From the Department of Oncology-Pathology,
Cancer Centrum Karolinska, Karolinska Institutet
at Karolinska University Hospital, Solna
Stockholm, Sweden

Pharmacological and molecular investigations on the mechanisms underlying

resistance of human leukaemia cells to the antimetabolites

methotrexate, 6-mercaptopurine and 6-thioguanine

Alan Kambiz Fotoohi

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On March 16 of every year since 1988, Kurds have gathered in the southern Kurdistan town of Halabja to commemorate one of the worst atrocities of the 20th century: the gassing of some 5,000 of the town's residents with chemical weapons, including mustard gas. Such chemical attacks against 40 Kurdish villages in 1987-88 was a part of a genocidal campaign against the Kurdish nation in Iraq which is estimated to have taken 182,000 lives….Even today, the victims of these gas attacks are suffering and dying of diseases including leukaemia and certain other types of cancer.

….And this is only part of the tragic history of the largest stateless nation in the world.

Halabja's horrific history is symbolised by a 30-metre-tall triangular structure in the centre of the town. Here, the names of the dead are inscribed in white on the black marble walls of a circular hall. There are also photos of disfigured residents and lifeless children piled on top of each other and statues depicting scenes from the attack.

For me, this monument symbolizes raised hands praying for *freedom* and *peace* in the world.

This work is dedicated to:

all my beloved people,

my family and my parents

ABSTRACT

The goal of this thesis has been to elucidate the mechanisms underlying resistance to methotrexate (MTX), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), the antimetabolites widely used in treatment of childhood leukemia, as well as to determine the influence of 7-hydroxymethotrexate (7-OHMTX), the major metabolite of MTX, on the therapeutic action of MTX.

Resistant sublines of leukemic cell lines were developed by long-term exposure to stepwise increasing concentrations of these different agents. The mechanism underlying resistance to MTX in cells exposed to this drug was a pronounced reduction (> 10-fold) in reduced folate carrier (RFC)–mediated uptake of MTX. In CCRF-CEM cells, this reduction was associated with transcriptional silencing of the RFC gene, due to attenuated or even abolished binding of various transcription factors to their *cis*-acting elements, including the CRE, E-box, AP1, Mzf-1 and GC-box. In contrast, resistance to 7-OHMTX was due solely to a dramatic decrement (> 95%) in folylpolyglutamate synthetase activity, which also conferred a greater than 100-fold increase in resistance to short-term exposure to MTX.

The levels of mRNA species originating from approximately 17000 genes present in MTX- and 7-OHMTX-resistant MOLT4 cells were compared. In the MTX-resistant subline, the levels of mRNA encoding proteins involved in DNA and RNA metabolism and in transport were altered most; whereas in the 7-OHMTX-resistant cells, mRNA species associated with metabolism and cell proliferation were affected more profoundly. In these 7-OHMTX-resistant cells, the 10-fold reduction in the level of the mRNA for adenosine deaminase (the major enzyme of purine catabolism), complete absence of mRNA for cystathionine β synthase, the 3-fold higher level of the mRNA for methylene tetrahydrofolate reductase (involved in methyl-tetrahydrofolate biosynthesis) and the 2-fold elevation in the level of the mRNA for glycinamide ribonucleotide formyltransferase (involved in purine biosyntheses), all revealed a pattern of preservation of pools of intracellular folates and of nucleotide biosynthesis.

Neither of the known mechanisms of resistance to thiopurines (i.e., alterations in the activity of hypoxanthine–guanine phosphoribosyl transferase or of thiopurine methyltransferase enzymes) was found to occur in 6-MP- or 6-TG-resistant cells. Instead, the primary mechanism of resistance was a pronounced reduction in cellular uptake of 6-MP. Selective down-regulation of the levels of mRNAs encoding two nucleoside transporters, the concentrative nucleoside transporter 3 (CNT3) and equilibrative nucleoside transporter 2 (ENT2), was detected in both resistant sublines. Moreover, silencing of the CNT3 and ENT2 genes by small interfering RNA attenuated both the transport and cytocidal effect of 6-MP.

Both of the thiopurine-resistant cell sublines exhibited a collateral enhancement in sensitivity to the cytotoxicity of methylmercaptopurine riboside (meMPR), an intra-cellular metabolite of 6-MP that is known to be a potent inhibitor of *de novo* purine biosynthesis. Transport of meMPR into these cells remained intact. These findings, together with the reduced rate of *de novo* purine biosynthesis and low levels of ribonucleoside triphosphates in these cells, can easily explain their enhanced sensitivity to meMPR. An additional investigation revealed that transfection of wild-type cells with small interference RNA molecules targeting the gene encoding the first member of the family of equilibrative nucleoside transporters (ENT1) reduced the initial rate of meMPR uptake.

In summary, our present findings clearly demonstrate the major involvement of defective transport in the development of resistance to MTX, 6-MP and 6-TG. Long-term exposure of leukemic cells to 7-OHMTX can impair the clinical efficacy of MTX. The disparate patterns of gene expression exhibited by MTX- and 7-OHMTX-resistant cells further confirms that these agents act in different ways. These results may help to improve individualization of MTX treatment on the basis of plasma levels of 7-OHMTX. The collateral enhancement in the sensitivity of thiopurine-resistant cells to the cytotoxicity of meMPR suggests that administration of meMPR or its analogues to patients with ALL experiencing relapse or resistance might be beneficial.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals:

I. Fotoohi AK, Jansen G, Assaraf YG, Rothem L, Stark M, Kathmann I, Gregorczyk J, Peters GJ, Albertioni F. Disparate mechanisms of antifolate resistance provoked by methotrexate and its metabolite 7-hydroxymethotrexate in leukemia cells: implications for efficacy of methotrexate therapy. *Blood. 2004;104:4194-201.*

II. Fotoohi AK, Assaraf YG, Moshfegh A, Hashemi J, Jansen G, Peters GJ, Larsson C, Albertioni F. Gene expression profiling of 7-hydroxymethotrexate-resistant human leukemia cells reveals a pattern of preservation of intracellular folates and nucleotide biosynthesis.
Manuscript, in preparation

III. Fotoohi AK, Lindqvist M, Peterson C, Albertioni F. Involvement of the concentrative nucleoside transporter 3 and equilibrative nucleoside transporter 2 in the resistance of T-lymphoblastic cell lines to thiopurines. *Biochem Biophys Res Commun. 2006; 343:208-15.*

IV. Fotoohi AK, Wrabel A, Moshfegh A, Peterson C, Albertioni F. Molecular mechanisms underlying the enhanced sensitivity of thiopurine-resistant T-lymphoblastic cell lines to methylmercaptopurine riboside. *Biochem Pharmacol. 2006; 72:816-23.*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AZA	Azathioprine
BCRP	Breast cancer resistant protein
cDNA	Complementary DNA
DHFR	Dihydrofolate reductase
dUMP	deoxyuridine monophosphate
dTGDP	Deoxy-6-thioguanosine diphosphate
dTGTP	Deoxy-6-thioguanosine triphosphate
dTMP	deoxythymidine monophosphate
FPGH	Folypolyglutamate hydrolase
FPGS	Folypolyglutamate synthetase
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GARFT	Glycinamide ribonucleotide formyltransferase
GMPS	Guanosine monophosphate synthetase
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
7-OHMTX	7-Hydroxymethotrexate
IMPDH	Inosine 5´-monophosphate dehydrogenase
ITPase	Inosine triphosphate pyrophosphatase
6-MP	6-Mercaptopurine
meMP	Methyl mercaptopurine
meTG	Methyl thioguanine
meTIMP	Methylthioinosine 5'-monophosphate
meTGMP	Methylthioguanosine 5'-monophosphate
MRP	Multidrug resistance-associated protein
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
MTX	Methotrexate
MTXGLs	methotrexate polyglutamates
NDPK	Nucleoside diphosphate kinase
PCR	Polymerase chain reaction
PDNS	*de novo* Synthesis of Purine
P-gp	P-Glycoprotein
RFC	Reduced folate carrier
RR	Ribonucleotide reductase
SAM	S-Adenosylmethionine
SAH	S-Adenosylhomocysteine
siRNA	Small interfering RNA
6-TG	6-Thioguanine
TGDP	Thioguanosine diphosphate
TGMP	Thioguanosine monophosphate
TGNs	Thioguanine nucleotides
TGTP	Thioguanosine triphosphate
TIMP	Thioinosine monophosphate
TITP	Thioinosine triphosphate
TPMT	Thiopurine methyltransferase
TS	Thymidylate synthetase
TXMP	Thioxanthosine monophosphate
XO	Xanthine oxidase

INTRODUCTION

Leukemia, a term derived from a Greek word meaning “white blood” and first used by Virchoiw in 1845, is a group of diseases characterized by uncontrolled proliferation of white blood cells. The major forms of leukemia are divided on the basis of their morphological characteristics into four categories, i.e., acute and chronic myelogenous and lymphocytic leukemia. Until recently, being diagnosed with this disease was tantamount to receiving a death sentence, but today the majority of these patients can be cured by chemotherapy, in combination with intensive supportive care. Originally involving the use of a single agent, today such chemotherapy is complex and multidisciplinary, involving combinations of drugs. Despite the availability of effective treatment, leukemia remains the second major cause of death in children in the Western world.

1. Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) develops from acquired damage to the DNA of a single cell in the bone marrow, resulted in uncontrolled and extensive growth and accumulation of so called lymphoblasts or leukemic blasts, which do not function as normal blood cells together with blockage of the production of normal marrow cells and deficiencies in erythrocytes (anemia), platelets (thrombocytopenia) and normal leukocytes (in particular neutrophils, i.e., neutropenia) in the circulating blood. Leukemic cells can spread from the bone marrow to the blood, lymph nodes, spleen, liver, central nervous system and other organs.

ALL is the most common malignancy that occurs in childhood, accounting for approximately 25% of all paediatric malignancies. The incidence in the Nordic countries is 3.9 per 100,000 children (<15 years of age) annually, with 175-200 new cases being diagnosed each year (Gustafsson, 2000). The five-year rate of event-free survival for children with ALL is nearly 80% (Gustafsson, 2000; Pui, 2003, a ; Schrappe, 2000; Silverman, 2001) and corresponding value for adults is approximately 40% (Gokbuget, 2002; Kantarjian, 2000; Linker, 2002). Unfortunately, in developing countries the rate of cure for this disease is often less than 10% (Pui, 2003, b). Methotrexate (MTX) and mercaptopurine (6-MP) are cornerstones in ALL chemotherapy. Intensified consolidation particularly with methotrexate

at a high-dose (HD-MTX), is one key reason for the improvement in survival attained in recent years.

Chemotheraputic treatment of ALL involves: 1) induction of remission with a combination of drugs; 2) a consolidation phase that includes high-dose systemic administration of drugs together with treatment designed to eliminate the disease in the central nervous system; and 3) continued therapy to prevent relapse and achieve a total cure. A combination of 3-8 medications, possibly including MTX and 6-MP, is typically used in this connection. Remission can be induced with daily administration of prednisone orally and weekly treatment with vincristine intravenously, in combination with an anthracycline or asparaginase. Cytarabine, etoposide, and cyclophosphamide may be also introduced early in treatment regimen. In some cases, a high dose of MTX followed by rescue with leucovorin is employed. In the case of leukemic infiltration to the meninges, prophylaxis and treatment including intrathecal administration of MTX at high-dose, together with cytarabine and corticosteroids may be advantageous. For patients at high risk for spread of the disease to the central nervous system, irradiation of brain may be required.

Following intensive treatment for induction of remission and subsequent consolidation, ALL patients usually receive maintenance therapy, most often involving MTX and 6-MP, for 2.5 - 3 years. For a patient exhibiting continuous and complete remission for a period of 2.5 years, the risk for relapse, which usually occurs within one year after cessation of therapy, is approximately 20%. Thus, when therapy can be terminated, most patients have been cured. Relapse usually occurs in the bone marrow, but may also develop simultaneously or even alone in the testes or CNS. At this point complete remission or even cure can still be obtained in a small proportion of the patients, especially children, by combination therapy or bone marrow transplantation.

The clinical response of ALL patients to treatment is determined by several different factors. The pharmacokinetics (i.e. absorption and distribution) and pharmacodynamics (metabolism and elimination) of the drugs administered are obviously of considerable importance in this context and may vary markedly among the patients. Optimization of the plasma level of a drug on an individual basis improves outcome (Evans, 1998) while reducing the risk for toxicity (Wall, 2000). At the cellular level the response to anti-malignancy agents is influenced by: influx and efflux mechanisms; intracellular metabolism; intracellular sequestration; alterations in the intended drug target(s); efficacy of DNA repair processes; and the case with which the tumor cells can be induced to undergo apoptosis.

2. Antimetabolites

By definition, antimetabolites, which are a major family of cytotoxic drugs, disturb or block one or more of the metabolic pathways involved in DNA synthesis. Drugs of this type were among the first introduced into cancer chemotherapy. The antimetabolites are subdivided into antagonists of folate (antifolates) and nucleoside (purine or pyrimidine) analogues. On the basis of their structural similarity, the thiopurines 6-MP and 6-thioguanine (6-TG) are classified as purine analogues.

2.1. Antifolates

In the early 1940's the observation of serum folate deficiency in patients with acute leukemia gave rise to the hypothesis that a deficiency in this B-vitamin might actually be the cause of acute leukaemia. This hypothesis was supported further by the finding that megaloblasts resembling leukemic blasts predominate in the bone marrow of folate-deficient patients. The isolation of pteroylglutamic acid (folic acid) from spinach in 1941 (Mitchell, 1941) and subsequent development of procedures for organic synthesis of this compound in 1945 (Angier, 1945), allowed investigators to test this idea by administrating folate to patients with leukemia.

It was soon recognized that not only was such administration ineffective, but it appeared to even accelerate the course of disease in patients with chronic myelocytic leukemia and acute leukaemia (Heinley, 1948). Consequently efforts turned to treating these leukemias by creating folate deficiency. The report that temporary remission could be obtained in 5 of 16 patients with ALL with aminopterin (4-amino-PGA; AMT) (Farber, 1948) provided the first evidence that an antimetabolite could be an effective antineoplastic agent and represented a landmark in cancer chemotherapy.

In mice carrying the L1210 leukemia, MTX (4-amino-N^{10}-methylpteroylglutamic acid) (**Figure 1**) has a more favourable therapeutic index than AMT. As a result, MTX has replaced AMT for clinical approach in recent years.

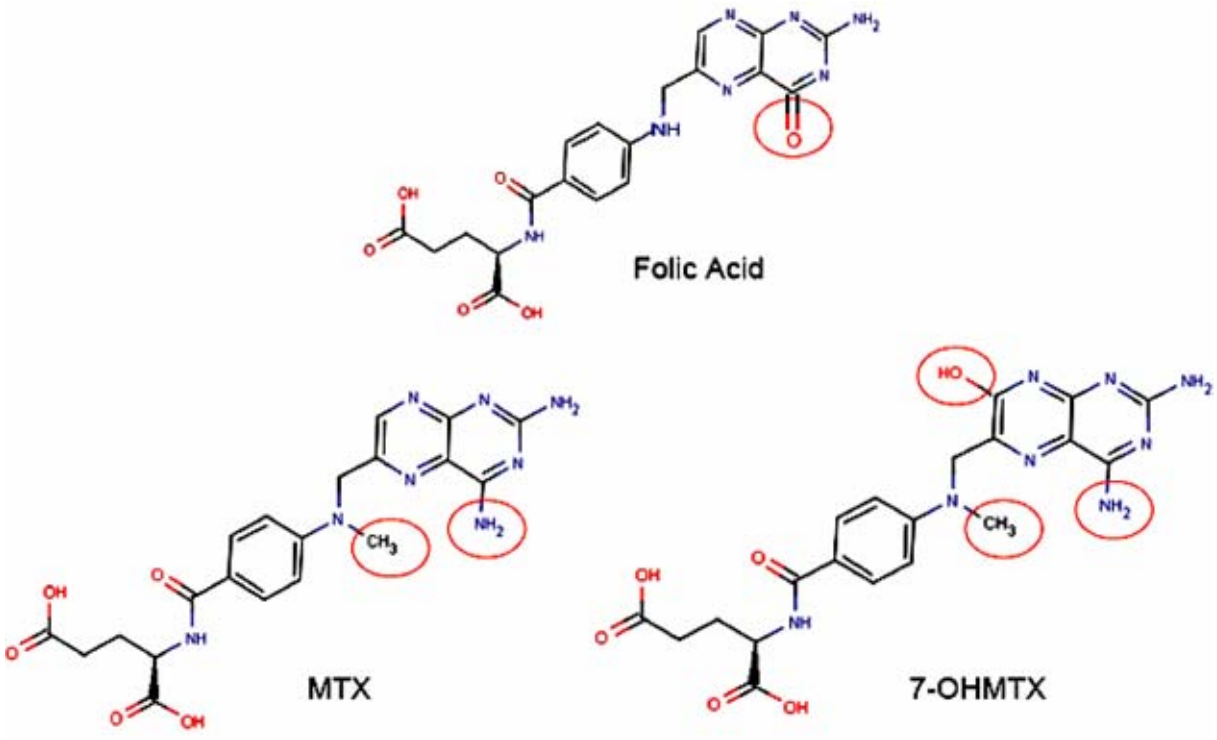

Figure 1. *The chemical structures of folic acid, methotrexate (MTX) and 7-hydroxymethotrexate (7-OHMTX)*

Although introduction of MTX to ALL therapy has improved the outcome of patients dramatically, the cancer cells of at least 20% of these patients are not susceptible of the cytotoxic effects of this antifolate. Observations concerning both inherent and acquired resistance to MTX have motivated a search for a new generation of antifolates which either themselves inhibit the metabolism of folate or folate-mediated reactions, or overcome cellular resistance to MTX.

2.1.1 Methotrexate

The highly versatile MTX has been one of the most widely administered anticancer drugs during the past half-century. Not only is MTX used to treat malignancies such as choriocarcinoma; lung, mammary gland and ovarian cancer, seminoma, osteogenic sarcoma

and ALL; but also for treatment of various autoimmune diseases, such as psoriasis, rheumatoid arthritis, lupus erythematosis and inflammatory bowel disease, primary biliary cirrhosis and intrinsic asthma, as well as for prevention of graft-versus-host disease. The first cure of a solid tumor, in this case choriocarcinoma, by MTX was reported in 1961 (Hertz, 1961), and represented yet another landmark in cancer chemotherapy, that prompted a number of investigators to focus more of their efforts on chemotherapeutic treatment of cancer. Further clinical studies revealed that MTX does not cause long-term toxic effects (Rustin, 1983), a highly advantageous characteristic for use in adjuvant therapies. To induce remission in patients with ALL, a high dose of MTX (1-30 g/m^2 of body surface) is administered by infusion, with subsequent administration of leucovorin as an antidote; whereas for maintenance therapy and prevention of relapse, a low dose of this drug (15-50 mg/m^2 of body surface – week) is administered orally or by intramuscular injection 1-3 times per week for a period of 2.5-3 years).

2.1.1.1 The clinical pharmacology and pharmacokinetics of methotrexate

Following oral administrations, doses of MTX up to 25 mg/m^2 are readily absorbed from the gastrointestinal tract. Larger doses are not absorbed completely via this route and are therefore administered intravenously. High-dose infusion results in peak plasma concentrations of 0.1 - 1 mM or higher. The drug disappears from the plasma in a triphasic fashion (Sonneveld, 1986): first, via rapid distribution; secondly, by renal clearance (with a $t_{1/2}$ of approximately 2 - 3 hours); and finally, in an elimination phase with a half-life of 8 - 10 hours. The half-life of this third phase is prolonged in patients who suffer from renal failure, which can result in toxic effects on the bone marrow and gastrointestinal tract. Thus, the dose administered to such patients should be adjusted in an appropriate manner.

