect of the serine protease inhibitors TPCK and TLCK on Mtx-induced apoptosis

Cells (17241) were seeded at $2x10^5$ cells/ml in folate-free RPMI containing 100nM Mtx and TPCK (100μM) or TLCK (200μM) as indicated. After 72h incubation, samples were processed and apoptosis measured by cell cycle analysis (A) and Annexin V staining (B). Data are the means +/- SD of 3 independent experiments. Data were analysed by one-way ANOVA with Tukey's post-tests. Bars without a common letter are significantly different, $p < 0.05$.

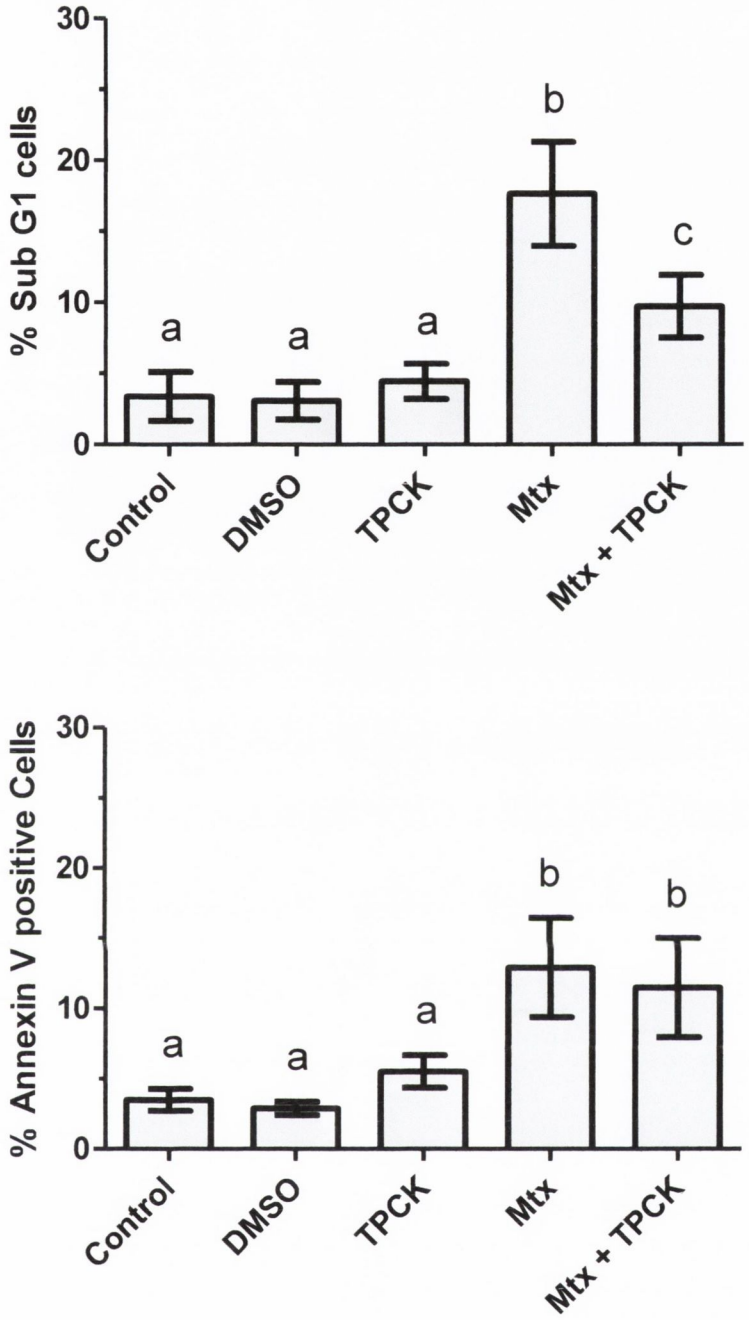

Figure 4.14 The effect of the serine protease inhibitor TPCK on Mtx-induced apoptosis after 40h incubation

Cells were seeded at 2x10 ^{5} cells/ml in folate-free RPMI containing 100nM Mtx and/or TPCK as indicated. After 40h incubation, samples were processed and apoptosis measured by cell cycle analysis (A) and Annexin V staining (B). Data are the means +/- SD of six independent experiments for cell cycle analysis, and 4 independent experiments for annexin V staining. Data were analysed by one-way ANOVA with Tukey's post-tests. Bars without a common letter are significantly different, $p < 0.05$.

was seen as in the previous experiment. TPCK caused a significant reduction in Mtx-induced apoptosis when measured by cell cycle analysis ($P < 0.001$, one-way ANOVA). When apoptosis was examined by Annexin V staining, there was no reductive effect of TPCK on Mtx-induced apoptosis.

A further experiment was carried out by adding TPCK and Z-VAD-fmk together to cells incubated with 100nM Mtx. Each of these compounds has been shown to partially block Mtx-induced apoptosis, and the aim was to investigate if the two compounds together would have a combinatorial effect on Mtx-induced apoptosis. Cells of cell line 17241 which had previously been cultured in standard RPMI medium were harvested by centrifugation and counted as described in section 2.2.1.2. Cells were washed three times in folate-free RPMI to remove residual folate. Cells were then seeded at $2x10^5$ cells/ml in a 12-well tissue culture plate, at a total volume of 2.5ml per well. The wells contained folate-free RPMI with either Z-VAD-fmk (50μM) or TPCK (100μM), or both. Control wells were also included with folate-free RPMI alone and with 0.0026% DMSO as a vehicle control, because the stock solutions of TPCK and TLCK were made up using DMSO as a solvent. After pre-incubation at 37°C 5% CO_2 for 1 hour, Mtx was added to one well of each condition to a final concentration of 100nM Mtx. After incubation at 37°C, 5% CO_2 for 40h and 72h samples from each condition were harvested by centrifugation. The samples were split in half, and processed for cell cycle analysis and Annexin V/PI staining as described in chapter 2. The percentage of apoptotic cells in each sample was measured by flow cytometry. The results from this experiment are presented in figure 4.15. The level of apoptosis in all groups was compared using one-way ANOVA with Tukey's post-tests. By the cell cycle analysis method, both TPCK and Z-VAD-fmk when used individually partially reduced apoptosis compared to Mtx only ($P < 0.01$ for both time points), as observed previously. The combination of TPCK and Z-VAD-fmk together was sufficient to reduce apoptosis to control levels. However, there were still differences observed depending on the assay method used. By the Annexin V staining method, TPCK did not reduce apoptosis caused by Mtx, and the combination of the two compounds did not reduce apoptosis to control levels.

TPCK has previously been shown to inhibit DNA fragmentation in response to certain apoptotic stimuli (Fabian et al. 2009). If TPCK inhibits DNA fragmentation in response to Mtx, but does not affect other processes associated with apoptosis, this could explain why the two different assay methods employed in the current study produce conflicting results in relation to TPCK. In order to test this hypothesis, cells treated with Mtx with or without TPCK were examined for DNA fragmentation by agarose gel electrophoresis. Cells treated with staurosporine were also included as a positive control for DNA laddering. Cells of cell line 17241 were seeded at $2x10^5$

cells/ml in 25cm^3 tissue culture flasks, in a total volume of 10ml folate-free RPMI. Mtx to a final concentration of 100nM, and TPCK to a final concentration of 100μM were added as required. Flasks were also set up with folate-free RPMI only and for treatment with 1μM staurosporine for 4h, as a positive control for DNA fragmentation. After incubation for 48h at 37^{0}C, 5% CO_2, DNA was extracted from samples by the phosphate-citrate method, as described in section 2.2.6.2. The DNA extracts were separated on a 1.5% agarose gel, and visualized using the UVP-GelDoc system.

A photograph of the gel is presented in figure 4.16. DNA isolated from untreated control cells is not fragmented, and there is fragmentation of DNA observed in Mtx-treated cells. However, fragmentation is not seen in DNA from cells treated concurrently with Mtx and TPCK. This indicates that TPCK blocks DNA fragmentation in response to Mtx in transformed B-lymphocyte cell lines.

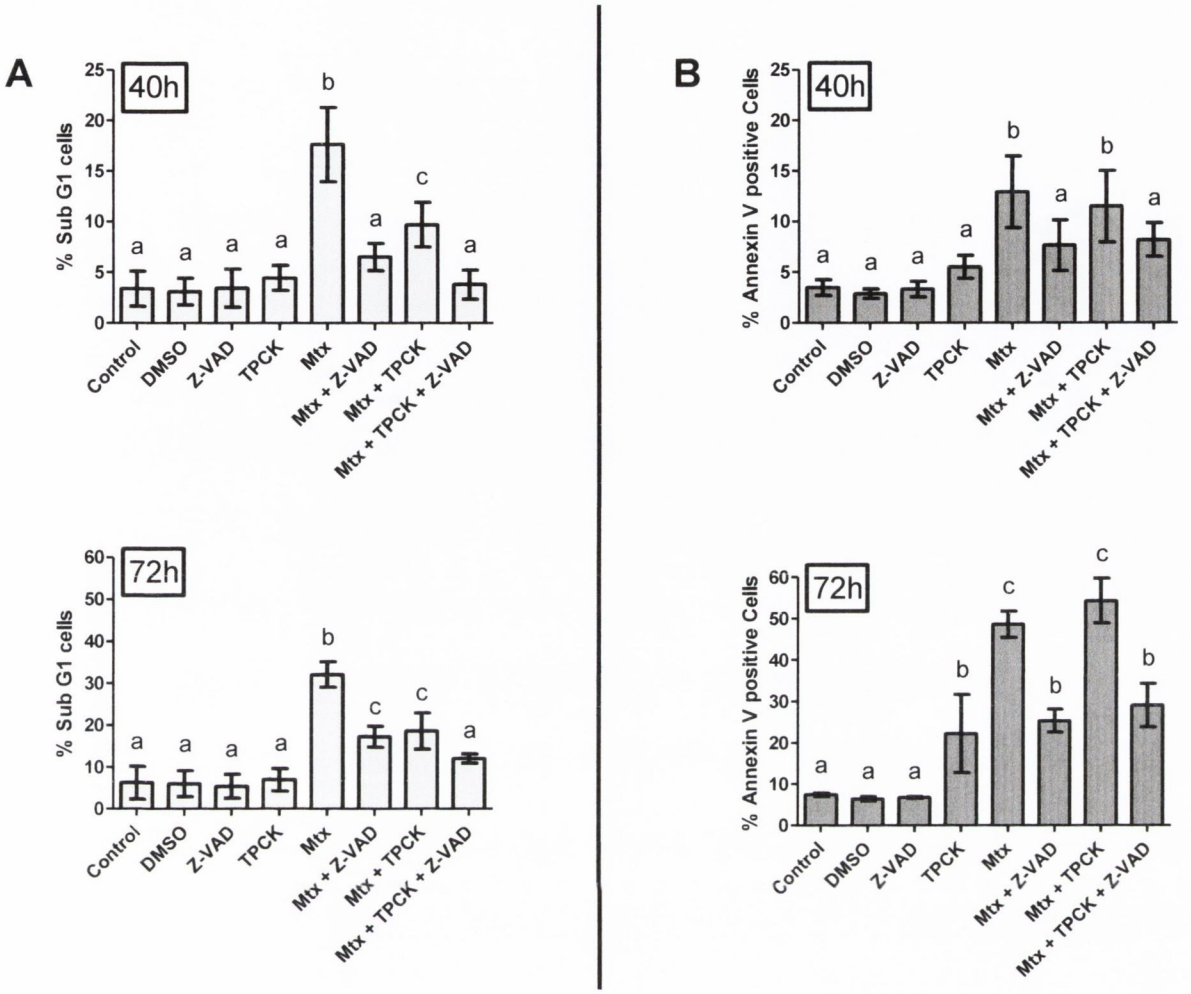

Figure 4.15 The effect of a combination of caspase inhibition and serine protease inhibition on Mtx-induced apoptosis

Cells were incubated with Mtx (100nM) and TPCK (100μM) and/or Z-VAD-fmk (50μM) as indicated for 40h and 72h. Samples were split in two and analysed for apoptosis by cell cycle analysis (A) and annexin V staining (B). Data are the means +/- SD of 4 separate experiments. One-way ANOVA with Tukey's post-tests was used to compare groups. Bars without a common letter are significantly different, p = 0.05.

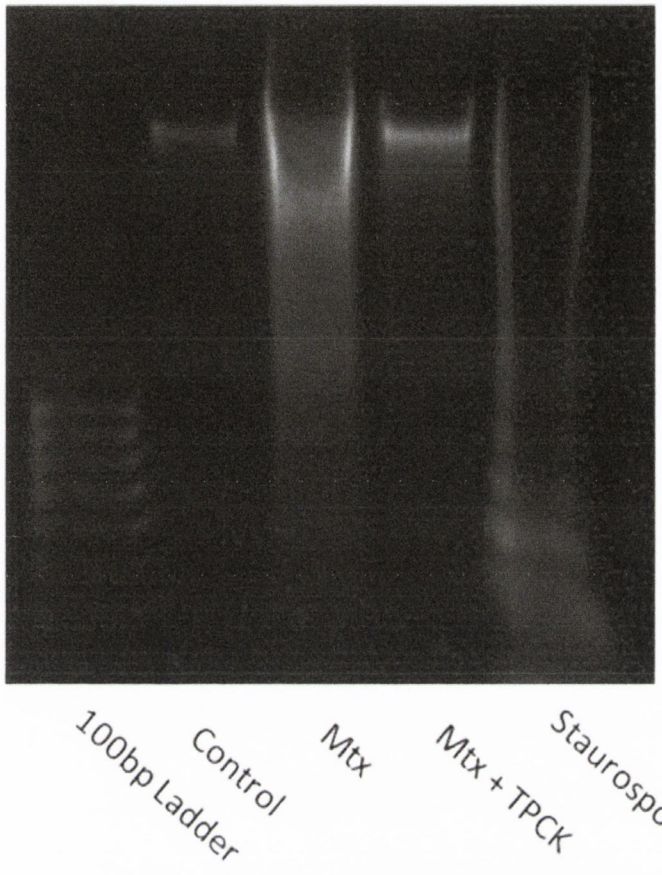

Figure 4.16 Investigation of DNA fragmentation induced by Mtx and the serine protease inhibitor TPCK

Cells (17241) were seeded at $2x10^5$ cells/ml in RPMI with Mtx, TPCK or staurosporine as indicated. After 48h, DNA was extracted by the phosphate-citrate method, and was run on a 1.5% agarose gel containing ethidium bromide. The gel was visualised under UV light. This gel represents a typical result of an experiment which was repeated 3 times.

