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Pharmacological studies on uptake, metabolism, and resistance to anti-cancer drugs: insights into the treatment of leukemia

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“Science never solves a problem without creating ten more”

George Bernard Shaw
(1856 – 1950)

Dedicated to my family

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ABSTRACT

Leukemia is blood cancer that begins in the bone marrow and characterized by abnormal production of white blood cells. Due to advances in treatment, there has been a dramatic increase in survival rate for patients with different types of leukemia. Among cytotoxic agents for leukemia, anthracyclines and thiopurines represent two highly effective groups of such therapy.

Anthracyclines are potent broad-spectrum cytotoxic drugs used for treatment of numerous cancers, including acute myelogenous leukemia (AML). However, side effects like dose-limiting bone marrow toxicity and the characteristic cumulative cardiotoxic effects, limit their clinical applications. These side effects are mainly caused by uptake of the drugs by normal cells, and the mechanisms behind their cellular uptake are not completely understood. Knowledge about uptake mechanisms could be beneficial to increase the selectivity of these drugs.

Thiopurines like 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) have been used extensively in the treatment of acute lymphoblastic leukaemia (ALL). Despite their wide-spread use, intrinsic and acquired resistance to thiopurines is a major problem. Understanding the mechanisms of resistance to these drugs should help optimize treatment and improve the survival rate.

In this thesis we investigated mechanisms and factors playing role in resistance to these two anti-leukemic groups by studying transport and intracellular metabolism by different enzymes:

In **Study I** we studied the uptake patterns of, daunorubicin (DNR), doxorubicin (DOX), epirubicin (EPI), idarubicin (IDA), and pirarubicin (PIRA) by human leukemic HL-60 cells in the presence of various inhibitors and investigated the possible involvement of specific carriers. Our data support the involvement of nucleoside transporters (NTs) in transmembrane transport of DNR, IDA, and PIRA. The results also showed a strong inhibitory effect of suramin on anthracycline uptake and cytotoxicity which requires further study. The significant reduction of cellular anthracycline uptake at low temperature strongly supports energy dependent carrier mechanisms.

In **Study II** we employed high-through-put characterization of genetic aberrations, including both expression microarrays and array-CGH, to elucidate the mechanisms underlying acquisition of resistance to thiopurines by human acute T-lymphocytic leukemia MOLT4 cells in an attempt to identify new markers and genes that may serve as valuable drug targets in the future.

The downregulation of the two nucleoside transporters, CNT3 and ENT2 in both 6-MP-and 6-TG-resistant cells indicates that impairment of the transport of these agents contribute to drug resistance. In addition, elevated expression of the human terminal transferase enzyme, encoded by the *DNTT* gene, was found in both 6-TG- and 6-MP-resistant cells as compared to the wild-type cells. Specific inhibitors of this enzyme might be developed into a novel class of antitumor agents.

In **Study III** we evaluated three different methods for thiopurine methyltransferase (TPMT) phenotyping, including an HPLC-based assay modified and optimized in our lab, which is able to measure TPMT activity in RBC of patients and leukemic cell lines with requirement of only one million cells.

We found significant relationships between the three methods and the distribution pattern of TPMT activity in RBC from 198 patients as determined by radiochemical, HPLC-UV, and HPLC-radiometric methods showed the classical trimodal distribution. Furthermore, the results indicated that the activity of TPMT enzyme is not changed in 6-MP-and 6-TG-resistant MOLT4 cells.

In **Study IV** we knocked down the expression of the TPMT enzyme in human MOLT4 leukemia cells employing specifically designed siRNA, in order to investigate the potential role of TPMT in the metabolism and thus, cytotoxicity of 6-MP and 6-TG. Our results indicate a 34% increase in sensitivity of MOLT4 cells to 1 μM 6-TG after treatment with TPMT-targeting siRNA, as compared to cells transfected with non-targeting siRNA, while sensitivity of the cells toward 6-MP was not affected significantly. We concluded that TPMT has a differential role in cytotoxicity of 6-MP and 6-TG, and probably inhibition of *de novo* purine synthesis (DNPS) by methylthioinosine monophosphate (meTIMP) makes a significant contribution to the cytotoxic action of 6-MP.

LIST OF PUBLICATIONS

I. **Karim H**, Bogason A, Bhuiyan H, Fotoohi AK, Lafolie P, Vitols S.
Comparison of uptake mechanisms for anthracyclines in human leukemic cells.
Curr Drug Deliv. 2012 Oct 8

II. **Karim H**, Hashemi J, Larsson C, Moshfegh A, Fotoohi AK, Albertioni F.
The pattern of gene expression and gene dose profiles of 6-mercaptopurine- and 6-thioguanine-resistant human leukemia cells.
Biochem Biophys Res Commun. 2011 Jul 22;411(1):156-61

III. **Karim H**, Malin Lindqvist Appell, Alan K. Fotoohi.
Comparison of three methods for measuring thiopurine methyltransferase activity in red blood cells and human leukemia cells.
Manuscript.

IV. **Karim H**, Aram Ghalali, Pierre Lafolie, Sigurd Vitols, Alan K. Fotoohi.
Differential role of thiopurine methyltransferase enzyme in the cytotoxic effects of 6-mercaptopurine and 6-thioguanine on human leukemia cells
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

6-MP	6-mercaptopurine
6-TG	6-thioguanine
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
AZA	Azathioprine
CGH	Comparative genomic hybridization
CNT	Concentrative Nucleoside Transporter
DMSO	Dimethyl sulfoxide
DNPS	*de novo* purine synthesis
DNR	Daunorubicin
DNTT	Deoxynucleotidyltransferase
DOX	Doxorubicin
DP	Dipyridamole
EDTA	Ethylene diamine tetraacetic acid
ENT	Equilibrative nucleoside transporters
EPI	Epirubicin
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
GMPS	Guanosine monophosphate synthetase
HGPRT	Hypoxanthine-guanine phosphoribosyl transferas
HPLC	High-performance liquid chromatography
IDA	Idarubicin
IMPDH	Inosine monophosphate dehydrogenase;
ITPase	Inosine triphosphate pyrophosphatase
LC-MS	Liquid chromatography–mass spectrometry
meMP	Methylmercaptopurine
meTIMP	Methylthioinosine monophosphate
NBMPR	Nitrobenzylthioinosine
NT	Nucleoside transporters
PI	Propidium iodide
PIRA	Pirarubicin
pRBC,	packed red blood cells
RT-PCR	Real-time polymerase chain reaction
SAM	S-adenosyl-L-methionine
siRNA	small interference RNA
SNP	Single nucleotide polymorphism
TGN	Thioguanine nucleotide
TIMP	Thioinosine-monophosphate
TPMT	Thiopurine methyltransferase

INTRODUCTION

According to the world health organization, cancer is a leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of all deaths) in 2008.
Deaths from cancer worldwide are expected to rise continuously, with an estimated 13.1 million deaths in 2030.
Among the types of cancer, leukemias represent a substantially important group with various types and clinical outcomes.
Due to advances in treatment, there has been a dramatic improvement in survival for people with acute lymphocytic leukemia (ALL), from a 5-year relative survival rate of 41% in 1975 to 1977 to 67% in 2001 to 2007 in adults. Survival rates for children with acute lymphocytic leukemia have increased from 58% to 91% over the same time period.

Leukemia

Leukemias can be defined as malignant neoplasms involving cells that are originally derived from hematopoietic precursor cells in bone marrow characterized by an abnormal increase in the number of immature white blood cells [1].
The term Leukemia or "white blood" was first described by the German pathologist Rudolf Virchow in 1845. He observed an abnormally large number of white blood cells in a blood sample from a patient.
It has been proposed that leukemias may be initiated by transforming events that take place in hematopoietic stem cells or they may originate from more committed progenitors caused by mutations and/or selective expression of genes (Figure 1).
Clinically and pathologically, leukemia is subdivided into 4 main categories, based on the type of white blood cell affected (lymphoid versus myeloid) and characteristics of the disease (acute versus chronic):

- Acute Lymphoblastic Leukemia (ALL)
- Acute Myeloid Leukemia (AML)
- Chronic Lymphoid Leukemia (CLL)
- Chronic Myeloid Leukemia (CML)

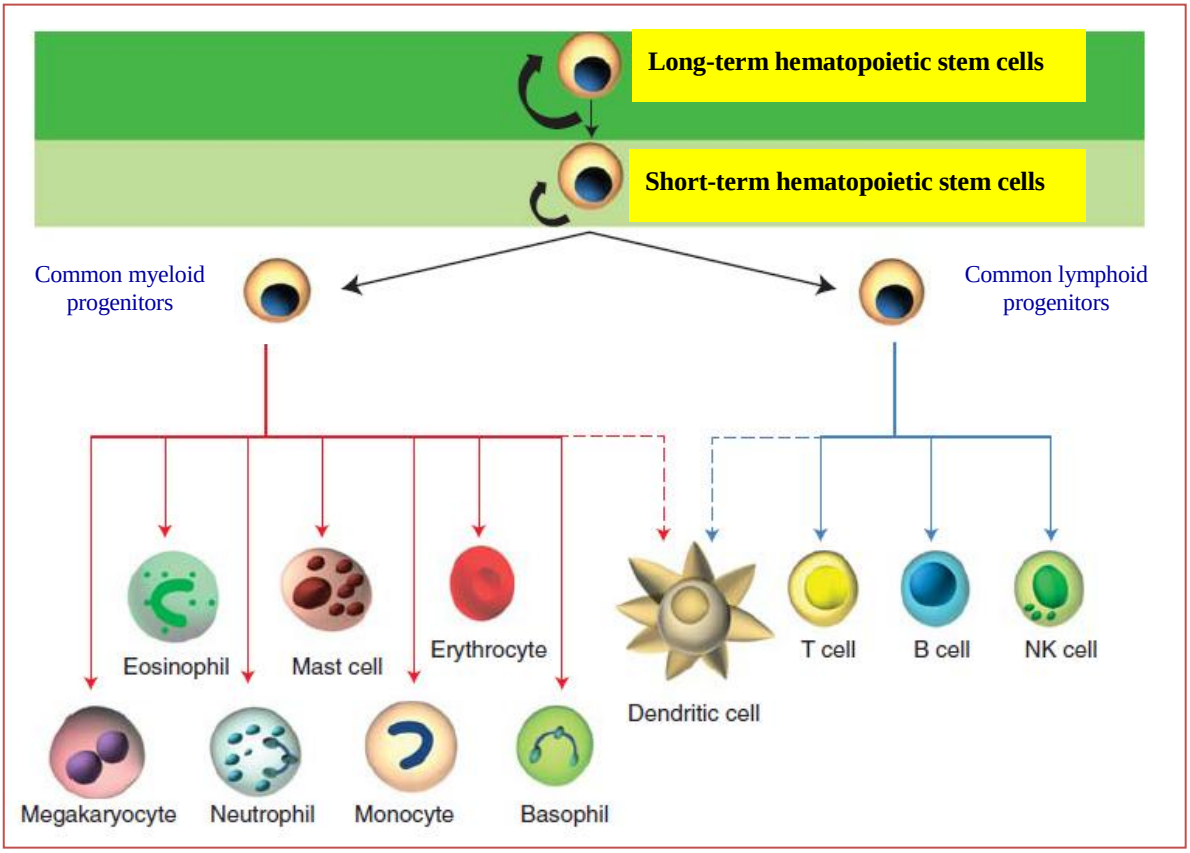


Figure 1. The hematopoietic stem cell hierarchy in adult bone marrow, (*Cold Spring Harb Perspect Biol 2013;5:a008011)*, with permission from publisher. The dashed lines indicate partial lineage connections.

Acute Leukemias

Acute leukemias (ALL and AML) are generally aggressive types of diseases in which cancerous transformation occurs at early stages in the development of the affected blood cell and if left untreated, these diseases can be rapidly fatal.