At pharmacological concentrations, approximately 50% of serum MTX is bound to plasma proteins. A number of other drugs, including sulfonamides, salicylates, tetracycline, chloramphenicol and phenytoin, can displace MTX from albumin and, accordingly, treatment in combination with non-steroidal anti-inflammatory drugs has been associated with severe toxicity caused by MTX. Biliary excretion accounts for approximately 10% of the overall clearance of this drug.

In humans, following low-dose administration, metabolism of MTX is minimal, so that of the amount taken up, approximately 90% is excreted unchanged in the urine within 2 days. In connection with high doses, however, metabolites, including 7-OHMTX and diamino-2,4-N-^{10}-methylpteroic acid (DAMPA), accumulate. In such cases 20-46% of the MTX absorbed is excreted in the urine as 7-OHMTX, the major metabolite, within 12-24 hours after the start of infusion. 7-OHMTX the main metabolite is formed through the action of hepatic aldehyde oxidase (AO) (Jacobs, 1976) and can precipitate in the kidneys and is considered to be potentially nephrotoxic.

Although the cytotoxicity of 7-OHMTX has been estimated to be at least 100-fold less than that of MTX, its polyglutamated metabolites inhibit the folate-dependent enzymes thymidylate synthase (TS) and aminoimidazole carboxamide transformylase (AICART) with a potency similar to that of MTX polyglutamates. The concentration of 7-OHMTX in plasma a few hours after initiation of high-dose therapy with MTX rises to 1-100 μM, which may be high enough for this metabolite and/or its polyglutamated forms to exert cytotoxic effects. However, the potential cytotoxicity of 7-OHMTX and underlying mechanisms have not yet been examined in detail. The inactive metabolite DAMPA is produced in smaller amounts via degradation of MTX by bacterial carboxypeptidase in the lumen of the gut (Donehower, 1979).

High-dose administration

A dose of MTX that is greater than 0.5 g/m^2 body surface area is considered to be high. Such high doses are applied for treatment of ALL, as well as of lymphomas and osteogenic sarcoma. High-dose administration of MTX is followed by vigorous hydration, alkalization of the urine and administration of an adequate dose of leucovorin as the rescue agent, in order to prevent potentially lethal side-effects.

In connection with high-dose therapy, the plasma pharmamacokinetics of MTX can be used to predict both efficacy and toxicity and, consequently, close monitoring of plasma levels of MTX is essential in this connection (Evans, 1979; Perez, 1978; Stoller, 1977). Furthermore, the choice of the rescue dose of leucovorin is based on the plasma concentration of MTX. Unfortunately, the rapid automated procedures presently used routinely, suffer from cross-reactivity with metabolites of MTX (Albertioni, 1996; Fotoohi, 2005). The resulting over-estimation of concentrations of MTX may lead to the patient being hospitalized for an

unnecessarily long period of time, as well as receiving an overdose of leucovorin that increases the risk for relapse (Fotoohi, 2005; Skarby, 2006).

Toxicity

The most common and serious toxic side-effect associated with administration of MTX is myelosuppression. Furtheremore, intratubular precipitation of MTX and its metabolites, 7-OHMTX and DAMPA, in acidic urine causes nephrotoxicity, which can prevented by vigorous hydration and alkalization of the urine. The elevations in the levels of hepatic enzymes frequently seen in the serum of patients receiving high doses of MTX are usually reversible (Exadaktylos, 1994; McIntosh, 1977; Perez, 1979; Slordal, 1987). Gastrointestinal mucositis is also a side effect of treatment with MTX.

2.1.1.2 Mechanisms of action and resistance

Following its entry into the cell, primarily via the reduced folate carrier (RFC) (Fabre, 1984), MTX is polyglutamylated in an adenosine triphosphate (ATP)–dependent reaction catalyzed by folylpolyglutamate synthetase (FPGS) (Fabre, 1983). Both MTX and its polyglutamate metabolites inhibit dihydrofolate reductase (DHFR). As a consequence of this inhibition, regeneration of tetrahydrofolate from dihydrofolate is impaired, leading to a deficiency in tetrahydrofolate and formation of various one-carbon adducts in replicating cells. This, in turn, results in inhibition of the synthesis of both purines and thymidine, inhibition of DNA replication and, finally, cell death. Moreover, the polyglutamates (MTX-PGs) can inhibit TS, as well as certain other enzymes involved in the *de novo* biosynthesis of purines (PDNS), e.g., glycinamide ribonucleotide formyltransferase (GARFT) and AICART (Allegra, 1987). Since the polyglutamated metabolites of MTX remain in the cell much longer than the parent drug itself, polyglutamylation determines the efficacy of MTX as a cytotoxic agent.

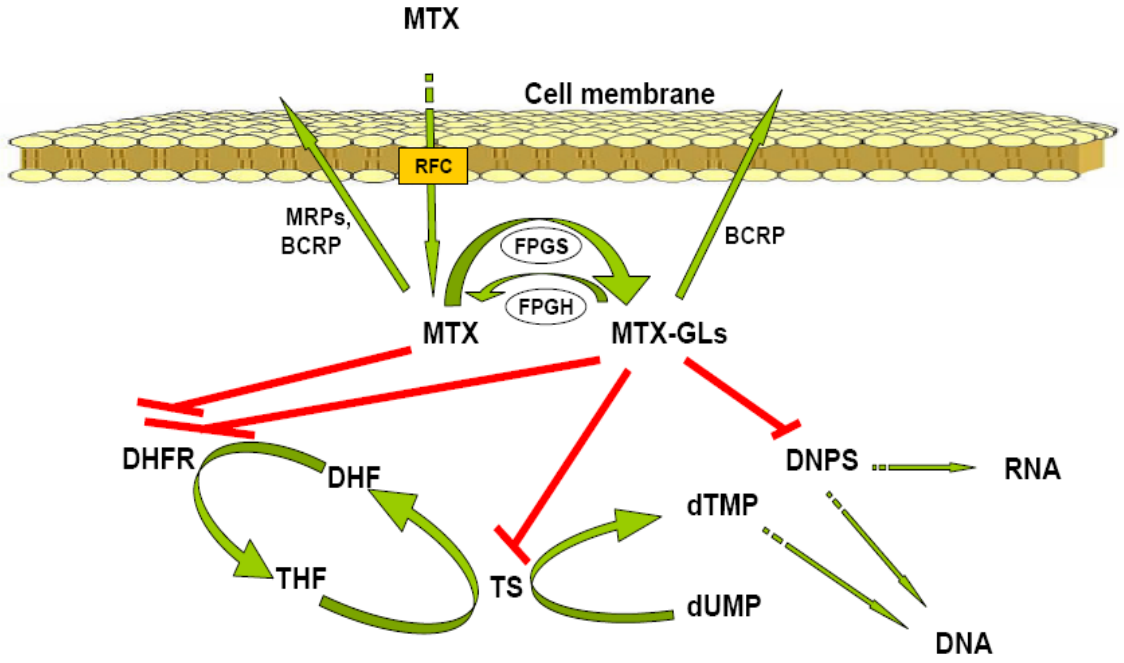

Figure 2. *Summary of the intracellular metabolism and targets of MTX. MTX is transported actively into the cell by reduced folate carrier (RFC), where it undergoes polyglutamylation and exerts its toxic effects by inhibiting dihydrofolate reductase (DHFR), thymidylate synthetase (TS) and de novo biosynthesis of purines (PDNS). DHF, dihydrofolate; THF, tetrahydrofolate; dTMP, deoxythymidine monophosphate, dUMP, deoxyuridine monophosphate; FPGS, Folypolyglutamate synthetase, FPGH, Folypolyglutamate hydrolase; MRPs, multidrug resistance-associated proteins; BCRP, breast cancer resistance protein; MTXGLs, methotrexate polyglutamates.*

2.1.1.3 Transport of MTX

A. Influx mechanisms

Folate carriers

Uptake of MTX into human cells is mediated primarily by the human reduced folate carrier (hRFC) (**Figure 2**), which has long been known to play a major role in the uptake of both reduced folate cofactors and this drug (Goldman, 1968). RFC (SLC19A1) is a member of the superfamily of major facilitators, a large group of carriers that transport various organic and inorganic compounds across the membranes of both prokaryotic and eukaryotic cells (Pao, 1998).

RFC actually transports MTX more efficiently than folic acid in its oxidised form. At least one additional transport system for folic acid capable of also transporting MTX exists, but this system does not appear to play a role in resistance to MTX (Spinella, 1995). Both of these systems are energy-dependent, i.e., perform active uptake. Moreover, at high concentrations MTX can diffuse across the cell membrane (Goldman, 1985).

Several years after the introduction of antifolates as anti-cancer agents, impaired cellular uptake was recognized as one mechanism of resistance to these agents (Hill, 1979; Sirotnak, 1968). Today, a defect in RFC-mediated transport has been established as the primary and most frequent mechanism of resistance to MTX, not only in human tumor cell lines (Jansen, 1998), but also in the clinic (Gorlick, 1997; Trippett, 1992). Molecular basis for such defective transport has been examined by several different groups.

For instance, in the case of the MTX-resistant L1210 cell line MTX uptake was restored completely by an A130P mutation in the third transmembrane domain of RFC (Brigle, 1995). In a CCRF-CEM cell line Jansen et al (Jansen, 1998) found that a Glu45Lys mutation in the first transmembrane domain of this transporter enhanced uptake of folic acid, resulting in several-fold elevation in the size of the intracellular pools of folate and, consequently, an abolition of polyglutamylation that conferred resistance to antifolates whose action are dependent on such polyglutamylation, e.g., ZD1694, DDATHF (5,10-dideazatetrahydrofolic acid) and AG2034, and even to lipophilic antifolates trimetrexate and pyrimethamine. Roy et al (Roy, 1998) discovered that the intrinsic resistance of sarcoma 180 cells to MTX, in comparison to L1210 leukemia cells, is due to a single difference in the amino acid sequence of RFC (Ser in L1210 and Asn in the sarcoma 180 cells). A novel mechanism of antifolate resistance, described by Rothem and co-workers (Rothem, 2003) involves altered expression and function of transcription factors that results in transcriptional silencing of the RFC gene. Later on the same group demonstrated that antifolate-resistance can be acquired due to loss of function of CREB-1 as a result of lack of CREB-1 phosphorylation (Rothem, 2004). This resulted in a markedly impairment of antifolate transport due to down-regulation of RFC gene expression, the latter of which is highly dependent on CREB-1 function. Antifolate-resistant cells frequently display loss of CRE-binding due to impaired signaling via the cAMP-Protein Kinase A (PKA) pathway (Rothem, 2003; Rothem, 2004).

Defective transport also plays a role in resistance to other hydrophilic antifolates. Thus resistance to PT523 and ZD9331 acquired in vitro has been shown to be due to a defect in RFC-mediated transport. Moreover, the efficiency of transport by RFC correlates well with

the pharmacological profiles of antifolate drugs (Goldman, 1985; Grant, 1993; Sirotnak, 1987).

Another mechanism of influx for antifolates is via folate receptors (FRs). However, the affinity of RFC to antifolates is much higher, and FRs usually doesn't play an important role in transport of MTX, when RFC presents at sufficient levels (Spinella, 1995).

Other transporters

Several organic anion carriers that are expressed at the highest levels in the liver and kidneys can also transport MTX, some with affinities comparable to that of RFC. However, the contribution of these transporters to the actions of MTX remains to be elucidated. The liver specific transporter-2 (LST-2) is expressed exclusively in the liver under normal physiological conditions, but is also present at high levels in gastric, colon, and pancreatic cancers. Transfection of mammalian cells with the LST-2 gene has been shown to potentiate their sensitivity to MTX (Abe, 2001).

B. Efflux mechanisms

Multidrug resistance-associated proteins (MRPs)

Although RFC is a bidirectional carrier, capable of transporting MTX both into and out of cells, the latter process is not energy-dependent. However, in 1965 Hakala and co-workers (Hakala, 1965) demonstrated the occurrence of ATP-dependent efflux of MTX from sarcoma-180 cells, an observation that was subsequently confirmed (Schlemmer, 1992; Schlemmer, 1995). Today, it is well known that the multidrug resistance-associated proteins (MRPs), of which 9 are presently known, are one of the families of energy-dependent transporters capable of mediating efflux of MTX.

In 1997, the Masuda et al. (Masuda, 1997) reported that the clearance and secretion of MTX by hyperbilirubinemic rats, which lack MRP2, are impaired and, furthermore, that canalicular membranes prepared from the livers of the same animals are incapable of ATP-dependent transport of MTX. The major amino acid residue in MRP2 of major importance in connection to transport of MTX is Trp^{1254} (Ito, 2001). Transfection of human ovarian 2008 carcinoma cells with the genes encoding MRP1 or MRP2 enhanced the tolerance of these cells to short- term (1-4-hour) exposure to MTX (Hooijberg, 1999). Later on NIH 3t3 cells transfected with MRP4 were found to be approximately 5-fold more resistant to short-term exposure to MTX than untransfected cells (Lee, 2000).

To date, at least four members of MRP family (MRP1-4) have been found to mediate the efflux of MTX but not of the polyglutamated forms of MTX out of cells (Chen, 2002; Zeng, 2001). Interestingly, transfection of cells with these MRPs confers resistance to MTX primarily in connection with brief exposure (4 hours) (Hooijberg, 1999; Kool, 1999; Lee, 2000). Active efflux by MRPs reduces intracellular levels of free MTX, and consequently, the rate of formation of polyglutamated forms of this drug, which can not transported out. Later, during the withdrawal period, MTX-monoglutamate will be pumped out rapidly by MRPs, thus preventing cytotoxicity. However, the significance of MRPs in connection with resistance to MTX has not yet been established in clinical situations.

P-glycoprotein (Pgp)

The capability of the permeability (p-)glycoprotein an ATP-dependent integral membrane transporter encoded by the human multidrug resistance 1 (MDR1) gene, to export many hydrophobic xenobiotics out of cells provides protection from toxic substances and metabolites. The existence of an active exporter in cell membrane was first reported by Dano in 1973 (Dano, 1973). Later, in 1976, Juliano and Ling (Juliano, 1976) found that the alterations in cell membrane permeability exhibited by a multi-drug-resistant Chinese hamster ovary cell were due to a glycoprotein with a molecular weight of 170 K-daltons, which they called the Permeability glycoprotein (Pgp). Pgp carries out efflux of lipophilic compounds, but not hydrophilic drugs such as MTX. Accordingly, cell lines selected for resistance to the lipophilic antifolate trimetrexate overexpress Pgp, but demonstrate no cross-resistance to MTX (Arkin, 1989). Apparently, resistance to MTX associated with elevated expression of PgP occurs solely under circumstances where RFC-mediated transport of this drug is defected (de Graaf, 1996; Gifford, 1998).

Breast cancer resistance protein (BCRP)

In the early 1990's observation of transport-mediated resistance to mitoxantrone in a few cell lines without altered expression of MDR1 and MRP1 (Dietel, 1990; Nakagawa, 1992; Taylor, 1991) encouraged a search for new transporters. In 1992, Nawagata and co-workers (Nakagawa, 1992) examined a mitoxantrone-resistant human MCF-7 breast cancer cell line that exhibited cross-resistance to doxorubicin and etoposide, but not to vinblastine which is well-known to be transported by Pgp. The reduced intracellular accumulation of mitoxantrone in these cells is associated with enhanced drug efflux and is reversed by both an inhibitor

(sodium azide) and uncoupler (2, 4-dinitrophenol) of mitochondrial oxidative phosphorylation, indicating that the efflux is energy-dependent.

A similar pattern of MDR was observed in another breast cancer cell line, MCF-7, that was made resistant to adriamycin in the presence of the verapamil (Chen, 1990), which do not overexpress either Pgp or MRP1. Later on, in 1997, Lee and colleagues (Lee, 1997) reported enhanced ATP-dependent efflux and reduced accumulation of daunomycin and rhodamine in this resistant cell line. This provided further evidence for the existence of a novel ATP-dependent xenobiotic transporter.

In 1998, Doyle et al. (Doyle, 1998) finally identified this transporter and named it the breast cancer resistance protein (BCRP). It is now known that BCRP is a member of the ATP-binding cassette superfamily of transporters that confer cellular resistance to a wide range of antineoplastic agents, including mitoxantrone, doxorubicin, daunorubicin and topotecan. The specificity of this protein is strongly influenced by the nature of the amino acid 482.

Ignorance of this fact initially caused doubt about the ability of BCRP to export MTX. Thus, Volk and co-workers (Volk, 2000) observed cross-resistance to MTX in a mitoxantrone-resistant breast cancer cell line and tried unsuccessfully to confirm the involvement of BCRP by transfection of these cells with BCRP cDNA that carried a mutation in codone 482. At first, these investigators postulated the existence of a novel MTX-specific efflux pump, but they subsequently found that MTX resistance is correlated with over-expression of the wild-type, but not of the R482T or R482G mutant forms of BCRP (Volk, 2002). With the exception of a 5-fold enhancement in resistance to AG337, the mitoxantrone-resistant cells that overexpressed BCRP showed no cross-resistance to other antifolates (e.g. ZD1694 and DDATHF), suggesting that these are poor ligands for BCRP-mediated efflux (Volk, 2000).

Interestingly, in contrast to the ability of MRP1-4 to export only monoglutamate forms of folates and MTX, BCRP (ABCG2) has recently been reported to transport mono-, di-, and triglutamate conjugates of folic acid and MTX out of cells in vitro (Chen, 2003; Volk, 2003) (**Figure 2**). This unique ability may explain the long-term resistance of cells transfected with BCRP cDNA to MTX (Volk, 2002). However, the effect of BCRP on accumulation of the polyglutamated forms of MTX (MTXGLs) in intact cells remains unclear (Rhee, 2005). Successful strategies designed to reduce the expression and/or activity of BCRP have been developed and may prove useful in overcoming drug resistance in the clinic (Kowalski, 2002).

2.1.1.4 Impact of covalent conjugation with polyglutamate residues on the effect of antifolates

A. Polyglutamylation by folylpolyglutamate synthetase

Following influx across the cell membrane MTX undergoes polyglutamylation, in the same manner as physiological folate coenzymes. This process involves sequential γ-linkage of as many as 6 glutamyl residues to the terminal glutamyl moiety of the molecule, and is catalyzed by the cytoplasmic enzyme FPGS (McGuire, 1981). The MTXGLs, like those of the natural folate coenzymes, are metabolized further only slowly.

MTXGLs exhibit much longer intracellular half-lives than the original monoglutamated MTX (MTX-GL1) and can thus be accumulated for longer periods of time. This is due to the fact that these congeners are transported by almost none of the folate transport systems (McBurney, 1974), with the exception of BCRP/ABCG2, which has recently been shown to export both mono- and polyglutamates of folates and MTX (Chen, 2003). MTXGLs retain ability to inhibit DHFR (Jolivet, 1982) and, in addition, inhibit several other enzymes involved in the *de novo* biosynthesis of deoxythymidylate and purines, such as TS, GARFT and AICART.