4.7 Discussion

The experiments in this chapter show that Mtx causes apoptotic cell death in transformed B-lymphocyte cell lines. This is the first demonstration of apoptosis in response to Mtx in this cell type. Incubation of these cells with 100nM Mtx causes activation of caspase-3 and cleavage of the caspase substrate PARP, as demonstrated by Western blot. Mtx also induces changes in cell morphology which are characteristic of apoptosis, and causes DNA degradation and phosphatidylserine exposure on the cell surface, two other biochemical features of apoptosis. In chapter 3, the folate cofactors 5-formyl THF, 5-methyl THF and also the combination of thymidine (Thy) and hypoxanthine (Hx) were found to restore cell proliferation to control levels in the presence of Mtx. These same interventions were able to block Mtx-induced apoptosis, demonstrating that apoptosis caused by Mtx is a direct consequence of disruption of the folate cycle and inhibition of dTMP and purine synthesis. Additionally, Mtx-induced apoptosis in transformed B-lymphocytes was found to involve caspases, and also serine protease activity. The involvement of serine proteases in Mtx-induced apoptosis has not been previously reported.

In section 4.3, cell lines of MTHFR 677 TT genotype were found to have higher basal levels of apoptosis than cells of CC genotype in folate-free conditions (without Mtx). Addition of Mtx at concentrations of 10-1000nM caused an increase in apoptosis compared to controls in all cell lines, but there was no difference in the level of apoptosis by genotype when Mtx was present. This indicates that the small difference in the basal level of apoptosis due to MTHFR 677 genotype was overshadowed by the greater apoptotic influence of Mtx. This is consistent with results from chapter 3, where differences observed in cell proliferation by genotype were less significant at the higher concentrations of Mtx tested. The observation that cells of TT genotype have an increased basal level of apoptosis compared to CC cells suggests that the small decrease in cell viability due to TT genotype observed in chapter 3 could be attributed to apoptotic cell death. In a mouse model of altered MTHFR expression, Celtikci et al. (2009) observed that both over and underexpression of MTHFR led to increased apoptosis of spleen cells in Mtx-treated animals compared to wild-type controls (Celtikci et al. 2009). The presence of the MTHFR 677C→T polymorphism is associated with lower enzyme activity, and based on the findings of Celtikci et al. (2009), cells of TT genotype would be expected to be more susceptible to Mtx-induced apoptosis than cells of CC genotype. The findings of the current study do not support this, because cells of CC and TT genotype were equally susceptible to Mtx-induced apoptosis, despite a difference in the basal level of apoptosis by genotype. This difference in findings is likely to be due to differences in observation of apoptosis in a cellular model as opposed to a whole-animal model,

and also may be related to different effects of over and under-expression of the MTHFR gene compared to the influence of a polymorphic form of the enzyme.

The results presented here indicate that Mtx-induced apoptosis in transformed B-lymphocyte cell lines closely parallels effects of the drug on cell proliferation. The doubling time for these cell lines is approximately 48h, and this reflects the time at which apoptosis due to Mtx treatment becomes statistically significant (figure 4.2). The addition of reduced folates or a combination of Thy plus Hx to the culture medium, interventions which were shown in chapter 3 to restore proliferation to control levels in the presence of Mtx, were also able to completely block Mtx-induced apoptosis. In addition, Mtx treatment caused a blockade of the cell cycle at the G1/S transition (figure 4.4). These features suggest that the events which lead to apoptotic cell death may be linked to aborted attempts at cell division or DNA replication. This is in keeping with studies that show that Mtx-induced apoptosis is dependent on p53, a key factor in the cellular response to DNA damage (Huang et al. 2010). It is also consistent with studies which have shown that exposure to Mtx induces apoptosis only in cells which are actively proliferating, and not to resting cells (Genestier et al. 1998; Nielsen et al. 2007). In the results presented in section 4.5, addition of Hx to the culture medium had no effect on Mtx-induced apoptosis, in contrast to Thy which partially prevented Mtx-induced apoptosis. This suggests that inhibition of dTMP synthesis by Mtx is more critical for induction of apoptosis than inhibition of purine synthesis. Hx is a substrate for the purine salvage pathway, which results in the production of IMP. It is possible that increased availability of purines due to Hx could stimulate DNA synthesis, but in the absence of dTMP this might lead to greater misincorporation of uracil into DNA. In order to investigate this possibility, a modification of the TUNEL assay to allow quantitation of uracil in DNA was tested. The results of the standard TUNEL assay confirmed that exposure to Mtx led to DNA strand breaks in this cell culture system, but the modification of the assay did not provide consistent results and so it was not possible to determine if Mtx treatment led to increased misincorporation of uracil into DNA.

The experiments carried out in this chapter using the general caspase inhibitor Z-VAD-fmk clearly demonstrate that Mtx-induced apoptosis in these cell lines is partially caspase dependent. When specific inhibitors for caspase-8 and caspase-9 were used, there were less significant inhibitions of Mtx-induced apoptosis observed than for the pan-caspase inhibitor. Inhibition of caspase-8 was slightly more effective at preventing Mtx-induced apoptosis than inhibition of caspase-9, but neither inhibitor had a dramatic effect on Mtx-induced apoptosis. This suggests that both caspase-8 and caspase-9 may have a partial role in Mtx-induced apoptosis in these cell lines, and does not exclude the possibility that other caspases are involved in the initiation of such

apoptosis. Others have reported that Mtx-induced apoptosis is largely due to generation of ROS, although this has been seen to vary depending on cell type (Herman et al. 2005). The use of the anti-oxidant compound NAC in section 4.6.2 produced a small decrease in Mtx-induced apoptosis, but could not fully block the process. This does not support the idea that oxidative stress is the major trigger of Mtx-induced apoptosis in transformed B-lymphocyte cell lines, however it may play a partial role in the progression of the apoptotic response. Two inhibitors of serine proteases were employed to investigate the role of serine proteases in Mtx-induced apoptosis. TLCK is an inhibitor of trypsin-like serine proteases, and was found to cause substantial amounts of apoptosis by itself, and did not influence Mtx-induced apoptosis. In contrast, the chymotrypsin-like serine protease inhibitor TPCK did not have an inherent pro-apoptotic effect, and was observed to partially inhibit Mtx-induced apoptosis. This indicates that chymotrypsin-like serine protease activity features in the mechanism of apoptosis in transformed B-lymphocyte cell lines.

The fact that apoptosis in response to Mtx in these cell lines does not occur simultaneously in all cells in the population under study causes some difficulties in experimental quantification of apoptosis. The Annexin V assay tends to detect a higher proportion of apoptotic cells than the cell cycle analysis method, even for the same sample. The fact that this effect was consistent during many different experiments using these methods shows that this difference is not random. A possible explanation for the difference could be that the Annexin V assay measures cells that are in an early stage of apoptosis (Martin et al. 1995), and may not yet have developed significant DNA fragmentation. This could explain why the Annexin V assay detects a higher proportion of apoptotic cells than the cell cycle analysis method.

Conflicting results for the two apoptosis assays were also noted in experiments investigating the effect of the chymotrypsin-like protease inhibitor TPCK on Mtx-induced apoptosis. When the cell cycle analysis method was used, TPCK appeared to protect from Mtx-induced apoptosis. However, when apoptosis was measured using the Annexin V assay on the same samples, there was no protective effect of TPCK. On further investigation of the literature concerning the effect of TPCK in apoptosis, the following study was found where TPCK was shown to inhibit DNA fragmentation (Fabian et al. 2009). In order to test if this was true in transformed B-lymphocyte cell lines, cultures were set up with untreated cells, cells treated with Mtx (100nM) or Mtx + TPCK (100μM), and also cells treated with staurosporine as a positive control for DNA fragmentation. It was found that TPCK inhibits DNA fragmentation in transformed B-lymphocytes. This provides an explanation for the difference in results depending on which apoptosis assay was used. As the cell cycle analysis method is essentially a measurement of DNA fragmentation, any factor which inhibits this process is likely to result in a lower amount of apoptosis being detected by this

method. This is an excellent example of why it is important to use more than one experimental method to measure apoptosis, but it also points to a role for chymotrypsin-like serine proteases in regulation of DNA fragmentation in Mtx-induced apoptosis. This is especially interesting because the mechanism of action of Mtx involves inhibition of dTMP and purine synthesis, and this leads to inhibition of DNA synthesis. It is not known how these effects of Mtx lead to initiation of apoptosis as opposed to simply a block in cell proliferation. The action of TPCK may prevent some type of initial DNA damage caused by Mtx, or it may simply block the downstream DNA fragmentation which is an inherent process during apoptosis. There are numerous reports of TPCK inhibiting DNA fragmentation due to various apoptotic stimuli. Murn et al. (2004) found that TPCK prevented internucleosomal DNA fragmentation in WEHI 231 cells triggered to undergo apoptosis by exposure to IgM (Murn et al. 2004). TPCK was also observed to prevent DNA fragmentation induced by etoposide or cycloheximide in THP-1 cells (Zhu et al. 1997). It is therefore likely that the inhibition of DNA fragmentation by TPCK observed in the current study is downstream of any effect on DNA caused by Mtx. This is the first description of the serine protease inhibitor TPCK blocking DNA fragmentation in Mtx-induced apoptosis. Along with the other evidence described above, the results of the current study point to a generalized role of serine protease activity in apoptotic DNA fragmentation. The precise nature of the serine protease enzyme involved in DNA fragmentation is not yet known. Experiments with isolated nuclei from rat thymocytes revealed the presence of a caspase-independent mechanism for DNA degradation localised to the nucleus, which was inhibited by TPCK (Ajiro et al. 2008). O'Connell et al. (2006) described a 16kDa chymotrypsin-like protease which was activated independently of caspases during cell death (O'Connell et al. 2006). The same research group identified 50kDa and 60kDa proteins which had chymotrypsin-like serine protease activity and were activated downstream of caspase-3 during staurosporine-induced apoptosis (O'Connell et al. 2006). However, further characterisation of these proteases has not yet been carried out, and therefore the identification of the serine proteases involved in apoptotic DNA fragmentation and elucidation of their niche in the apoptotic machinery remains a goal for the future.

In conclusion, the experiments in this chapter provide evidence that Mtx causes apoptosis in transformed B-lymphocyte cell lines. Similarly to the effects of the drug on cell proliferation, the ability of Mtx to induce apoptosis is related to its antifolate properties, and subsequent inhibition of purine and pyrimidine synthesis. MTHFR 677C→T genotype does not influence sensitivity to Mtx-induced apoptosis in these cell lines, although there was a difference in basal apoptosis levels with TT cells having a slightly higher rate of apoptosis. The mechanism of Mtx-induced apoptosis in these cell lines involves caspase activation, and also the activity of chymotrypsin-like serine

proteases. Inhibition of chymotrypsin-like serine protease activity inhibits DNA fragmentation in Mtx-induced apoptosis, to a lesser extent than phosphatidylserine exposure. The mechanisms by which Mtx leads to signalling for apoptosis are not fully understood. In the following chapter, the hypothesis that Mtx-induced apoptosis is triggered by intracellular events involving transmethylation reactions is explored.

5 Chapter 5: Investigation of the role of ICMT in methotrexate-induced cytotoxicity

5.1 Introduction

In addition to its inhibitory effects on purine and pyrimidine synthesis, methotrexate (Mtx) treatment is also associated with changes in the methylation capacity of the cell. Early work investigating the effect of Mtx on MCF-7 cells demonstrated that treatment with the drug led to a decrease in the intracellular concentration of 5-methyl-THF, demonstrating decreased flux through MTHFR (Allegra et al. 1986). There is only one reaction within the cell which requires 5-methyl THF as a substrate, the remethylation of homocysteine (Hcy) to methionine via methionine synthase (MS). Decreased supply of 5-methyl-THF therefore reduces the rate of the MS reaction, leading to a build up of Hcy. The preferred direction of the SAH hydrolase reaction is towards Hcy breakdown when Hcy is present at high concentration (Yi et al. 2000), therefore an accumulation of Hcy promotes build up of SAH, which is a potent inhibitor of methyltransferase reactions (Ueland 1982).

The mechanism described above whereby Mtx reduces the flux through MTHFR, thereby reducing the availability of 5-methyl THF for the methylation cycle explains observations of cellular hypomethylation in response to Mtx in vitro and in vivo. Kishi et al. (2000) measured the SAM/SAH ratio in the CSF of patients undergoing treatment with Mtx and in healthy controls. The SAM/SAH ratio is regarded as a measurement of the ability of the cell to carry out transmethylation reactions, because SAM is the universal methyl donor for such reactions, and SAH is a product and feedback inhibitor of the same. The SAM/SAH ratio was found to be lower in the Mtx-treated group compared to the untreated individuals, providing evidence that Mtx treatment alters cellular methylation capacity in vivo (Kishi et al. 2000). Another study of ALL patients treated with Mtx demonstrated that CSF levels of 5-methyl THF and SAM were below the normal range following Mtx treatment, providing further evidence that Mtx treatment affects the methylation cycle (Vezmar et al. 2009). Nesher et al. (1991) noted that incubation with Mtx inhibited chemotaxis and production of superoxide by human monocytes. Low-methionine conditions exacerbated these effects of Mtx, while addition of SAM abolished the Mtx-mediated effects, suggesting that the influence of Mtx on these cells was occurring through methylation reactions (Nesher et al. 1991). The ability of Mtx to alter the concentration of cofactors required for methylation leads to the possibility that some effects of Mtx may be mediated by altered activity of intracellular methylation reactions. There are some intriguing hints that this may be

the case, for example Mtx was found to reduce the level of methylated protein phosphatase 2A in cultured rat neurons (Yoon et al. 2007).