Acute leukemias are caused by damage to stem cells or cells in the early stages of development in the bone marrow (Figure 1). Mutations involving the control of cell division, differentiation, and cell death lead to the accumulation of early blood cell precursors known as *blast cells.*

Acute Lymphoblastic Leukemia

ALL is the most common form of leukemia diagnosed in children [2-4]. The incidence of ALL reaches peak between the ages of 3-7, falls by 10 years of age and followed by a rise again after the age of 40.

Acute Myeloid Leukemia

AML comprises of 10-15% of leukemias diagnosed in childhood and represents the most common type of acute leukemia diagnosed in adults [5,6], with about 320 cases per year in Sweden.

Chronic leukemias

Chronic leukemias are characterized by a slower progression than acute leukemias. These leukemias are difficult to cure radically, so the approach to therapy is often conservative aiming at controlling symptoms (palliative treatment).
Chronic lymphoid leukemias generally are diseases characterized by the accumulation of fully developed B or T lymphocytes in the blood. These diseases are closely related to lymphomas, in which lymphocytes accumulate in lymph nodes and vessels.

Chronic Lymphocytic Leukemia

It represents the most common type of chronic lymphoid leukemia and involves B lymphocytes. CLL is mainly disease of elderly individuals, with a peak incidence between 60 and 80 years of age. It is the most common form of leukemia in Western countries [7]. CLL follows a variable course, with survival ranging from months to decades [7].

Other types of chronic lymphoid leukemias include:

- Prolymphocytic leukemia (PLL)
- Hairy cell leukemia (HCL)
- Plasma cell leukemia
- Large granular lymphocytic leukemia
- T-cell prolymphocytic leukemia (T-PLL)

Chronic Myeloid Leukemia

CML accounts for approximately 15% of leukemias, it is seen most frequently between the ages of 40 and 60 years. It is characterized by increased numbers of cells belonging to the myeloid cell line (monocytes, neutrophils, basophils, eosinophils) at different stages of development circulating in the blood stream.

Treatments of leukemia generally include:

- ✓ **Chemotherapy.**
- ✓ **Radiation therapy.**
- ✓ **Bone marrow transplantation.**
- ✓ **Biological therapy.**
- ✓ **Targeted therapy.**

Treatment of ALL is divided into several phases:

- Induction chemotherapy

The primary goal is to achieve bone marrow remission. For adults, standard induction protocols include **prednisone, vincristine,** and an **anthracycline** ; other protocols may include **L-asparaginase** or **cyclophosphamide**. For children with low-risk ALL, standard induction therapy usually comprises **prednisone, L-asparaginase**, and **vincristine**.

- Consolidation therapy or intensification therapy

The goal is to eliminate any remaining leukemic cells. There are a variety of approaches to achieve consolidation, but it is typically a high-dose, multi-drug treatment protocol that is undertaken for several months. Patients with low- to average-risk ALL receive therapy with one of the **anti-metabolites** such as **methotrexate, 6-mercaptopurine (6-MP),** or **6-thioguanine (6-TG).** High-risk patients may receive higher doses of these drugs, plus additional necessary drugs.

- Central nervous system prophylaxis

This type of treatment prevents the leukemia from spreading to the brain and nervous system in high-risk patients. Standard prophylaxis may include radiation of the head and/or delivering of drugs directly into the spine.

- Maintenance treatments

Maintenance therapy is performed with chemotherapeutic drugs to prevent disease recurrence after remission has been achieved. Maintenance therapy usually involves lower drug doses, and may continue for few years.

- Alternatively, allogeneic bone marrow transplantation may be appropriate for high-risk or relapsed patients.

Chemotherapeutic drugs used for treatment of AML

Individuals with AML are usually treated with an anthracycline in combination with the antimetabolite **cytosine arabinoside (Ara-C).**

Most AML patients are treated with one of the **anthracyclines** [8,9] such as **daunorubicin,** or **idarubicin,** they are considered as golden standard for AML therapy since 1970s. Other drugs may be added or substituted for higher-risk, refractory or relapsed patients. According to Swedish counsel of Technology Assessment in Health Care (SBU), standard induction therapy for AML patients, consisting of **daunorubicin** and **Ara-C** in conventional doses , results in a complete remission rate of 50-60% in an unselected population and a long term survival of about 10-20%.

Treatment of Acute Promyelocytic Leukemia

Treatment for patients with APL, which represents the M3 subtype of AML, is different from treatment for patients with other AML subtypes. As a matter of fact, APL is one of the most frequently cured subtypes of AML. APL affects marrow cells called promyelocytes, which form after myeloblast development. The promyelocytes have abnormal chromosomal changes, usually a translocation between the chromosomes 15 and 17.

Drugs commonly used to treat APL are:

- all-*trans* retinoic acid (ATRA)
- arsenic trioxide, (ATO)

About 70 percent to 80 percent of APL patients go into remission after being treated with **ATRA** and an **anthracycline**, such as **idarubicin.**

The ideal duration of maintenance therapy consists of 2 years of **6-MP, methotrexate,** and **ATRA**.

Treatment of CLL

Fludarabine is considered one of the most effective types of chemotherapy treatment for CLL. Other standard chemotherapy drugs that physicians use to treat CLL as a single agent or in combination include:

Cyclophosphamide, **doxorubicin**, prednisone, vincristine, cladribine, bendamustine, and chlorambucil.

ANTHRACYCLINES

Anthracyclines (or anthracycline antibiotics) are a class of drugs widely used in cancer chemotherapy derived from Streptomyces bacterium *Streptomyces peucetius var. caesius.* They are potent broad-spectrum cytotoxic drugs [8,10-14] that are characterized by their strong red colors, examples are:

Doxorubicin (DOX), daunorubicin (DNR), epirubicin (EPI), idarubicin (IDA), pirarubicin (PIRA), valrubicin.

Mechanisms of action for anthracyclines:

The anthracyclines are the class of antitumor drugs with the widest antitumor spectrum of activity in human cancers, they are amongst the most effective cytotoxic drugs ever developed [8,11].

They exert their effects through a variety of mechanisms like:

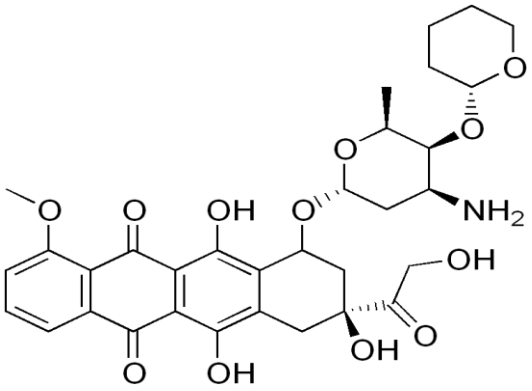
- ✓ Blockage of DNA/ RNA synthesis through interfering between base pairs of the DNA/RNA strand, thus inhibiting the replication of rapidly-growing cancer cells.
- ✓ Generation of free oxygen radicals, leading to both DNA damage and lipid peroxidation which damages the cell membranes [15].
- ✓ Inhibition topoisomerase II enzyme, preventing the relaxing of supercoiled DNA and thus blocking DNA transcription and replication [16,17].

DOX and IDA have also been reported to inhibit topoisomerase I [12,18] Furthermore, anthracyclines like many other genotoxic substances, can induce apoptosis through activation of different pathways and through activation of p53 [19,20].

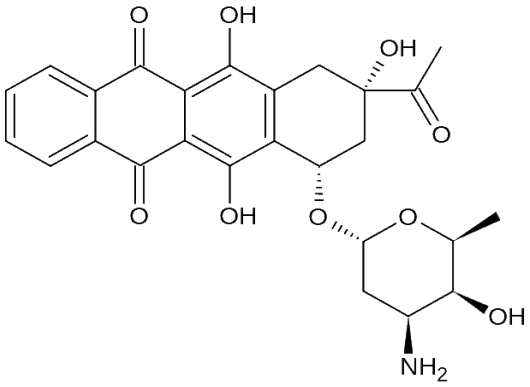
However, there are some major problems associated the use of these drugs that limit their clinical use like a dose- limiting bone marrow toxicity and the characteristic cumulative cardiotoxic effect (Figure 3) that irreversibly leads to congestive heart failure [13,21-23].

The mechanisms behind the cardiotoxicity of anthracycline are not completely understood but their C-13 metabolites generate reactive oxygen species (ROS) which might be particularly harmful for cardiac cells [13,21] (Figure 3).

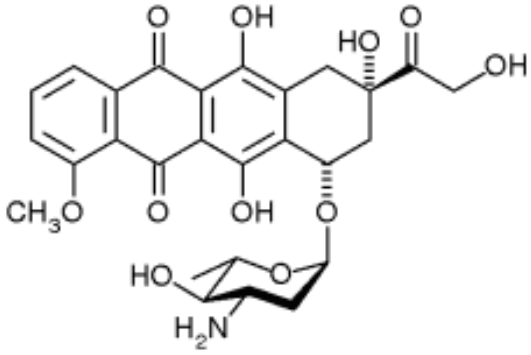
These agents can kill cells by inducing either apoptosis (programmed cell death) or necrosis (uncontrolled/spontaneous cell death) [15,24].



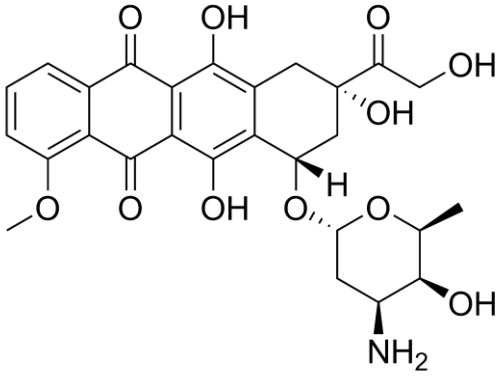
Pirarubicin



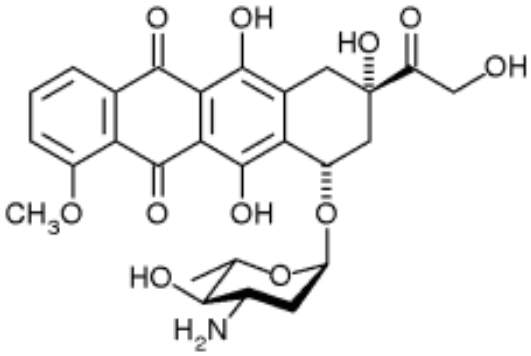
Idarubicin



Daunorubicin



Doxorubicin



Epirubicin

Figure 2. *Chemical structure of the five Anthracyclines used in study I*

The liposomal formulations of DNR and DOX are known to be less toxic to the cardiac cells as compared to non-liposomal preparations because a lower proportion of drug administered in the liposome form is delivered to the cardiac tissue [25].

Anthracyclines are known to be lipophilic compounds that distribute rapidly in body tissues, readily binding to plasma proteins and cell membranes. They have a high volume of distribution most of them exceeding 500 L/m^2 and IDA, being the most lipophilic one, exceeding 1800 L/m^2 while having a plasma protein binding ranging from 50 to 85 %, meaning that most part of a given dose is located in the tissues outside the bloodstream.

Anthracyclines are cleared from circulation through liver metabolism with a terminal half-life that ranges from 16-48 hours and are excreted primarily via the hepatobiliary tract [26-29].