Thus, the extent and rate of polyglutamylation is as a determining factor for MTX cytotoxicity (Fabre, 1984). However, in the case of other antifolates, the impact of polyglutamylation differs, although in all cases polyglutamylation enhances accumulation and usually increases the degree of binding to enzymes which require tetrahydrofolate as a cofactor. For instance, polyglutamylation significantly increases the affinity of TS for ZD1694 and pemetrexed for TS (Shih, 1997) and of GARFT for pemetrexed and DDATHF (Sanghani, 1997; Shih, 1997).

Impaired polyglutamylation contributes to MTX resistance most significantly when exposure is limited to a few hours, since MTX-monoglutamate is itself a potent inhibitor of DHFR. However in connection with such brief exposure, which resembles clinical pulse therapy, only MTX that is retained intracellularly, i.e., polyglutamated forms, contribute to the toxic effects of this drug. A number of studies have revealed that attenuated polyglutamylation is one mechanism underlying acquired resistance to MTX (Cowan, 1984; Li, 1992).

In this connection, the intrinsic resistance of acute non-lymphocytic cells to MTX in comparison to ALL cells, particularly during short-term exposure as well as the more pronounced tolerance to MTX and poorer prognosis associated with T- than B-lymphoblastic leukaemia has been explained on the basis of lower capacities to produce long-chain polyglutamated forms of MTX (Barredo, 1994; Lin, 1991; Rodenhuis, 1986).

In attempts to explain the molecular basis for the difference accumulation in long-chain MTXGLs in ALL and acute myeloblastic leukemia (AML) cells, Longo and coworkers (Longo, 1997) observed two fold higher K*m* values of FPGS for MTX in the AML cells. This finding may be explained by the presence of alternative splice variants of the FPGS gene. Moreover, the isoforms of FPGS are probably expressed (Chen, 1996; Jansen, 1992; Roy, 1997), or/and subject to posttranslational modifications (Leclerc, 2001) in a tissue-specific manner.

B. Hydrolysis by folypolyglutamate hydrolase

As in the case for folate-polyglutamates, MTXGLs are hydrolysed in lysosomes, following uptake by a facilitated transport system (Barrueco, 1992). The enzyme involved is active only at very low pH and has been given several different names, e.g., folypolyglutamate hydrolase (FPGH), γ-glutamyl hydrolase and γ-Glu-X carboxypeptidase. Since FPGH counteracts the reactions catalyzed by FPGS, enhanced activity of this hydrolase can be expected to lower intracellular accumulation of polyglutamated MTX and thereby confer different degrees of tolerance. Indeed since 1993, several reports have emphasized the contribution of high FPGH activity to cellular resistance to MTX (Li, 1993; Rhee, 1993), as well as to other non-classic antifolates DDATHF whose cytotoxicity requires polyglutamylation (Pizzorno, 1995; Rhee, 1993).

However, transfection of HT-1080 (a human fibroblastoma) or MCF-7 (a breast cancer) cell lines with FPGH cDNA does not confer resistance to short-term exposure to MTX (Cole, 2001). Since in both of these cell lines, the alterations in the accumulation of MTX resulting from overexpression of FPGH were accompanied by changes in accumulation of folate, these investigators proposed that the ratio of MTX/folate may be a better predictor of MTX cytotoxicity than the level of either these substances alone. Clinical studies on patients with leukaemia have revealed that the ratio of FPGS/FPGH activity at the time of diagnosis is a useful predictor of response to MTX and therapeutic outcome (Longo, 1997; Rots, 1999).

2.1.1.5 Influence of the size of tetrahydrofolate (THF)-cofactor pools on the response to antifolates

Modulation of the activity of antifolates as a consequence of alterations in the size of THF-cofactor pools are well known to occur (Johnson, 1988; Nimec, 1983; Zhao, 2001). This pool exerts a regulatory effect on the synthesis of MTX polyglutamates (Balinska, 1986) and thereby the toxicity of antifolates (Balinska, 1988). Large pools are associated with resistance in particular to antifolates that require polyglutamylation for their effect (Assaraf, 1997), while cells grown in the presence of low levels of folic acid are more sensitive to MTX and many other antifolates (Jansen, 1998). In one such study, where L1210 cells were cultured in the presence of different concentrations of 5-methylenetetrahydrofolate (5-CHO-THF), the intracellular concentrations of DDATHF and pemetrexed attained were negatively correlated to intracellular levels of folate, with enlargement of the folate pool resulting in much higher IC_{50} values for these agents (Zhao, 2001). In contrast, the size of the folate pool had no influence on the cytotoxicity of the polyglutamate-independent antifolate ZD9331 and the effect on MTX toxicity was slight.

In fact, the influence of intracellular THF cofactor pools on the cytotoxicity of Polyglutamylation-dependent drugs is more pronounced when the period of exposure is too short to allow significant accumulation of the polyglutamated forms of these drugs (Zhao, 2001). Interestingly, various groups have reported that cells which are resistant to MTX, as a consequence of a defect in RFC-mediated transport exhibit enhanced sensitivity to lipophilic antifolates whose uptake is not impaired by this defect. For instance, transport- defective cells resistant to MTX are more sensitive to DDATHF (Dixon, 1991), as well as to two other lipophilic antifolates, AG377 and trimetrexate (Rothem, 2002). Under such circumstances, the defect in RFC leads to depletion of the intracellular folate pool and, in this way to lesser competition for interaction with DHFR.

In the clinic, the levels of folate in the plasma or erythrocytes prior to treatment have not been useful in predicting which patients will develop toxicity following administration of antoifolates. However supplementation with folic acid can protect patients receiving low doses of MTX from toxicity without lessening the efficacy of the drug (Dijkmans, 1995; Morgan, 1994). In a similar manner, oral co-administration of folic acid diminishes the clinical toxicity of DDATHF (Wedge, 1995) and pemetrexed (Calvert, 2002).

2.1.1.6 Dihydrofolate reductase

DHFR catalyses the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, an essential step in *de novo* synthesis of glycine and of purines and deoxythymidine phosphate, which are precursors for DNA synthesis. MTX is believed to exert its cytotoxicity primarily through inhibition of DHFR (Jolivet, 1983). Such inhibition should decrease intracellular levels of reduced folates, augment accumulation of dihydrofolate, attenuate *de novo* synthesis of precursors required for DNA synthesis and lead finally to cell death.

At the same time sustained exposure to MTX can evoke an increase in the activity of DHFR (Alt, 1976) as a result of gene amplification, thereby altering the response to MTX (Alt, 1978; Schimke, 1988; Srimatkandada, 1983). This phenomenon has been observed in tissue specimens of the patients with ALL (Carman, 1984; Horns, 1984) and ovarian cancer (Trent, 1984), treated with ALL and probably contributes to clinical resistance to this drug. An alternative mechanism for such resistance are mutations which decrease the affinity of DHFR for MTX (Jackson, 1976) without decreasing the catalytic activity of this enzyme to the same extent (McIvor, 1990; Melera, 1984; Miyachi, 1995; Simonsen, 1983). However, such mutations have not to date been shown to be clinically relevant.

2.1.1.7 Thymidylate synthase (TS)

TS catalyses the conversion of 5,10- methylene THF to dihydrofolate, as well as of deoxyuridine monophosphate to deoxythymidine monophosphate. Since the latter reaction is the sole source of deoxythymidylate for DNA biosyntesis, TS has become an important target for cancer chemotherapy. Inhibition of this enzyme leads to depletion of deoxythymidine triphosphate enhanced misincorporation of uracil into nucleic acids subsequent chromosomal damage and apoptotic cell death (Van Triest, 2000; Welsh, 2000). MTXGLs can inhibit TS, by binding directly to this enzyme, whereas the monoglutamate form of this drug inhibits TS indirectly by diminishing the level of 5,10-methylene THF.

Augmentation of TS activity, which is usually due to gene amplification, is a common mechanism underlying resistance to MTX (Ayusawa, 1981), and the major mechanism of resistance to other agents that target TS i.e. fluorouracil, pemetrexed, AG337, ZD1694 and ZD9331 (Kitchens, 1999; O'Connor, 1992; Tong, 1998). Alterations in the stability of the TS

polypeptide accompany, indeed, even contribute to resistance to inhibitors of this enzyme acquired by colon tumor cells (Kitchens, 1999). Such resistance can result from mutations in the TS gene that alter drug binding (Barbour, 1992) or produce an unstable enzyme molecule that is rapidly degraded, lowering TS levels (Kitchens, 1999).

In a clinical study Krajinovic and colleagues (Krajinovic, 2002) demonstrated an association between a tandem-repeat polymorphism in the promoter of the TS gene with a homozygous triple repeat leading to enhanced expression of this enzyme and poorer outcome for children with ALL treated with MTX. These investigators concluded that genotyping might help to provide appropriate individualized treatment for patients with ALL. In agreement with their findings, a higher frequency of the homozygous triple repeat is present in patients with rheumatoid arthritis who require higher doses of MTX for successful treatment (Kumagai, 2003).

Recent studies have revealed that certain mutations in TS lead to highly selective differences in the binding of various inhibitors. Thus an HT1080 cell line resistant to AG337 and carrying Lys47Glu and Asp49Gly mutations was cross-resistant to fluorouracil, but not to ZD1694 or GW1843U89 (Tong, 1998). Such selective effects have been confirmed by site-directed mutagenesis of highly conserved residues. With this approach, Ile108Ala mutation was found to confer high level of resistance to ZD1694 and AG337, but not to GW1843U89; while a Phe225Trp mutation resulted in a 17-fold elevation in resistance to GW1843U89, but less enhancement of resistance to fluorouracil (Tong, 1998).

2.1.1.8 Pharmacogenetic determinants of the response to MTX

The predictive value of several genetic variants and gene-gene interactions with respect to response to MTX therapy has been examined. The genes studied most extensively in this context are those encoding 5,10-methylenetetrahydrofolate reductase (MTHFR) and TS. MTHFR converts 5,10-methylene-THF into 5-methyl-THF, the major circulating form of folate and the source of the methyl group for homocysteine methylation (**Figure 3**).

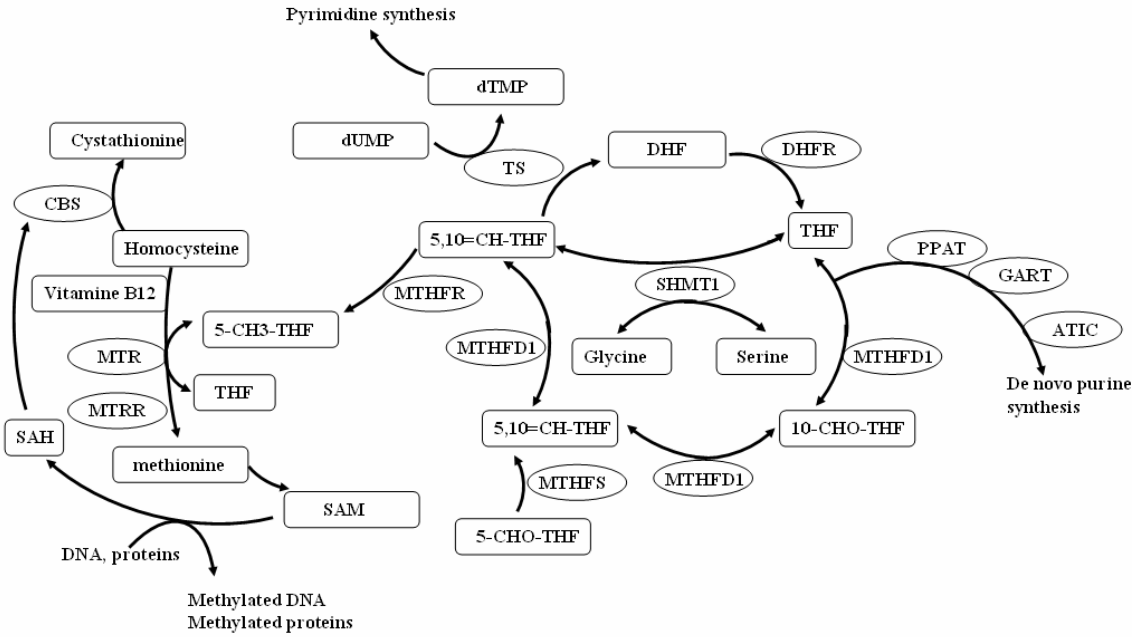

Figure 3. *Schemate illustration of folate pathway and its relationship to purine, pyrimidine and methionine biosynthesis. DHFR, dihydrofolate reductase; DHF, dihydrofolate; THF, tetrahydrofolate; SAM, S-adenosylmethionine; TS, thymidylate synthetase; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; SAH, S-adenosylhomocysteine; MTHFD 1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, 5,10-methylenetetrahydrofolate reductase; GARFT, phosphoribosylglycinamide formyltransferase; MTHFS; 5,10-methenyltetrahydrofolate synthetase, MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; ATIC (AICART), 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; SHMT 1, serine hydroxymethyltransferase 1; PPAT, phosphoribosyl pyrophosphate amidotransferase; CBS, cystathionine β-synthase; 10-CHO-THF, 10- formyltetrahydrofolate; 5,10=CH-THF, 5,10 methylen tetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; 5-CH3-THF, 5 methyl tetrahydrofolate*

MTHFR plays a vital role in channelling single-carbon units to methylation reactions (Schwahn, 2001) and alterations in the activity of this enzyme can lead to an imbalance in the folate pools (Bailey, 1999), which, as explained above, might result in altered sensitivity to MTX. Two common polymorphisms in the MTHFR gene i.e., a C677 to T (Frosst, 1995) and an A1298 to C (van der Put, 1998; Weisberg, 1998) transition, have been described.

Homozygous TT677 or CC1298 cells exhibit reduced enzyme activity, while in heterozygous individuals this activity is also decreased, but to a lesser extent (Frosst, 1995; van der Put, 1998; Weisberg, 1998). The influence of these polymorphisms and, in particular that of the C677T substitution on the pharmacodynamics of antifolates, both in vitro and in connection with clinical response to and outcome of MTX treatment, have been characterized by a number of investigators (Taub, 2002; Schmeling, 2005). Possibly, other polymorphisms in the MTHFR or other genes (e.g. those encoding cystathionine-β-synthase and methionine synthase) might alter the reduced folate pathway and thereby the cytotoxicity of MTX (Taub, 2002).

Furthermore, in CCRF-CEM cells a change of G to T at position 1037 in FPGS cDNA resulting in the substitution of Phe for the native Cys at residue 346 (Cys346Phe), can give rise to resistance to polyglutamylation-dependent antifolates (Cys346Phe) (Liani, 2003). The potential influence of this mutation on clinical response to MTX therapy and the risk for relapse is not yet known.

Since the primary mechanism underlying the cytotoxicity of MTX involves tight binding to and inhibition of the enzyme DHFR, an elevation in the intracellular level of DHFR would be expected to cause resistance to MTX. Indeed, the copy number of the DHFR gene may be a determinant for the response to and toxicity associated with MTX treatment (Banerjee, 2002). On the other hand, a minor study has indicated that mutations in DHFR are unlikely to be a major mechanism for acquired resistance in patients exposed to MTX (Spencer, 1996).

No polymorphism in GARFT or AICART, the two enzymes essential for PDNS, has yet been described.

In addition to alterations in genes whose products are involved in the metabolism of folate and MTX, changes in certain other genes, e.g., those whose products regulate the cell cycle or participate in the apoptotic cascade have been reported to contribute to resistance to MTX. Thus, in 1995 Li and coworkers (Li, 1995) observed several-fold higher IC_{50} values for MTX and 5-fluorodeoxyuridine in two different cell lines expressing no or non-functional retinoblastoma protein, which is involved in regulating the S-phase of the cell cycle. The same cell lines that lacked retinoblastoma protein, expressed high levels of free E2F, which binds to the DHFR and TS promoters and activates transcription of these genes. Moreover, elevated expression of Bcl-XL and Bcl-2, which regulate apoptosis (Simonian, 1997), as well as mutations in the p53 gene can provide various degrees of tolerance to the toxic effects of MTX (Yeager, 1998).

2.1.1.9 Interactions between MTX and its metabolites

In light of the fact that, like MTX and reduced folate cofactors, 7-OHMTX is taken up by cells via the RFC (Fabre, 1983), this metabolite can compete for such uptake, as well as for intracellular pathways of metabolism. Concentrations of 7-OHMTX that are approximately 3-fold higher than those of MTX have been detected in the bone marrows of children 24 hours after receiving an oral dose of MTX (Sonneveld, 1986). This metabolite is a good substrate for FPGS, being polyglutamylated at least at the same rate and at least to the same extent as MTX (Fabre, 1984; Fabre, 1983), and is therefore speculated to compete with MTX and natural folates for polyglutamylation. Furthermore, the half life of 7-OHMTX in the body is about 3 times longer than that of MTX (Breithaupt, 1982) which allows this metabolite to interfere with the effects of MTX for the entire period that this drug remains in the body.

2.1.1.10 7-OHMTX may be a cytotoxic metabolite

Due to the low affinity of its binding to DHFR, 7-OHMTX has been considered to be an inactive metabolite of MTX (Johns, 1967; Redetzki, 1966). Indeed, 7-OHMTX is more than 100-fold less cytotoxic towards cells than MTX. However, as mentioned above, the half-life of 7-OHMTX is much longer (Breithaupt, 1982) and following HDMTX therapy, the plasma concentration of 7-OHMTX can be several-fold higher (>1 μM for as long as a few days) than that of MTX (Lankelma, 1980), i.e., sufficient to exert toxic effects. Furthermore, polyglutamylated form of 7-OHMTX are much better inhibitors of DHFR than the monoglutamated form (Drake, 1987). Thus, the ki values for inhibition of TS and AICART by 7-OHMTXGLs are <1μM, so that these enzymes of PDNS can also be inhibited. Despite all these indications that 7-OHMTX may act as a cytotoxic agent following HDMTX therapy, the possible mechanisms of action and ability of this metabolite to induce resistance in leukemic cells have simply been neglected.

2.1.2 Novel antifolates

On the basis of the observations described above, newer antifolates are designed to possess one or more of the following properties: (1) more effective transport into the cell due either to increased affinity for the RFC or enhanced lipophilicity which lessens dependency on RFC-mediated transport (e.g., trimetrexate and AG337); (2) cytotoxicity that is independent of polyglutamylation (e.g., trimetrexate, ZD9331 and AG337); and/or (3) more extensive polyglutamylation due to higher affinity for FPGS and, consequently, more potent inhibition of DHFR and/or TS, and/or enhanced ability to inhibit a number of other targets (such as enzymes responsible for purine and pyrimidine synthesis) (e.g., edatrexate, pemetrexed, lometrexol and LY309887). All novel antifolates in various stages of development exhibit their own unique clinical pharmacology. In 2004, the FDA approved the use of pemetrexed for treatment of malignant pleural mesothelioma in patients who are not candidates for resection or curative surgery.

Although the major target in connection with the development of novel antifolates has been DHFR and TS, other critical folate-dependent enzymes, such as methionine synthetase, MTHFR and FPGS, should also be considered in this context.

However, despite the fact that some promising results with novel antifolates have been achieved during the past two decades, the most significant improvements in cancer chemotherapy have been based on optimization of available protocols rather than administration of new drugs.

2.2. Thiopurines

The antimetabolite thiopurines 6-MP and 6-TG have been commonly used as cytotoxic agents against leukaemia for the past 45 years. Azathioprine (AZA), a derivative of 6-MP, is a commonly used immunosuppressive drug. 6-MP and 6-TG were originally synthesized by Elion and Hutchins in 1951 (Elion, 1951) by replacement of the oxygen atom at carbon 6 of hypoxanthine or guanine, respectively, by sulfur (**Figure 4**).