One type of protein methylation which is essential for function of a number of proteins involved in signal transduction is catalysed by the enzyme isoprenylcysteine-O-carboxymethyltransferase (ICMT). This enzyme catalyses the methylation of proteins during post-translational addition of isoprenyl groups. Certain proteins are targeted for isoprenylation by a C-terminal CAAX motif, where C indicates a cysteine residue, A indicates an aliphatic amino acid and X stands for any other amino acid. The methylation step catalysed by ICMT is part of a 3-step post translational modification. The first step is the addition of either a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid group to the cysteine residue of the CAAX motif. The second step in the process is proteolytic removal of the 'AAX' tripeptide by the enzyme RCE1 (Otto et al. 1999). Finally, the isoprenylated cysteine residue is methylated on the carboxyl group by ICMT (Clarke 1992). ICMT is a 33kDa protein which is located in the endoplasmic reticulum in all cell types (Dai et al. 1998). Isoprenylation is associated with targeting of proteins to the cell membrane, and the final methylation step is necessary for correct location of the protein. This was demonstrated by Bergo et al. (2000), who observed that in cells without ICMT activity, the normally membrane-associated K-Ras protein was mislocalised to the cytosol (Bergo et al. 2000). Many proteins which are involved in signal transduction cascades undergo post-translational processing in this way, including the Gγ subunits of heterotrimeric G proteins and the small GTPase proteins such as Ras and Rho (Solski et al. 2002). Knockout of the ICMT gene in mice is embryonic lethal (Bergo et al. 2001), demonstrating the critical importance of ICMT and the apparent lack of a redundant pathway for carboxymethylation of isoprenylated proteins in mammals.

Winter-Vann et al. (2003) suggested that disruption of carboxymethylation of the signalling protein Ras is a potential mechanism of action of Mtx. Mtx treatment of mouse embryonic fibroblasts (MEFs) led to increased intracellular SAH, and this correlated with decreased methylation of Ras, mislocalisation of Ras to the cytosol, and decreased signalling through the MAPK/Akt pathway which is downstream of Ras (Winter-Vann et al. 2003). MEFs expressing wild-type ICMT exhibited reduced proliferation on incubation with Mtx. In contrast, cells lacking ICMT expression were resistant to growth inhibition caused by Mtx. The authors concluded that Mtx led to inhibition of ICMT activity by increasing intracellular levels of SAH, which then inhibited Ras localisation and signalling (Winter-Vann et al. 2003). The fact that cells without ICMT expression were resistant to Mtx led to the suggestion that inhibition of ICMT and downstream signalling pathways was a major route of action of the drug in their experimental system. It is not known if this may be a feature of Mtx action in other systems.

N-acetyl-S-geranylgeranyl-L-cysteine (AGGC) is a competitive inhibitor of ICMT, owing to its structural similarity to the isoprenoid groups which are the physiological substrates of ICMT. According to the work of Winter-Vann et al. (2003), cells without active ICMT are resistant to Mtx (Winter-Vann et al. 2003). Following on from this, inhibition of ICMT by addition of the competitive inhibitor AGGC may oppose the effects of Mtx in transformed B-lymphocyte cell lines. The study by Winter-Vann et al. (2003) did not investigate if Mtx caused apoptosis in the experimental system used. Ras is known to regulate a complex network of intracellular signal transduction pathways which collectively control cell proliferation, cell cycle progression and apoptosis (Cox and Der 2003). It is therefore possible that inhibition of ICMT by addition of AGGC might inhibit Mtx-induced apoptosis in transformed B-lymphocyte cell lines. In addition, any effect of AGGC could be further influenced by folate status because low folate availability will further impact on cellular transmethylation reactions.

5.1.1 Aims

- To investigate if inhibition of ICMT by AGGC can inhibit growth inhibition by Mtx in transformed B-lymphocytes.
- To investigate if inhibition of ICMT using AGGC can protect transformed B-lymphocytes from Mtx-induced apoptosis.
- To determine if folate availability has an influence on any effect of AGGC.

5.2 Investigation of the ability of AGGC to interfere with the cytotoxic effect of methotrexate in transformed B-lymphocyte cell lines

The aim of this initial experiment was to investigate if AGGC can protect against the cytotoxic effects of Mtx in cultured B-lymphocyte cell lines. The MTS assay was used for this purpose because it can sensitively detect changes in cell proliferation over a 48h timeframe. Dilutions of AGGC stock solution in standard RPMI were prepared, at twice the final assay concentration required. 50 µl of each of these AGGC solutions was pipetted into the wells of a 96-well plate. Triplicate wells were prepared for each condition. Cells of cell line 17241 were counted as described in section 2.2.1.2, and a cell suspension was prepared at 6×10^5 cells/ml in standard RPMI. A 45µl aliquot of this cell suspension was added to each prepared well in the 96-well plate. This resulted in a final cell density of 0.27×10^5 cells/well, and a range of AGGC concentrations from 0-1000µM. The plate was incubated for 1 hour at 37°C, 5% CO_2. After this pre-incubation with AGGC, 5µl of a solution of 2µM Mtx in Standard RPMI was added to the appropriate wells, giving a final Mtx concentration of 100nM. The plate was incubated for 48h at 37°C, 5% CO_2.

After this time, 20µl MTS assay reagent was added to each well and the plate returned to the incubator for 4h. Absorbance at 490nm was then determined using a plate reader.

The results of this experiment are shown in figure 5.1. As observed in previous experiments, 100nM Mtx caused a significant decrease in cell proliferation compared to untreated control ($P < 0.001$, Student's t-test). When compared to the Mtx-only condition, the level of cell proliferation in the presence of 100µM AGGC + Mtx was higher by a small but significant amount ($P = 0.0004$, Student's t-test). Lower concentrations of AGGC did not have this protective effect, and the highest concentrations of AGGC tested were more cytotoxic in combination with Mtx than for Mtx alone ($P < 0.001$ for Mtx + 500µM AGGC and Mtx + 1000µM AGGC compared to Mtx alone, Student's t-test). Although the effect of AGGC observed in this experiment is small, it is notable because previous work which has investigated the effect of ICMT activity on response to Mtx has used cell lines completely lacking ICMT expression. The results of this initial experiment were surprising in that they showed that AGGC does have a slight but significant inhibitory effect on cytotoxicity due to Mtx, in a cell line with no modifications in ICMT expression. The effect is only evident only at a narrow concentration range. Below this threshold AGGC has no effect, and above it the combination of AGGC and Mtx is more cytotoxic than Mtx alone.

In order to confirm the interaction of AGGC with Mtx-induced cytotoxicity, the above experiment was repeated three times with cell line 17241 with a refined range of AGGC concentrations. The procedure used to carry out the experiment was exactly as described above, except that the final concentrations of AGGC tested were between 0 and 200µM. In addition, control conditions with AGGC but without Mtx were included to test if AGGC had an intrinsic cytotoxic effect. The results of this experiment are presented in figure 5.2 (A). In this case, 150µM AGGC was the only concentration which had a statistically significant partial effect on Mtx-induced cytotoxicity ($P = 0.006$, paired t-test), although in the individual replicates of the experiment both 100µM and 150µM AGGC were effective ($P < 0.01$, Student's t-test). The effect of AGGC at 200µM is quite different. There is no partial protective effect of AGGC against Mtx-induced cytotoxicity at this concentration, and it appears that the combination of Mtx + 200µM AGGC is more cytotoxic than Mtx alone, although this difference is non-significant when the 3 replicates are combined. The control conditions without Mtx show that AGGC up to 100µM does not reduce proliferation significantly compared to the untreated control, but AGGC at the higher concentration of 200µM reduces proliferation to a similar degree as Mtx alone. Cells incubated in the presence of 200µM AGGC alone have proliferation values of only 19.5% of control cells, and cells with Mtx alone have 20.8% proliferation values compared to control cells.

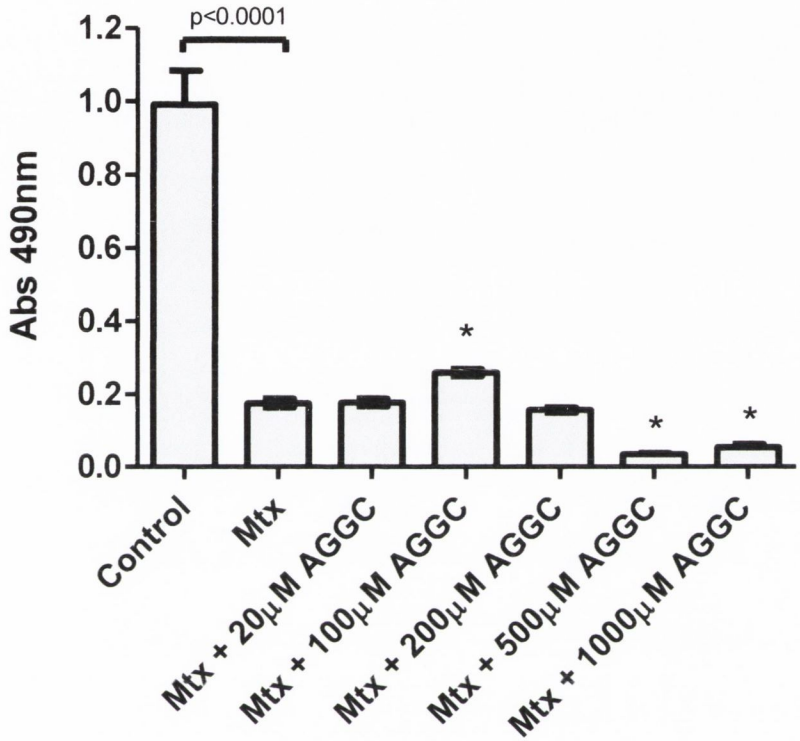

Figure 5.1 Initial experiment to assess the ability of a range of AGGC concentrations to interfere with the cytotoxic effect of Mtx

Cells (17241) were seeded at 2 x 10^5 cells/ml in standard RPMI with Mtx (100nM) and AGGC concentrations as shown. After 48h incubation, cell proliferation was measured by MTS assay. Data shown are the means +/- SD of a triplicate experiment. Bars marked with * differ significantly from the Mtx-only condition, Student's t-test $P < 0.001$. P-value for the effect of methotrexate by Student's t-test is also shown.

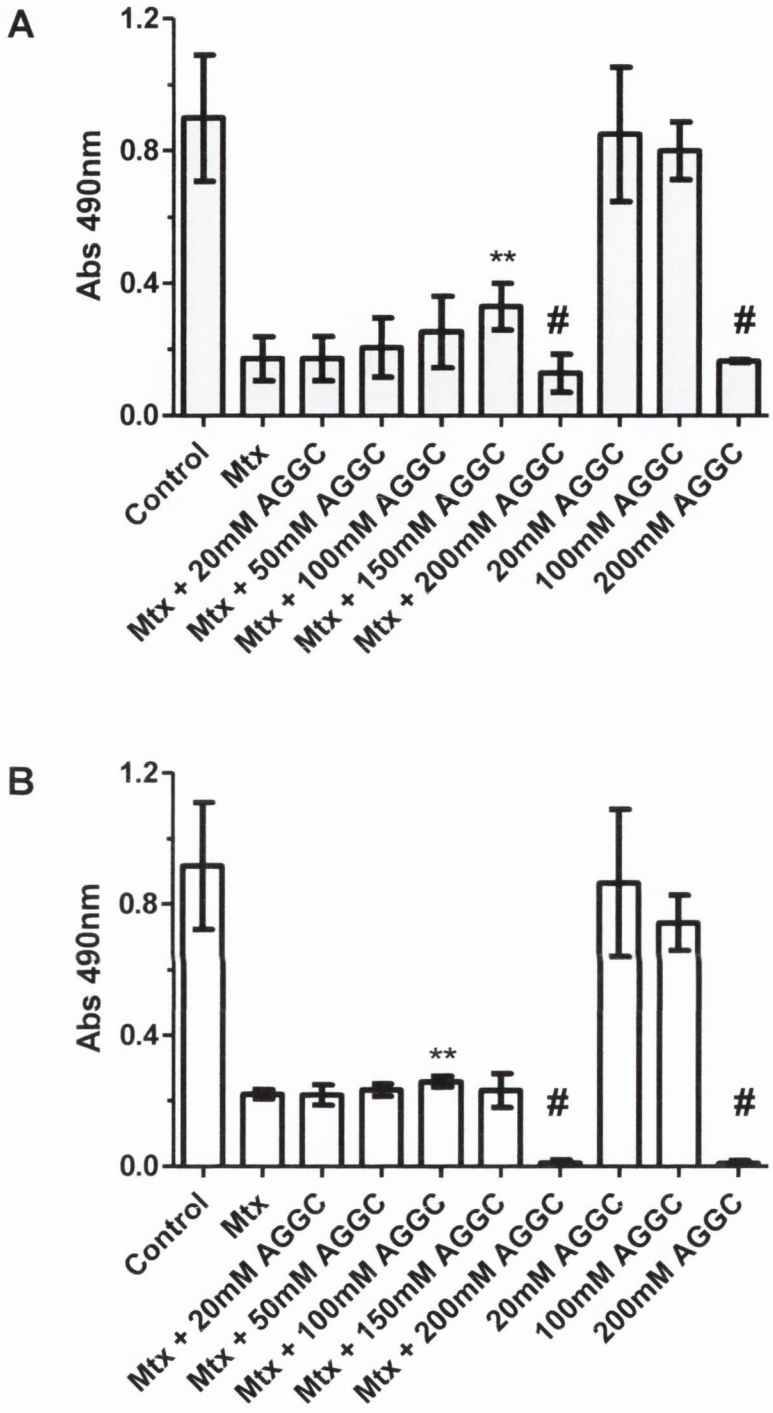

Figure 5.2 Investigation of the interaction between AGGC and Mtx in folate-free and folate sufficient conditions in a cell line of MTHFR 677 CC genotype

Cells (17241) were seeded at 2 x 10^5 cells/ml in either standard RPMI (A) or folate free RPMI (B) with 100nM Mtx and AGGC concentrations as shown. After 48h incubation, cell proliferation was measured by MTS assay. Data are the means +/- SD of 3 independent experiments. Bars marked with ** differ significantly from the Mtx only condition, paired t-test $P < 0.05$. Bars marked with # are significantly different between graphs, Student's t-test $P < 0.01$.