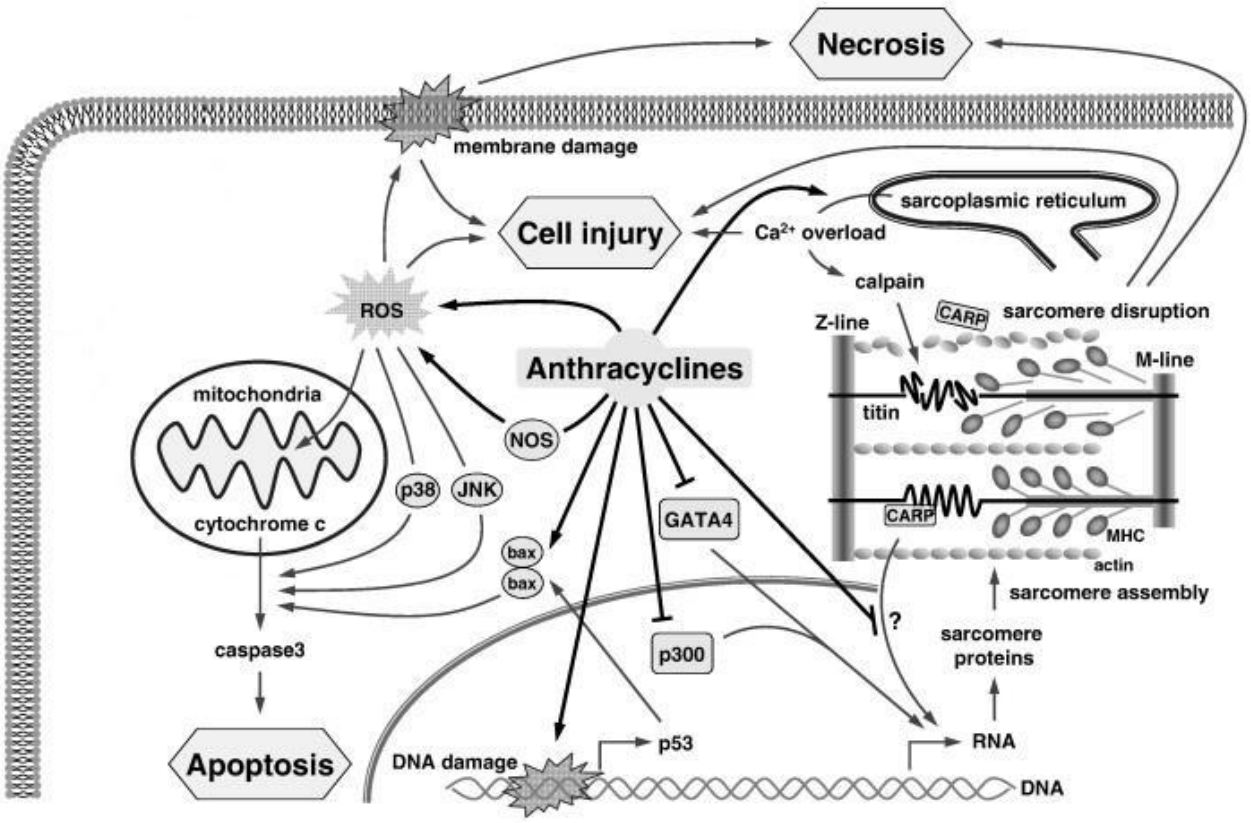


Figure 3. Anthracyclines promote myocyte injury and death via multiple mechanisms. This is balanced by intracellular signaling (e.g. GATA4, Akt) that is responsive to paracrine factors such as neuregulin (nrg). Anthracyclines also disrupt cardiac sarcomere structure by accelerating the degradation of key sarcomeric proteins (e.g. titin) and inhibit new sarcomere protein synthesis via alteration of transcriptional regulation.
D.B. Sawyer et al. / Progress in Cardiovascular Diseases 53 (2010) 105–113, with permission from publisher.

Initial studies suggested that anthracyclines are transported via passive diffusion into the cells [30,31]. However, recent studies have shown that transport proteins might be involved in cellular anthracycline uptake [32,33].

However, evidence for such transport mediated uptake was not found in normal mononuclear blood cells suggesting the possibility of different mechanisms behind their uptake in normal and malignant cells [32,33].

Results from previous studies also indicate that different transport mechanisms could be involved in the uptake of DOX and PIRA in Ehrlich ascites carcinoma cells, however, the specific transporters were not identified [34].

This is why studies on cellular anthracycline uptake using protein inhibitors such as suramin, a purinergic-2-receptor blocker, and nucleoside transport inhibitors such as nitrobenzylthioinosine (NBMPR) and dipyridamole are important to conduct and helps to provide more insights into the transport of these drugs in cancer cells.

Metabolism of anthracyclines

There is a pronounced inter- and intra individual variation in plasma levels of anthracyclines in AML patients, despite standardized dosing based on body surface area [8,9]. The alteration in pharmacokinetics is most likely due to a variation in systemic metabolism of the drugs [35,36].

All commonly used anthracyclines are mainly metabolized by a cytoplasmic reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent aldoketo reductase to a glycoside metabolite (i.e., doxorubicinol, epirubicinol, daunorubicinol) that has antitumor activity [37,38].

The plasma concentrations of e.g., daunorubicinol exceed those of the parent substance within a few minutes, and the metabolite is retained in plasma much longer. Due to their reduced lipid solubility, the cellular uptake of these metabolites is low and they are generally not considered to be of importance for the clinical effect of the drugs [37].

Three enzymes were reported to be capable of anthracycline carbonyl reduction in human liver: aldoketoreductases AKR1A1, AKR1C2, and, the short-chain dehydrogenase /reductase, carbonyl reductase 1 (CR1) [38,39]. Currently it is believed that AKR1A1 and CR1 are the

major anthracycline metabolizing enzymes and that enzyme specificity might vary with anthracycline type [38,40-42].

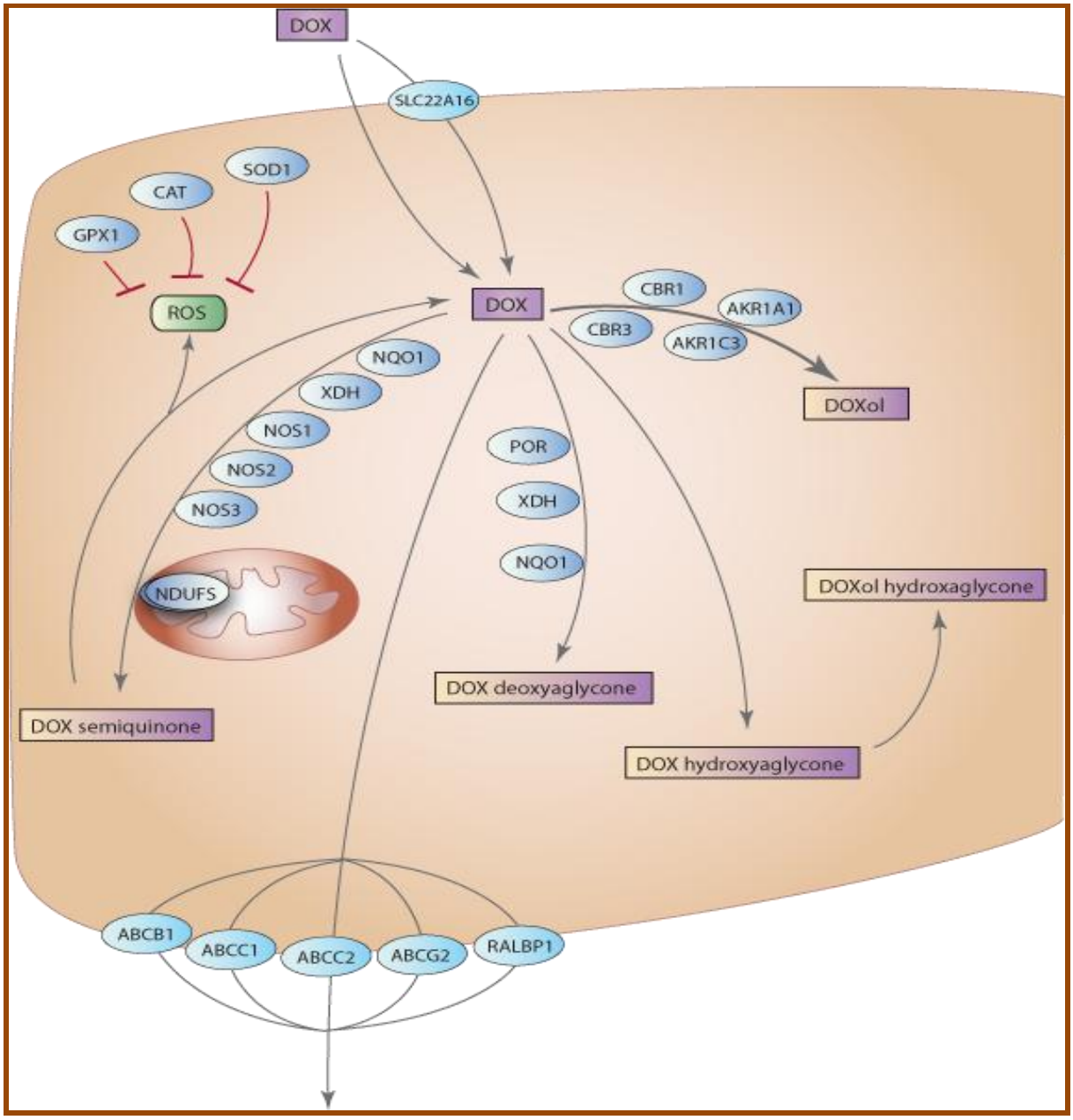


Figure 4. Diagrammatic representation of the transport and metabolism of doxorubicin. *Thorn Caroline F, Oshiro Connie, Marsh Sharon, Hernandez-Boussard Tina, McLeod Howard, Klein Teri E, Altman Russ B. "Doxorubicin pathways: pharmacodynamics and adverse effects" Pharmacogenetics and genomics (2010).* Permission has been given by PharmGKB and Stanford University.

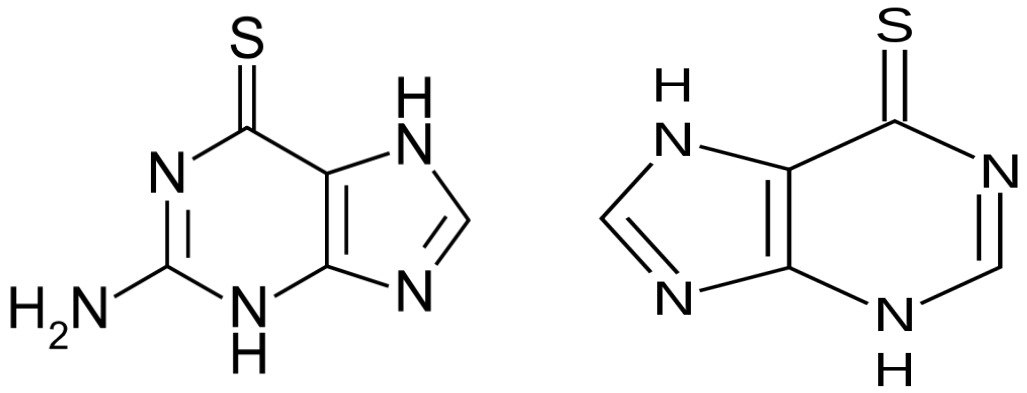
THIOPURINES

The thiopurine drugs, azathioprine (AZA), 6-MP, and 6 TG are widely used to treat malignancies, rheumatic diseases, dermatologic conditions, inflammatory bowel diseases, and solid organ transplant rejection [43-52].

Two of these drugs, 6-MP and 6-TG, have been used extensively in the treatment of acute leukemia. 6-MP has been preferred over 6-TG during continuing (maintenance) therapy in childhood ALL, whereas 6-TG has been used during consolidation and in remission induction in AML [45,52].

The third drug, AZA is widely used as an immunosuppressant for the treatment of autoimmune conditions like inflammatory bowel disease, and following transplantation.

Like many cytotoxic agents, the therapeutic dose range for the thiopurine drugs is relatively narrow with potential life-threatening side-effects primarily in the form of hepatotoxicity and myelosuppression [43,46-51,53].



6-TG **6-MP**

Figure 5. Chemical structure of the two major thiopuirne antimetabolites.