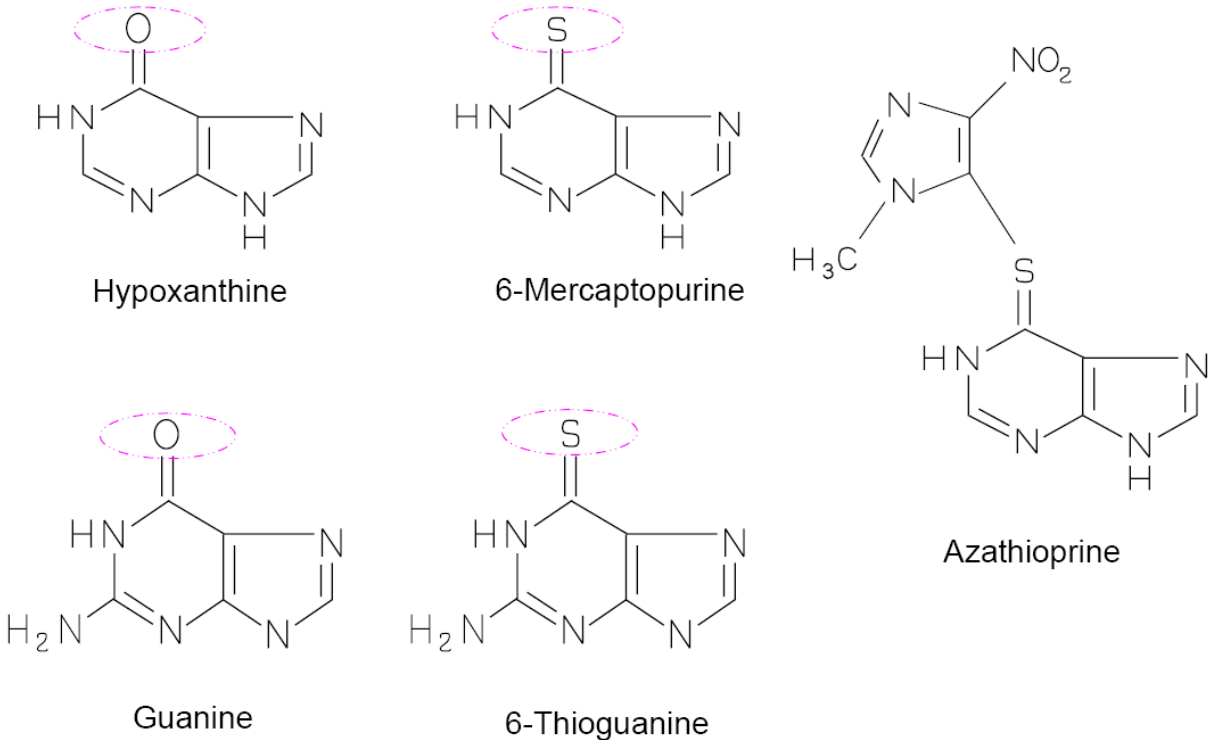

Figure 4. *Chemical structures of the thiopurines and the corresponding endogenous purines*

The first clinical trial with 6-MP involved oral administration and demonstrated a beneficial effect in connection with treatment of acute leukaemia in children (Burchenal, 1953). Subsequently, extensive experience in the administration of 6-MP was obtained and in

the 1960's this drug was established as the backbone of orally administered maintenance therapy of ALL (Elion, 1967).

In 1948, Elion, and Hitchings found that 6-TG is effectively inhibits the growth of both the bacterium *Lactobacillus (L.) casei* and tumors in mice. Later, 6-TG was shown to produce good clinical remission of granulocytic leukemia in two adults, although with severe side-effects, including nausea, vomiting and suppression of the bone marrow (Burchenal, 1951). On the basis of empirical evidence, 6-TG very soon became part of the routine treatment for AML (Brox, 1981).

Combination of 6-MP with MTX and steroids (the only drugs available for treatment of acute leukemia at the time of introduction of thiopurines) extended the median survival time from 3 to 12 months. Only two years after its original synthesis and the initial anti-microbiological investigations, 6-MP was approved by the authorities for use in the treatment of childhood ALL (Elion, 1989).

In 1958 Schwartz et al. (Schwartz, 1958) demonstrated the immunosuppressive ability of 6-MP to prevent an antibody response in rabbit injected with an antigen. Shortly thereafter, the Hitchings-Elion laboratory synthesized numerous 6-MP derivatives, including azathioprine (AZA, 6-(1-methyl-4-nitro-5-imidazolylthio), a prodrug that is reduced non-enzymatically to 6-MP *in vivo* (Lennard, 1992; Remy, 1963; Woodson, 1983) (**Figure 4**). The first comparison of AZA and 6-MP as immunosuppressors was performed on 10 dogs that had received renal transplants. AZA had the better effect in this system (Calne, 1962) and was soon found to be an effective immunosuppressive agent in humans as well (Murray, 1963).

Today, thiopurines are widely used to treat ALL of childhood, inflammatory bowel disease and autoimmune diseases, as well as to prevent rejection of organ transplants (Coulthard, 2005; Lennard, 1992). These drugs, like many cytotoxic agents, have a relatively narrow therapeutic index, with potential life-threatening drug-induced toxicity, primarily in the form of myelosuppression (Evans, 1991; Lennard, 1992; Lennard, 1989). The other major toxic effect of thiopurines is their hepatotoxicity (Einhorn, 1964) which has been reported to be related to the amounts of thioguanine nucleotides (TGNs) (Rulyak, 2003), or methylmercaptopurine (meMP) (Dubinsky, 2000) in erythrocytes and to the accumulation of 6-MP and its metabolites in the liver (Berkovitch, 1996). 6-MP is now used as a routine component of all modern protocols for maintenance therapy of children with ALL (Evans, 2001) and the combination of high-dose MTX and 6-MP is commonly employed for consolidation therapy of childhood ALL (Nachman, 1998; Schrappe, 2000). 6-TG is

administered for induction of remission and maintenance treatment of patients with AML (Coulthard, 2002).

2.2.1. Metabolism

Thiopurines are inactive prodrugs that exert their cytotoxicity only after intracellular metabolism to active metabolites (Lennard, 1983). Following oral administration, 6-MP undergoes extensive intestinal and hepatic metabolism by aldehyde oxidase (AO) and xanthine oxidase (XO), which convert this substance into rather inactive metabolites, leaving only about 16% of the total dose of 6-MP available for systemic distribution. The XO activity of bone marrow is insignificant, and plays a negligible role in the intracellular metabolism of thiopurines in leukemic cells. XO can oxidase 6-TG only after its prior conversion to 6-thioxanthine by guanase (Coulthard, 2002) (**Figure 5**).

The intracellular metabolites contribute to the effect of thiopurines either by inhibiting PDNS or by being incorporated into DNA (Elion, 1989). In the case of oral administration of AZA, the main product is 6-MP, but humans can metabolize as much as up to 12% of this compound to hypoxanthine and methyl-4-nitro-5-thioimidazole, which may contribute to the immunosuppressive effects (Elion, 1972). 6-MP requires activation to 6-thioinosine 5′ monophosphate (TIMP) by hypoxanthine-guanine phosphoribosyl transferase (HGPRT); followed by a multi-step conversion to therapeutically active TGNs in order to exert its cytotoxity effects (Tidd, 1974, a).

TIMP is converted to 6-thioguanosine 5′-monophosphate (TGMP) in a two-step process involving inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase (GMPS) (Elion, 1989) that is dependent on glutamine and energy (Adamson, 1993). The important difference between this metabolic pathway for 6-MP with metabolism of TG is that TIMP undergoes methylation by thiopurine methyltransferase (TPMT) to produce methylthioinosine monophosphate (meTIMP), which inhibits PDNS potently (Bokkerink, 1993; Tay, 1969). 6-TG is converted by HGPRT directly into 6-thioguanosine 5′-monophosphate (TGMP), which is then phosphorylated further by two kinases to yield 6-thioguanosine 5’triphosphate (TGTP), which can either be incorporated into RNA or, following enzymatic reduction to deoxy-6-thioguanosine 5’triphosphate (dTGTP), incorporates into DNA (**Figure 5**).

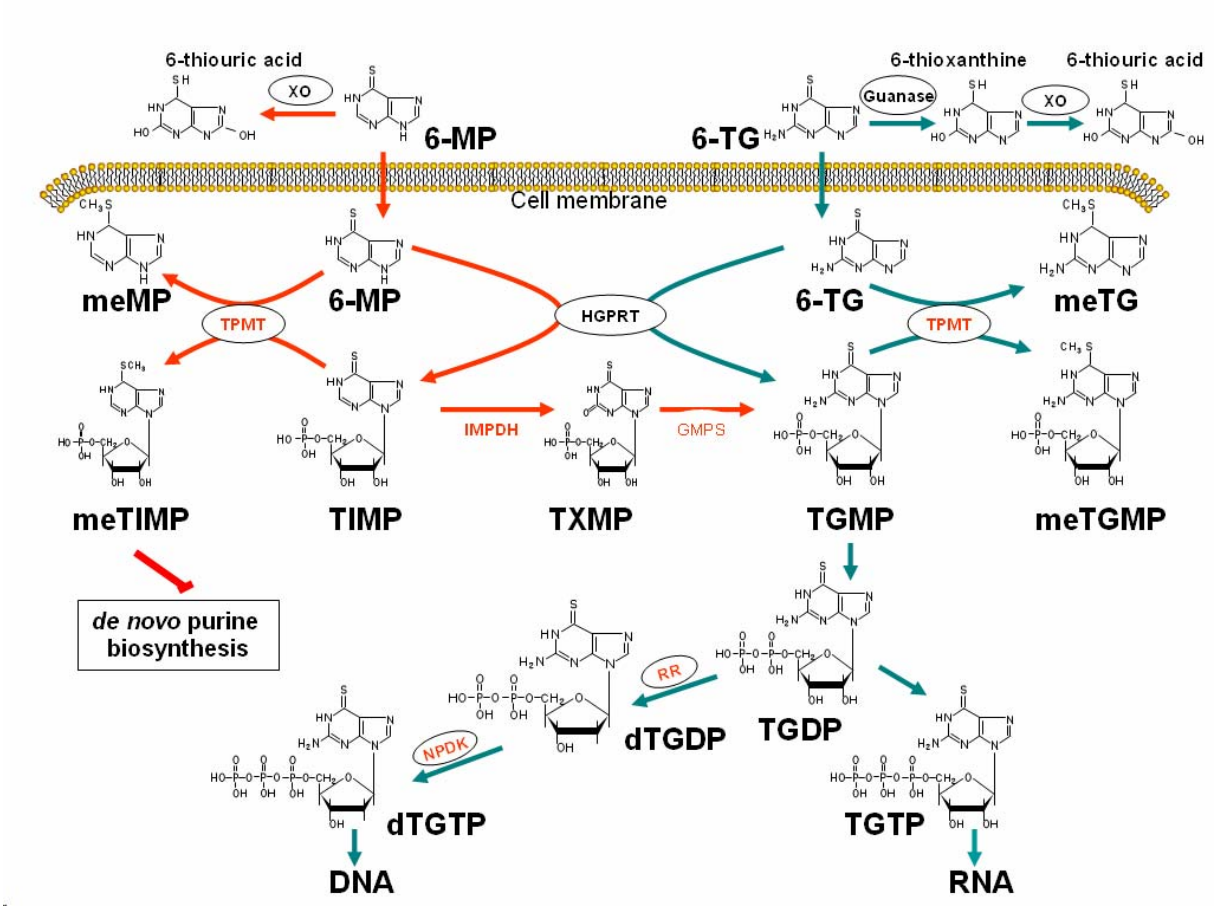

Figure 5. *Pathways for metabolism of thiopurines*

XO, xanthine oxidase; meMP, methyl mercaptopurine; TPMT, thiopurine methyltransferase; HGPRT, hypoxanthine–guanine phosphoribosyl transferase; IMPDH, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; meTIMP, methylthioinosine monophosphate; TGMP, 6-thioguanosine-5'-monophosphate; meTG, methyl thioguanine; TIMP, 6-thioinosine-5'-monophosphate; RR, ribonucleotide reductase; NDPK, nucleoside diphosphate kinase; TXMP, 6-thioxanthosine-5'-monophosphate; TGDP, 6-thioguanosine-5'-diphosphate; TGTP, 6-thioguanosine-5'-triphosphate; dTGDP, deoxy-6-thioguanosine-5'-diphosphate; dTGTP, deoxy-6-thioguanosine-5'-triphosphate; meTGMP, S-methylthioguanosine-5'-monophosphate.

Incorporation of TGNs into DNA triggers cell cycle arrest and apoptosis by a process involving the mismatch repair (MMR) pathway (Swann, 1996). In this connection at least three enzymes compete with HGPRT (Fig. 2): first, 6-MP and 6-TG can undergo methylation by the enzyme TPMT to produce the inactive metabolites meMP and 6-methylthioguanine (meTG) (Dervieux, 2001), which are not substrates for HGPRT. Secondly, XO can metabolise 6-MP to inactive thiouric acid (Elion, 1967), which, however, does not happen to

any significant extent in the hematopoietic tissue. And, third, AO can convert 6-MP and 6-TG into 8-hydroxythioguanine (Coulthard, 2002). In the case of 6-MP, it can apparently oxidise only the methylated form, i.e., convert meMP to me-8-hydroxypurine, but not 6-MP itself (Keuzenkamp-Jansen, 1996; Kitchen, 1999).

Because of the lower cytotoxic potency of meMP, methylated metabolites were considered at first to be less cytotoxic (Stet, 1993). Later on, several groups established that certain methylated metabolites are actually more cytotoxic and it is now clear that methylation can contribute to the cytotoxic effects of thiopurines, both *in vivo* and *in vitro* (Dervieux, 2001). One of the methylated metabolites of 6-MP, meMPR, is a well-characterized and potent inhibitor of PDNS and cytotoxic agent that contributes significantly to the cytocidal effects of 6-MP.

2.2.1.1. Hypoxanthine-guanine phosphoribosyltransferase (HGPRT)

HGPRT is a purine salvage enzyme that catalyses the conversion of hypoxanthine and guanine to their respective mononucleotides. A partial deficiency in this enzyme, often caused by a single base mutation (Wilson, 1983), can result in overproduction of uric acid, leading to hyperuricemia, hereditary gouty arthritis and nephrolithiasis (Kelley, 1967); whereas virtual absence of HGPRT activity (i.e., the X-linked recessive Lesch-Nyhan syndrome) is characterised by hyperuricaemia, mental retardation, choreoathetosis and compulsive self-mutilation (Lesch, 1964). AZA and 6-MP are not cytotoxic in these patients (Nyhan, 1968). HGPRT is expressed widely throughout the human body, with highest levels in the central nervous system, 4-8-fold lower levels in erythrocytes and lymphocytes, and 16-20-fold lower levels in the liver, kidney and spleen (Cory, 1986).

In children with leukaemia this enzyme activity increases in response to long-term treatment with 6-MP. When Lennard et al. (Lennard, 1993) compared erythrocyte HGPRT activity in 86 control children and 63 children with ALL, the leukaemic children were found to demonstrate significantly higher activity, but this activity was not correlated to the production of 6-TGNs. Since HGPRT catalyses the initial metabolic activation of 6-MP to 6-TGNs, lack of this activity results in resistance of leukemic cells to both 6-MP and 6-TG (Lennard, 1992).

2.2.1.2. Thiopurine methyltransferase (TPMT)

TPMT is a cytosolic enzyme whose physiological role, despite extensive investigations remains unclear (Evans, 1999). However, this enzyme is known to catalyse S-methylation of aromatic and heterocyclic compounds, preferentially thio compounds such as 6-MP and 6-TG (Remy, 1967; Woodson, 1983). It has a molecular mass of 26 kDa and is expressed in the liver, kidneys, intestine, erythrocytes, leukocytes and a number of other tissues.

The discovery that levels of TPMT activity in human tissues are influenced by a common genetic polymorphism represents the most important example of the influence of pharmacogenetics on anti-cancer therapy as well as one of the dearest examples of the potential importance of pharmacogenetics to clinical medicine in general (Weinshilboum, 1980) Specifically, it is now known that a reduction in TPMT activity, caused by such genetic polymorphism results in severe and sometimes fatal haematological toxicity in patients treated with standard doses of thiopurines (Evans, 1991; Schutz, 1993) so the dose must be decreased for patients with heterozygous or homozygous mutation in the TPMT gene. On the other hand, patients with very high TPMT activity may be undertreated.

Pronounced inherited variations in TPMT activity, ranging from high to virtually undetectable, were first observed in human tissues more than two decades ago. The frequency of distribution of erythrocyte TPMT activity in 298 control subjects was found to be trimodal. Approximately one in 300 individuals (0.3%) has low or undetectable levels of this activity, which is intermediate in approximately 10% of the general population (Weinshilboum, 1980).

The level of TPMT activity in erythrocytes reflects the corresponding levels in the kidney, liver and lymphocytes (Coulthard, 1998; Szumlanski, 1992; Van Loon, 1982; Woodson, 1982). Cloning and characterization of the human TPMT cDNA and gene revealed that these phenotypic variations were primarily from variation in the sequence of the gene itself (Honchel, 1993; Szumlanski, 1996; Tai, 1996). A total of 21 such genetic polymorphisms have been identified which are or may be associated with decreased levels of TPMT enzyme activity and/or enhanced toxicity of thiopurines have now been identified (Salavaggione, 2005).

Variant human alleles associated with decreased catalytic activity of TPMT involve point mutations in the open-reading frame or at intron/exon splice sites. The wild-type allele, TPMT*1, encodes the fully active enzyme; while TPMT*2 (238G4C), TPMT*3A (460G4A, 719A4G) and TPMT*3C (719A4G) are the most prevalent accounting together for (80–95%) of the polymorphic alleles that lead to a significant reduction in enzyme activity (Yates,

1997). TPMT*3B (460G4A) is much rare and the remaining TPMT variants can be considered to be family-specific ('private') mutations found in individuals belonging to various ethnic groups (Hon, 1999; McLeod, 1994; Otterness, 1997; Spire-Vayron de la Moureyre, 1998).

The important impact of TPMT polymorphism on ALL treatment is connected to the inverse relationship between the TPMT activity and TGN concentrations in the erythrocytes of children treated for leukaemia (Lennard, 1987). High erythrocyte concentrations of TGNs are correlated with the degree of leucopenia and a good prognosis (Lilleyman, 1994); whereas low concentrations are associated with an increased risk for relapse (Bostrom, 1993). Other alterations detected in the TPMT gene include deletion of exons six and nine (Krynetski, 1997) and polymorphisms in the variable number of tandem repeats (Alves, 2001; Spire-Vayron de la Moureyre, 1999; Yan, 2000).