The experiment was repeated in folate-free RPMI, in order to investigate if there was a difference in the observed influence of AGGC depending on folate availability. The experiment was set up as detailed above, except that folate-free RPMI was used in place of standard RPMI, and cells were rinsed three times in folate-free RPMI before counting, to remove residual folate. Proliferation was determined by the MTS assay after 48h incubation at 37^{o}C, 5% CO_2. This experiment was repeated on three separate occasions with cell line 17241.

The results of this experiment are presented in figure 5.2 (B). It appears that the protective effect of AGGC is far less pronounced in FF RPMI compared to standard RPMI, although there are no significant differences between the FA-containing and folate-free conditions by Student's t-test. In the folate-free condition, there is still a small but statistically significant protective effect of 100μM AGGC compared to Mtx alone ($P = 0.009$, paired t-test). None of the other AGGC concentrations had a significant effect.

Although AGGC has a protective effect in both standard RPMI and folate-free conditions, there is evidence that supply of folate changes the scale of this protective effect. Firstly, the concentration of AGGC which produces a statistically significant protective effect in Standard RPMI (150μM) is higher than the protective concentration in folate-free RPMI (100μM). Secondly, the magnitude of the protective effect is smaller in folate-free conditions. In standard RPMI, the proliferation of cells in the presence of Mtx is 20.81% of the control value, and for Mtx + 150μM AGGC it is 38.73%. In folate-free RPMI the proliferation of cells in the presence of Mtx is 24.95% of the control value, and for Mtx + 100μM AGGC it is 29.36%. The cytotoxic effect which was noted with higher concentrations of AGGC is also significantly different between the two folate conditions. In folate-free RPMI, 200 μM AGGC leads to complete cell death as the proliferation value is only 1.18% of the control value when folate is not present in the growth medium. In standard RPMI, a small level of cell viability is measurable in the presence of 200μM AGGC, with a proliferation value 19.5% of the control value. The difference between 200μM AGGC conditions in the standard RPMI and folate-free conditions is significant by Student's t-test, $P < 0.01$. This indicates that cells are more sensitive to the toxic effects of AGGC when folate is limited.

The above experiment was repeated using a cell line of MTHFR 677 TT genotype (17274), in order to investigate if the observed effect of AGGC on Mtx-induced cytotoxicity might be affected by MTHFR C677T genotype. The experiment was set up exactly as described above, and was carried out three times in both standard RPMI and folate-free RPMI. Results for the TT cell line are presented in figure 5.3. In standard RPMI, two concentrations of AGGC were found to be result in higher levels of proliferation in the presence of Mtx compared to Mtx alone - 100μM AGGC

(P = 0.027, paired t-test) and 150μM (P = 0.042, paired t-test). As observed in the CC cell line, 200μM AGGC did not have a protective effect compared to Mtx alone, and was in fact cytotoxic. Under folate-free conditions, again 100μM AGGC reduced the level of cytotoxicity compared to Mtx alone (P = 0.038, paired t-test), but 150μM AGGC did not. Comparing the two folate conditions, the effects in the TT cell line were similar to those for the CC cell line. The magnitude of the AGGC protective effect was lesser in folate-free conditions than in standard RPMI. Under standard folate conditions, cells exposed to Mtx alone had 31.35% proliferation compared to the control value, and cells with Mtx + 150μM AGGC had 48.55% proliferation compared to the control. In folate-free medium, cells exposed to Mtx alone had 37.03% proliferation compared to the control value, and cells with Mtx + 150μM AGGC had 41.10% proliferation compared with the control. Also similar to results observed in the CC cell line, 200μM AGGC had a greater cytotoxic effect in folate-free RPMI than in standard RPMI. For the TT cell line, cells grown in standard RPMI with 200μM AGGC had 20.57% proliferation compared to control cells, and in folate-free RPMI with 200μM AGGC the proliferation value was 2.85% of control cells.

In order to compare the CC and TT cell line, absorbance values obtained from the experiments above were expressed as a percentage of the control value, and the mean of these values was calculated for each cell line. Results from this analysis of the data are presented together in figure 5.4. There is a trend towards TT cells having a higher level of cell proliferation when Mtx is present, while in control samples without Mtx, values for the two genotypes appear similar. However, when an unpaired Student's t-test was used to compare the result between the CC and TT cell line under each treatment condition, there were no significant differences found by genotype under any of the conditions tested.

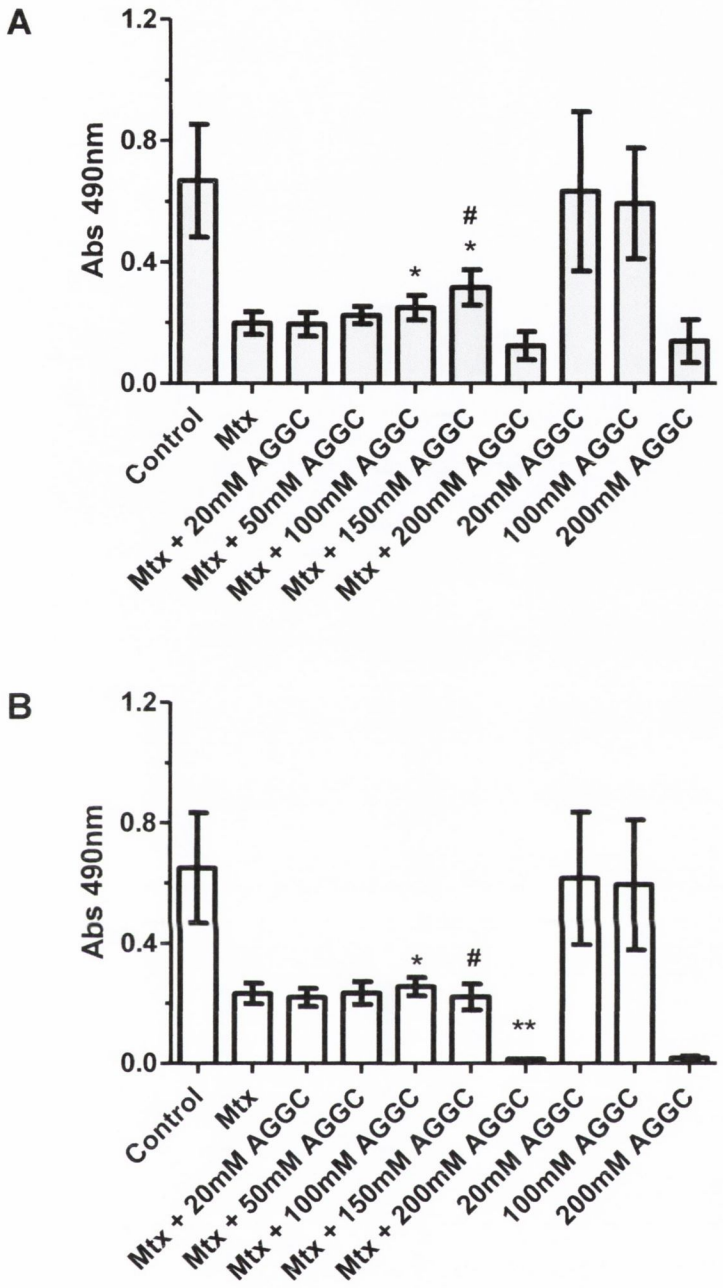

Figure 5.3 Investigation of the interaction between AGGC and Mtx in folate-free and folate sufficient conditions in a cell line of MTHFR 677 TT genotype

Cells (17274 TT) were seeded at 2 x 10^5 cells/ml in either standard RPMI (A) or folate free RPMI (B) with 100nM Mtx and AGGC concentrations as shown. After 48h incubation, cell proliferation was measured by MTS assay. Data are the means +/- SD of 3 independent experiments. Bars marked with * differ significantly from the Mtx only condition, paired t-test * = $P < 0.05$, ** = $P < 0.01$. Bars marked with # are significantly different between graphs, Student's t-test, $P < 0.05$.

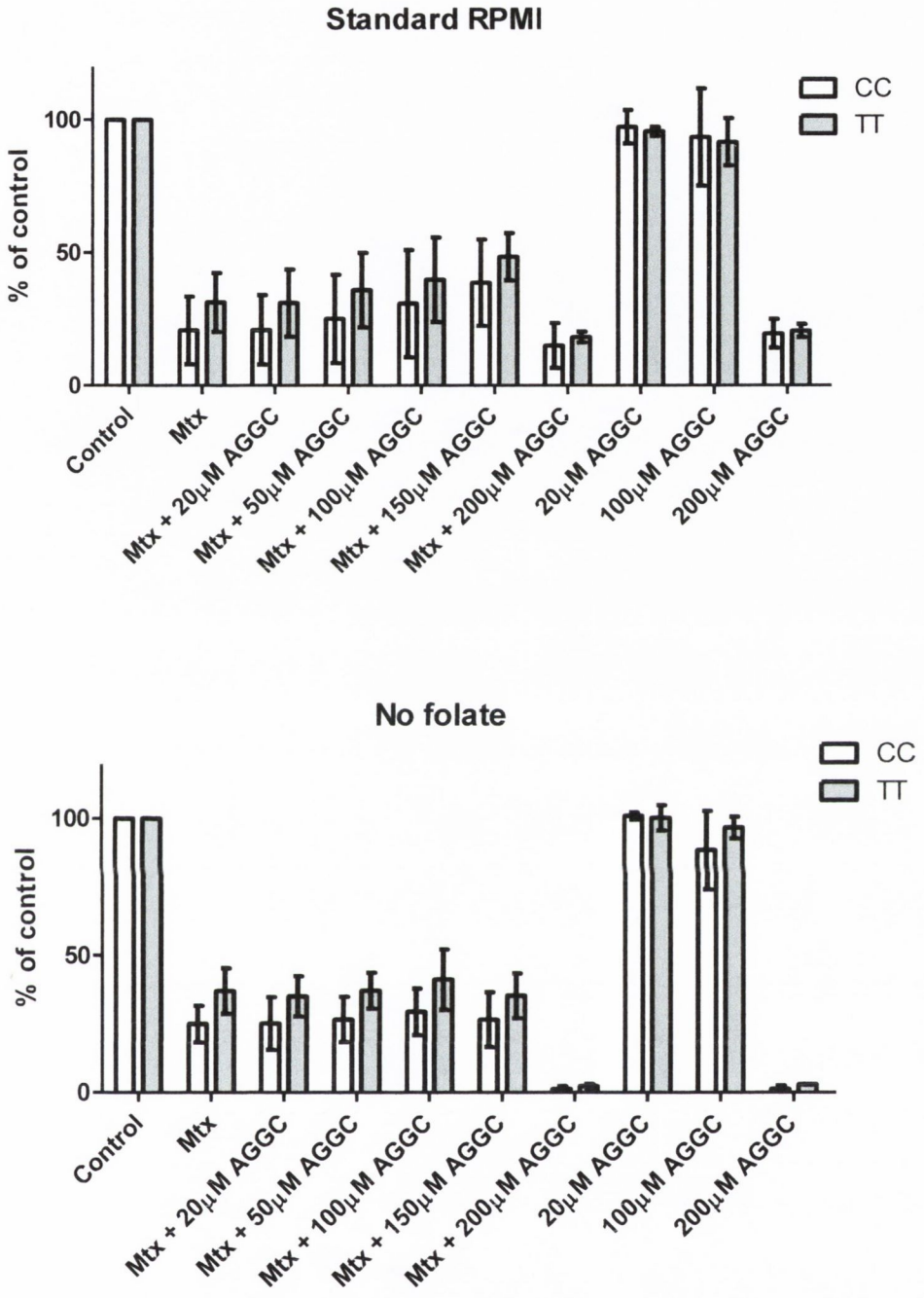

Figure 5.4 Comparison of the effect of AGGC on transformed B-lymphocyte cell lines of MTHFR 677 CC and TT genotype

This figure shows the data from figures 5.3 and 5.4, where proliferation has been presented as a percentage of the control in order to compare results between the CC and the TT cell line. Data shown are the means +/- SD of 3 independent experiments with each cell line. Student's t-test was used to compare between genotypes at each condition, there were no significant differences.

5.3 Investigation of the effect of AGGC on methotrexate-induced apoptosis in transformed B-lymphocyte cell lines

In section 5.2 it was observed that a defined concentration of AGGC could partially support cell proliferation in the presence of 100nM Mtx. It was also of interest to investigate if the protective effect of AGGC noted above could be detected at the level of apoptosis. As has been discussed previously, the Annexin V/PI staining and cell cycle analysis methods are both suitable methods to use for quantification of two different apoptosis parameters, DNA fragmentation and phosphatidylserine exposure. As noted in Chapter 4, while the two measurements are complementary and often used together, it is possible that other factors in the experiment could influence these parameters to a different extent. For this reason, apoptosis was quantified using both of these methods.

Cells which had previously been cultured in standard RPMI medium were harvested by centrifugation and counted as described in section 2.2.1.2. Cells were washed three times in folate-free RPMI to remove residual folate. Cells were then seeded at $2x10^5$ cells/ml in a 12-well tissue culture plate, at a total volume of 2.5ml per well. The wells contained either standard RPMI or folate-free RPMI, with or without 150μM AGGC. After pre-incubation at 37°C, 5% CO2 for 1 hour, Mtx was added to one well of each condition to a final concentration of 100nM Mtx. Control wells were also included with standard RPMI or folate-free RPMI without Mtx or AGGC. After incubation at 37°C, 5% CO2 for 48h, the cells of each condition were harvested by centrifugation. The samples were split in half, and processed for cell cycle analysis and Annexin V/PI staining as described in chapter 2. The percentage of apoptotic cells in each sample was measured by flow cytometry. This experiment was carried out 5 separate times with two cell lines, one of CC genotype (17241) and one of TT genotype (17274).

The results of this experiment are presented in figures 5.5 for the CC cell line and 5.6 for the TT cell line. One-way ANOVA with Tukey's post tests was used to compare the groups within each graph. From figure 5.5, it can be seen that treatment with Mtx causes substantial apoptosis compared with controls ($P < 0.001$), as observed previously in these cell lines. By both types of analysis, the level of apoptosis for the AGGC only condition are significantly higher than the control value ($P < 0.01$). This indicates that AGGC in itself causes a substantial amount of apoptosis. This is not surprising, as results from the MTS assays with AGGC indicated that the compound has a cytotoxic effect at sufficient concentration. In spite of this, for the CC cell line in standard RPMI the level of apoptosis observed in the AGGC + Mtx condition was significantly lower compared to the Mtx-only condition ($P < 0.05$). This correlates with the results of the MTS

cell proliferation assay in section 5.2, where 100-150μM AGGC resulted in a reduction of the cytotoxic effects of Mtx. However, AGGC did not reduce the the level of Mtx-induced apoptosis in folate-free conditions. This suggests that the protective effect of AGGC may be weakened under conditions where folate supply is limited.