Cellular uptake of Thiopurines

The most extensively studied transporter proteins that transport thiopurines are the nucleoside transporters (NT), which can be subdivided into two major classes: equilibrative (facilitated) transporters and concentrative or Na^+-dependent transporters [53-56].

The human family of equilibrative transporters (ENT) so far comprising four members of which two are well-characterized, namely, ENT1 and ENT2 that exhibit similar broad specificities for purine and pyrimidine nucleosides. On the other hand, the family of concentrative nucleoside transporters (CNT) consists of three subtypes of sodium-dependent transporter proteins: CNT1 transports pyrimidine nucleosides preferentially, CNT2 prefers purine nucleosides, while CNT3 can transport both purine and pyrimidine nucleosides across the cell membrane.

On the other hand, the multidrug resistance protein 4 (MRP4/ABCC4), which is also thought to be involved in nucleoside drug transport, has been shown recently to provide protection against thiopurine-induced hematopoietic toxicity by actively exporting thiopurine nucleotides [53].

Metabolism of Thiopurines

Thiopurines are pro-drugs and have to be metabolized inside the cells (Figure 6) in order to exert their cytotoxic action [57,58]. Thiouric acid is the principal catabolite of 6-MP and 6-TG found in urine [59,60]. For both 6-MP and 6-TG, activation is catalyzed by the hypoxanthine-guanine phosphoribosyl transferase (HGPRT), followed by multi-step conversion (Figure 6) to thioguanine nucleotides (TGNs) that can be incorporated into DNA or RNA [61]. In case of 6-MP, this process is accompanied by production of methylated metabolites such as methylthioinosine monophosphate (MeTIMP), that strongly inhibit *de novo* purine synthesis (DNPS) [55,61]. However, there is a competition between such activation by HGPRT and deactivation through methylation of thiopurines by thiopurine methyltransferase (TPMT), which is characterized by several common genetic polymorphisms [50,55,62-64]. The observed variation in TPMT activity is now known to be largely the result of single nucleotide polymorphisms (SNPs) [62,63,65].

So far, at least 40 sequence variants have been identified in the TPMT gene [66-68]. TPMT*3A, TPMT*3C and TPMT*2 genotypes are the most common mutant alleles which cause 80-95% of intermediate or deficient TPMT phenotypes in white Caucasians and African-Americans [66,69,70].

TPMT represents the predominant inactivation pathway of thiopurines in hematopoietic cells; thus, patients who inherit TPMT deficiency accumulate excessive concentrations of the active TGNs in blood cells when treated with conventional doses of these medications [43,44], Conversely, patients with very high TPMT activity may be undertreated and have an increased risk of relapse [48,62].

Recently, genetic polymorphisms have also been identified in the gene regulating inosine triphosphate pyrophosphatase (ITPase) enzyme. This enzyme phosphorylates the intermediary metabolite 6-thioinosine-5'-monophosphate (TIMP) to 6-thioinosine triphosphate. Potentially toxic 6-thio-ITP is predicted to accumulate in ITPase-deficient patients [71]. Polymorphisms in the ITPase gene may account for non–TPMT-related adverse effects from AZA/ 6-MP therapy such as influenza-like symptoms, rash, and pancreatitis [43].

The TPMT phenotype and genotype can be measured by different methods, for the phenotype there are a number of assays using radiochemical activity assay, high performance liquid chromatography (HPLC) or Liquid chromatography–mass spectrometry (LC-MS) [48,64,65,72-75].

Commonly, all methods employ S-adenosyl-L-methionine (SAM) as methyl donor [76] thereafter the product 6-methylmercaptopurine (6-meMP) is extracted and its concentration measured either by scintillation counting (for radiochemical assay) or by ultraviolet, radiometric, or mass spectrometry detectors (for liquid chromatography methods).

A quite similar HPLC method for TPMT activity quantification has been described in which the methyl group is transferred from SAM to 6-TG i.e. based on the conversion of (6-TG) to 6-methylthioguanine (6-mTG) [77].

The choice of which method / assay to use probably depends on the availability of the HPLC / LC-MS instrumentation or the availability of a license and guidelines for handling radioactive substances under safe conditions.

In spite of the long term application of the thiopurines in the clinic, intrinsic and acquired resistance still remains as a major problem. The mechanisms underlying the acquired resistance of leukemic cells to 6-MP and 6-TG are still poorly understood [55].

The most extensively characterized mechanism is a reduction or lack of HGPRT activity. In addition, altered TPMT activity can influence sensitivity to 6-MP and 6-TG. Furthermore, it has been shown that in MOLT4, CCRF-CEM, and Jurkat cell lines, inactivation of the mismatch repair system leads to pronounced thiopurine resistance.

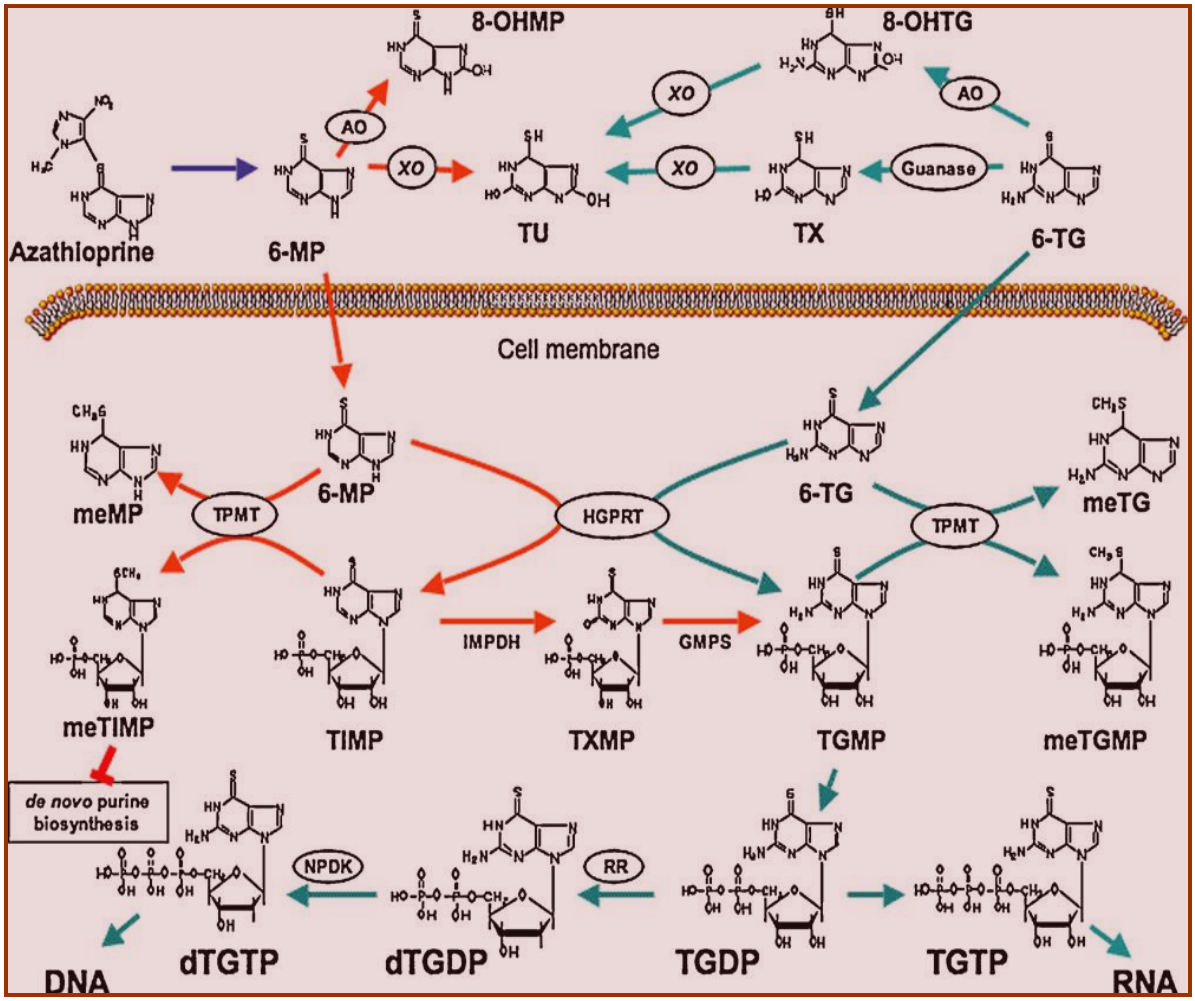


Figure 6. Metabolism of thiopurines. Thiopurines are catabolized by xanthine oxidase (XO), guanase and aldehyde oxidase (AO) in the extracellular space. When inside the cell 6- TG is converted directly by hypoxanthine–guanine phosphoribosyl transferase (HGPRT) through addition of ribose-5-phosphate to 6-thioguanosine-5'-monophosphate (TGMP), 6-MP is converted first to 6-thioinosine-5'-monophosphate (TIMP) by HGPRT then to 6-thioxanthine-5'-monophosphate (TXMP) by inosine monophosphate dehydrogenase (IMPDH) and finally to TGMP by guanosine monophosphate synthetase (GMPS). Both 6-MP and 6-TG and their respective monophosphates (TIMP and TGMP) are extensively inactivated inside the cell by thiopurine-S-methyltransferase (TPMT). Methylthioinosine monophosphate (meTIMP) is a strong inhibitor of DNPS. The remaining TGMP is converted to 6-thioguanosine-5'-diphosphate (TGDP), reduced to deoxy-6-thioguanosine-5'-diphosphate (dTGDP) by ribonucleotide reductase (RR) and phosphorylated by nucleoside diphosphate kinase (NDPK) to dTGTP, a DNA polymerase substrate, *(Fotoohi AK, et al. Thiopurines: Factors influencing toxicity and response.Biochem Pharmacol (2010),* doi:10.1016/j.bcp.2010.01.006. With permission from publisher.

Screening for TPMT activity in clinical laboratories:

Several studies have focused on TPMT genotype-phenotype relationships, and overall concordance rates in different population studies range from 76 to 99% [43,48]. These genotype-phenotype discrepancies could be interpreted by the possible contribution of genetic factors involving regulatory elements of the TPMT gene or other loci as well as with epigenetic factors such as TPMT inhibition by co-administered medications [43].

Sometimes phenotyping of TPMT is preferred over genotyping for the following reasons: there is a two- to three-fold variation in TPMT activity among heterozygous individuals, which could be reflected in the thiopurine tolerance and can not be predicted by the genotype [48,77]. Furthermore, a number of homozygous "wild-type" individuals reveal very high TPMT activity, and they might need treatment with higher than standard doses of the drugs. In addition, there is evidence that chronic disease, medications like diuretics and benzoic acid derivatives can alter the baseline TPMT activity [77] without any changes detectable by genotypic methods [76,78]. The activity of TPMT may also be affected by anemia (e.g. deficient RBC production) and age of RBC [79].

Phenotypic measurements are usually carried out on whole blood from patients at the time of diagnosis or, if they have had a transplant, at least 60 days following the transplant. Samples for genotyping are usually taken at diagnosis with the assumption that blast cell genotype is the same as somatic genotype [65].

At the beginning of therapy with 6-MP, 6-TG, or AZA, measurement of TPMT activity categorizes patients into high, intermediate, and low activity groups, where there is a high risk of side effects such as severe myelosuppression in the low activity group when treated with standard doses of thiopurines [65,80].

Thiopurines do not induce TPMT enzyme activity. However, TPMT gene expression decreased during thiopurine treatment, but no change in enzyme activity was observed [81]. Although erythrocyte TPMT activity correlates with the levels of TPMT activity found in other cells and tissues, including liver, kidney, platelets, lymphocytes and lymphoblasts [43,72], some people prefer TPMT activity in peripheral blood mononuclear cells (pMNC) rather than red blood cells (RBCs) , since frequent transfusions for anemia are required during thiopurine treatment and this might interfere with the readings [72], and the optimum

point in the treatment for measurement of TPMT activity in a prospective study remains to be determined.