2.2.1.3. Inosine 5'-monophosphate dehydrogenase (IMPDH)

IMPDH catalyzes the first rate-limiting step in guanine nucleotide biosynthesis (Jackson, 1975); i.e., the conversion of inosine monophosphate to xanthosine monophosphate, (with a km value of 14 μM for IMP (Holmes, 1974)). Human IMPDH activity is catalysed by two separate 56-kDa enzymes, termed type I and II, which exhibit virtually identical catalytic activities, substrate affinities and ki values and are 84% identical at the amino-acid level (Carr, 1993; Collart, 1988; Hager, 1995; Natsumeda, 1990). Variation in the expression of either of these forms can be expected to exert a significant influence on thiopurine metabolism, with increased activity promoting toxicity and reduced activity predicting a poor clinical response. IMPDH activity increases in connection with cell proliferation and transformation (Collart, 1992; Jackson, 1975; Natsumeda, 1988) and is higher in acute leukemic blasts than in a mixture of normal bone marrow cells (Price, 1987). Inhibition of this enzyme in HL60 myeloid cells results in a low intracellular concentration of GTP and terminal differentiation of the cells (Lucas, 1983) and chemical inhibition of this enzyme in leukemic cells hampers their growth at micromolar concentrations of inhibitor (Yamada, 1990).

Since TIMP, the major intracellular metabolite of 6-MP, is a substrate for IMPDH, the activity of this enzyme may play an important role in connection with treatment of patients with antipurines. Approximately 9% of patients with inflammatory bowel disease who are

resistant to AZA may carry mutations in enzymes involved in drug metabolism, including IMPDH. Indeed a 9-bp insertion into the IMPDH1 P3 promoter was detected in one patient who exhibited severe resistance to AZA (Roberts, 2006). However, to date no significant correlation between altered IMPDH activity and resistance to thiopurines has been demonstrated, either *in vitro* or *in vivo*.

Mizoribine and mycophenolic acid are highly specific inhibitors of IMPDH, that block cell proliferation directly by causing depletion of guanine ribonucleotides. 6-MP, on the other hand, exerts mixed effects on the pools of adenine and guanine ribonucleotide, while azathioprine inhibits cell proliferation via a mechanism completely independent of its effects on purine metabolism. Inhibitors of IMPDH have potential for use as specific immunosuppressive agents (Mitchell, 1993).

2.2.1.4. Guanine monophosphate synthetase (GMPS)

GMPS catalyzes the amination of xanthosine 5'-monophosphate to guanosine monophosphate, and like IMPDH, is a crucial enzyme in the *de novo* biosynthesis of guanine nucleotides. The level of mRNA expression is substantially higher in rapidly proliferating, such as neoplastic and regenerating tissues (Weber, 1983; Weber, 1992). Since inhibition of this enzyme results in depletion of guanine nucleotides and cell proliferation in lymphocytes (Yu, 1989; Mitchell, 1993), it can be a target for immunosuppression and cancer chemotherapy. GMPS converts TXMP to TGMP in the process of intracellular metabolism of 6-MP. However, contribution of alterations of activity of this enzyme with efficacy of 6-MP has not been studied.

2.2.2. Transport

Earlier studies indicated that cellular uptake of 6-MP occurs primarily by passive diffusion (Bieber, 1964; Sasaki, 1986), but Plagemann and collageous (Plagemann, 1981) discovered facilitated diffusion of this compound that can be competitively inhibited by hypoxanthine. These investigators found that 6-MP is rapidly transported into cells and phosphoribosylated, after which the main rate-determining step in its incorporation into nucleic acids is further conversion to 6-MP riboside 5'-monophosphate. Employing in vitro

and in situ techniques, Ravis and coworkers (Ravis, 1984) demonstrated the absence of active secretion or absorption of 6-MP by the rat intestine.

Since the chemical structures of 6-MP and 6-TG are very similar to those of hypoxanthine and guanine, these drugs can be expected to be taken up into and exported from cells by the same transporters that translocate these nucleobases. The most extensively characterised families of transporters that mediate uptake of nucleosides and nucleobases are the nucleoside transporters, which can be subdivided into two major classes: equilibrative (facilitated) transporters (the SLC29 family) that mediate the net flux of nucleoside molecules across the plasma membrane only down a concentration gradient; and concentrative or Na^+-dependent transporters (the SLC28 family) which use an electrochemical ion gradient to drive active uptake even against a concentration gradient (Baldwin, 1999). The human family of equilibrative transporters contains four members for which the well-characterized ENT1 and ENT2 exhibited similar broad specificities for purine and pyrimidine nucleosides, with ENT2 possessing the ability to transport nucleobases as well (Baldwin, 2004). Nanomolar concentrations of nitrobenzylthioinosine (NBTI) or dipyridamole potentially inhibit ENT1, whereas NBTI cannot inhibit ENT2 efficiently, even at higher concentrations (Baldwin, 1999). The family of concentrative nucleoside transporters consists of three subtypes of sodium-dependent transporters: CNT1 transports pyrimidine nucleosides preferentially, CNT2 prefers purine nucleosides, and CNT3 transports both these types of nucleosides across the cell membrane (Gray, 2004) (**Figure 6**).

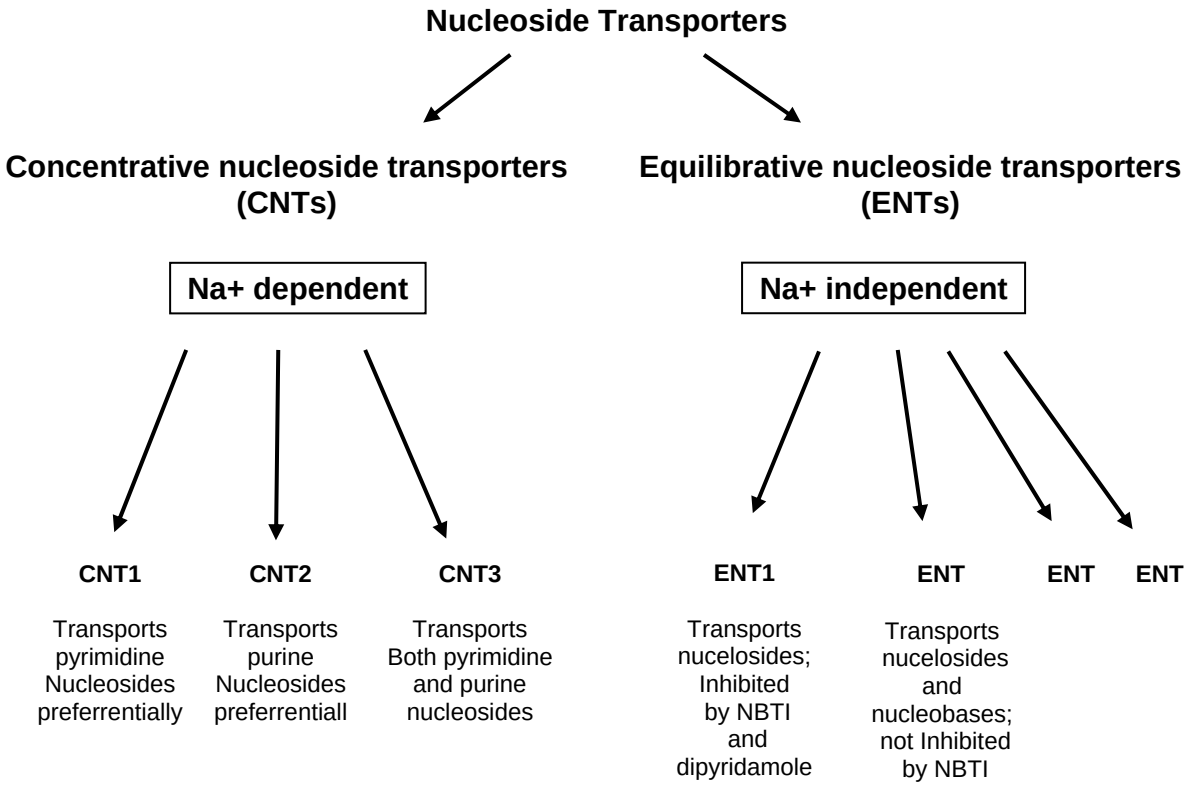

Figure 6. *Classification of the human nucleoside transporters*

Recently, Zaza and colleagues (Zaza, 2005) identified 60 species of mRNA whose level of expression was significantly correlated with accumulation of TGNs in ALL patients treated with 6-MP. Down-regulation of the gene encoding ENT1 was associated with low intracellular concentrations of TGNs. This same study revealed that inhibition of ENT1 by NBTI causes a significant reduction of the level of TGNs in ALL cells *in vitro*, suggesting that this transporter protein plays a role in clinical response to 6-MP therapy.

The family of MRPs now consists of nine multi-specific drug transporters, designated, MRP1–9 (see the review by Borst et al. (Borst, 2000)). Although the ability of several of these transmembrane proteins to transport a wide variety of anticancer drugs out of cells and their expression by many different types of tumor cells make them prime suspects in unexplained cases of drug resistance, proof that they contribute to clinical drug resistance is still lacking. Overexpression of the related MRP4 (Chen, 2001) and MRP5 (Wijnholds, 2000) does confer a certain degree of resistance against 6-MP and 6-TG. Furthermore, the resistance of human embryonic kidney cells to 6-MP and 6-TG resulting from transfection with MRP4 and MRP5 cDNAs appears to reflect extrusion of metabolites of 6-MP by these transporters (Wielinga, 2002).

Several years ago, Deguchi and colleagues (Deguchi, 2000) proposed that the limited accumulation of 6-MP in the brain is due to efficient efflux of this drug from this organ. The organic anion transporter-3 expressed by cells of the blood-brain barrier is likely to be involved in the transport of anionic drugs such as 6-MP and acyclovir (Ohtsuki, 2003). Brain-to-blood efflux of thiopurine nucleobase analogues via this transporter may be one mechanism underlying the limited accumulation of these drugs in the brain, and could contribute to the proliferation of leukemic cells in this organ and relapse during chemotherapy (Mori, 2004).

2.2.3. Other pharmacogenetic influences on thiopurine metabolism

Systematic consideration of each of the enzymes involved in the degradation of thiopurines reveals a number of plausible candidate enzymes whose levels of expression might vary as a result of allelic polymorphism. Among these, a deficiency in 5´-nucleotidase enzymes is associated with enhanced thiopurine toxicity (Kerstens, 1995), but the genetic basis for this phenomenon has not been determined. Inhibition of XO and AO by allopurinol also potentiates thiopurine toxicity (Cummins, 1996), but genetic deficiencies in this enzymes, manifested as xanthinuria, are very rare. However, the level of expression of XO in the liver does vary considerably between individuals and an as-yet-undiscovered polymorphism may be involved here (Guerciolini, 1991).

Inosine triphosphate pyrophosphatase (ITPase) converts inosine triphosphate (ITP) back to IMP, thereby preventing accumulation of ITP. Recently, Marinaky and coworkers (Marinaki, 2004) predicted that in ITPase-deficient patients treated with thiopurine drugs, the metabolite 6-thio-ITP would accumulate and give rise to toxicity. Indeed, these investigators found that an *ITPA* 94 C>A heterozygous genotype was significantly associated with adverse effects of AZA, especially flu-like illness, pancreatitis and rash. However, not all researchers have observed this same association (Allorge, 2005; Gearry, 2004).

Mechanisms of cytotoxicity

It has been postulated that the primary cytotoxic effect of 6-MP results from negative feed-back on PDNS (Hamilton, 1954). At the same time, feedback inhibition of PDNS by TIMP and TGMP reduces endogenous levels of purines, which has been proposed to be the mechanism of action of 6-MP (Skipper, 1954). Purine ribonucleotide pools can also be decreased in size by methylated metabolites (Loo, 1968).

Tay et al. (Tay, 1969) have reported that meTIMP, the predominant intracellular methylated metabolite of 6-MP, inhibits phosphoribosyl pyrophosphate (PRPP) amidotransferase, the first enzyme in PDNS, much more potently than does TIMP. Since inhibition of this enzyme gives rise to accumulation of PRPP and PRPP is a co-substrate for the conversion of 6-MP to TIMP, such inhibition might enhance production of the cytotoxic TIMP. The other mechanism via which inhibition of PDNS by meTIMP might contribute to cytotoxicity involves depletion of intracellular pools of nucleotides, which are vital for the survival of rapidly proliferating cells. Diminished pools of purine nucleotides can also lead to elevated pyrimidine synthesis, leading thereby to imbalanced cell growth and, finally, cell death (Bokkerink, 1993).

In 1972 Tid and coworkers (Tidd, 1972) demonstrated that the delayed cytotoxic effect of 6-MP following brief exposure to a high concentration of this drug cannot be explained by alterations in intracellular pools of purine nucleotides (Tidd, 1974, a). These investigators observed a relationship between the extent of incorporation of TGN into DNA and RNA and the delaying toxicity and therefore proposed that this was the biochemical mechanism involved (Tidd, 1974, b).

Metabolites of 6-MP can also inhibit DNA-dependent RNA-polymerase (Kawahata, 1980). Moreover, in 1980 Lee and colleagues (Lee, 1980) reported that TIMP potentiates the inhibition of DNA synthesis induced by 1-beta-D-arabinofuranosyl-ATP (ara-ATP). The mechanistic explanation for this phenomenon may be selective inhibition of the proofreading 3'- to -5' exonuclease activity of DNA polymerase by the metabolite of 6-MP, which would prevent removal of newly incorporated araAMP from the 3'- termini of elongating DNA chains.

When Chinese hamster ovary fibroblasts are exposed to 6-TG, specific and drastic morphological changes in the chromosomes in the G2 phase of the cell cycle become evident 28 hours later (Maybaum, 1981). This effect is dose dependent, which indicates that unilateral

chromatid damage may play a central role in the delayed cytotoxicity of 6-TG (Maybaum, 1983). However, no such gross chromosomal deformation occurred when this same cell line exposed to 6-MP (Maybaum, 1985). Furthermore the delay in 6-MP-induced cytotoxicity is associated with arrest in the G1 or G1/S phase, whereas in the case of 6-TG this delay is associated with arrest in the late S/G2 phase (Maybaum, 1985).

It has been proposed that the delayed cytotoxicity of 6-TG involves the postreplicative DNA-MMR system. Following incorporation into DNA, 6-TG is methylated non-enzymatically by S-adenosylmethionine to form S6-methylthioguanine. During DNA replication, this S6-methylthioguanine directs incorporation of either thymine or cytosine into the growing complementary DNA strand and the resulting S6-methylthioguanine-thymine pairs are recognized and repaired by the postreplicative mismatch system (Swann, 1996).

Direct induction of apoptosis by TGN via a mitochondrial pathway has recently been described. This induction requires co-stimulation with CD28 and is mediated by specific inhibition of Rac1 activation by the binding of TGTP instead of GTP to Rac1 (Tiede, 2003). In this manner activation of Rac1 target genes, such as mitogen-activated protein kinase, NF-kappaB, and bcl-x(L), is suppressed by azathioprine, giving rise to apoptosis via a mitochondrial pathway (Tiede, 2003).

2.2.5. The clinical pharmacology of thiopurines as anti-cancer agents

The routine dose of 6-MP employed for chemotheraputic maintenance of ALL is 75 mg/m^2 body surface area. Following oral administration, peak plasma concentrations of 0.3 - 1.8 μM achieved after a mean of 2.2 hours (Zimm, 1983). This drug has a poor and variable bioavailability (5 - 37%) and a short half-life (21 minutes in children) (Loo, 1968).

When 6-TG is administered orally at the usual dose of 20 mg/m^2 to patients with AML, peak plasma levels of 0.03 - 5 μM are observed 2 - 4 hours later (Brox, 1981). The bioavailability of this drug ranges between 14 and 46% (LePage, 1971) and its half-life in plasma is 90 minutes (Konits, 1982).

Because of the pronounced interindividual variation in response to 75 mg 6-MP /m^2 administered either orally or intravenously (Zimm, 1983), as well as variations in concentration with time observed in one and the same patient who receives the same dose on repeated occasions (Hayder, 1989), it has been difficult to develop a reliable strategy for

determining the dosage of 6-MP required to achieve the desirable effect while avoiding both relapse and severe myelotoxicity. Prolonged intra-venous administration of a high-dose of 6-MP (50 mg/m^2- for 48 hours) to children with refractory cancers, which can be tolerated, yields a mean steady-state plasma concentration of 6.9 μM a concentration within the cytotoxic range *in vitro* (Zimm, 1985) with little interpatient variation. Unfortunately, this approach did not produce a therapeutic response in connection with a phase II paediatric trial involving 40 children with ALL (Adamson, 1990). Actually, as a prodrug, 6-MP undergoes extensive metabolism and intracellular formation and persistence of TGNs in the target cells is therefore more relevant for optimization of therapy than the plasma concentration of 6-MP (Rundles, 1984).

There is a negative correlation between TPMT activity and the intracellular concentration of TGNs (Lennard, 1987). Since determination of this concentration in lymphoblasts in the bone-marrow is difficult, erythrocyte concentrations of TGNs and TPMT activities are measured as reflections of the corresponding parameters in the target leukemic cells. Today these erythrocyte values serve as prognostic markers of 6-MP metabolism and TPMT activity in children with ALL. Patients with a low concentration of TGNs might be at higher risk for relapse (Lennard, 1990), while patients with high levels may experience myelosuppression and hepatotoxicity. The efficiency of 6-TGN on disease control is independent of the WBC count, sex, age, the immunological cell type affected, the French-American-British type, variations in other antineoplastic therapy, and the duration of remission at the time when erythrocyte 6-TGN is assayed (Lennard, 1989).

The most important factor influencing intracellular accumulation of TGN following administration of 6-MP is the activity of TPMT resulting from the well-characterized genetic polymorphisms (see above). There is a clear negative correlation between TPMT activity and intracellular concentration of TGNs, with high activity and the associated lower concentration being connected with a higher risk of failure in treatment of children with ALL (Lennard, 1987; McLeod, 1995).

2.2.6. Resistance to thiopurines

A low level or absence of HGPRT activity is the most extensively characterized mechanism underlying the resistance of leukemic cells to 6-MP and 6-TG (Lennard, 1992; Rosman, 1973), but this mechanism is rarely observed in leukemic cells obtained from

patients with ALL. One report did demonstrate a 100-fold variation in HGPRT activity in the lymphoblasts of children with ALL at the time of relapse (Zimm, 1983). In a study involving 83 children with untreated ALL, low HGPRT activity was correlated with a poorer prognosis in those patients with precursor B-ALL (Pieters, 1992). However, no currently available evidence supports a significant contribution of this mechanism to clinical resistance to thiopurines, probably because a very low level of HGPRT activity can generate sufficient levels of the cytotoxic nucleotides of thiopurines.

Alterations in TPMT activity influence the sensitivity of cells to the cytotoxic effects of both 6-MP and 6-TG, but in opposite directions (Dervieux, 2001). A decrease in the activity of this methylating enzyme can result in lower levels of meTIMP following exposure to 6-MP, thus conferring resistance to this drug. On the other hand, such a decrease could enhance sensitivity to 6-TG by lowering the level of meTGMP and elevating the levels of DNA-TGNs (Coulthard, 2002).

Different types of antineoplastic drugs act directly or indirectly by damaging DNA and evidence are accumulating that resistance to a wide range of such drugs, including 6-MP and 6-TG, can be acquired through loss of MMR activity. This resistance arises from an attenuation of MMR-dependent stimulation of signal-transduction pathways that lead to programmed cell death (Lang, 2001).

There are also indications that alterations in transport can influence the efficacy of thiopurines at the cellular level. Human colon carcinoma cells resistant to high concentration (30 μM) of 6-TG, but with unaltered levels of HGPRT activity, were found to exhibit significant alterations in the kinetics of 6-TG uptake (Bemi, 1999). As discussed in more detail above, several reports indicate that the levels of certain influxing (e.g., ENT1) and/or effluxing (OAT3, MRP4 and MRP5) transporters can influence thiopurine cytotoxicity. However, the extent to which thiopurine transport contributes to the clinical response is not yet clear and alteration in trans-membrane transport has not been established as a mechanism for resistance to thiopurines *in vivo*.