Figure 5.6 shows the results for this experiment in the cell line of MTHFR 677 TT genotype. The purpose of carrying out the experiment in two cell lines was to determine if the same effect of AGGC would also be observed in a TT cell line. In this case, in both standard and folate-free RPMI, the level of apoptosis in the presence of Mtx, AGGC or a combination of both Mtx + AGGC was significantly higher than control levels ($P < 0.001$ for all conditions compared to control, one-way ANOVA with Tukey's post-tests). In contrast to the observation in the CC cell line, there was no protective effect of AGGC against Mtx-induced apoptosis in either standard or folate-free RPMI. In fact by cell cycle analysis in folate-free conditions, AGGC + Mtx appeared to result in a higher level of apoptosis than Mtx alone, although this was non-significant (Figure 5.6 A, lower panel).

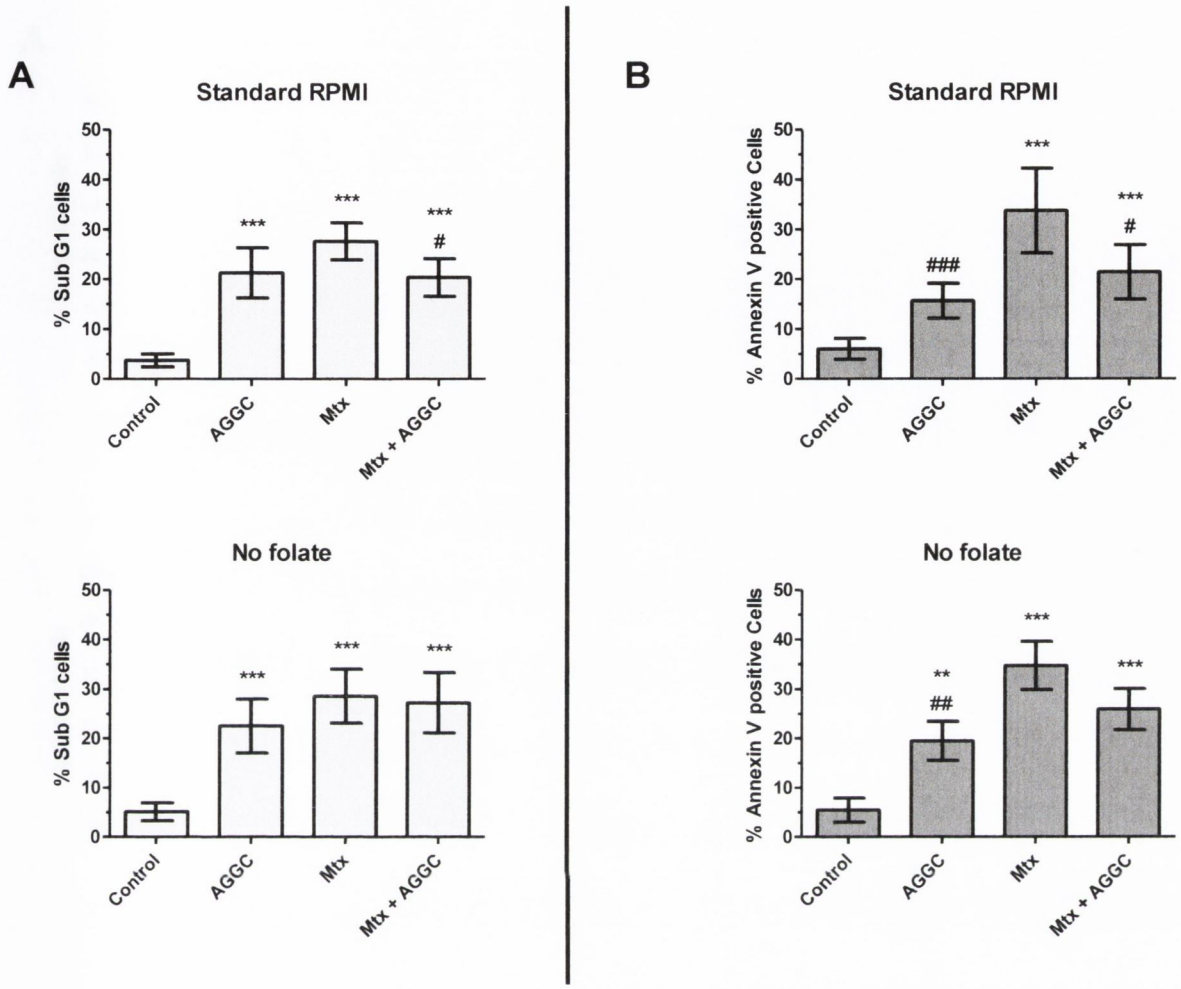

Figure 5.5 Investigation of the effect of AGGC on Mtx-induced apoptosis in a transformed B-lymphocyte cell line of MTHFR 677 CC genotype

Cells (17241 CC) were incubated with 100nM Mtx or 150μM AGGC as indicated, in standard RPMI medium or folate-free RPMI. After 48h, cells were collected and each sample was analysed for apoptosis by cell cycle analysis (A) and annexin V staining (B). Data are the means +/- SD of 5 independent experiments. One-way ANOVA with Tukey's post-tests was used to compare the groups within each graph. * = differs from control value, # = differs from Mtx-treated value. One symbol = $P < 0.05$, two symbols = $P < 0.01$, three symbols = $P < 0.001$.

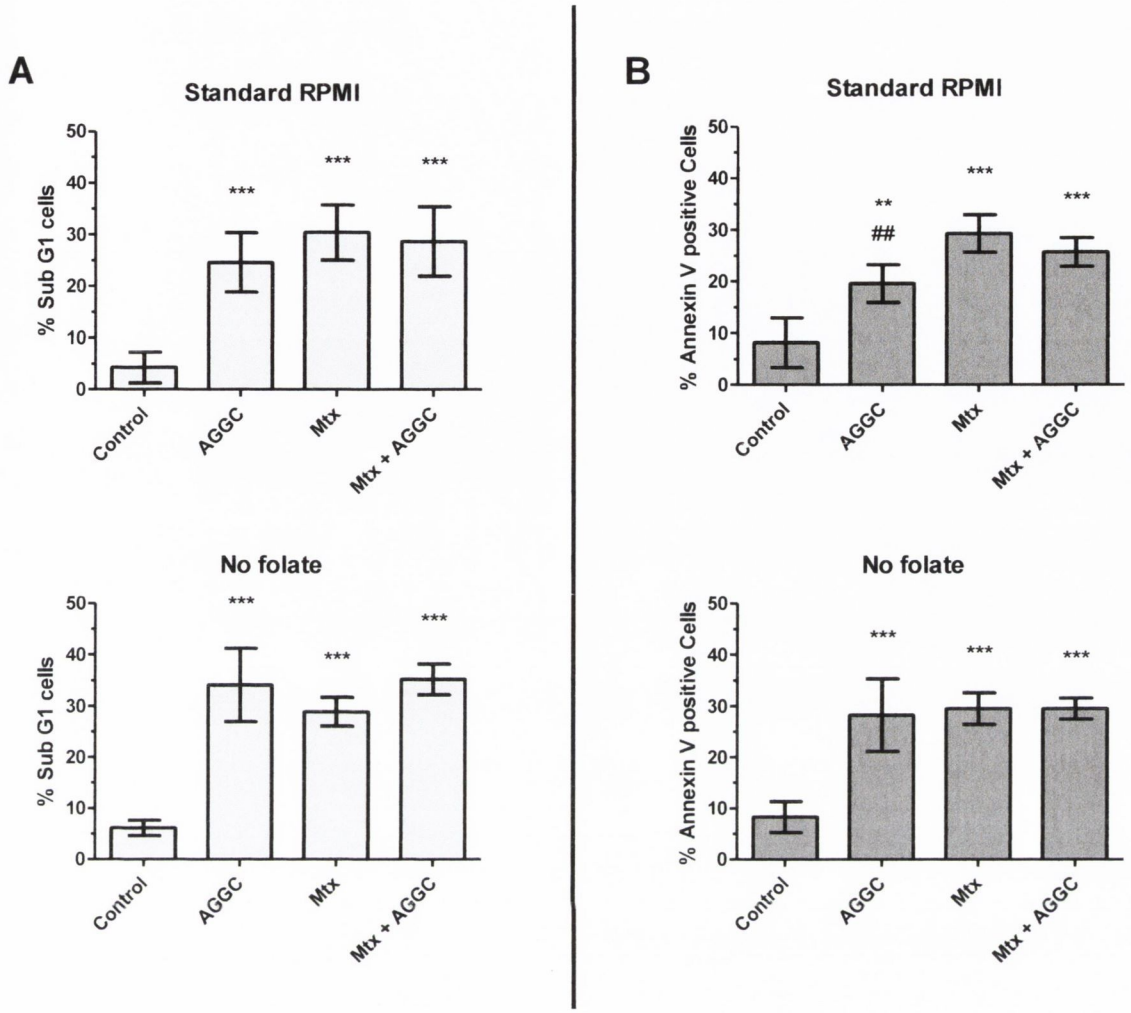

Figure 5.6 Investigation of the effect of AGGC on Mtx-induced apoptosis in a transformed B-lymphocyte cell line of MTHFR 677 TT genotype

Cells of cell line 17274 TT were incubated with 100nM Mtx or 150μM AGGC as indicated, in standard RPMI medium or folate-free RPMI. After 48h, cells were collected and each sample was split in two and analysed for apoptosis by cell cycle analysis (A) and annexin V staining (B). Data are the means of 5 separate experiments. Error bars show SD. One-way ANOVA with Tukey's post-tests was used to compare the groups within each graph. * = differs from control value, # = differs from Mtx-treated value. One symbol = $P < 0.05$, two symbols = $P < 0.01$, three symbols = $P < 0.001$.

5.4 Discussion

The results in this chapter demonstrate that inhibition of ICMT by AGGC can obstruct Mtx induced cytotoxicity in transformed B-lymphocyte cell lines, measured by the MTS cell proliferation assay and by two standard assays for apoptosis. In addition, this interference is folate dependent. This finding is novel because previous experiments demonstrating the involvement of ICMT inhibition on the anti-proliferative effect of Mtx have been carried out in mouse cells with altered expression of ICMT. The results presented here show that inhibition of ICMT confers resistance to Mtx in human cells with unaltered ICMT and Ras signalling pathways. The influence of ICMT inhibition on Mtx-induced apoptosis has not previously been described. Winter-Vann et al. (2003) showed that MEF cells lacking ICMT were resistant to the growth–inhibitory effects of Mtx (Winter-Vann et al. 2003), but did not investigate Mtx-induced apoptosis. The folate dependence of the effect of AGGC on Mtx-induced cytotoxicity and apoptosis has not previously been observed.

The results presented in section 5.2 show that at a defined concentration of AGGC, there is a statistically significant effect of AGGC which reduces the growth inhibitory effect of Mtx. Compared to treatment with 100nM Mtx, cells incubated in the presence of Mtx plus 150μM AGGC had a higher level of cell proliferation ($P = 0.006$, paired t-test). The effect is small, but nevertheless this demonstrates that AGGC is capable interfering with the very powerful effect of Mtx. Results obtained from the MTS assay are an expression of the number of metabolically active cells present after 48h exposure to Mtx and AGGC. This type of assay cannot directly show the mechanism of how AGGC affects the cell, but the results clearly indicate an interaction between AGGC and Mtx, and it is noteworthy that this can be detected at the level of cell proliferation.

The results in section 5.3 show that AGGC partially inhibits Mtx-induced apoptosis in a transformed B-lymphocyte cell line of CC genotype. The percentage of apoptotic cells in samples treated with Mtx plus AGGC was lower than in cells treated with Mtx alone ($P < 0.05$, one-way ANOVA with Tukey's post-tests, n=5). This observation confirms the small but significant effect of AGGC observed in the MTS assays. This is also interesting because inhibition of Mtx-induced apoptosis by AGGC has not been previously demonstrated. Although AGGC reduces Mtx-induced apoptosis, the results presented here show that AGGC also causes apoptosis in transformed B-lymphocyte cell lines itself. This is consistent with the results of Kramer et al, who showed that AGGC causes apoptosis in bovine pulmonary artery endothelial cells (Kramer et al. 2003). Taken together, these results suggest that concentrations of AGGC in the region of 100 -150μM can

partially block apoptosis due to Mtx, but that this effect is slight and is influenced by the fact that AGGC can cause apoptosis by itself.

The apoptotic effect of AGGC, and its influence on apoptosis induced by mechanisms other than Mtx have previously been investigated by others. Kramer et al. found that AGGC induced apoptosis in bovine pulmonary artery endothelial cells, and also decreased Ras methylation and downstream AKT/ERK signalling (Kramer et al. 2003). A reduction in Ras methylation also occurred during apoptosis in response to exogenous adenosine/homocysteine, a treatment which increases intracellular SAH and would also have the effect of inhibiting methyltransferase reactions (Kramer et al. 2003). Wang et al. observed that inhibition of ICMT by another inhibitor, cysmethynil, caused cell death with features of autophagy and apoptosis in HepG2 cells (Wang et al. 2010). The results presented in this chapter also show that AGGC causes apoptosis and this is in agreement with these studies. However, the observation that AGGC can reduce Mtx-induced apoptosis is surprising.