Comparison between metabolism of 6-MP and 6-TG

Intracellularly, 6-MP is converted by HGPRT into thioinosine monophosphate (TIMP) which can be converted subsequently into thioguanosine monophosphate (TGMP) involving two additional enzymes, inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthetase (GMPS) [43,53,82].
The less direct metabolism of 6-MP to TGMP than that of 6-TG (Figure 6), may be the reason behind the stronger cytotoxicity of 6-TG, as compared to 6-MP , in terms of apoptosis induction [83].
TPMT on the other hand is a major player in this process and metabolizes both 6-MP and 6-TG to different methylated metabolites including meTIMP and methylthioguanosine monophosphate (meTGMP), respectively, with different suggested pharmacological and cytotoxic properties.

It has been suggested that DNPS inhibition by meTIMP may make a significant contribution to the cytotoxic action of 6-MP [45], however, the equivalent of meTIMP in 6-TG metabolism, namely, meTGMP is a twelve-fold less potent inhibitor of DNPS when compared to meTIMP [72,84].
Thus it seems likely that significant DNPS inhibition by MeTIMP can be achieved in vivo after oral thiopurines and can contribute to the cytotoxic action of AZA/6-MP [85].

In this way, 6-MP exerts a cytotoxic effect even by another mechanism independent of TGN production, i.e. through inhibition of DNPS by MeTIMP, while the mechanism of cytotoxicity for 6-TG is solely dependent on DNA-TGN incorporation rather than inhibition of DNPS [45].

Coulthard et al. showed a 4.4-fold increment in sensitivity to 6-MP, rise in intracellular levels of meTIMP, and a decrease in levels of DNA-TGN after induction of TPMT activity in the embryonic kidney cell line. On the contrary, induction of TPMT led to a 1.6-fold reduction in sensitivity to 6-TG, a reduction in levels of DNA-TGN, and an increase in levels of meTGMP [45].

Moreover, Dervieux et al. demonstrated that human CCRF-CEM cell lines that overexpress TPMT were more sensitive to 6-MP and less sensitive to 6-TG than cells not overexpressing TPMT [86].

However, a recent study by Misdaq et al. which is technically closer to our work, through transfection of a TPMT-specific short hairpin RNA (shRNA) expressing plasmid in T-lymphocytes, showed that responses to 6-MP treatment were not much affected by TPMT status in wild type and knock-down cells when considering the IC_{60} concentrations for wild type and knock-down cells, 4.6 µM/L and 4.7 µM /L, respectively.
While they concluded that 6-TG efficacy was more affected than 6-MP by TPMT levels as reflected by IC_{60} concentrations for wild type and knock-down cells, 2.7 µM/L and 0.8 µM /L, respectively [83]. These results lead to a common conclusion that TPMT affects each of 6-TG and 6-MP differentially, by metabolisisng each drug to pharmacologically different metabolites with variable cytotoxic properties.

AIMS OF THE PHD PROJECT

The primary aims of this thesis were to shed light on the various mechanisms and factors playing role in resistance to anticancer drugs, especially anti-leukemic medications, from different aspects like transport and intracellular metabolism by different enzymes.
The goals of the studies were:

1. To study the transport and uptake mechanisms of different anthracyclines by human leukemic cells and check for the involvement of carriers / transporter proteins in this process.

2. To elucidate the pattern of gene expression and gene dose profiles of 6-mercaptopurine- and 6-thioguanine-resistant human MOLT4 leukemia cells with the aim of finding novel therapeutic targets such as transporters and enzymes .

3. To compare three clinical assays for the measurement of TPMT activity in red blood cells and human leukemia MOLT4 cells and optimize one of the methods.

4. To investigate the differential role of TPMT enzyme, involved in metabolism of thiopurines, in tolerance of human leukemia MOLT4 cells to the cytotoxic effects of 6-MP and 6-TG.

MATERIAL AND METHODS

Cell lines (Study I II III IV)

- Human acute promyelocytic leukemia HL-60 cells
- Human acute lymphoblastic leukemia MOLT-4 cells
- Human breast adenocarcinoma MCF-7 cells
- Two resistant cell lines MOLT4/6-MP and MOLT4/6-TG derived from wild-type

cell line (American Type Culture Collection) were generated by continuous exposure to increasing concentrations of 6-MP and 6-TG, respectively until the final concentration of 5µm was reached.

The resistant cells were then cultured in drug-free medium for three passages before using them during logarithmic phase growth [56].

The cells were counted using a Coulter Multisizer (Coulter Electronics, Luton, United Kingdom) and harvested during the logarithmic phase of their growth.

Each cell line was cultured in a humidified incubator containing 5% CO_2 at 37 ^{o}C in the recommended cell culture medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml).

Human blood samples (Study III)

198 human samples were used in the evaluation of the methods for the measurement of TPMT enzyme activity in RBC, the samples were anonymous to the investigators and the patients underwent enzyme investigation as a part of their treatment.

Analysis of the uptake of anthracyclines (Study I)

During the uptake experiments HL-60 cells were incubated at a concentration of 10^6/ml in 8 ml RPMI 1640 growth medium at 37 ^{o}C for 1 h (pulse incubation) with 0.1, 0.5, 1, 5, and 10 µM DOX, EPI, PIRA, DNR, or IDA. The control MCF-7 cells were seeded in 6-well plates 24 hours before the uptake experiments. In other experiments, HL-60 cells were preincubated for 20 min with suramin (2-500 µM), nucleosides (100 µM), dipyridamole (DP, 1µM), nitrobenzylthioinosine (NBMPR, 1µM), verapamil (100 µM) (Sigma Aldrich AB,

Stockholm, Sweden) or kept at 0 ^{o}C (for the duration of the experiment) before being incubated with anthracyclines in 12-well plates at a density of 10^6/ml and total volume of 2ml growth medium. Immediately after the anthracycline incubation, portions were taken (2x 1ml for drug determination), cells were collected by centrifugation at 550 g for 5 minutes at 4 ^{o}C, followed by washing twice with ice-cold PBS (pH 7.4). For DNA fragmentation quantification, the remaining cells were washed twice with PBS and then resuspended and cultured in drug-free medium for 24 h after which an aliquot of the cells was used for the assay.

Anthracycline measurement by HPLC (Study I)

Cells were lysed in ice-cold water followed by sonication for 10 seconds using an ultrasonic processor, VCX 400 (Sonics & Materials, Danbury, CO, USA), and proteins were precipitated with 60% acetonitrile for drug analysis. Anthracycline concentrations in cells was determined by HPLC using a phenyl-µ-Bondapak column (3.9 x 150 mm, 5 mm. Waters Associates, Milford, MA, USA) eluted with acetonitrile and 0.2% ammonium formate pH 4 (60:40 v/v) at a flow rate of 1.5 ml/min. PIRA was used as internal standard for DOX and DNR while DOX was used as an internal standard for IDA, PIRA and EPI. The anthracyclines were quantified by a fluorescence detector model Shimadzu RF-551 fluorescence HPLC monitor at $\lambda_{excitation}$ 485 nm and $\lambda_{emission}$ 560 nm. The detection limit of the assay was $5*10^{-3}$ µM and the range of quantification was 0.03-20 µM, with a coefficient of variation (CV) of less than 7 %. Cellular drug concentration was expressed in µM.

Determination of apoptosis by flow cytometry (Study I)

Propidium iodide (PI)-based staining procedure was used for detection of apoptosis, as previously described [87]. As a marker of apoptosis, the appearance of cells less intensively stained than G1 cells (sub-G1 or A_o cells) in flow cytometric DNA histograms was used. In brief, a cell pellet containing 10^6 cells was gently resuspended in 0.5 ml PI staining solution (PI 50µg/ml in 0.1% (w/v) sodium citrate plus 0.1% (v/v) Triton X-100) in 12 x 75 mm polypropylene tubes. The tubes were stored at 4°C in a dark room for 1 to 3 days before running with FACS machine. Flow cytometry FACScan (Becton and Dickinson, Mountain View, CA, USA) with a single 488-nm Argon laser was employed for measuring the PI fluorescence of individual nuclei. The red fluorescence due to PI staining of DNA was detected in the FL-3 channel and the data was registered on logarithmic and linear scales.

Both forward scatter and side scatter of particles were measured simultaneously. The flow rate was set at 12 µl /second and at least 10,000 target events were collected for each sample. Analysis of the results was performed by using Cell Quest™ software.

RNA extraction and cDNA synthesis (Study I & IV)

Total RNA was extracted from cultured cells using the RNeasy Midi kit and the protocol recommended by the manufacturer (RNeasy Midi Handbook; Qiagen, KEBO Lab, Spånga, Sweden) according to the manufacturer's instructions. RNA concentrations and quality were determined using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE), RNA with an OD ratio of 1.99–2.0 at 260/280 was regarded as of acceptable purity.

Complementary DNA was produced using Invitrogen SuperScript™ III first strand synthesis system as recommended by the manufacturer's instructions.

Messenger RNA expression by RT-PCR (Study I & IV)

The expression of selected genes in *study I* was determined employing commercially available quantitative real-time PCR analyses (Applied Biosystems, Stockholm, Sweden). The mRNA levels thus obtained were related to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA (*GAPDH*) [56].

In *study IV,* Real-time PCR reactions were carried out using the 7500 real-time PCR system and reagents from Applied Biosystems (Foster City, CA).

The cDNA samples were used as templates whereas an endogenous housekeeping gene *(GAPDH)* was quantified as a positive control and used for normalization of the different template values.

Each real-time TaqMan PCR reaction mixture (20 µl) contained 9 µl cDNA template, 1 µl TaqMan® Gene Expression Assay and 10 µl TaqMan Universal PCR Master Mix (according to manufacturer's instruction ''TaqMan® Gene Expression Assays'' Applied Biosystems).

The mRNA expression of TPMT in transfected cells was related to the expression in cells treated with the non-targeting negative control siRNA.

Genotyping of wild-type and resistant MOLT4 cells (Study II)

Both parental and resistant MOLT4 sublines were submitted to genotyping in order to check for single nucleotide polymorphism (SNP) at the Mutational Analysis Facility (MAF), Karolinska Institutet, Stockholm, using a panel of 47 markers and analysis on a SequenomTM mass 156 spectrometer as described by Hannelius and colleagues [88]. Pair-wise comparison showed identical genotypes in parental MOLT4 and 6-MP-resistant cells and 88% identity between parental and 6-TG-resistant cells.

Gene expression by Microarray (Study II)

The gene expression microarrays and all other reagents required for these analyses were purchased from Affymetrix (Affymetrix Inc., Santa Clara, CA, USA). The wild-type MOLT4, 6-TG-resistant and 6-MP-resistant sublines were analyzed in triplicate in accordance with the manufacturer's instructions (Technical manual of Affymetrix GeneChip products). The experimental steps and analyses procedures have also been described in detail in one of our previous publications [89]. The expression data were analyzed using the GeneSpring software (Agilent) and subsequently categorized with the Ingenuity software for normalization and exclusion of probe sets that did not meet criteria for detection. Probe sets with low expression below 50 in intensity value were excluded. Statistical calculations were performed by Analysis of variance (ANOVA) with $p < 0.05$ as the cut-off for statistical significance. Categorization into selected groups for some differentially expressed genes was carried out by Ingenuity software. All of the microarray data were submitted and are available at http://www.ncbi.nlm.nih.gov/geo/.

DNA isolation and comparative genomic hybridization (array-CGH) (Study II)

Genomic DNA was purified using the GenElute^{TM} kit (Sigma-Aldrich, Inc.). NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used for quantification and purity control of DNA. Array-CGH was carried out and analyzed basically according to a previously published method [90].