Alterations in the activities of other enzymes involved in the metabolism of thiopurines to their active nucleotides or in the metabolism or catabolism of natural purines or their nucleotide derivatives (involving, e.g., 5′-nucleotidase, XO, AO, IMPDH, GMPS, nucleotide diphosphate kinase, alkaline phosphatase, ITPase and ribonucleotide reductase) have not been reported to be mechanisms of resistance to thiopurines.

AIMS OF THE PRESENT STUDY

The present investigations were designed to characterize the mechanisms of action and resistance to antifolates and thiopurines in leukemic cell lines; to explore the involvement of metabolites in the development of resistance; to suggest strategies for bypassing the mechanisms of resistance and to develop procedures to individualize cancer chemotherapy.

Specific aims:

1. To investigate the mechanisms of resistance of leukemic cells to MTX.
2. To examine the ability of 7-OHMTX, the dominant metabolite of MTX present in plasma, to induce resistance in leukemic cells and to determine whether resistance to this metabolite contributes to resistance to MTX.
3. To explore the patterns of gene expressions in MTX- and 7-OHMTX- resistant cells and compare these to the corresponding patterns in the parental wild-type cells, in order to further understanding of the MTX-resistant genotype and find new potential targets for MTX therapy.
4. To investigate the mechanisms of action by and resistance to thiopurines 6-MP and 6-TG and factors that can influence the efficacy of these drugs.
5. To characterize the toxic effects of the metabolites of thiopurines, including methylated metabolites, on thiopurine-resistant leukemic cells and clarify the molecular basis for variations in response.

RESULTS AND DISCUSSION

Disparate mechanisms of resistance to MTX and 7-OHMTX (Paper I)

Development of resistance to MTX is a serious obstacle to the success of ALL therapy. In order to explore the mechanism(s) underlying acquired resistance to MTX, we developed two MTX-resistant leukemic cell lines and explored the reasons for their tolerance to the cytocidal effects of MTX.

Following high-dose administration of MTX in connection with ALL therapy, the plasma concentration of its major metabolite 7-OHMTX reaches to levels sufficient to exert cytotoxic effects and, due to its long half-life, remains at these levels for several hours or even days. We hypothesised that this cytotoxic metabolite can itself evoke resistance in leukemic cells. Accordingly our aim was to induce resistance in leukemic cells by long-term exposure to 7-OHMTX, explore the mechanisms underlying the acquired resistance and, finally, establish whether this phenomenon could affect the efficacy of MTX treatment. For this purpose, we exposed human CCRF-CEM and MOLT4 leukemia cells to gradually increasing concentrations of 7-OHMTX (from 50 nM to 30 μM), and, in parallel, to MTX (from 1 nM to 300 nM) for 12 - 18 passages.

Mechanism of resistance to MTX: The CCRF-CEM and MOLT4 cell lines selected for resistance to MTX were more than 50-fold more resistant than their parental cells to a 72-hour exposure to MTX, (**Table 1**).

Table 1. *Growth inhibitory effects of various antifolates on human CCRF-CEM and MOLT4 leukemia cells with acquired resistance to MTX and 7-OHMTX*

Cell line	MTX[1,2,5] (nM)	7-OHMTX (μM)	TMQ[2] (nM)	ZD1694[1,3,5] (nM)	ZD9331[1,3] (nM)	AG2037[1,4,5] (nM)
CCRF-CEM/WT	12.6 ± 0.2	17.7 ± 5.5	23.1 ± 4.6	3.9 ± 0.4	10.4 ± 2.6	13.1 ± 1.6
CCRF-CEM/MTX	1175 ± 36	>250 *(>14)*	9.1 ± 1.0 *(0.4)*	176 ± 5 *(45)*	601 ± 195 *(58)*	83.5 ± 24.2 *(6.3)*
CCRF-CEM/7-OHMTX	15.9 ± 2.1	>250 *(>14)*	6.5 ± 2.1 *(0.3)*	106 ± 64 *(27)*	11.5 ± 2.9 *(1.1)*	37.3 ± 9.2 *(2.8)*
Molt-4/WT	13.5 ± 2.1	3.5 ± 1.4	19.1 ± 2.1	4.5 ± 0.3	15.4 ± 1.6	41.6 ± 2.6
Molt-4/MTX	962 ± 42	>250 *(>71)*	3.9 ± 0.7 *(0.2)*	667 ± 38 *(148)*	1304 ± 182 *(85)*	842 ± 204 *(20)*
Molt-4/7-OHMTX	15.6 ± 3.6	>250 *(>71)*	8.3 ± 2.9 *(0.4)*	111 ± 56 *(25)*	10.7 ± 2.3 *(0.7)*	36.1 ± 1.5 *(0.9)*

[#] Growth inhibitory effects were analyzed after 72 hours of drug exposure and expressed as the IC_{50}, i.e., they concentration of drug required to inhibit cell growth by 50%. Results are presented as the mean ± S.D. of 3-5 independent experiments. The values in parentheses depict the resistance factor, i.e., the ratio of the IC_{50} value of the resistant cells line to that of the parental cell line.

[1] RFC-dependent antifolate; [2] Primary target, DHFR; [3] primary target, TS; [4] Primary target, GARFT; [5] Polyglutamylation-dependent antifolate.

The pattern of cross-resistance of MTX-resistant CCRF-CEM and MOLT4 cells to antifolates (i.e., resistance to antifolates whose growth inhibitory effect is dependent on transport by RFC as well as hypersensitivity to the lipophylic antifolate trimetrexate (TMQ)) indicated on alteration in RFC-mediated uptake by these cells. We confirmed that the initial rates of MTX transport into these resistant cells were 13- and 11-fold slower than into the parental cells, respectively, and that accumulation of MTX by these resistant cells over a period of 60 minutes was profoundly impaired in comparison to that by the parental and 7-OHMTX–resistant cells. Since 7-OHMTX is also transported into cells by RFC, the reduction in such uptake by the MTX-resistant cells can easily explain the cross-resistance of these cells to the metabolite as well **(Table 1).** Indeed, defective transport via RFC is a well-established (Mauritz, 2002; Mini, 1985; Rothem, 2002; Zhao, 2003) and the most common mechanism of resistance to MTX, both in human tumour cell lines (Jansen, 1998) and in the clinic (Gorlick, 1997; Trippett, 1992).

The molecular basis for the defect in transport in the MTX-resistant CCRF-CEM cells appeared to involve simultaneous loss of the binding of multiple transcription factors to *cis*-acting elements present in the promoter region of RFC gene, resulting in a pronounced reduction in the level of RFC mRNA, and consequent impaired antifolate transport. In contrast, the defect in MTX transport by MOLT4/MTX cells was associated neither with inactivating mutations in the RFC gene nor with dramatic changes in the level of RFC mRNA (**Figure 7**).

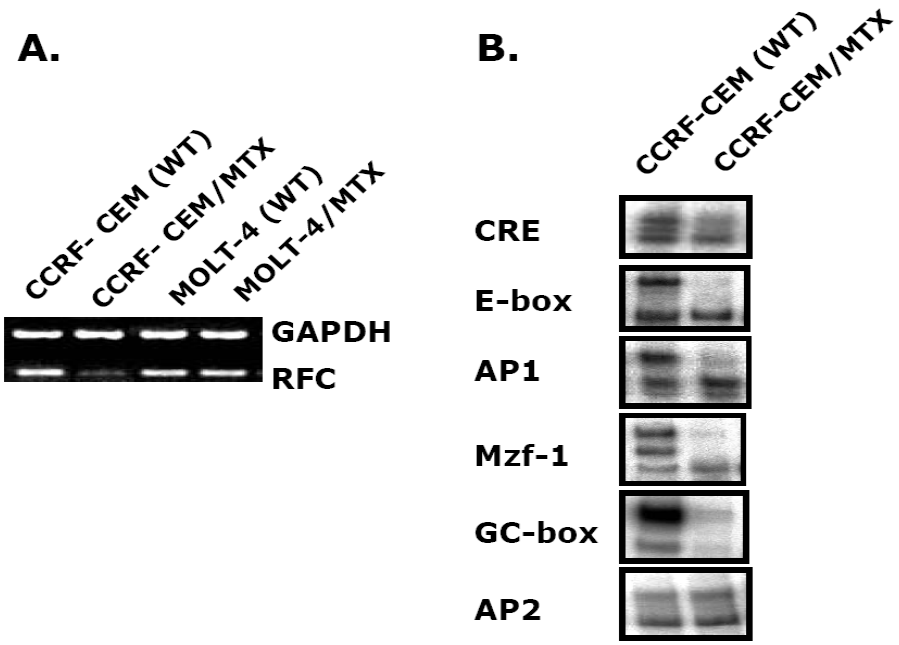

Figure 7. *Expression of RFC mRNA as determined by semi-quantitative RT-PCR (A) and the electrophoretic mobility shift assay (B), indicates loss of binding of multiple transcription factors to cis-acting elements in the promoter of the RFC gene of the CCRF-CEM/MTX-resistant cell sublines*

Mechanism of resistance to 7-OHMTX: The cell lines selected for resistance to 7-OHMTX were more than 15-times more resistant to this compound than the wild-type cells, but nonetheless retained sensitivity to long-time (72-hour) exposure to MTX as shown in the **Table 1**. Their pattern of cross-resistance revealed tolerance to antifolates whose ctotoxicity is dependent on polyglutamylation. To further evaluate the hypothesis that this resistance to 7-OHMTX was due to impaired intracellular polyglutamylation, we found that the activities of FPGS in the CCRF-CEM and MOLT4 cells resistant to 7-OHMTX were 56- and 92-fold lower, respectively, than in the corresponding parental cells.

Although 7-OHMTX itself is not a potent cytotoxic agent, its polyglutamated derivatives are more potent inhibitors of the enzyme DHFR (Drake, 1987). Moreover 7-OHMTXGLs can also inhibit TS, AICART (Sholar, 1988) and GARFT (Baggott, 1994), the enzymes involved in *de novo* biosynthesis of purines. Thus, the tremendous reduction in FPGS activity in the 7-OHMTX-resistant cells can easily explain their tolerance to this compound and other polyglutamylation-dependent antifolates. Defective polyglutamylation associated with a pronounced decrease in FPGS activity is a common mechanism of resistance to antifolates that rely heavily on polyglutamylation for their inhibitory effect on folate-dependent enzymes (Liani, 2003; Mauritz, 2002; Takemura, 1999). In the present case we observed no major reduction in the level of FPGS mRNA in our 7-OHMTX-resistant cells, suggesting that the decrease in FPGS activity is due to posttranscriptional or posttranslational alterations possibly involving protein instability (McGuire, 1998).

Interestingly, when exposure to MTX was limited to 4 hours rather than 72 hours, the 7-OHMTX-resistant CCRF-CEM cells were > 100-fold more resistant to growth inhibition by MTX than the corresponding parental cells (**Figure 8** A-B).

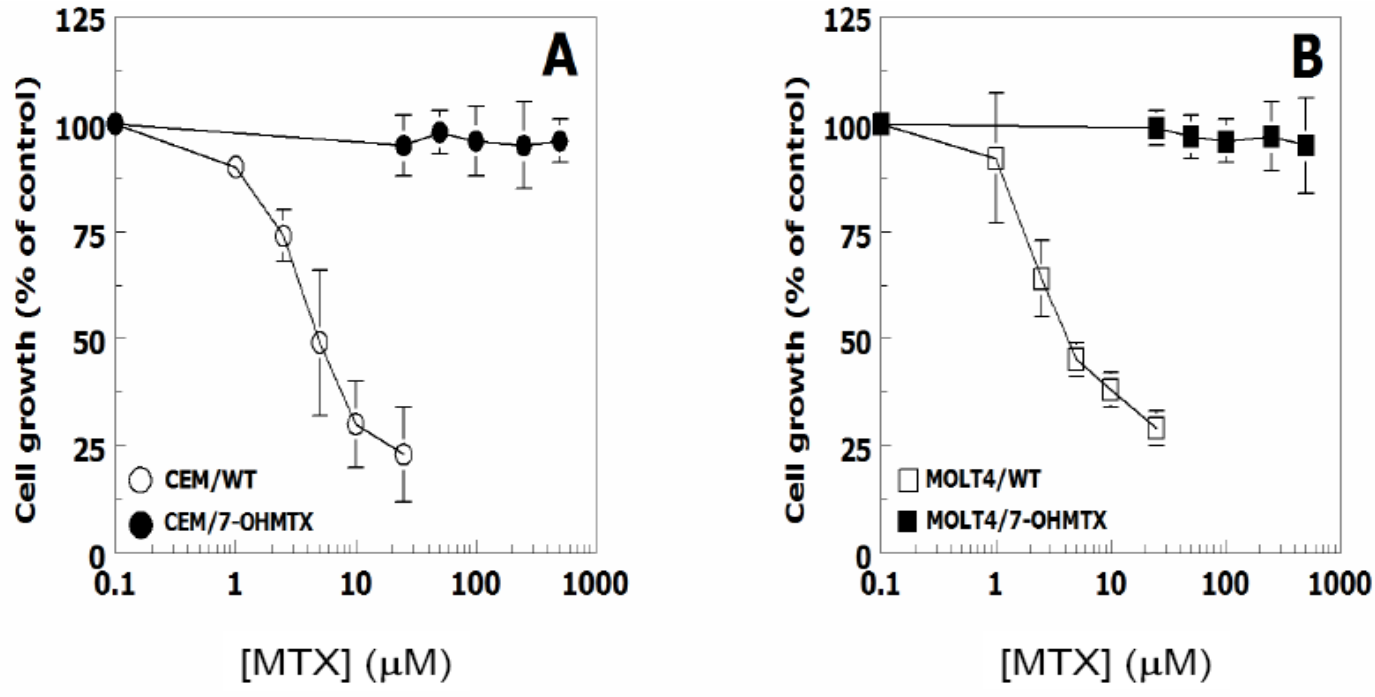

Figure 8. *Growth-inhibitory effects of MTX on parental and 7-OHMTX-resistant sublines of CCRF-CEM and MOLT4 cells after short-term (4-hour) exposure. (A) Parental CCRF-CEM cells (○) and the 7-OHMTX-resistant subline (●) and (B) parental MOLT4 cells (□) and the 7- OHMTX-resistant subline (■)*

This phenomenon may be a consequence of low levels of retainable polyglutamated forms of MTX in the 7-OHMTX-resistant cells following short-term exposure. The washing process following such exposure may remove non-polyglutamated MTX, so that no cytosidal effect is detected after 72 hours.

Strikingly, the two different procedures applied resulted in completely different mechanisms of drug resistance. Thus, the development of different mechanisms for resistance to MTX (defective cellular uptake via RFC) and 7-OHMTX (defective polyglutamylation) may be particularly prevalent after repeated cycles of high-dose MTX therapy. In a clinical setting, this development can impair the efficacy of MTX and various other antifolates that depend on transport and/or polyglutamylation for their cytotoxic activity.

Comparison of the patterns gene expression by MTX- and 7-OHMTX-resistant MOLT4 cells (Paper II)

Comparison of the pattern of gene expression by 7-OHMTX-resistant cells to that by MTX-resistant cells can hopefully provide additional information concerning the mechanisms of action by and resistance to these two compounds, as well as help to specify and, possibly, identify new targets for MTX therapy. Accordingly, we performed a global analysis of the expression of approximately 17,000 genes by wild-type MOLT4 cells and sublines with acquired resistance to MTX or 7-OHMTX. In this context, we first selected all species of mRNA whose levels were altered by at least 2-fold. Thereafter, we classified these different mRNA species into ten different functional groups (transport, cell proliferation, DNA/RNA metabolism, etc.) on the basis of the activities of the proteins which they encode. Finally, we determined the ratios of the number of mRNA species that fell into each of these ten groups to the number of all more than two-fold-changed mRNA species, and exporessed them by percentages. This analysis led to the conclusions that in the case of the MTX-resistant subline, the levels of mRNA encoding proteins involved in DNA/RNA metabolism and transport were altered more profoundly; whereas the most pronounced changes in the 7-OHMTX-resistant cells involved regulation of metabolism and proliferation (**Figure 9**).

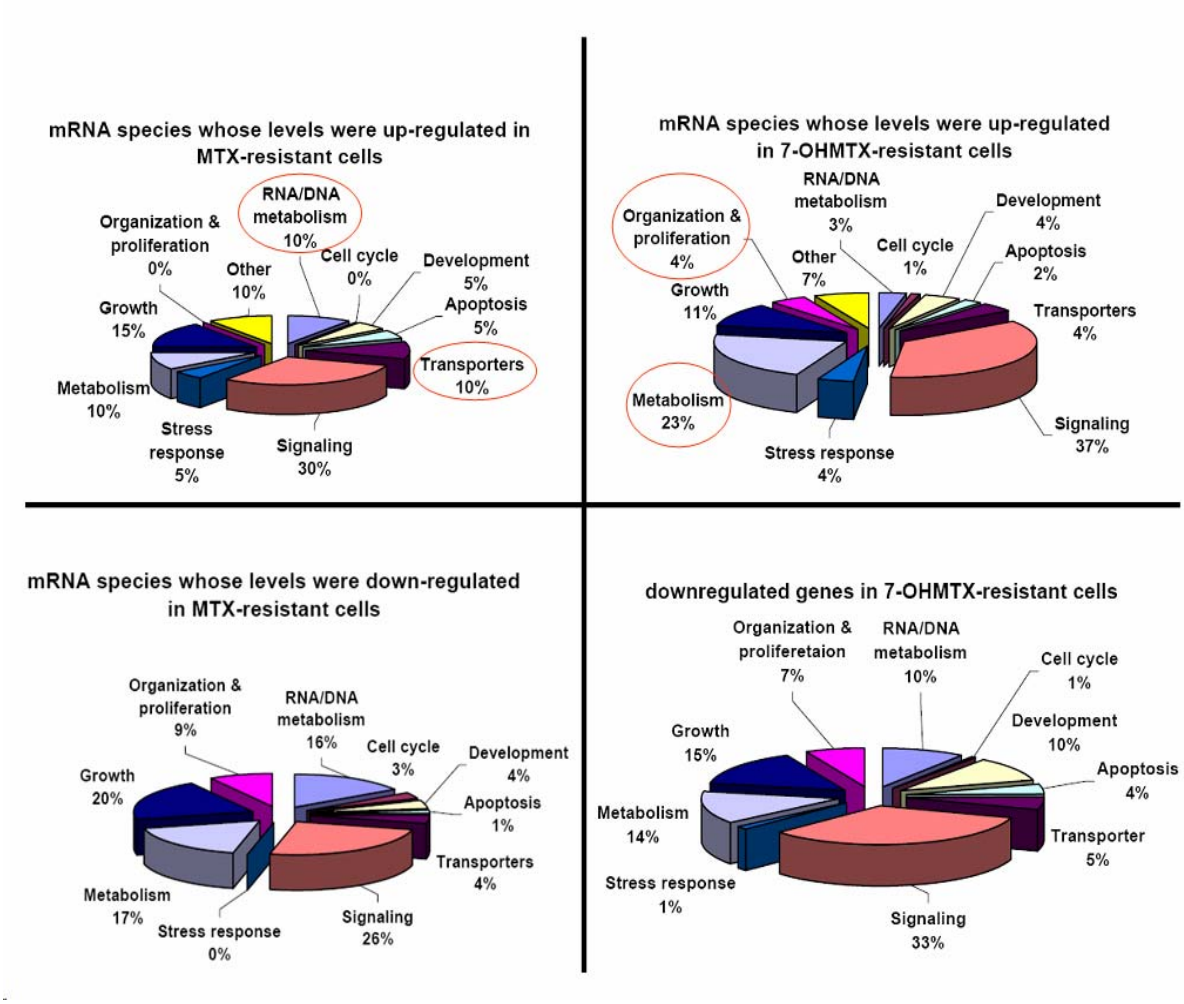

Figure 9. *Classification of mRNA species whose levels were at least 2-fold decreased or increased in MTX- and 7-OHMTX-resistant sublines of MOLT4 cells, on the basis of the biological activities of the proteins they encode (The ratio of the number of mRNA species in each group to all mRNA species, expressed in percentages)*

The 7-OHMTX-resistant cells displayed a 10-fold decrease in the level of mRNA encoding adenosine deaminase the major enzyme of purine catabolism. Consistent with this finding, the mRNA for cystathionine β synthase was completely absent, whereas the mRNA levels for the biosynthetic enzymes MTHFR and GARFT, involved in methyl-tetrahydrofolate and purine biosyntheses, respectively, were elevated 3- and 2-fold, respectively (**Table 2**). The differences in the levels of a relatively large number of mRNA species in the 7-OHMTX- to MTX-resistant cells, indicates the occurrence of distinct and independent effects. Actually, this mRNA profiling suggests that intracellular folate and nucleotide biosynthesis is preserved to a greater extent in 7-OHMTX – than in MTX-resistant cells.