Winter-Vann et al. showed that mouse embryonic fibroblasts (MEFs) lacking ICMT were resistant to growth inhibition caused by Mtx (Winter-Vann et al. 2003). Transfection of MEFs with a form of Ras that does not require carboxyl-methylation for its membrane localisation made cells with normal ICMT resistant to Mtx. They reasoned that Mtx causes an increase in intracellular SAH, leading to inhibition of ICMT, which reduces the methylation and correct membrane localisation of Ras. Their paper also showed that Mtx blocked phosphorylation of MAPK and AKT, and cells without ICMT were not sensitive to this effect of Mtx. They suggested that this interference with Ras signalling was a major part of the mechanism of growth inhibition associated with Mtx, but they did not investigate Mtx-induced apoptosis. The results presented here do not support the idea that interference with ICMT activity is a major part of the mechanism of Mtx in this cell culture system, because AGGC only partially prevented the effects of Mtx, and it is clear from the preceding chapters that the effect of Mtx is also related to inhibition of dTMP and purine synthesis. Winter-Vann et al. concluded that inhibition of DNA synthesis was not an important factor in their cell model, based on the observation that addition of 1μM thymidine only partially prevented the antiproliferative effect of Mtx. However, they did not investigate the effects of thymidine plus hypoxanthine, which have been shown here and elsewhere (Refsum et al. 1991; Budzik et al. 2000) to completely overcome growth inhibition caused by Mtx.

An interesting aspect of the results presented in this chapter is that the effects of AGGC are influenced by the supply of FA in the cell culture medium. In the MTS cell proliferation assays, the magnitude of the protective effect of AGGC was greater when 2μM FA was present in the culture

medium, than in folate-free conditions. In the apoptosis assays, the inhibitory effect of AGGC on Mtx-induced apoptosis was observed in a cell line of MTHFR 677 CC genotype under standard folate conditions, but not under folate-free conditions. The protective effect of AGGC in terms of apoptosis was not observed in a cell line of MTHFR 677 TT genotype in either folate condition. Since the effect of AGGC on the response to Mtx is less pronounced in low folate conditions, this suggests that folate status is an important determinant of Mtx cytotoxicity. Due to the fact that there was no influence of AGGC on Mtx-induced apoptosis in a TT cell line, this raises the possibility that MTHFR 677C→T genotype may also have an influence on the effect of AGGC. The MTHFR 677C→T polymorphism would be expected to reduce the availability of 5-methyl-THF and thereby reduce the flux through the methylation cycle. Such a reduction in cellular methylation capacity would be expected to further augment inhibition of ICMT, however the opposite effect was observed in the current study. The possibility that there is an influence of MTHFR 677C→T genotype on ICMT activity requires further investigation.

Due to the fact that AGGC has cytotoxic effects in itself, it is clear that throughout these experiments the overall level of apoptosis observed is due to the balance between the cytotoxic effects of AGGC and Mtx, and the inhibitory effect of AGGC on the cellular response to Mtx. The effect of AGGC is observed to be less effective in folate-free conditions. This could be because folate depletion makes cells more susceptible to Mtx, or it could also be that the inherent cytotoxic effect of AGGC is more potent when folate is insufficient. AGGC acts by inhibiting a methyltransferase enzyme. Folate depletion is also likely to inhibit transmethylation reactions because the supply of methyl groups will be restricted. Therefore the observed influence of AGGC might be less pronounced when folate is limited, because methylation is already inhibited in the cell.

The work of Winter-Vann et al. (2003) focused on the effect of ICMT inhibition on methylation of one protein, Ras. However, ICMT has many cellular substrates in addition to Ras, for example the small GTPase proteins Rac and Rho. Inhibition of the activity of ICMT either directly via a specific inhibitor, or indirectly via reduced supply of the methyl donor SAM could also inhibit cell signalling and other processes which are mediated through any number of ICMT target proteins. An example of another cellular effect of ICMT inhibition is reduced cell migration and adhesion of a metastatic breast cancer cell line, mediated by decreased methylation of the Ras-related proteins Rac1 and RhoA (Cushman and Casey 2009). Folate depletion has also been shown to reduce actin turnover and cell motility (Oleinik et al. 2010), and it is possible that reduced methylation of these signalling proteins could be involved in this effect.

In conclusion, the experiments in this chapter demonstrate that inhibition of ICMT by AGGC can interfere with the antiproliferative and apoptotic effects of Mtx in transformed B-lymphocyte cell lines. The availability of folate in the culture medium appears to further interact with this effect. However, the interpretation of these results is complicated by the fact that AGGC also has inherent cytotoxic properties. Further investigation is required in order to establish the signalling events which are affected by AGGC in this cell culture system.

6 Chapter 6: General Discussion

6.1 Discussion

The folate antagonist methotrexate (Mtx) has been in use in treatment of cancer for over 60 years. In that time, new uses for the drug have been discovered in treatment of autoimmune and inflammatory diseases. Despite attempts to define the mechanism of action and the design of several other folate analogue drugs, Mtx still remains a key component of clinical treatment regimens. Although the effect of Mtx on various folate-dependent enzymes is well understood, the precise downstream mechanisms by which this drug works are still the subject of much research. Even though Mtx is a widely used and successful drug, it is also associated with significant undesirable side effects and toxicity. This is of particular importance for individuals who undergo treatment with Mtx for chronic autoimmune or inflammatory conditions, and therefore may be exposed to the drug for many months or years.

For any drug, there will always be some individuals who respond well, and others for whom the drug either does not have the desired effect, or creates undesirable side-effects. The aim of pharmacogenomic research is to understand how individual genetic differences affect response to a drug, ultimately allowing optimisation of efficacy and minimization of toxicity on a personalised level. Mtx acts by inhibiting several enzymes in the folate metabolic pathway, therefore genetic differences which affect folate metabolism are likely to affect individual response to Mtx. The MTHFR 677C→T polymorphism is known to affect the activity of one of the key regulatory enzymes of folate metabolism (Frosst et al. 1995), which regulates the partitioning of folate cofactors between DNA synthesis and the methylation cycle. Mtx acts by inhibiting the recycling of DHF to THF, and thereby inhibiting synthesis of dTMP and purines. Due to its ability to alter the intracellular folate cofactor distribution, the MTHFR 677C→T polymorphism may influence response to Mtx.

Most of the evidence for the possible link between MTHFR 677C→T genotype with Mtx toxicity has come from association studies. However, the results of such studies are not consistent. Several studies have reported that TT genotype is a risk factor for increased toxicity during Mtx treatment of acute lymphoblastic leukaemia (ALL) (Aplenc et al. 2005; Ongaro et al. 2009; D'Angelo et al. 2011; Faganel Kotnik et al. 2011). Increased toxicity associated with TT genotype has also been reported in Mtx treatment of ovarian cancer (Toffoli et al. 2003), RA and psoriatic arthritis (van Ede et al. 2001a; Chandran et al. 2010; Xiao et al. 2010). In other cases no association has been found between MTHFR 677C→T genotype and Mtx-related toxicity in

treatment of RA (Wessels et al. 2006; Owen et al. 2010; Stamp et al. 2010), juvenile idiopathic arthritis (Yanagimachi et al. 2011), and osteosarcoma (Windsor et al. 2011). The inconsistency in the results from the above studies may be explained by differences in the treatment regimen of the patient cohort under study, or the specific outcome reported. For example in some cases toxicity is defined as elevated liver enzyme test (van Ede et al. 2001a), while other cases report any type of adverse drug event (ADE) (Stamp et al. 2010).

Meta-analyses attempt to overcome the problem of low sample size associated with small studies by integrating results from several studies of the same phenomenon to produce one statistical result. This has the advantage of improving statistical power, but there are also limitations of this approach. One limitation is that the quality of the meta-analysis is only as good as the individual studies which make it up. Heterogeneity in the design and methodology of the individual studies may introduce variability into the data available for meta-analysis, affecting the accuracy of the result. A further limitation which is particularly relevant to meta-analyses of observational association studies is that confounding factors which are not accounted for but which affect the observed outcome may skew the result. The result of the meta-analysis cannot give direct evidence of causality and therefore it is essential that relationships identified by association studies or meta-analysis thereof are investigated for causality by experimental methods. There are two meta-analyses of the relationship between Mtx and the MTHFR 677C→T polymorphism. Both cases included data from almost the exact same list of individual studies, but nonetheless produced somewhat different results. Fisher & Cronstein (2009) reported that presence of the MTHFR 677C→T polymorphism was associated with increased risk of Mtx toxicity in a fixed effects model (OR 1.71, 95% CI 1.32-2.21) but not in a random effects model (OR 1.60, 95% CI 0.90-2.86) (Fisher and Cronstein 2009). Lee & Song found no association between MTHFR 677C→T genotype and Mtx toxicity or efficacy in a meta-analysis including data from 1514 RA patients from eight studies (Lee and Song 2010). The latter study attempted to address heterogeneity by subdividing the data into results from Asian and non-Asian populations, but no association was found in any subgroup. The conclusion of both studies was that heterogeneity of Mtx dose, folate supplementation and definition of toxicity in the individual studies was sufficient to confound the results of meta-analysis. In the end, both groups are in agreement that currently there is insufficient data from association studies to make clear conclusions about the relationship between MTHFR 677C→T genotype and Mtx toxicity or efficacy.

The current study sought to establish if the MTHFR 677C→T polymorphism can influence sensitivity to Mtx in vitro, by examining the effect of the drug on growth rate, viability and apoptosis of transformed B-lymphocyte cell lines of either MTHFR 677 CC or TT genotype. Two

complementary systems were used to measure cell proliferation; trypan blue staining and cell counting which quantifies the number of live and dead cells, and the functional MTS cell proliferation assay which is based on measurement of metabolically active cells. By the cell counting method, cell lines of TT genotype were found to have a lower proliferation rate than cells of CC genotype when incubated in folate-free growth medium both with and without Mtx. The difference in proliferation rate between cells of CC and TT genotype cannot be attributed solely to a response to Mtx treatment, because this difference was also observed when Mtx was not present. The cell counting method also allows calculation of the percentage of live cells, and TT cells showed lower cell viability compared to CC cells under control conditions. Although Mtx caused a dose and time-dependent decrease in cell viability, there was no difference by genotype when the cells were exposed to Mtx. Cell proliferation was also assessed by the functional MTS cell proliferation assay. In this case proliferation was normalised to control values without Mtx, which should take into account the basal difference in proliferation rate observed in the cell counting experiment. Despite this correction, a lower proliferation rate of TT cells compared to CC cells was noted in the presence of all concentrations of Mtx tested. However, the difference in proliferation rate was most pronounced at the lowest Mtx concentration used (10nM), and was less significant at higher drug concentrations. This indicates that the difference in proliferation rate observed between genotypes is maintained in the presence of low concentrations of Mtx, but becomes less pronounced under the increasing effect of higher concentrations of the drug. Differences by genotype observed under control conditions may be attributed to decreased stability and activity of the 677T form of MTHFR in low folate conditions (Yamada et al. 2001). The influence of MTHFR 677C→T genotype on the apoptotic response to Mtx was also investigated in these cell lines. Cells of TT genotype were found to have a level of apoptosis approximately 5% higher compared to cells of CC genotype after 24h incubation with Mtx, however at this time point the effect of Mtx itself was not statistically significant. After 48h incubation with Mtx, the increase in apoptosis due to Mtx was statistically significant, but there was no longer a significant effect of MTHFR genotype. Taken together, the results of the current suggest that in this cell culture system, the effect of Mtx is sufficiently strong to obscure basal differences in cell proliferation and apoptosis which are attributable to genotype. These results therefore do not provide evidence that MTHFR 677C→T genotype affects sensitivity to Mtx, and so are in agreement with the association studies mentioned above which did not find an effect of the polymorphism.

Sohn et al. (2004) previously investigated the effect of the MTHFR 677C→T polymorphism on Mtx toxicity of cells in vitro. Their model involved use of breast cancer and colon cancer cell lines

which were treated to overexpress either the wild-type or 677T form of MTHFR. Overexpression of the 667T form of MTHFR in the breast cancer cell line decreased the cytotoxic effect of Mtx compared to cells overexpressing wild type MTHFR. In the colon cancer cell line, there was no difference in Mtx cytotoxicity between cells overexpressing wild type or 677T MTHFR (Sohn et al. 2004). The authors concluded that decreased sensitivity to Mtx in cells overexpressing 677T MTHFR was due to altered distribution in folate cofactors favouring formation of DNA precursors. Although previous work in this laboratory has shown a difference in folate distribution related to MTHFR 677C→T genotype in transformed B-lymphocyte cell lines (McCarthy 2005), the current study did not find a difference in Mtx cytotoxicity attributable to MTHFR 677C→T genotype. There are several differences between the current study and the work of Sohn et al. which may explain the difference in findings. The current study examined cell lines of CC or TT genotype with normal expression of MTHFR rather than overexpression of each form of the protein, which is likely to have a different effect on intracellular folate metabolism. In support of differences between the current cell culture model and that of Sohn et al, the latter noted an increased proliferation rate of cells overexpressing 677T MTHFR, which is in contrast with the decreased proliferation rate associated with TT genotype in the current study. A second difference between the two studies is that the current study was conducted in folate-free conditions, in contrast to Sohn et al. where standard RPMI growth medium which contains 2μM FA was used. The presence of increased folate is known to stabilise the 677T form of MTHFR (Yamada et al. 2001), therefore differences by genotype are more likely to be observed under conditions of folate deficiency. Finally, the cell lines under study in Sohn et al. were tumour-derived, and so may have other alterations which affect cytotoxicity of Mtx in addition to altered MTHFR function.

Association studies have the advantage of being applicable to large data sets, but the disadvantage of not directly testing the effect of the polymorphism on cellular metabolism. In contrast, mechanistic studies investigating the question of interaction between MTHFR 677C→T and the effect of Mtx are few in number and consist of smaller sample sizes. A limitation of the current study and of other cellular and mechanistic studies is the low number of cell lines which it is feasible to test. The current study included three cell lines from individuals of MTHFR 677 CC genotype and three cell lines of MTHFR 677 TT genotype, the same number as similar studies such as that of Lathrop-Stern et al. (2003). A further limitation of cellular studies is the difficulty in extrapolation of results from an in vitro system to effects on the whole organism. The transformed B-lymphocyte cell lines used in this study were suitable for the purpose of investigating Mtx cytotoxicity because they undergo rapid proliferation and therefore have an ongoing requirement of functional folate metabolism for DNA synthesis. However, there are

other sites in the body which also have high levels of folate metabolism, particularly the liver. The liver is sensitive to Mtx toxicity, as are rapidly proliferating tissues such as the skin and gastrointestinal tract. The results obtained in this study of transformed B-lymphocyte cell lines are more likely to reflect an effect on cells of lymphocyte lineage than toxicity affecting hepatic or gastrointestinal cells. This study therefore cannot exclude the possibility that differences in folate metabolism due to the MTHFR 677C→T polymorphism are relevant in development of Mtx toxicity in vivo. A more ambitious approach to cellular experiments involving high-throughput methods or the coordinated efforts of a large number of researchers would allow the analysis of cells from a far larger number of individuals, or a variety of different tissue types, and might provide more comprehensive mechanistic evidence to exhaustively test the findings of the association studies.