Cellular DNA marked with Cy3-dCTP and reference DNA (Promega, USA) labeled with Cy5-dCTP were pooled, mixed with human Cot-1 DNA, and hybridized to tiling 38K BAC arrays (SCIBLU Genomics Centre at Lund University, Sweden; www.lu.se/sciblu) for 72 hours at 37°C. The slides were subjected to washing and drying then followed by scanning in

a GenePix 4200A (Axon Instruments Inc., Union City, CA) and they were analyzed by GenePix Pro 6.0 (Axon Instruments, Wheatherford TX, USA), the data were uploaded into the BioArray Software Environment, BASE (http://www.base.thep.lu.se/) [91]. Normalization was performed with the pin-based LOWESS algorithm [92] and relative copy numbers were identified with CGH plotter [93] applying: >0.25 (gain), >1.0 (amplification), <-0.25 (loss) and <-1.0 (homozygous loss) as cut-offs for log2 ratios. The cytogenetic localization of clones with mapping information were achieved according to the UCSC genome browser (http:/www.genome.ucsc.edu/; July 2004 freeze). The sex chromosomes were excluded from the analyses and aberrations in telomeric regions were interpreted carefully.

Preparation of erythrocyte lysates (Study III)

To prepare red blood cell (RBC) lysates, blood was collected from 198 anonymous patients who were undergoing investigation as a part of their treatment, in tubes containing ethylenediamine tetra-acetic acid (EDTA) and was kept at room temperature or at 4ºC prior to isolation. The RBCs were washed twice in 0.9 % NaCl and the hematocrit was determined. The packed red blood cells (pRBC) were then lysed using ice-cold water. The prepared cell lysates were kept at -80 ºC until analysis [80].

Preparation of cell line lysates (Study III)

One to ten millions of both sensitive and resistant leukemic MOLT4 cells were centrifuged at 1500 rpm for 10 minutes and the supernatants were removed. The cells were washed with PBS, kept on ice, and then lysed by extracting buffer (50 mM Tris-HCl, 2mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol and 0.5% NP40) and 3 times sonication with duration of 10 seconds and interval of 20 seconds. Followed by centrifugation 30 000g for 10 min in cold centrifuge.

Protein content of lysed cells was quantified by Bio-Rad, DC protein assay (Bio-Rad laboratories, CA 94547). The prepared cell lysates were kept at -80 ºC until analysis.

HPLC instruments and chromatographic conditions (Study III)

The chromatographic condition setup included an automated HPLC with a Gynkotek 580 G Pump, a Hypersil Duet C18/SAX Column which Diameter and particle size are 250 x 4.6 mm, 5 μm respectively, an autosampler (waters 717), a Packard Bioscience Radiomatic detector and an UV detector (Lamda Max 481). Sample cooler of autosampler was kept at 4 ºC. Mobile phase consisted of $NH_4H_2PO_4$ (37.5 mM) containing 20% methanol (pH 3.4). The UV detection wavelength was set up at 290-nm absorbance. Mobile phase and scintillation fluid flow rate was set up at 2 and 6 ml/min, respectively. The peaks of the relevant compounds were identified employing both retention time and UV spectra. The UV spectra were constructed using standard 6-meMP and retention time was set up by measuring peak of standard in UV detector. Radiometric peak and retention time were compared with UV spectra to confirm peak identity for all samples.

HPLC-based TPMT Assay (UV and radiometric) (Study III)

The TPMT activity was determined by measuring the 6-meMP formed in the enzymatic reaction by the HPLC method. The TPMT assay was a modification of the radiochemical enzymatic assay described by Weinshilboum et al [63] in which they used radioactive SAM as methyl donor, but here in our optimized HPLC-UV assay we replaced the radioactive SAM with a non-radioactive SAM.

Briefly, the enzymatic reaction was initiated by the addition of a mixture containing 25 μl of phosphate buffer and cocktail, 10 μl of 6-MP (29.25 mM) in DMSO and 100 μl of cell lysates at 37 °C for 1 hour. The reaction was stopped by the addition of 200 μl of 100 mM borate buffer (pH 10). Toluen (2.5 ml) with 20% isoamylalcohol was used to extract 6-meMP. Organic phase was evaporated and the residue was reconstituted with 200 μl of mobile phase (20% methanol in 37.5 mM $NH_4H_2PO_4$, pH 3.7). Thereafter, an aliquot of 150 μl was injected into the HPLC column.

Radiochemical assay (Study III)

The TPMT activity in RBC was measured radiochemically according to the classical method described by Weinshilboum et al [75] and modified by Klemetsdal et al [64], which is based on the conversion of 6-MP to 6-meMP with radiolabeled S-adenosyl-L-[methyl-

^{14}C]methionine (SAM) as methyl donor. Detection was performed with scintillation counter.

Transfection of TPMT gene with siRNA in MOLT4 Cells (Study IV)

Prior to siRNA transfection cells were washed in PBS and resuspended in 400 µl volume of RPMI-1640. This was followed by addition of siRNA at a final concentration of 100 nM targeting the TPMT gene or of non-targeting siRNA at room temperature in 0.4 cm electroporation cuvettes.

The whole mixture was pulsed using an electroporator (Gene Pulser Xcell Electroporation System, Bio-Rad) at 340 V for 10 ms, repeated once after 24h [56,94].

After electroporation, cells were transferred from the cuvettes to pre-heated (37 °C) 25 cm^2 tissue culture flasks containing post-electroporation medium (RPMI-1640 supplied with L-glutamine (2 mM) and 20% FCS).

The siGENOME® siRNA against human TPMT gene and non-targeting siGENOME® siRNA (negative control) not binding to any human mRNA sequences were used.

A non-transfected control (wild-type MOLT4) sample was always included in parallel with the rest of the samples to show the effect of siRNA transfection *per se* on the cells and the cytotoxicity of the drugs used during the experiments.

The knockdown of the TPMT gene and transfection efficiency was evaluated by measuring TPMT mRNA levels 24 h, 48 h and 72 h after transfection to study if and when an acceptable level of down-regulation is achieved.

Antibiotics were avoided during electroporation since they could be toxic to the electroporated cells, and the recovery medium used after electroporation contained 20% FCS to increase the down-regulation and enhance cell viability [94].

Western Blotting (Study IV)

Cells were washed with PBS and lysed in IPB-7 (1mg/mL phenylmethylsulfonyl fluoride, 0.1mg/mL trypsin inhibitor, 1mg/mL aprotinin, 1mg/mL leupeptin, 1mg/mL pepstatin, 1mmol/L Na3VO4, and 1mmol/L NaF). The samples were subjected to SDS-PAGE and thereafter blotted onto a PVDF membrane (Bio-Rad). The protein bands were probed using antibodies against Actin and TPMT, from Santa Cruz Biotechnology. (Montgomery, TX. Proteins were visualized with the ECL procedure (Amersham Biosciences). The Western blotting results were analyzed with NIH Image 1.62 software.

TPMT activity measurement (Study IV)

The TPMT activity was determined by measuring the 6-meMP formed in the enzymatic reaction by Liquid chromatography–mass spectrometry (LC-MS) method which is based on the conversion of 6-MP to 6-meMP with SAM as methyl donor.

MOLT4 cell lysates were prepared and enzyme activity assay was carried out according to a method modified in our laboratory from a previously described method for TPMT phenotyping by Ford et al. [95].

Results were expressed as units per five million MOLT4 cells, where one unit of enzyme activity represents the formation 1 nmol of 6-meMP per hour.

Standard curve was made from cells of 2, 5 and 10 millions suspended in eppendorf tubes containing 200 µL cold dilute saline (0,9 g/l sodium chloride in water), the mixture was vortexed and sonicated on ice. The samples were kept at -80 °C until analysis.

Cytotoxicity Assays (Study IV)

Following siRNA transfection, the MOLT4 cells were allowed to recover at 37 °C for 24 hours before starting the cytotoxicity experiments, and the cells were incubated with different concentrations of both 6-MP and 6-TG for the durations of 24, 48, and 72 hours in 24-well tissue culture plates.

Annexin V and propidium iodide flow cytometry apoptosis analysis (Study IV)

After treatment with drug, the cells were centrifuged, washed in PBS, and resuspended in 100 µl of binding buffer (10 mM HEPES, 0.9% NaCl, 2.5 mM CaCl2, 0.1% BSA) containing 5 µl of phycoerythrin-conjugated Annexin-V (Annexin-V-PE; PharMingen, San Diego, CA) and incubated at room temperature in the dark for 15 min, followed by the addition of 500 µl of binding buffer containing 5 µl of propidium iodide. Apoptosis was assessed by gating 10000 events with a FACS machine (Becton Dickinson, San Jose, CA) using annexin V and propidium iodide staining, the cells that had been transfected with non-specific siRNA were used as controls.

RESULTS AND DISCUSSION

Study I: cellular uptake of anthracyclines

Since the mechanisms behind cellular anthracycline uptake are not completely understood and others have suggested that some anthracyclines might be taken up into cells via NTs, we studied the uptake patterns of DNR, DOX, EPI, IDA, and PIRA by human leukemic cells in the presence of various transport inhibitors and investigated the possible involvement of specific carriers. Knowledge about uptake mechanisms could be beneficial to increase the selectivity of these drugs.

Dose-uptake studies

Cells were incubated for one hour at 37°C since previous uptake studies by us and others have shown that an uptake plateau was reached in one hour in leukemic cells [96,97]. Generally, the tested drugs had similar cellular uptake at medium concentrations of 0.1-1.0 µM. However, uptake of IDA, DNR and PIRA increased markedly at medium concentrations > 1.0 µM. A reason for the higher uptake of IDA could be that it is more a lipophilic drug but it could also be explained by its lower affinity for P-gp as compared with other anthracyclines.

Since the uptake of IDA by HL-60 cells was not linear, we attributed the sharp increase in the accumulation of IDA at extracellular concentrations > 4 µM to saturation of the efflux pump P-glycoprotein (P-gp). We therefore studied if verapamil, a known P-gp inhibitor, influenced cellular accumulation of IDA. Verapamil significantly increased cellular accumulation of IDA by approximately 40 %.

Effect of suramin on DOX uptake

Suramin, significantly inhibited uptake of DOX in a dose dependent manner. The highest suramin concentration, 500 µM, inhibited DOX uptake by 95 %. There was a simultaneous decrease in DOX induced cell death. Virtually no cell death could be detected in cells exposed to 200 and 500 µM suramin.

To exclude the possibility of complex formation between suramin and DOX in the medium, we incubated DOX with suramin, at the same concentrations and under same conditions as in the uptake experiments, and compared the DOX peak-area measured by HPLC with standard medium containing DOX only, and found no significant variation in the value of DOX peak-area between the two cases (data not shown).

The strong inhibitory effect of suramin on DOX uptake and apoptosis in HL60 cells is interesting considering that previous studies have shown that suramin enhances the cytotoxic effects of DOX in prostate cancer cells [98,99]. This dissimilarity may be due to tissue and tumour specific mechanisms behind anthracycline uptake [34,100] and suramin might not affect DOX uptake in prostate cancer cells in the same way as in leukemic cells.

Effect of temperature and various transport inhibitors on anthracycline uptake

Furthermore, we compared the cellular uptake of the various anthracyclines at 0°C, 37°C, and in the presence of Dipyridamole (DP) or nitrobenzylthioinosine (NBMPR), two known ENT inhibitor. The uptake of all five tested anthracyclines was markedly reduced at 0°C and DP inhibited the uptake of DNR only. Next, we studied the effects of adding suramin. Uptake of all anthracyclines, except PIRA, was strongly inhibited by suramin. Actually none of the transport inhibitors affected the uptake of PIRA. NBMPR did not show any significant effect on the cellular uptake of any of the five studied drugs.

Effect of nucleosides on anthracycline uptake

No significant effect of nucleosides on DOX or EPI uptake was found. However, DNR, IDA and PIRA uptake was inhibited to some degree by nucleosides. DNR uptake was inhibited by adenosine, cytidine, thymidine and uridine while PIRA was inhibited by 80 % and by 40 % after exposure to thymidine and uridine, respectively. IDA uptake was inhibited by 65 % by adenosine.