The disparate pattern of gene expression by 7-OHMTX-resistant cells indicates that MTX is not the only cytotoxic agent of relevance in connection with high-dose MTX therapy. Indeed, the pattern of gene expression associated with such therapy (Cheok, 2003) resembles a combination of those obtained upon exposure to MTX or 7-OHMTX in our study. These findings may help to identify the specific targets for MTX therapy and provide additional insight into cellular responses to 7-OHMTX.

Table 2. *Ratios of the levels of selected species of mRNA in MTX- and 7-OHMTX-resistant sublines to those in the wild-type MOLT4 cells*

Gene symbol	Gene product	Ratios of mRNA gene expressions	
		MTX-resistant/wild type	7OHMTX-resistant/wild type
MTHFD1	methylenetetrahydrofolate dehydrogenase 1	**1.4**	**1.5**
MTHFD2	methylenetetrahydrofolate dehydrogenase 2	**1.2**	**1.8**
DHFR	dihydrofolate reductase	**1.4**	**1.0**
TS/TYMS	thymidylate synthetase	**0.6**	**0.7**
FPGS*	folylpolyglutamate synthase	**1.0**	**1.0**
MTRR	5-methyltetrahydrofolate-homocysteine methyltransferase reductase	**1.1**	**0.9**
MTHFS	5,10-methenyltetrahydrofolate synthetase	**1.0**	**0.9**
FPGH/GGH*	folylpolygammaglutamyl hydrolase, gamma-glutamyl hydrolase	**1.2**	**3.0**
MTR*	5-methyltetrahydrofolate-homocysteine methyltransferase	**2.8**	**1.3**
GCH1	GTP cyclohydrolase 1	**1.4**	**0.9**
ATIC*	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase	**1.0**	**1.0**
SLC19A2	solute carrier family 19 (thiamine transporter), member 2	**1.4**	**2.1**
SLC19A1 (RFC)*	solute carrier family 19 (folate transporter), member 1	**0.4**	**0.9**
SHMT1	serine hydroxymethyltransferase 1	**1.1**	**2.0**
GART*	phosphoribosylglycinamide formyltransferase,	**1.6**	**1.8**
BCRP/ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	**1.7**	**0.4**
PFAS	phosphoribosylformylglycinamidine synthase (FGAR amidotransferase)	**1.9**	**2.5**
GCHFR	GTP cyclohydrolase I feedback regulator	**0.6**	**7.7**
ITPA	inosine triphosphatase (nucleoside triphosphate pyrophosphatase)	**0.6**	**0.9**
dGK/ DGUOK	deoxyguanosine kinase	**1.0**	**1.8**
APRT	adenine phosphoribosyltransferase	**0.4**	**0.6**
ADA	adenosine deaminase	**0.9**	**0.1**
PPAT	phosphoribosyl pyrophosphate amidotransferase	**1.1**	**1.3**
MTHFR*	5,10-methylenetetrahydrofolate reductase (NADPH)	**4.3**	**4.8**
MRP1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	**1.5**	**0.7**
MRP2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	**0.8**	**0.8**
MRP3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	**1.0**	**1.0**
MRP4*	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	**1.4**	**0.9**
MRP5*	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	**1.6**	**1.2**
P-glycoprotein	ATP-binding cassette, sub-family B (MDR/TAP), member 1	**1.2**	**0.9**
CBS	cystathionine β-synthase	**0.5**	**0.0**

* mRNA species whose relative level of expression has been confirmed by real-time quantitative PCR

Involvement of the CNT3 and ENT2 in the resistance of T-lymphoblastic cell lines to thiopurines (Paper III)

The mechanisms underlying the resistance of leukemic cells to 6-MP and 6-TG are poorly understood. The most extensively characterized mechanism in this context is a reduction in or lack of HPGRT activity. In addition, alterations in TPMT activity can influence the degree of cellular sensitivity to the cytotoxicity of 6-MP and 6-TG. Development of thiopurine-resistant cell sublines can provide models for improving our understanding of the mechanisms underlying resistance to this family of antimetabolites with the long-term goal of finding strategies to overcome this resistance. Consequently, in this investigation, we obtained two thiopurine-resistant sublines of MOLT4 cells utilizing a classic approach i.e., exposure to stepwise increasing concentrations (from 50 nM to 5 μM) of 6-MP or 6-TG. Employing a 72-hour exposure, the cells selected in this manner for resistance to 6-MP were 7-fold more resistant to 6-MP and 6-fold more resistant to 6-TG, than wild-type MOLT4 cells. Similarly, the cell subline selected for resistance to 6-TG was approximately 20-fold more resistant to both 6-TG and 6-MP.

Neither of the well-characterized mechanisms of resistance to thiopurines, i.e., absence of HGPRT activity or altered TPMT activity, was involved in the resistance of these cell sublines to 6-MP and 6-TG. Instead, defective cellular uptake was found to be the primary mechanism underlying this resistance. Quantitation of the levels of mRNA encoding nucleoside transporters revealed significant reductions with respect to the third member of the concentrative family and the second member of the equilibrative family of nucleoside transporters (CNT3 & ENT2) in the thiopurine-resistant cells in comparison to their wild-type parental cells. In order to verify the involvement of these nucleoside transporters in cellular uptake of 6-MP, targeting of the genes encoding these transporters in wild-type MOLT4 cells with siRNAs resulted in a significant reduction in the initial rate of 6-MP transport as well as an enhanced resistance to this agent (**Figure 10**).

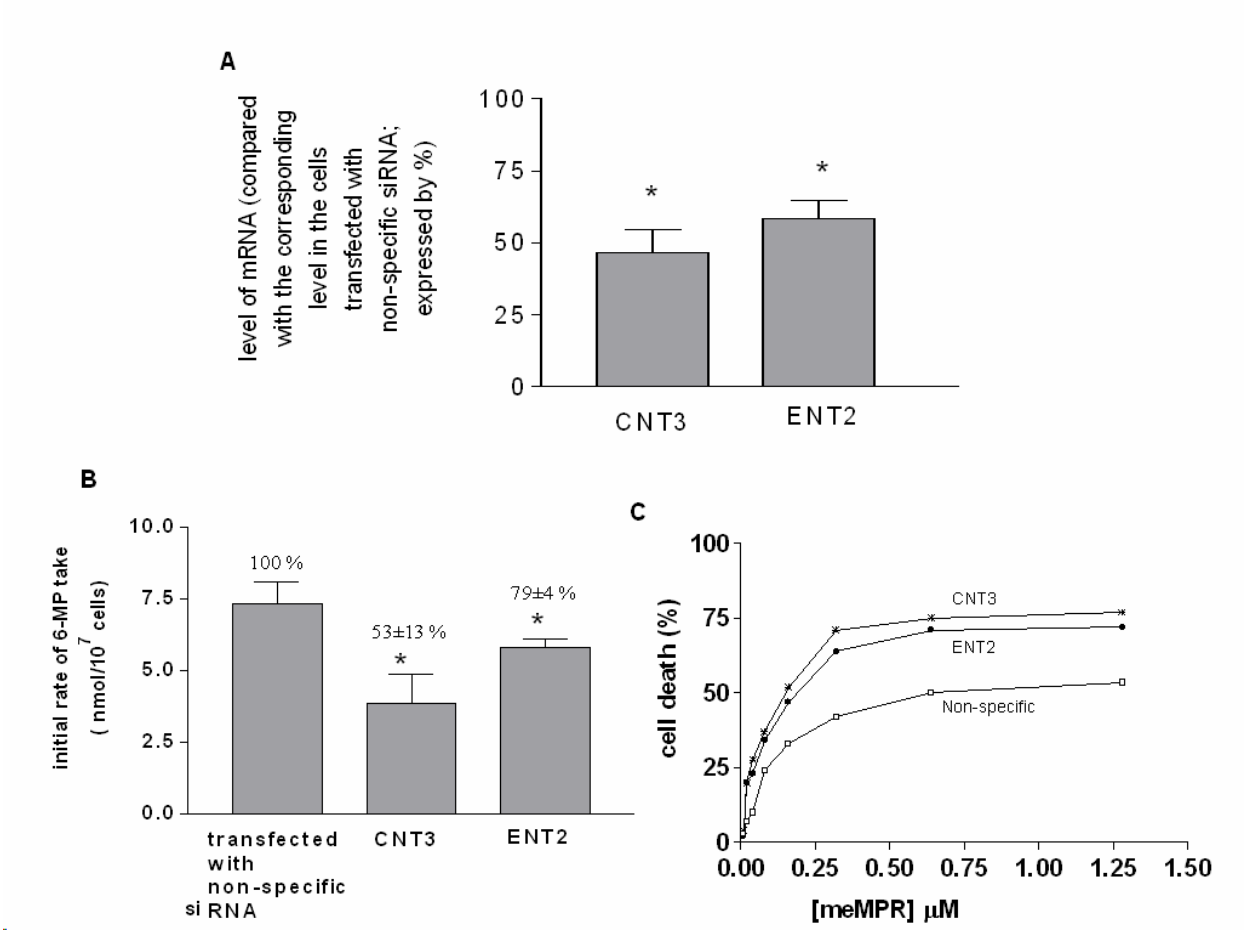

Figure 10. *Transfection of wild-type MOLT4 cells with siRNA targeting the CNT3 and ENT2 genes resulted in lower levels of the corresponding mRNAs (A), as well as decreased transport of (B) and enhanced resistance to 6-MP (C)*

High concentrations of NBTI and dipyridamole, well-known inhibitors of ENT1, did not influence the uptake of 6-MP by wild-type MOLT4 cells, ruling out involvement of this transporter in such uptake. In contrast, this uptake was significantly attenuated in the absence of Na^+ ions, further verifying the involvement of a concentrative nucleoside transporter in this process. The present study provides the first evidence that impairment of transport as a consequence of decreased expression of CNT3 and ENT2 can in itself confer resistance to thiopurines.

Mechanisms underlying the collateral sensitivity of thiopurine-resistant cells to methyl mercaptopurine riboside (Paper IV)

The 6-MP- and 6-TG-resistant sublines of MOLT4 cells characterized in Paper III, exhibited a collateral enhancement in sensitivity to one of the methylated metabolites of 6-MP, methyl mercaptopurine riboside (meMPR). The uptake of meMPR to resistant cells was unchanged. At the same time the resistant cells exhibited a greater than 50% reduction in their rate of PDNS and 30-52% reductions in their levels of mRNA encoding AICART, GARFT and GMPS, enzymes involved in this synthesis. Furthermore, there was a significant reduction in the size of the ribonucleoside triphosphate pools in the thiopurine-resistant cell sublines. Since meMPR is a potent inhibitor of PDNS, these reductions can render these sublines more sensitive to the cytotoxicity of this metabolite.

Since meMPR is an analogue of adenosine (**Figure 11**), we went on to determine whether nucleoside transporters are involved in the influx of meMPR into cells.

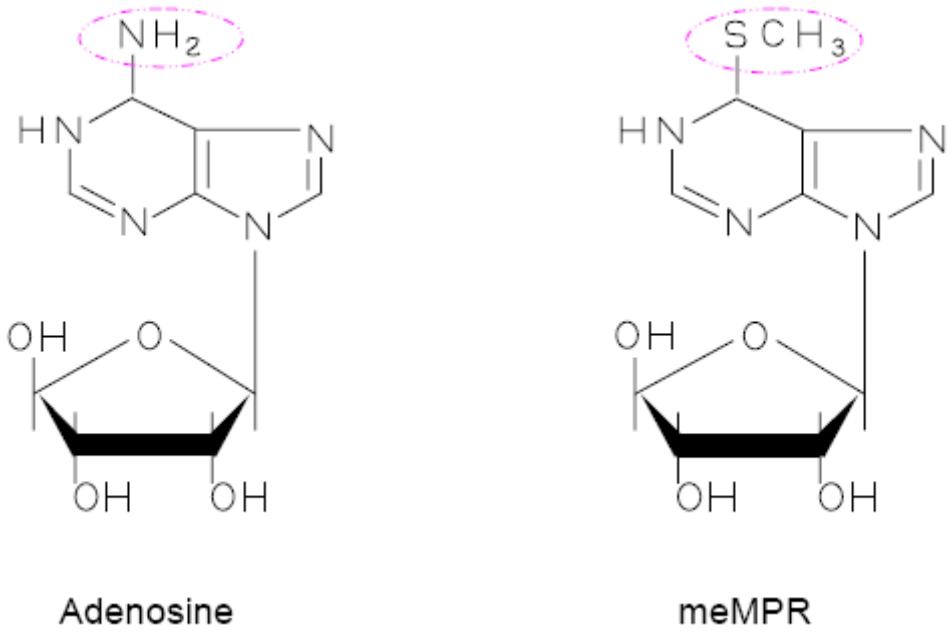

Figure 11. *Chemical structures of adenosine and its analogue methyl mercaptopurine riboside (meMPR)*

Substrate-inhibition assays performed on wild-type MOLT4 cells revealed that adenosine, dipyridamole and nitrobenzylthioinosine all inhibit the uptake of meMPR to a significant

extent, indicating the involvement of ENT1 in this uptake by leukemic cells. The lack of any influence of Na^+ ions on this influx rules out any participation of CNTs in this process. Moreover, transfection of the leukemic cells with siRNA molecules targeting the ENT1 gene strongly reduced the initial rate of meMPR transport. Together, these findings indicate that administration of meMPR to patients experiencing relapse or resistance following thiopurine therapy may be beneficial; a hypothesis that demands testing in the clinic.

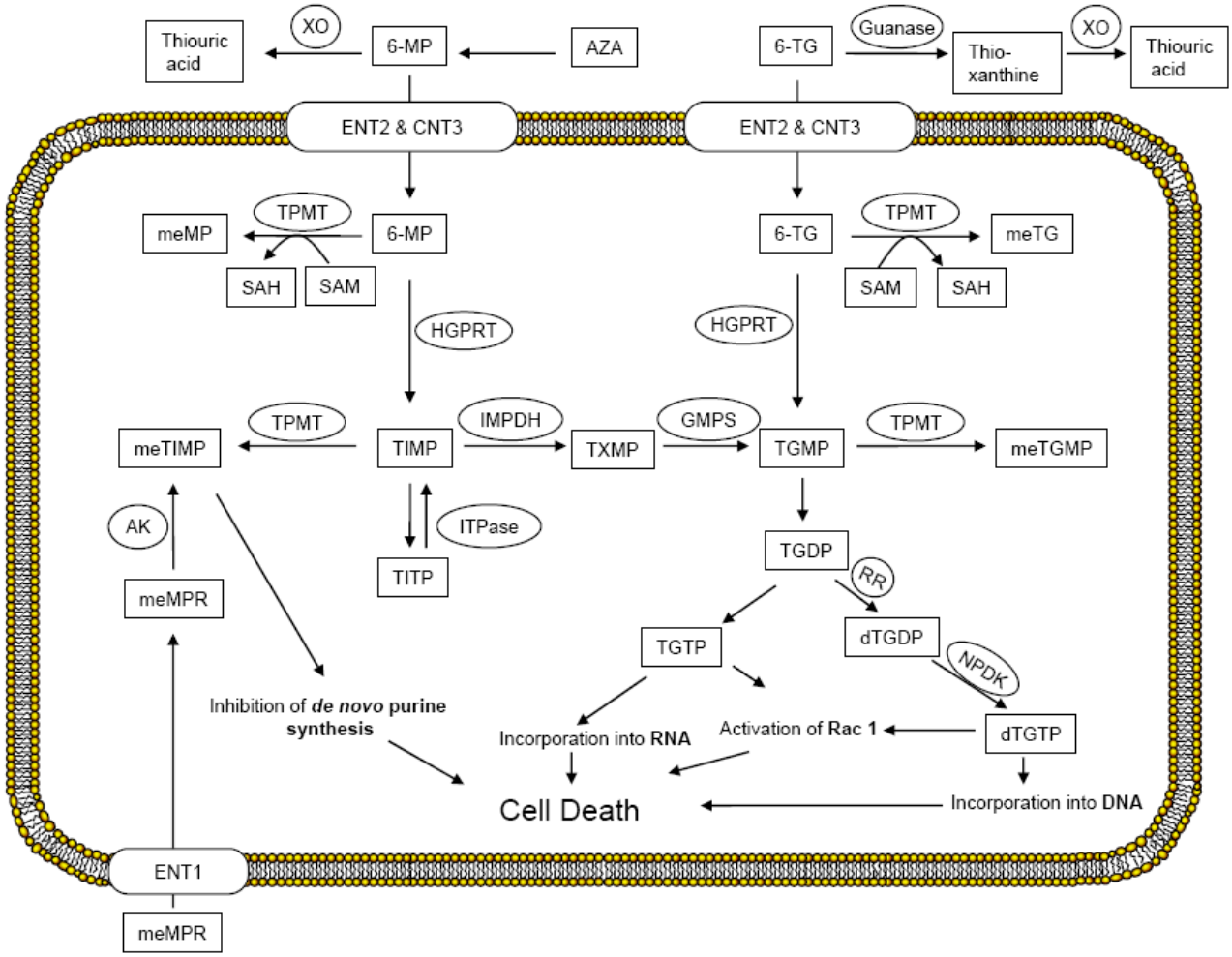

Figure 12. *Schematic illustration of the metabolism, influx, and modes of cytotoxicity of 6-MP and 6-TG in mammalian cells*

XO, xanthine oxidase; AZA, azathioprine; meMP, methyl mercaptopurine; TPMT, thiopurine methyltransferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; HGPRT, hypoxanthine–guanine phosphoribosyl transferase; IMPDH, inosine monophosphate dehydrogenase; GMPS, guanosine monophosphate synthetase; meTIMP, methylthioinosine monophosphate; TGMP, 6-thioguanosine 5'-monophosphate; meTG, methyl thioguanine; TIMP, 6-thioinosine 5'-monophosphate; TITP, 6-thioinosine 5'-triphosphate; ITPase, inosine triphosphate pyrophosphatase; RR, ribonucleotide reductase; NDPK, nucleoside diphosphate kinase; TXMP, 6-thioxanthosine 5'-monophosphate; TGDP, 6-thioguanosine 5'-diphosphate; TGTP, 6-thioguanosine 5'-triphosphate; dTGDP, deoxy-6-thioguanosine 5'-diphosphate; dTGTP, deoxy-6-thioguanosine 5'-triphosphate; meTGMP, S-methylthioguanosine 5'-monophosphate

CONCLUSIONS

The following general conclusions can be drawn from the investigations presented here:

The mechanism underlying the resistance of both of our cell sublines resistant to MTX was a pronounced decrease (greater than 10-fold) in RFC– mediated cellular uptake of this drug. This reduction was associated with transcriptional silencing of the RFC gene in MTX-resistant CCRF-CEM cells. The MTX-resistant cells demonstrated cross-resistance to 7-OHMTX and certain novel antifolates that are taken up into cells by RFC, but a collateral enhancement in sensitivity to the cytotoxicity of the lipophilic antifolate TMQ, whose entry into cells is not mediated by RFC. These findings emphasize the major role played by transport in connection with the cytotoxic effects of MTX and other antifolates taken up via RFC.