A notable feature of the results presented in this study is that differences were observed between cells of CC and TT genotype when cells were maintained in folate-free growth medium without Mtx. These results suggest that TT genotype confers greater sensitivity to folate stress compared to CC genotype. Association studies have shown that TT genotype is significantly correlated with higher serum Hcy, particularly in individuals who have low folate status (Harmon et al. 1996; Jacques et al. 1996; Devlin et al. 2006). Through its link with elevated Hcy, TT genotype is also associated with increased risk of cardiovascular disease. A meta-analysis found that the most significant risk of coronary heart disease was associated with the group with TT genotype and low folate status (OR 1.44, 95% CI 1.12-1.83) (Klerk et al. 2002). The observation presented here that TT cells exhibit reduced proliferation rate, cell viability, and increased apoptosis compared to CC cells in low-folate conditions are in agreement with the above studies which have shown that effects of TT genotype are more pronounced when folate status is low.

MTHFR 677 TT genotype is regarded as protective against some types of cancer, most notably colorectal cancer (Chen et al. 1996a; Huang et al. 2007) and childhood ALL (Yan et al. 2011), but in contrast has been associated with increased risk of other cancers including oesophageal and gastric cancer (Zacho et al. 2010). Huang et al. reported that the 677T allele was associated with reduced risk of colorectal cancer but was not associated with the earlier stage of the disease, colorectal adenoma, suggesting that the protective effect was linked to prevention of cancer progression (Huang et al. 2007). Decreased cell proliferation rate and increased apoptosis associated with TT genotype as observed in the results presented here could be beneficial if these features contribute to inhibition of cancer progression. However, if the increased apoptosis is a reflection of increased DNA damage this could have a cancer-promoting effect. Due to the effects of the MTHFR 677C→T polymorphism on folate metabolism, it is biologically plausible that MTHFR

677C→T genotype and folate status may interact to affect cancer risk. There is epidemiological evidence to suggest that this may be so. In the Physicians' Health Study, TT genotype was associated with decreased risk of colorectal cancer compared to CT/CC genotype in men with adequate folate status, but there was no protective effect of genotype in folate deficiency (Ma et al. 1997). In a case-control study of colorectal adenoma, TT genotype was associated with increased risk of disease in comparison to CC or CT genotype in the lowest folate quartile (OR 2.04, 95% CI 0.6-7.0), and with decreased risk in the highest folate quartile (OR 0.82, 95% CI 0.32-2.10) (Levine et al. 2000). A similar relationship was observed in a case-control study of colorectal cancer (CRC) in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort. In this case TT genotype was also associated with increased risk of CRC in the lowest folate quintile, but with decreased disease risk in the quintile with highest folate status (Eussen et al. 2010).

DNA damage and altered DNA methylation, biochemical features which are associated with cancer, have been observed to correlate to folate status in experimental studies. Leopardi et al. (2006) investigated the effects of folate depletion and MTHFR 677C→T genotype in human lymphocytes. Folate deficiency was found to increase markers of DNA damage regardless of genotype, and cells grown in lower folate medium also exhibited genomic hypermethylation. TT genotype was associated with higher levels of nucleoplasmic bridges, one of the three DNA damage markers assessed in this study (Leopardi et al. 2006). Crott et al. (2001) investigated the influence of folate depletion and MTHFR 677C→T genotype on misincorporation of uracil into genomic DNA in vitro. Folate deficiency caused increased uracil misincorporation, but there was no effect of MTHFR 677C→T genotype observed (Crott et al. 2001). Regarding global DNA methylation, TT genotype has been associated with global DNA hypomethylation when folate status is also low (Stern et al. 2000; Friso et al. 2002). The biochemical effect of the MTHFR 677C→T polymorphism could have a dual effect on cancer risk through alteration of both DNA methylation patterns and DNA damage. The 677T form of the enzyme would be expected to increase the availability of folate cofactors necessary for DNA precursor synthesis. This might explain the protective effect of TT genotype in adequate folate status. However in low folate conditions the reduced activity of 677T MTHFR could hamper cellular methylation capacity, leading to alterations in genomic methylation and consequent changes in gene expression. Using their MTHFR overexpression model, Sohn et al. showed that DNA methylation varied with MTHFR genotype and folate status to a different extent in colon cancer cells to breast cancer cells (Sohn et al. 2009). The influence of the MTHFR 677C→T polymorphism on cancer risk may therefore vary in a site-specific manner, and may be further influenced by folate status. These factors suggest an explanation for the inconsistent association of the MTHFR 677C→T polymorphism with

various cancer subtypes, and suggest that folate status should be considered in investigations of associations between MTHFR 677C→T polymorphism and cancer. The work presented here is useful because it provides in vitro evidence that cells of CC and TT genotype respond differently to folate stress at a cellular level, in a cell culture model with unaltered MTHFR expression. The observed difference between CC and TT genotypes is not of great magnitude, but this is to be expected because the effect of the polymorphism is to reduce enzyme activity rather than completely abolish activity. The minor allele of the MTHFR 677C→T polymorphism is present at a reasonably high level in the global population, with a T allele frequency varying between 6-41% depending on geographic origin of the population studied (Botto and Yang 2000). This high prevalence of the polymorphism would not be expected if TT genotype had a strongly deleterious effect.

Consumption of folic acid (FA) has increased greatly in recent years, due to the introduction of mandatory FA fortification of grains in certain countries, along with increased intake of vitamin supplements and the trend for production of vitamin-fortified foods. An unexpected consequence of increased FA availability in foods is that some individuals consume more than the recommended daily amount of FA (Crane et al. 1995). Consumption of large amounts of FA results in the appearance of unmetabolised FA in the serum, and there is evidence that this occurs in settings both with and without mandatory folate fortification (Kalmbach et al. 2008; Sweeney et al. 2009). Unmetabolised FA does not normally occur in circulation, and its potential effects are unknown. There are some concerns that unmetabolised FA in serum may interfere with antifolate therapy. One study demonstrated that the average Mtx dose for the treatment of RA had increased in the US in the period after the introduction of mandatory folate fortification (Arabelovic et al. 2007). The current study investigated if FA, 5-formyl-THF, or 5-methyl THF could interfere with the cytotoxic effect of Mtx on transformed B-lymphocytes. The results show that addition of FA does not prevent the cytotoxic effect of Mtx, in contrast to both 5-formyl THF and 5-methyl THF, which in sufficient concentration can allow normal cell proliferation in the presence of Mtx. This observation suggests that the presence of unmetabolised FA in serum is unlikely to directly affect the actions of Mtx, at least on cells of lymphocyte lineage. However, FA taken orally in recommended amounts is converted to 5-methyl THF during absorption and first-pass metabolism, thereby increasing levels of the physiological form of folate. Increasing the concentration of 5-methyl THF can affect the action of Mtx in vitro, therefore an increase in folic acid intake from supplements or fortified foods could interfere with the action of Mtx by virtue of increasing the level of 5-methyl THF in the bloodstream. This is in agreement with the study by Arabelovic (2007) which suggests that introduction of mandatory FA fortification of foods may

increase the average dose of Mtx necessary to treat RA (Arabelovic et al. 2007), but suggests that the increase is due to fortification leading to an increase in the level of physiological plasma folate rather than to effects of unmetabolised FA.

Mtx-induced apoptosis has been described in a number of cell types, including primary neutrophils (Weinmann et al. 2007) and eosinophils (Dahlman-Ghozlan et al. 2008), activated primary human lymphocytes (Genestier et al. 1998), and a number of tumour derived cell lines including choriocarcinoma (Chen et al. 2009), leukemia (Ehrhardt et al. 2007), hepatoma (Müller et al. 1997), cervical cancer (Mazur et al. 2009), lung cancer (Huang et al. 2010), and head and neck cancer (Kraljevic Pavelic et al. 2008). The experiments in chapter 4 represent the first examination of Mtx-induced apoptosis in transformed B-lymphocytes. The ability of Mtx to induce apoptosis in this cell type could explain part of the beneficial effect of Mtx in treatment of autoimmune disease, because activation of B-cells is a feature of several autoimmune diseases for which Mtx is a treatment including RA (Edwards and Cambridge 2001). Further understanding of Mtx-induced apoptosis is also relevant in the context of cancer treatment, for which induction of apoptosis in malignant cells is a treatment goal. In the current study, Mtx-induced apoptosis in transformed B-lymphocytes was found to be caspase-dependent. This is in accordance with Mazur (2009) who reported activation of the central effector caspase-3 in cervical cancer cells and normal rat kidney cells after exposure to Mtx (Mazur et al. 2009). In some cell types, Mtx-induced apoptosis has been linked to increased expression of caspase-8 (Ehrhardt et al. 2007), and enhanced sensitivity to the extrinsic apoptotic pathway (Müller et al. 1998). Others have reported that Mtx-induced apoptosis occurs by the mitochondrial pathway involving activation of caspase-9 (Chen et al. 2009). The results presented in chapter 4 show that although a general caspase inhibitor significantly reduced the level of Mtx-induced apoptosis, specific inhibition of either caspase-8 or caspase-9 only partially repressed Mtx-induced apoptosis. This suggests that other mechanisms in addition to these particular initiator caspases may be involved in Mtx-induced apoptosis in these cell lines. Experiments using TPCK, an inhibitor of chymotrypsin-like serine proteases, demonstrated that this compound could also inhibit Mtx-induce apoptosis. The effect of TPCK was observed most clearly in measurements of DNA degradation, in agreement with others who have reported inhibition of apoptotic DNA degradation by TPCK (Zhu et al. 1997; Murn et al. 2004). This is the first demonstration of chymotrypsin-like serine protease activity in Mtx-induced apoptosis, and lends support to the idea that serine proteases may play an important role in apoptosis independently of caspase activity.

A number of studies have shown that Mtx induces apoptosis preferentially in cells which are actively proliferating. Nakajima et al. studied the effect of Mtx on growth of a number of cell

types, and observed that fibroblast cell lines with slower proliferation rates were resistant to Mtx, compared to fast-proliferating fibroblasts (Nakajima et al. 1996). Genestier et al. (1998) examined Mtx-induced apoptosis in human primary T-lymphocyes in vitro, and reported that Mtx induced apoptosis in activated, but not in resting T-cells (Genestier et al. 1998). Two lines of evidence suggested that progression to the S-phase was required for Mtx-induced apoptosis; Mtx did not reduce the number of cells in G1 phase, and inhibition of IL-2 (which is required to stimulate proliferation of primary T-lymphocytes in vitro) reduced Mtx-induced apoptosis. In addition, resting cells which had been incubated with Mtx underwent apoptosis when subsequently activated in Mtx-free growth medium (Genestier et al. 1998). This is likely due to retention of Mtx-polyglutamates within the cell. Activation-dependent apoptosis of peripheral blood mononuclear cells exposed to Mtx has also been reported (Swierkot et al. 2004).

An interesting observation from this study was that addition of hypoxanthine (Hx) plus thymidine (Thy), 5-methyl THF, or 5-formyl THF, interventions which allowed cell proliferation to continue in the presence of Mtx, also completely blocked apoptosis. Genestier et al. also noted that 5-formyl THF and thymidine prevented Mtx apoptosis, although they did report that FA also had this effect, albeit less effectively than 5-formyl THF (Genestier et al. 1998). In the current study, the timing of Mtx-induced apoptosis also coincided with the doubling time of the cell lines. These features suggest that the triggering event for apoptosis is closely linked to disruption of DNA replication secondary to the inhibition of dTMP and purine synthesis by Mtx. In chapter three it was observed that Thy did not support cell proliferation in the presence of Mtx, but appeared to prevent cell death. In comparison, Hx did not support cell proliferation or prevent cell death in the presence of Mtx. In chapter 4, addition of Thy to culture medium partially prevented Mtx-induced apoptosis while Hx alone had no effect. These results suggest that while inhibition of purine synthesis may prevent cell proliferation, it is the inability to synthesise dTMP that leads to cell death via apoptosis rather than growth arrest. This is supported by a similar effect observed in primary T-lymphocytes, where Mtx and 5-fluorouracil, another inhibitor of TS, induced apoptosis while inhibitors of purine synthesis arrested cell growth but did not induce apoptosis (Quemeneur et al. 2003).

The link between inhibition of dTMP synthesis and apoptosis is interesting in the context of studies from many years ago which describe a phenomenon known as thymineless death (TLD). This process was originally described in bacteria, whereby cells deprived of thymine or unable to synthesise dTMP due to genetic mutations undergo cell death (Cohen and Barner 1954). This type of cell death occurs only in cells which are otherwise capable of undergoing DNA synthesis, and non-proliferating cells are not affected. Cell death occurring due to thymine starvation has also

been reported in yeast (Brendel and Langjahr 1974) and in mammalian cells (Ayusawa et al. 1983). TLD has been associated with the accumulation of DNA strand breaks in all of these cellular systems, although in mammalian cells distinction between DNA damage which may arise from impaired DNA synthesis and damage secondary to initiation of apoptosis is problematic. The precise mechanism leading from dTMP depletion to cell death is not fully understood. It is thought to involve mechanisms of DNA repair, as reviewed in (Ahmad et al. 1998), or altered balance of nucleotide pools. The cellular response to a lack of thymine is also associated with an increased rate of homologous replication (Waldman et al. 2008), and it is thought that this process may result in accumulation of lethal DNA lesions, although the mechanisms by which such lesions may lead to cell death are unknown. A further possibility is that inhibition of TS leads to nucleotide imbalance, with increased levels of dUTP and decreased dTTP. Accumulation of dUTP within the cell results in increased incorporation of uracil into DNA and subsequent incease in DNA repair, or failure of replication if excessive uracil is incorporated. In support of a role of uracil misincorporation in the mechanism of action of Mtx, overexpression of deoxyuridine triphosphate nucleotidohydrolase (dUTPase) which reduces dUTP accumulation has been shown to confer resistance to Mtx and 5-Fluorouracil (an inhibitor of TS), but only for a limited time (Parsels et al. 1998). Further support for the triggering of DNA repair processes by Mtx come from reports of p53 induction after Mtx exposure (Müller et al. 1998; Ehrhardt et al. 2007; Huang et al. 2010), and the observation that cells deficient in mismatch repair (MMR) mechanisms were less sensitive to Mtx-induced apoptosis compared to cells with active MMR capability (Frouin et al. 2001). The observation in the current study that Mtx-induced apoptosis is linked to inhibition of dTMP synthesis and is temporally correlated with cell division suggests that the mechanism of Mtx-induced apoptosis may be closely related to observed features of TLD. In order to investigate if exposure to Mtx resulted in increased uracil misincorporation into DNA in these cell lines, a modification of the TUNEL assay was attempted as described in chapter 4. Unfortunately this method did not prove to be suitable for such a measurement.