To further support the idea of involvement of CNTs in the uptake of anthracyclines by HL-60 cells, we studied the effect of addition of nucleosides on the uptake of anthracyclines by MCF-7 breast cancer cells which are known to lack functional CNTs. The results show no significant effect of nucleosides on the uptake process.

Study II: Gene expression a of 6-Mercaptopurine- and 6-Thioguanine-resistant human leukemia cells

In this study, we employed high-through-put characterization of genetic aberrations, including both expression microarrays and array-CGH, to elucidate the mechanisms underlying acquisition of resistance to thiopurines by leukemic cells in an attempt to identify new markers and genes that may serve as valuable drug targets in the future.
The varying responses to 6-MP and 6-TG observed here may help identify novel cellular targets and modalities of resistance to thiopurines, as well as indicating new potential approaches to individualization therapy with these drugs.

The expression profiles of 6-MP- and 6-TG-resistant cells

The expression of certain genes of interest by the resistant cells, namely, *CNT1*, *CNT2*, *CNT3*, *ENT1*, *ENT2*, *MRP4* and *MRP5* in the microarray data was compared with previously published qRT-PCR analyses, using *GAPDH* mRNA as an internal standard [56]. According to the qRT-PCR analysis, the level of the *CNT3* mRNA was reduced by 1.7- and 4.3-fold in 6-MP- and 6-TG-resistant cells, respectively. Similarly, the level of *ENT2* mRNA was 3.3- and 5.0-fold lower in both types of cells, respectively. By microarray, we found 1.3- and 1.7-fold reductions in the level of expression of *CNT3*, as well as, 5.3- and 2.8-fold reductions in *ENT2* mRNA in 6-MP- and 6-TG-resistant cells, respectively. However, we observed no significant changes in expression of the *ENT1*, *MRP4*, *MRP5*, *CNT1* or *CNT2* genes.

Determination of alteration in DNA copy number changes by array-CGH

DNA copy number results by array-CGH, for the parental and resistant cells showed aberrations in form of frequent gains and losses, but no amplifications. Homozygous deletions in the 14q11.2 region were detected in both parental and 6-TG-resistant cells. The 14q11.2 region encompasses the genes *OR4E2, OR10G2, TRAJ17, MGC40069, TRAV20, TRA@*, and 7q34 include genes *TRBV19, PRSS1, PRSS2, TRBV21-1, TRBV3-1,* and *TRBC1*. The chromosomal regions in which losses occurred included 1q, 3q, 6, 7p, 14q, 15q, 17p, 18q, 20 and 22q. Moreover losses within the 6p11.2-q21, 15q14, 17p11.2-p13.1, 20p12, 20q21.3 and 22qter chromosomal regions were also acquired by one of the resistant sublines. Gains in copy number were detected on all chromosomes, with the exception of chromosome

15. The majority of these gains were identical or highly similar in the parental and resistant cells. Acquired gains occurred at loci within the 7q22.1, 7q21.11-qter, 9q33.3-qter, 10q11.1-q12, 14q32.2-qter, and 18qter chromosomal regions.

Comparison of the gene categories between the two resistant cell sublines

In terms of numbers, 610 differences were seen between parental and 6-MP-resistant cells; 1,082 differences between parental and 6-TG-resistant cells; and 1,606 differences between the wild-type and both types of resistant cells.
Functional categorization of selected mRNA species revealed highly similar patterns for both 6-TG- and 6-MP-resistant sublines.

Initial studies characterizing the MOLT4 cell line have reported activity of the terminal transferase (TdT) [101] also known as terminal deoxynucleotidyl transferase (DNTT). Here, expression of mRNA for this specialized nuclear enzyme was 122- and 93-fold higher in 6-TG- and 6-MP-resistant cells, respectively.
TdT is a special intranuclear DNA polymerase which catalyzes the template-independent addition of deoxynucleotides to the 3'-hydroxyl terminus of oligonucleotide primers [102] . Normally, TdT is expressed only by lymphoid precursors of the B- and T-cell lineage [102] and it serve as a useful marker for distinguishing ALL from mature lymphoid neoplasms [103]. Indeed, more than 90% of ALL cells and approximately 30% of chronic myelogenous leukemia cells exhibit elevated TdT activity, which is associated with a poor prognosis and response to chemotherapy and reduced survival time [104]. Accordingly, specific inhibitors of this enzyme might be developed into a novel class of antitumor agents [104].

The adenylate kinase 3-like 1 gene (*AK3L1*) which encodes a member of the adenylate kinase family of enzymes was down-regulated in both resistant sublines. Adenylate kinases play major role in the regulation of adenine and guanine nucleotide compositions within cells through transferring of phosphate group among these nucleotides in a reversible manner, thus maintaining homoeostasis of these nucleotides in cells which is vital for performing various cellular functions [105].

Study III: Measurement of thiopurine methyltransferase enzyme activity in red blood cells and human leukemia cells

Determination of TPMT activity before administration of thiopurines is the key predicting factor for the both efficacy and toxicity before starting the treatment and continuing chemotherapy with thiopurines.

Although TPMT assay is performed routinely in RBC in many clinical settings, the activity is influenced by transfusions and anemia [72]. Therefore measuring TPMT activity in pMNC seems more reasonable.

However, because of remission, the leukemic patients are leukopenic at the time of maintenance therapy, and achieving the minimum required amount of white blood cells for TPMT activity assay is often problematic [72,106]. Therefore, Keuzenkamp-jansen et al. postulated that TPMT assay in pMNC following high-dose 6-MP infusions can only be investigated further when a method that requires less than 5×10^6 pMNCs has been developed [72].

Keizer et al developed a method based on HPLC that is able to measure TPMT activity in less than 5×10^6 (i.e. 2×10^6) cells [106]. However, with the present modified HPLC-UV method, we carry out the assay using as less as one million cells by measuring the TPMT activity in MOLT4 cell lines. The new method shows a good correlation with radiochemical and HPLC-radiometric methods regarding RBC TPMT activity.

Here we describe an evaluation of three different methods including an HPLC-based assay modified and optimized in our lab, which is able to measure TPMT activity in RBC of patients and leukemic cell lines. The method is reliable and shows good selectivity that makes it applicable to measure the activity of the TPMT enzyme.

Development of the HPLC-UV method

The method was developed based on the conversion of 6-MP to 6-meMP using non-radioactive methyl donor (SAM). We identified 6-meMP which separated well in the HPLC system with a UV detector.

The 6-MP and 6-meMP standards were detected at the wavelength of 330 and 290 nm at 1.30 min and 4.42 min, respectively. The retention time was less than 5 minutes and no interference with the compounds of interest was observed in the chromatographic profile.

Distribution of TPMT activity in RBC from patients

TPMT activity in RBC from 198 patients was determined by radiochemical, HPLC-UV, and HPLC-radiometric methods and the data were compared for the three methods. A small difference was observed between the radiochemical and HPLC-UV during the TPMT assay. The radiochemical method showed a higher background whereas HPLC-UV showed little or no background. This suggests that the background of radiochemical method may cause variation in the measured TPMT activity. However, we found a good correlation between the three methods in TPMT assay

Correlation between TPMT activity using HPLC-radiometric and HPLC-UV assay with radiochemical assay

In the correlation study, TPMT activity data determined by the radiochemical, HPLC-UV and HPLC-radiometric methods from the 198 patients were correlated. The linear regression analysis for TPMT assay by three methods showed a significant correlation.

Resistant cell lines

Two resistant cell lines (MOLT4/6-MP and MOLT4/6-TG) developed from MOLT4 wild-type cells were examined to determine TPMT activity. Cells of both sensitive and resistant MOLT4 cell lines (MOLT4/WT, MOLT4/6-MP and MOLT4/6-TG) were cultured separately for a week and everyday aliquot of cell lines were collected to examine TPMT activity. We found similar basal expression of TPMT in all resistant cell lines compared to wild-type cells. The inter-day TPMT activity determination assay showed no significant variation among sensitive and resistant cell lines.

These results indicate that the activity of TPMT enzyme is not changed in thiopurine resistant cell sub-lines.

Study IV: Differential Role of Thiopurine Methyltransferase Enzyme in the Cytotoxic Effects of 6-Mercaptopurine and 6-Thioguanine on Human Leukemia

In order to investigate the potential role of TPMT in metabolism and thus, the cytotoxic effects of 6-MP and 6-TG, we knocked down the expression of the gene encoding TPMT enzyme employing specifically designed small interference RNA (siRNA) in human MOLT4 leukemia cells. The knock-down was confirmed both at protein and enzyme levels. Furthermore, mRNA expression of TPMT in the transfected cells was determined by Real Time PCR and compared to its expression in cells transfected with non targeting siRNA. Cytotoxicity assays were performed using annexin V and propidium iodide staining and FACS analysis.

Transfection with siRNA and TPMT down-regulation

To evaluate the role of TPMT in the metabolism of 6-MP and 6-TG, we transfected MOLT4 cells with siRNA against TPMT. Different time points, 24 h, 48 h and 72 h were tested in order to evaluate the efficiency of electroporation process at different time points.

The level of TPMT mRNA was significantly reduced in response to siRNA treatment as assessed by real-time quantitative PCR with GAPDH as an internal standard.

Electroporation of MOLT4 cells with TPMT-siRNA significantly decreased the gene expressions at the mRNA level 48 h after transfection. The levels of mRNA expression of TPMT in siRNA-treated MOLT4 cells showed 88 % and 86% reduction, in comparison with wild-type cells and cells treated with non-targeting siRNA, respectively.

The knock-down of the TPMT gene was further confirmed at protein level by Western blotting which showed significant reduction at the protein level and confirmed the RT-PCR results.

Moreover, results of the enzyme activity as determined by LC-MS correlated well with the results of Western blotting and RT-PCR and indicated 71 % reduction in the activity of TPMT enzyme in MOLT4 cells transfected with siRNA targeting the enzyme, as compared to, cells treated with non-targeting siRNA.

Cytotoxicity assays and Flow cytometric analysis

The MOLT4 cells were incubated with a range of concentrations of either 6-MP and 6-TG for a period of 48 h under a humidified atmosphere containing 5% CO_2 at 37 ^{0}C. Then cell viabilities were determined and expressed as percentage of the viable control (non-drug treated) cells. Double negative (annexin V and PI negative) cells were considered as viable.
Since we observed no significant cytotoxic effects after 24 h of treatment with these agents, we chose 48 h incubations and this is consistent with the delayed effects of thiopurines [86].

Both transfected and non-transfected MOLT4 cells were more sensitive to 6-TG than to 6-MP. The sensitivity to 6-TG was significantly increased after treatment with a range of different concentrations of this drug, however, concentrations of 1, 2, and 3µM showed maximum increase in sensitivity of 34, 23 and 13 %, respectively, in cells transfected with TPMT siRNA compared to cells transfected with non-targeting siRNA.
However, 6-MP showed unaltered toxicity or even a non-significant level of resistance in cells transfected with TPMT siRNA, compared to cells transfected with non-targeting siRNA.
We investigated the effect of electroporation/ transfection *per se* on the cells and their sensitivity toward the agents used in this work by studying the cytotoxicity of Daunorubicin (DNR) on the MOLT4 cells.
As we expected, after 48 h incubation, there was no alteration in sensitivity between the non-transfected (wild-type MOLT4), cells transfected with non-targeting siRNA and cells transfected with siRNA against TPMT.