7-OHMTX, the major metabolite of MTX, can induce resistance in leukemic cells by a mechanism different from that by which MTX induces resistance. The resistance of both CCRF-CEM- and MOLT4 cell sublines to 7-OHMTX was solely due to a pronounced reduction (>95%) in FPGS activity. When exposure was limited to 4 hours, 7-OHMTX-resistant cells were > 100-fold more resistant to MTX. In a clinical setting, this phenomenon may further impair the efficacy of MTX therapy.

Differential expression of a relatively large number of genes in 7-OHMTX- and MTX-resistant cell sublines was observed. In the MTX-resistant subline the levels of mRNA encoding proteins involved in DNA/RNA metabolism and transport were more profoundly altered; whereas the 7-OHMTX-resistant cells demonstrated the most pronounced effects with respect to mRNAs encoding proteins participating in metabolism and cell proliferation. These disparate cellular responses to MTX and 7-OHMTX may illuminate pathways of action by these components, help identify more specific targets for MTX, and aid in the design of more individualized therapy.

The underlying mechanism in 6-MP- and 6-TG-resistant MOLT4 cells was reduction in the trans-membrane uptake of these drugs via the nucleoside transporters ENT2 and CNT3. Neither of the well-characterized mechanisms of resistance to thiopurines, i.e., alterations in the activities of the HGPRT or TPMT enzymes were detected in these resistant cells.

Thiopurine-resistant cells exhibited a collateral enhancement in sensitivity to the toxicity of meMPR, a metabolite of 6-MP, because: A. Uptake of meMPR by these cells was normal;

B. The size of ribonucleoside triphosphate pools and rate of PDNS in 6-MP- and 6-TG-resistant cells were reduced by greater than 50%, which can render these cells more sensitive to inhibition of PDNS by meMPR. This finding may indicate that administration of meMPR to patients with ALL experiencing relapse or resistance could be beneficial.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Drug resistance remains the most serious obstacle to treatment of ALL, as well as of most other forms of cancer. We have tried here to obtain more detailed information concerning the mechanisms of resistance to the widely used antimetabolites MTX, 6-MP and 6-TG, employing resistant sublines of leukemic cell lines as our model system. In the both resistant cell sublines the mechanism underlying resistance to 7-OHMTX was a dramatic reduction in FPGS activity; whereas acquired resistance to MTX involved a defect in the RFC-mediated transport of this drug. Whether 7-OHMTX can evoke resistance by a mechanism distinct from that of its parental drug in clinical situations as well now demands evaluation. For instance, retrospective surveys of the influence of the pharmacokinetics of 7-OHMTX on the response to MTX may reveal whether high concentrations of this metabolite are associated with an elevated risk for relapse or, more specifically, with relapse due to a low degree of polyglutamylation, the mechanism of resistance detected in both of our 7-OHMTX-resistant cell sublines.

Profiling of the levels of different mRNAs in 7-OHMTX-resistant cells revealed preservation of intracellular folates and nucleotides, in contrast to the pattern in MTX-resistant cells. Comparison of mRNA levels in MTX- and 7-OHMTX-resistant cells indicates that totally different modes of toxicity and development of resistance are associated with these two compounds. This observation suggests that the cellular pattern of gene expression following HDMTX therapy analyzed by Cheok and colleagues (Cheok, 2003) (with high levels of 7-OHMTX being simultaneously present in the plasma) cannot be considered as being induced by MTX alone, but may be influenced differently by the concentration of 7-OHMTX in different patients.

Significant alterations in the levels of enzymes involved in folate metabolism may indicate their participation in the development of tolerance to MTX and 7-OHMTX. The expression of MTHFR was up-regulated more than four-fold n in both MTX- and 7-OHMTX-resistant cells and this enzyme would thus appear to be a promising object for further study in this connection. The level of mRNA for the apoptosis-inducing BIK (a BCL2-interacting killer) is downregulated > 20-fold in the MTX-resistant cell sublines. Contribution of this modification to resistance to MTX is attractive to be studied, as well.

In our first investigation the MTT assay revealed a 3.5- to 4.9-fold increase in the sensitivity of both cell sublines resistant to MTX and both cell sublines resistant to 7-OHMTX to the cytotoxicity of TMQ, a lipophilic antifolate whose action is independent of transport by RFC or polyglutamylation. This phenomenon can be explained by the fact that deficiencies in RFC and FPGS lead to depletion of intracellular folate pools, thereby diminishing the competition for interaction of TMQ with DHFR. The collateral enhancement in the sensitivity of 6-MP- and 6-TG-resistant cells, with reduced intracellular ribonucleoside triphosphate pools, to meMPR, an inhibitor of PDNS whose uptake into cells is independent of thiopurine transporters, can be explained in the same way.

These findings suggest that administration of alternative agents with separate mechanisms of action and different targets in combination with antimetabolites or following relapse may be a useful strategy in connection with antimalignancy therapy. Thus administration of meMPR or its analogues to patients experiencing relapse following or resistance to thiopurine therapy, may be beneficial, as may treatment of patients resistant to MTX with TMQ, which bypasses the main primary mechanisms of resistance to MTX (reduced uptake via RFC) and 7-OHMTX (lack of polyglutamylation). Evaluation of the efficacy of meMPR as an antimalignancy agent in animal models and clinical trials is certainly warranted in this context.

TPMT activity contributes to the toxicity of 6-MP and 6-TG in different ways. Thus, Dervieux and colleagues (Dervieux, 2001) found that elevation of this activity in human CCRF-CEM cell lines, by retroviral gene transfer rendered the cells less sensitive to 6-TG, but more sensitive to 6-MP. These opposite effects can be explained by reduced incorporation of TGNs following treatment with 6-TG, and in enhanced production of the highly cytotoxic meTIMP (the phosphorylated form of meMPR) after treatment with 6-MP, respectively. Our preliminary results from an on-going study in our laboratory (unpublished data) reveal that suppression of expression of the TPMT gene utilizing siRNAs directed against the corresponding mRNA can increase the resistance of MOLT4 cells to the cytotoxic effects of 6-MP several-fold. This finding is in agreement with the observations by Dervieux and coworkers mentioned above. In contrast, we detected no change in sensitivity to 6-MP by siRNA targeting the mRNA encoding IMPDH.

In Paper IV we show that meMPR is a potent cytotoxic agent towards leukemic cells which have acquired resistance to 6-MP and 6-TG. These observations suggest that

methylation of 6-MP can contribute to the toxicity of this drug and that monitoring of meMPR in connection with 6-MP therapy might provide useful information.

The role of transport in connection with the efficacy of thiopurine treatment has not been investigated to any great extent. Our finding that of 6-MP is transported by ENT2 and CNT3 may provide increased incentive for such efforts in the future. The influence of nucleoside transporters on the thiopurine efficacy of thiopurine therapy should be examined in other cell lines that express these transporters, e.g., by transfection with appropriate cDNAs.

Studies on pharmacogenetics designed to improve drug safety and efficacy have been already proven to be advantageous in the case of the influence of genetic polymorphisms in the TPMT gene on response to thiopurines. However, the 15% - 28% occurrence of adverse reactions in patients receiving AZA or 6-MP (Lennard, 2002; Sandborn, 2000; Schwab, 2002) is more frequent than the prevalence of such polymorphisms, indicating that additional pharmacogenetic factors may be significantly involved in this context. Gene array technology should aid researchers in finding new targets for thiopurines and may help predict the response to these agents through comparison of the expression "finger-prints" of responsive and nonresponsive patients.

Since several phosphorylating enzymes are involved in the activation of thiopurines and their consequent incorporation into DNA and RNA, it is highly likely that alterations in the activities of certain kinases and phosphatases may influence the efficacy of thiopurine therapy profoundly. 5′-Nucleotidases are well known to deactivate nucleoside analogues, which require phosphorylation in order to exert their cytotoxic effects and a deficiency in these enzymes is associated with enhanced thiopurine cytotoxicity (Kerstens, 1995). Further investigations are required in this area. Furthermore, the consequence of alterations in the activities of other enzymes directly involved in or capable of influencing the metabolism of thiopurines and the natural nucleosides of which they are analogues (e.g., XO, AO, IMPDH, GMPS, nucleotide diphosphate kinase, alkaline phosphatase, ITPase, ribonucleotide reductase) for thiopurine therapy also require examination.

SUMMARY IN KURDISH

لە نێوان جۆرەکانی چارەسەری شێرپەنجە (کانسەر) کە لەبەردەستن، چارەسەری نەخۆشی شێرپەنجەی خوێنی تیژە (گروپێک لە نەخۆشیەکانی شێرپەنجەی خوێن کە زۆر بە خێرایی بڵاو دەبنەوە)، پشت دەبەستێ بە بە کار هێنانی دەرمانەکانی دژە شێرپەنجە. لە ناو ئەو دەرمانانە دا، دژە مێتابۆلیتە کان (Antimetabolites) یەکێ لە یەکەم گرووپە دەرمانەکانی دە کار کراون دژ بە شێرپەنجەی خوێن.

لە ناو دژە مێتابۆلیتە کان، دەرمانەکانی methotrexate (MTX) و 6-mercaptopurine (6-MP) ناوەرۆکی هەموو پرۆتۆکۆلەکانی چارەسەرکردنی چەشنێ لە نەخۆشیەکانی شێرپەنجەی خوێنی تیژە بە ناوی acute lymphoblastic leukemia (ALL) پێك دەهێنن، کە وابێ دەتوانرێ ناویان بە دەرمانی"بەردی بناغەی" چارەسەرکردنی ئەم نەخۆشیە دا بنرێت. بەداخەوە سەرەڕای گشت ئەوپێشکەوتنانەی بەدەست هاتووە لە بارەی چارەسەرکردنی کیمیاوی نەخۆشیەکانی شێرپەنجە لەم دەیان ساڵەی رابردوودا، هێشتا 20% شێرپەنجەی خوێنی منداڵان و 60% شێرپەنجەی خوێنی گەوران لە جۆری ALL ناتوانرێت چارەسەر بکرێت. بە داخەوە لەوڵاتانی پاش کەوتوو رادەی چارەسەری نەخۆشیەکە تەنانەت لە 10%یش کەمترە.

یەکێک لە گرنگترین کۆسپەکان لەم بارەوە ئەمەیە کە وا بەرگری دەستکەوتی لە خانە شێرپەنچەکان بەرامبەر بە دەرمانە دژە کانسەرەکان دروست دەبێت. پشکنینی زیاتر لەم بارەوە دەربارەی میکانیزمی کارکردنی دژە کانسەرەکان و هەروەها پشکنینی میکانیزمی بەرگری خانە کانسەرەکان بەرامبەر بەو دەرمانانە یارمەتی توێژەرەکان دەدات بۆ ئەوەی رێ و شوێنی باشتر بدۆزنەوە بۆ زاڵ بوون بەسەر ئەم بەرگریە دا و گەیشتن بەو ئامانجە کە ژمارەی چاک بووەکان لە نێوان نەخۆشەکانی شێرپەنجە ی خوێنهەر دەم زیادتر بکرێن.

لەم تێزە ئێمە هە لّستاوین بە پشکنینی جیاوازی لە نێوان خانە کانسەرەکانی بەرگریدارو هەستیاری خوێن بەرامبەر بە تاقمێک لە دژە مێتابۆلیتەکان ، کە بریتین لە MTX، 6-MP و 6-thioguanine (6-TG) و هەروەها پشکنینی زیاترمان کرد دەربارەی ئەو گۆڕانکاریانە ی کار دەکەنە سەر خانە کانسەرەکان بۆ بەرگری بەرامبەر بە دژە کانسەرەکان و تا رادەیەک شیمان کردوونەوە. MTX پاش دە کار کردنی، دە ناو جەرگ دا ئۆکسیدە دەبێ و دەبێت بە پاشەرۆکێک (مێتابۆلیتێک) بە ناوی 7-hydroxymethotrexate (7-OHMTX) کە زۆر کەمتر ژاراوییە لە MTX خۆی و توانایی کوشتنی خانە کانی کەمتر هەیە. ئێمە دە ئەم تێزە دا لێکۆڵینەوە مان کردووە دەربارەی ئەگەری شوێن دانانی 7-OHMTX لەسەر کاریگەری کوشندەیی MTX لە سەر خانەکان و ئەگەری دروست کردنی بەرگری خانە کانسەرەکانی خوێن دژ بە MTX.

ئێمە نیشانماندواوە کەوا بەرگری دەستکەوتوو دژ بە MTX بە شێوەیەکی سەرەکی دەگەڕێتەوە بۆ سە ر کەمایەسی لە وەرگرتنی MTX لە خانەکان بە هۆی کەمبونی چالاکی "هە لّگری فۆلێت" reduced folate carrier (RFC)، ئەمیش جۆرە پرۆتینێکی گواستنەوەیە، هە لّکەوتوو لە ناو دیواری خانەکان.

بە پێچەوانەی بەرگری بەرامبەر7-OHMTX کە دەگەڕێتەوە بۆ کەمبوونی چالاکی ئەنزیمی فۆلیپۆلیگلوتامایت ساینتەیس (FPGS) کە ئەمیش پرۆتێئینێکە توانای گۆڕانی 7-OHMTXی هەیە بە فۆرمە پۆلیگلوتامایتەکەی کەوا زۆر ژەهراوی ترن. ئەوەی زۆر سرنج راکێشە ئەوەیە کە خانەکانی بەرگریدار بەرامبەر 7-OHMTX هەروەهابەرگریدارن بەرامبەر بە ،MTX و ئەوەش لەوانەیە لە کلینیك ڕوو بدات و ببێتە هۆی لەناوچوونی کاریگەری MTX. شایانی باسە هەردووک خانە بەرگریدارەکان هەستیارن بەرامبەربە دەرمانی ترایمێترێکسێت (Trimetrexate, TMQ) . ترایمێترێکسێت یەکێکە لە دەرمانە دژە کانسەرەکان لە بنە ماڵەی MTX کە هەروەها پێیان دەوترێت دژە فۆلێت (Antifolates). ئەودەرمانە پێویستی بە RFC یان FPGS نیە بۆ بۆ ئە وەی بچێتە ناو خانەکان یان خود بۆ ئەوەی کارە ژەهراویەکەی خۆی بەرامبەر بە خانە کانسەرەکان بکارێ ئەنجام بدات.

هەر وەها سەلماندومانە کە وا شێوەی بەرگری خانەکانی شێرپەنجەی خوێن بەرامبەر بە MTX و پاشەرۆکەکەی بە میکانیزمی جیا دروست دەبێت و ئەمەش هانی ئێمەی دا کەوا زیاتر لەم بارەوە بکۆڵینەوە.

شێوەی چالاکی ژێن (gene)ە گرنگەکان لەپشت ئە و شێوازە دروست بونی بەرگریەیە لەناو خانەکان دژ بە هەردووک MTX و 7-OHMTX.

ئەم گۆڕانکاریانەی لە خانە بەرگرەکان دژ بە7-OHMTX بە شێوەیەکی وایە کە خانەکان ئاماده دەکەن بۆ پاشکەوتکردنی فۆلێت (ماددەیەکە کەوا زۆر پێویستە بۆ ژیانی خانەکان). ئێمە چەند جیاوازیەکی گرنگمان دۆزیەوە کە لەوانەیە گرنگبن بۆ پێکهاتنی بەرگری دەستکەوتوو دژ بە MTX و 7-OHMTX.
رەنگە دە داهاتو دا،بە دەستێوەردانی ئەم ژێنانە بتوانین گرنگیان لە پەیوەندی دەگەڵ بە رگری بەرامبەربە MTX هەڵبسەنگێنین.

بەرگری بەرامبەر بە هەردووك 6-MP و6-TG (کەهەردووک پێیان دەوترێت تیۆپیورینس Thiopurines) دەگەڕێتەوە بۆ کەمبونێکی بەرچاو لە وەرگرتنی ئەم دوو دەرمانە لەلایەن خانەکانەوە.
ئێمە کەم بونی چالاکی و دەرخستنی دوولە ژێنەکانی گواستنەکانمان لە خانە بەرگریدارەکان بەدی کرد: گۆزەری نیۆکلیۆسایدی هاوسەنگی 2(ENT2) گۆزەری نیۆکلیۆسیدی بەستراوی 3 و(CNT3)، کەوا کەم بون بەبەراوردکردنیان لەگەڵ خانە هەستیارەکان. بۆ ئەوەی بیسەلمێنین کەوا ئەم پرۆتێینانە رۆلێکی بەرچاویان هەیە لە توانای بەرگری بەرامبەر بە تیۆپیورینەکان، نیشانمان دا کە بە کەم کردنەوەی رادەی چێ بونی ئەم پرۆتێینانە رادەی چونی 6-MP بۆ ناو خانەکان کەم دەبێتەوە. هەروەها لە کۆتایشدا توانیمان بەو شێوەیە بەرگری دەستکرد دروست بکەین بەرامبەر بەئەو دەرمانە دە خانەکان دا.

شایانی باسە خانە بەرگریکەرەکان بەرامبەر بە تیۆپیورینس، هەستیارن بەرامبەر بە یەکێک لە پاشەرۆکەکانی 6-MP ، بە ناوی methyl mercaptopurineriboside (meMPR). ئێمە نیشانماندا کە وا meMPR پێویستی بە CNT3 یاخود ENT2 بۆ چونە ناو خانەکان نیە، بەڵام لە جیاتی ئەوە بە هۆی ENT1 دەگوازرێتەوە.
هەستیاربونی خانە بەرگریکارەکان بە meMPR، ئەم بیرۆکەیە دێنێتە ئاراوەکە دەکار کردنی ئەم فاکتەرە بۆ ئەو نەخۆشانەی کانسەری خوێنی تیژە یان هەیە و وەڵامێکی خراپ دەدەنەوە بەرامبەر بە دەرمانی تیۆپیورینس، لەوەانەیە سودی هەبێت. هەمان بیرۆکەش لەوانەیە پەیرەو بکرێت بۆ بەکارهێنانی ترایمێتریکسێت بۆ ئە نەخۆشانەی کە وا بە باشی وەڵامی یان نیە بەرامبەر بە MTX. ئەم بیرۆکەیەش دەتوانرێت بە لێکۆڵینەوی کلینیکی دە داهاتوو دا هە لبسەنگێنرێت.

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APPENDIX (PAPERS I-V)