Recent research has indicated that activity of TS and therefore synthesis of dTMP is localised to the nucleus (Anderson and Stover 2009) and the mitochondria (Anderson et al. 2011), the two cellular compartments where synthesis of DNA occurs. In contrast, purine synthesis occurs in the cytosol. It has been suggested that dTMP synthesis may occur directly at the replication fork as part of a multi-enzyme complex during DNA synthesis (Prem veer Reddy and Pardee 1980). It is possible that prevention of the action of TS at the site of DNA replication could initiate signalling events which lead to induction of apoptosis, possibly through interaction with other proteins involved in the DNA repair process. It may be of interest to study the effects of dTMP depletion

on mitochondrial DNA replication as well as genomic DNA, as the effects and consequences of impaired DNA synthesis in these two compartments may be different. Mitochondria play a central role in apoptosis, and therefore a link between dTMP depletion and apoptosis may be mediated through mitochondrial changes. Kronenberg et al. showed that in mice with genetic knockout of uracil DNA glycosylase (UDG), a key component of the base-excision repair (BER) pathway, rates of mitochondrial DNA mutagenesis in neurons were increased compared to wild-type, and a folate-deficient diet further increased such mitochondrial defects (Kronenberg et al. 2011). Yeast cells undergoing TLD also accumulate mitochondrial DNA mutations and defects in respiration (Barclay and Little 1978). Ricci et al (2008) described the involvement of oxidative damage of mitochondrial DNA in apoptosis of rat cardiomyocytes, where apoptosis was inhibited by overexpression of a mitochondrially-targeted DNA repair enzyme (Ricci et al. 2008). In their investigation of mitochondrial dTMP synthesis, Anderson et al. showed that Mtx could inhibit dTMP synthesis in isolated mitochondria, demonstrating that the drug can enter this compartment of the cell (Anderson et al. 2011). Further understanding of the pathway from dTMP depletion to initiation of apoptosis in both nuclear and mitochondrial compartments may clarify why Mtx is such a successful drug, and may also provide impetus for development of new therapies.

As well as its effects on DNA precursor synthesis, there is some evidence that Mtx may also act by altering the methylation capacity of the cell. Methotrexate treatment is associated with increased plasma homocysteine (Hcy) levels in patients (Van Ede et al. 2002), and has also been shown to increase Hcy export in cultured cells (Refsum et al. 1991). The increased production of Hcy in response to Mtx suggests that remethylation of Hcy to methionine is impaired. This was demonstrated in vitro by Fiskerstrand et al. (1997), who showed that cells protected from Mtx cytotoxicity by a combination of Thy and Hx had an increased rate of Hcy export, reduced levels of 5-methyl-THF and reduced methionine synthase activity compared to control cells not treated with Mtx (Fiskerstrand et al. 1997). Increased Hcy can lead to increased levels of intracellular SAH, a powerful inhibitor of transmethylation reactions (Ueland 1982). Winter-Vann et al. focused on the ability of Mtx to affect the methylation and thereby function of the signalling protein Ras (Winter-Vann et al. 2003). During post-translational processing, Ras is methylated by ICMT, and this modification is required for membrane localisation and correct function of Ras. Winter-Vann et al. showed that Mtx-treatment in vitro led to reduced Ras methylation, and mislocalisation of Ras to the cytosol. They also showed that mouse embryonic fibroblasts (MEFs) which did not express ICMT were resistant to growth inhibition by Mtx, in contrast to cells with normal ICMT expression. The authors concluded that the antiproliferative effect of Mtx was

mediated through inhibition of ICMT activity and subsequent alteration of Ras signalling pathways.

The experiments presented in chapter 5 aimed to investigate the observations of Winter-Vann et al. in transformed B-lymphocytes, using the competitive inhibitor AGGC to inhibit ICMT activity. If Mtx acts by inhibition of ICMT, addition of AGGC concurrent with Mtx exposure would be expected to be more toxic to the cell than either of these agents alone. In sufficient concentration, AGGC itself was found to prevent cell proliferation and induce apoptosis. Surprisingly, at concentrations below those found to be cytotoxic, AGGC was found to partially hinder the cytotoxic and apoptotic effect of Mtx, suggesting that signalling events involving methylation may influence the cellular response to Mtx. AGGC has been shown to induce apoptosis in bovine arterial endothelial cells, and this involved reduction of Ras methylation and impairment of MAPK signalling (Kramer et al. 2003). Signalling via the MAPK pathway is closely related to cell proliferation and cell fate determination during metabolic stress (Winter-Vann and Johnson 2007). It is possible that in the current study, AGGC at a particular concentration reduces cell proliferation by interference with MAPK signalling. Due to the close relationship between the effect of Mtx and induction of cell proliferation as discussed above, a halt in proliferation due to AGGC could appear in this cell culture system as a transient protection against the effects of Mtx. This could explain the observed interference of AGGC with Mtx action and apoptosis.

AGGC is a competitive inhibitor of ICMT, and hence only inhibits one type of methyltransferase activity. In contrast, Mtx treatment is proposed to increase SAH concentration which would be likely to affect all SAM-dependent methyltransferase reactions which occur within the cell. Methylation is increasingly recognised as a feature of signalling pathways and a regulator of gene expression. There are a number of studies which have shown that interference with protein methylation can prevent function of signalling proteins and alter several cell properties. Ras belongs to a protein superfamily whose members undergo similar post-translational processing involving isoprenylation and methylation by ICMT. Lu et al. (2004) showed that ICMT inhibition led to decreased carboxymethylation and activity of RhoA, and linked this to changes in endothelial monolayer permeability in vitro (Lu et al. 2004). ICMT inhibition has also been linked to a decrease in activity of Rac1, another protein in the Ras superfamily. In this case inhibition of Rac1 was associated with down-regulation of MAPK signalling and increased association of Rac1 with an endogenous inhibitory protein, RhoGDI (Papaharalambus et al. 2005). Collectively, the Ras superfamily proteins are involved in intracellular signalling which controls a number of cellular processes including cell proliferation (Drosten et al. 2010), apoptosis (Cox and Der 2003), cytoskeletal organisation and cell migration (Cushman and Casey 2011), and cell division

(Narumiya and Yasuda 2006). Interference with carboxymethlyation due to inhibition of ICMT therefore has the potential to modify cell function in an extremely complex manner. A functional role for N-terminal methylation of a mammalian protein has also been recently described for a protein called regulator of chromatin condensation 1 (RCC1), which requires methylation for its interaction with chromatin (Chen et al. 2007). Prevention of RCC1 methylation or knockdown of NMRT, the methyltransferase responsible for N-terminal methylation of RCC1, results in defects of mitotic spindle formation (Chen et al. 2007; Schaner Tooley et al. 2010). These examples show that protein methylation is involved in regulation of several cellular processes which are relevant to cancer progression and also to inflammation. The results presented here suggest that there may be an interaction between cellular effects of Mtx and methylation events mediated by ICMT. It is possible that such an interaction may alter processes in addition to cell proliferation and apoptosis, for example cell migration. This could form a part of the anti-cancer or anti-inflammatory action of Mtx.

Although it was not investigated in the current study, the role of methylation of DNA and histones in regulation of gene expression is a topic in which new concepts are rapidly emerging, and such cellular reactions may potentially be altered by mechanisms which interfere with folate metabolism and the methylation cycle. DNA and histones are extensively methylated in SAM-dependent transmethylation reactions, and these modifications are centrally involved in regulation of gene transcription. Until recently, methylation of DNA and histones was thought to be a stable epigenetic marker which was maintained through cell division and required for genome stability. More recently evidence has emerged that methylation of histones and DNA is reversible, and may play a dynamic role in regulation of gene expression in response to signal transduction. Histones are methylated by three classes of histone methyltransferases, all of which use SAM as a methyl donor. There are two classes of histone demethylases, those containing the LSD1 domain, or the JmjC domain (Teperino et al. 2010). Methylation of specific histone residues is associated with activation or repression of gene transcription. Polycomb (PcG) proteins and trithorax (TrxG) proteins are capable of mediating histone methylation, and are associated with repressive and activating modifications, respectively. There are a number of genes which are regulated by both PcG and TrxG systems, and it is thought that this type of dual regulation may allow expression of these genes to rapidly change in response to alterations in the balance of PcG/TrxG activity (Blomen and Boonstra 2011). Methylation of cytosine residues in CpG dinucleotides in DNA is also associated with repression of transcription, and this too may be a reversible modification. Active DNA demethylation has been implicated in regulation of BDNF transcription in postmitotic neurons (Martinowich et al. 2003), in IL-2 signalling in T-lymphocytes

(Bruniquel and Schwartz 2003), and in gene expression in response to oestrogen signalling (Metivier et al. 2008). Recent work suggests that active DNA demethylation may occur by conversion of 5-methyl cytosine (5meC) to 5-hydroxymethyl cytosine (5hmC) by TET enzymes, followed by replacement of the altered residue by unmethylated cytosine by BER. Guo et al. recently described active demethylation of specific promoter regions in mouse brain (Guo et al. 2011). Their evidence suggests a model for active demethylation involving conversion of 5meC to 5hmC by TET1, followed by deamination of 5hmC by AID/APOBEC deaminases and finally replacement of the altered residue with unmethylated cytosine by BER mechanisms (Guo et al. 2011).

The emerging evidence that methylation of proteins and DNA is a reversible process which responds to extracellular signals points to a potential role for agents such as Mtx in regulation of these events. Elevated intracellular SAH and subsequent interference with methylation reactions is not limited to the effects of Mtx treatment, but could also occur in folate deficiency. There is evidence that methylation events in the cell respond to changes in extracellular folate status, although most analyses of DNA methylation have been global in nature and are thus unable to report effects on expression of specific genes. The effects of in vitro folate deficiency on DNA methylation also differ in a cell-specific manner, for example human colonic epithelial cells grown in folate deficient conditions exhibited genomic hypomethylation (Duthie et al. 2000b), while growth of murine prostate cells in folate-deficient medium was associated with increased CpG island and histone methylation compared to cells grown in high FA (Bistulfi et al. 2010). Dietary folate can also influence DNA methylation patterns in the whole organism. A striking example of this is the agouti mouse, where a maternal diet rich in sources of methyl-groups results in increased methylation of the agouti promoter in offspring, and a long-term effect on phenotype (Cooney et al. 2002). Induction of subclinical folate deficiency in a human controlled-feeding study showed that folate deficiency was associated with decreased lymphocyte DNA methylation (Jacob et al. 1998). Inhibition of methylation events within the cell can clearly affect many processes, and therefore it is not surprising that factors affecting methylation (folate status, increased plasma homocysteine) have been found to influence a large number of cellular processes and diseases including NTDs, cardiovascular disease, cancer, inflammation and apoptosis. Further understanding of signalling events involving methylation, the role of methylation in gene expression, and how factors which affect methylation capacity of the cell may influence these processes will lead to improved understanding of the mechanisms of such diseases. From a clinical and public health perspective, such improved knowledge of the

molecular mechanisms of disease will provide new strategies to treat disease, or to avoid certain conditions by appropriate dietary recommendations.

6.2 Future directions

The experiments carried out in this study lead to several areas of interest which may further inform about the mechanisms by which Mtx leads to cell death, but which were beyond the scope of the current investigation.

The first is the mechanism by which inhibition of dTMP synthesis leads to apoptosis. It is clear from previous work in combination with results from the current study that inhibition of dTMP synthesis is the aspect of Mtx action which triggers apoptosis. The precise nature of the cellular signals which lead from this initiating event to the activation of the ordered process of apoptotic cell death are not known. Another observation of this study which is relevant to apoptosis in a more general sense is the observation that inhibition of chymotrypsin-like serine protease activity by TPCK inhibits DNA degradation associated with apoptosis, but affects other features such as phosphatidylserine exposure to a lesser extent. Others have reported that TPCK also inhibits DNA degradation caused by a number of alternative apoptotic agents. The precise nature of the TPCK-sensitive protease is not known. Further investigation of this mechanism may lead to greater understanding of how DNA degradation occurs in the latter stages of the apoptotic process.

A further area of interest arising from this study is the observation that inhibition of ICMT at least partially affected the effects of Mtx on transformed B-lymphoctye cell lines. ICMT has a large range of cellular targets, so it would be enlightening to directly examine the effect of ICMT inhibition or Mtx exposure on methylation of one or several of these target proteins. In addition it would be useful to investigate the intracellular signalling pathways which are activated downstream of ICMT target proteins to determine if Mtx exposure or ICMT inhibition affects these signalling pathways.

A final point of note is relevant to the design of further studies which aim to assess the contribution of the MTHFR 677C→T polymorphism to cellular metabolism or disease processes. In the work reported here, there was a consistent difference between cell lines of CC and TT genotype in terms of cell proliferation and viability which was observed in low folate conditions. In the cell culture system used here, the effect of Mtx tended to obscure this genotype effect. However, the observation of a difference between CC and TT cell lines in folate-free conditions is in keeping with many studies which have identified an interaction between this polymorphism

and folate status, and underscores the need to assess the contribution of folate status to such a genotype effect in any investigations of the cellular or physiological effect of this polymorphism.

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