CONCLUSIONS

- ✓ Carriers mediate uptake of the anthracyclines by human HL-60 cells.
- ✓ Our data support the involvement of NTs in the transport of DNR, IDA, and PIRA.
- ✓ The results also showed a strong inhibitory effect of suramin on the transport of anthracyclines by so far unknown mechanisms.
- ✓ Uptake of DNR is mediated by NTs and most likely CNT3.
- ✓ Impairment of the transport of 6-MP and 6-TG as a result of attenuated expression of nucleoside transporters CNT3 and ENT2 accounts for drug resistance.
- ✓ Since, human terminal transferase enzyme encoded by the DNTT gene was found to be highly expressed by both 6-TG- and 6-MP-resistant cells, specific inhibitors of this enzyme might be developed as a novel class of antitumor agents.
- ✓ The varying responses to 6-MP and 6-TG observed here may help identify novel cellular targets and modalities of resistance to thiopurines.
- ✓ The adenylate kinases including AK3L1 play major role in the regulation of adenine and guanine nucleotide compositions. Down-regulation of AK3L1 in both resistant sublines may play role in resistance to the thiopurines.
- ✓ We have developed a method capable of measuring TPMT assay in pMNC which requires as low as one million cells.
- ✓ The distribution pattern of TPMT activity in RBC from 198 patients as determined by radiochemical, HPLC-UV and HPLC-radiometric methods showed the classical trimodal distribution.
- ✓ The results indicate that the activity of TPMT enzyme is not changed in thiopurine resistant MOLT4 cell sub-lines.
- ✓ Both transfected and control MOLT4 cells were more sensitive to 6-TG than to 6-MP.
- ✓ Down-regulation of TPMT in MOLT-4 cells resulted in up to 34% higher sensitivity to 6-TG in comparison to cells transfected with non-targeting siRNA, however, 6-MP showed unaltered toxicity.
- ✓ TPMT differentially contributes to the metabolism and thus cytotoxicity of 6-MP and 6-TG.

SAMMANFATTNING PÅ SVENSKA

Cytostatika tillhörande grupperna antracykliner och tiopuriner är viktiga läkemedel vid behandling av patienter med olika former av leukemi. Antracykliner, som har brett antitumoralt spektrum, används vid akut myeloisk leuekmi, och också vid olika solida cancersjukdomar. Biverkningar såsom benmärssuppression och kardiotoxicitet begränsar deras kliniska användning. De cellulära upptagsmekanismerna är inte klarlagda för antracykliner. Kunskaper om upptagsmekanismer till tumörceller och normala celler skulle kunna användas till att öka medlens selektivitet.

Tiopurinerna 6-merkaptopurin (6-MP) och 6-tioguanin (6-TG) har en stor användning vid behandling av akut lymfatisk leukemi (ALL). De novo och förvärvad resistens är också ett stort kliniskt problem för dessa medel. Ökad förståelse om mekanismerna bakom resistens för dessa medel skulle kunna utnyttjas till att göra behandlingen effektivare.

I denna avhandling undersökte vi faktorer som kan ha betydelse för resistensmekanismer för dessa två grupper av viktiga cytostatika genom att studera transport och intracellulär metabolism.

Studie I.
Upptaget av antracyklinerna daunorubicin (DNR), doxorubicin (DOX), epirubicin (EPI), idarubicin (IDA) och pirarubicin (PIRA) av den leukemiska cellinjer HL-60 studerades i närvaro och frånvaro av olika inhibitorer och under olika förhållanden. Resultaten stöder att nukleosid transportörer medverkar i cellulärt upptag av DNR, IDA och PIRA. Suramin uppvisade kraftigt hämmande effekter på upptaget och cytotoxicitet av antracyklinerna via oklara mekanismer. Låg temperatur hämmade kraftig upptaget av alla studerade medel vilket stöder enrgiberoende upptagsmekanismer.

Studie II
Med målet att identifiera nya enzymer och transportörer av betydelse för resistens studerades DNA kopior (array CGH) och genexpression profiler (mikroarray) i leukemiskaT-lymfocyt MOLT4 celler som gjorts resistenta mot 6-MP och 6-TG genom långtidsexponering för gradvis ökande koncentrationer av respektive medel. Vi fann minskad genexpression av nukleosidtransportörerna CNT3 och ENT2 i båda cellinjerna vilket stöder att resistensen delvis är transportbetingad. Förhöjd expression av humant terminalt tranferas enzym, kodad av DNTT genen, observerades också i båda de resistenta cellinjerna, vilket skulle kunna vara ett potentiellt angreppsmål för nya terapier.

Studie III
Vi evaluerade tre olika metoder för tiopurin metyltransferas (TPMT) fenotypning som är av stor betydelse i kliniken. En HPLC baserad metod som utvecklats i vårt laboratorium kräver enbart en million celler totalt för analys. De tre metoderna (HPLC-UV, radiokemisk, HPLC-radiometrisk) visade goda överensstämmelser i 198 anonyma kliniska blodprover.

Studie IV
För att öka förståelsen för TPMT expression i metabolism och cytotoxicitet av 6-MP och 6-TG slogs expressionen ut av specifikt tillverkad ”silencing RNA” (siRNA) i MOLT4 celler. Känsligheten för de cytotoxiska effekterna av 6-TG ökade härvid ca 34 % men var oförändrat för 6-MP. Resultatet visar att TPMT har olika roller i cytotoxiciteten av 6-MP och 6-TG möjligen beroende på 6-MP´s metabolit, metyltiosinmonofosfat (meTIMP), hämmande effekt på *de novo* purinsyntesen.

SUMMARY IN KURDISH

شێرپەنجە یە کێکە لە نەخۆشیە کوشندەکان کە سەرەڕای تێپەڕبوونی ماوەیەکی زۆر، تا ئێستا چارەسەرێکی بنەڕەتی بۆ نەدۆزراوەتەوە.

بە گوێرەی سەرچاوە کانی ڕێکخراوی تەندروستی جیهانی (WHO) تەنها لە ساڵی ٢٠٠٨ دا نزیکەی ٧,٦ملیۆن مردن بەهۆی شێرپەنجەوە تومار کراوە ، وە چاوە ڕوان دەکرێت تێکڕای ژمارەی مردوان بە شێرپەنجە لە ساڵی ٢٠٣٠ دا بگاتە١٣,١ ملیۆن مرۆڤ.
لە ناو جۆرەکانی شێرپەنجەشدا، شێرپەنجەی خوێن گروپێکی گرنگن و توشی هەمو تەمەنێک، ڕەگەزێک، یان نەتەوەیەک دەبن بە بێ جیاوازی.
بەڵام خۆشبەختانە، بەهۆی پێشکەوتنە پزیشکیەکانەوە لە بواری چارەسەری شێرپەنجەی خوێن دا، بە تایبەتی جۆری (ALL)، توانراوە ڕێژەی چارەسەر(مانەوە بۆماوەی ٥ سا ڵ) بەرزبکرێتەوە بۆ نزیکەی ٩١%، بە تایبەتی لە منداڵان دا.

گروپێک لەو دەرمانانەی بۆ چارەسەری شێرپەنجەی خوێن بەکاردێن بریتین لە ئەنتراسایکلینەکان (Anthracyclines) کە پێک دێت لە (daunorubicin, doxorubicin, epirubicin, idarubicin, pirarubicin)، ئەمانە کۆمەڵێک دەرمانی بەهێزو فرە بەکارهێنانن و جگە لە شێرپەنجەی خوێن، بۆ شێرپەنجەی گەدەو مەمک یش بەکاردێن.

گروپی دووەم لەو دەرمانانەی بۆ چارەسەری شێرپەنجەی خوێن بەکاردێن بریتین لە دژە میتابۆلیتەکان (Antimetabolites) کە پێک دێت (6-mercaptopurine, 6-thioguanine, azathioprine) ، ئەم گروپەش چەند دەرمانێک دەگرێتەوە کە بەزۆری بۆ چارەسەری شێرپەنجەی خوێن بەکاردێن، لەگەڵ چەند بەکارهێنانێکی تری وەک دامرکاندنەوەی بەرگری لەش بەتایبەتی دوای چاندنی ئەندامەکانی لەش وەکو مۆخی ئێسک یان گورچیلە.

هەریەک لەم دەرمانانە لە ڕێگەی چەند پرۆتینێکی تایبەتیەوە دەگوازرێنەوەو دەبرێنە ناو خانەکانی لەشەوە بەتایبەتی خانە نەخۆشەکان بۆ ئەوەی کاری خۆیان بکەن و خانەکە لەناو بەرن.
وە هەریەکێک لەمانە ئەنزایمێک یان کۆمەڵە ئەنزایمێکی تایبەت بە خۆی هەیە کە دەرمانەکە لەناوخانە دا دەگۆڕێت و هەندێک جارچالاک تری دەکات.

لەم تێزی دکتۆرایەدا.. ئێمە وەک کۆمەڵێک دکتۆر لە زانکۆی کارۆلینسکا لە شاری ستۆکهۆڵم لە وڵاتی سوید هەستاین بە ئەنجام دانی چەند لێکۆڵینەوەیەکی پزیشکی لەسەر ئەم دەرمانانە لە پێناوی زیاتر تێگەیشتن لەو میکانیزمانەی دەبنە هۆی بەرگری خانەکان دژی ئەم دەرمانانە....وە ئایا چۆن خانە شێرپەنجەیی یەکانی خوێنی مرۆڤ خۆیان دەپارێزن لەم دەرمانانە و نامرن ؟..کە ئەمەش دەبێتە هۆی چاک نەبونەوەی نەخۆشەکە.

لە ئەنجامی توێژینەوەکانماندا توانیمان چەند میکانیزمێکی نوێ بۆگواستنەوەی دەرمانەکانی جۆری (ئەنتراسایکلین) بدۆزینەوە لە خانەکانی شێرپەنجەی خوێنی مرۆڤدا، کە دەتوانرێت لەم ڕێگەیەوە کۆنترۆلی دابەشبون و چونە ژورەوەی ئەم دەرمانانە بکرێت بۆ ناو خانە ساغ ونەخۆشەکانی لەشی مرۆڤ.

وە لەهەمان کاتدا پاش لێکۆڵینەوە لە سەر خانەی شێرپەنجەی خوێنی مرۆڤ کە بەرگرییان پەیدا کردبوو بەرامبەر دەرمانی دژەمیتابۆلیتەکان (6-mercaptopurine, 6-thioguanine)، بۆمان دەرکەوت کە گواستنەوەی دەرمانەکان لەڕێگای پرۆتینی تایبەتەوە و هەروەها بوونی ئەنزایمی تایبەتی لە ناو خانەکاندا هۆکاری سەرەکین بۆپەیداکردنی بەرگری دژی دەرمانەکان و نەمردنی خانە شێرپەنجەییەکان.

لە درێژەی کارەکانماندا لە سەر دەرمانی دژەمیتابۆلیتەکان، میتۆدێکی نوێمان دروست کرد وبەراووردمان کرد لەگەڵ دوو میتۆدی دیکە بۆ پێوانەکردنی چالاکی ئەنزایمی(TPMT) کە لێپرسراوە لە گۆڕین و چالاککردنی ئەم دەرمانانە لە خانەکانی لەشی مرۆڤدا. شایانی باسە کە پێوانەکردنی چالاکی ئەم ئەنزایمە کارێکی گرنگە پێش ئەوەی نەخۆشەکە ئەم دەرمانانە وەربگرێت وە لە زۆربەی نەخۆشخانەکاندا ئەنجام دەدرێت و بەگوێرەی ئەم چالاکی یە بڕی دەرمانی پێویست بە نەخۆشەکە دەدرێت.

لە کۆتایی دا هەستاین بە بەراوردکردنی ڕۆڵی ئەم ئەنزایمە (TPMT) لە کاریگەریەکانی هەریەک لە دەرمانەکانی (6-mercaptopurine, 6-thioguanine) لەسەر خانەکانی شێرپەنجەی خوێنی مرۆڤ و بۆمان دەرکەوت کە بە شێوەیەکی تایبەتمەندو جیاواز هەریەک لەم دەرمانانە دەگۆڕێت و کاریان تێدەکات.

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