Role of Multiple Glutathione Transferases in Bioactivation of Thiopurine Prodrugs

Studies of Human Soluble Glutathione Transferases from Alpha, Kappa, Mu, Omega, Pi, Theta, and Zeta Classes

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Dissertation presented at Uppsala University to be publicly examined in B21, BMC, Uppsala, Friday, September 22, 2006 at 10:15 for the degree of Doctor of Philosophy. The examination will be conducted in Swedish.

Abstract

A screening method was developed for identification of catalytically active enzymes in combinatorial cDNA libraries of mutated glutathione transferase (GST) derivatives expressed in E. coli. The method is based on spraying monochlorobimane (MCB) directly over bacterial colonies growing on agar. The substrate MCB become fluorescent under UV light, when the bacterial colony contains active GSTs catalyzing the conjugation with endogenous glutathione. Eleven out of twelve GSTs investigated where active with MCB. This method can be used to screen libraries generated from most cytosolic GSTs in the search for proteins with altered functions and structures. Azathioprine (Aza), a thiopurine that has been used clinically for 40 years was investigated with 14 GSTs. Three enzymes showed prominent catalytic activities with Aza and all of them are highly expressed in the liver. We estimated the contribution of the three enzymes GSTs A1-1, A2-2 and M1-1 bioactivation of Aza in the liver and concluded that it was about 2 orders of magnitude more effective than the uncatalyzed reaction. GST bioactivation of Aza could clarify aspects of idiosyncratic reactions observed in some individuals. Two other thiopurine prodrugs, cis-acetylvinyli thiopurine (cAVTP) and trans-acetylvinyli thioguanine (tAVTG), were investigated with the same 14 GSTs. The results displayed diverse catalytic activities. A mechanism of consecutive reactions was proposed. The studies contribute to knowledge under what conditions the drug should optimally be administered. A study of the same prodrugs with several mutants from the Mu class characterized by a point mutation of a hypervariable residue. We conclude that the effects of the mutations were qualitatively parallel for cAVTP and tAVTG, but they vary significantly in magnitude; steric hindrance may interfere with transition-state stabilization. From the evolutionary perspective the data show that a point mutation can alternatively enhance or attenuate the activity with a particular substrate and illustrate the functional plasticity of GSTs.

Keywords: Glutathione Transferases, Thiopurines, Prodrug, Bioactivation, Screening Method, Chemotherapy, Functional plasticity, Modulated activity

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List of Papers included

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


IV  Eklund, B. I. and Mannervik, B. (2006) Importance of a hypervariable active-site residue in human Mu class glutathione transferases catalyzing the bioactivation of chemotherapeutic thiopurine prodrugs Manuscript

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Contents

Introduction...................................................................................................11
Glutathione transferases and their substrates ................................................13
  Glutathione transferases ...........................................................................13
  Classification............................................................................................13
  Tissue distribution....................................................................................13
  Polymorphisms.........................................................................................15
  The structure of glutathione transferases..................................................15
  Scope of glutathione transferase substrates..............................................16
  Glutathione ...............................................................................................16
  Enzyme kinetics .......................................................................................17

Cancer, drugs and resistance.........................................................................18
  Cancer ......................................................................................................18
  Anticancer drugs ......................................................................................18
  Drug resistance .........................................................................................18
  Glutathione transferase and drug resistance ............................................19
  Glutathione and drug resistance ..............................................................19
  Glutathione depletion in cancer therapy ................................................19
  Anticancer drugs as prodrugs ...................................................................20
  Importance of in vitro studies ..................................................................20

Present investigation .....................................................................................21
  Paper I ......................................................................................................21
    Screening for recombinant glutathione transferases active with monochlorobimane ..............................................................21
  Paper II ....................................................................................................23
    Divergent activities of human glutathione transferases in the bioactivation of azathioprine ..........................................................23
  Paper III ....................................................................................................26
    Human glutathione transferases catalyzing the bioactivation of anticancer thiopurine prodrugs ......................................................26
  Paper IV ...................................................................................................30
    Importance of a hypervariable active-site residue in human Mu class glutathione transferases catalyzing the bioactivation of chemotherapeutic thiopurine prodrugs ........................................30
Summary.......................................................................................................34
Summary in Swedish ....................................................................................35
Glutationtransferaser............................................................................35
Cancer ..................................................................................................35
Pro-läkemedel ......................................................................................36
Enzymkinetik ........................................................................................36
Biverkningar och resistens.................................................................36
Glutationtransferaser i läkemedelsmetabolism ....................................37
Min forskning ...........................................................................................38
Artikel I .................................................................................................38
  Gallring av rekombinanta glutationtransferaser (GSTs) (aktiva med
  monoklorobiman) ................................................................................38
Artikel II ..................................................................................................39
  Divergenta aktiviteter av humana glutationtransferaser i dess
  bioaktivering av azatioprin .................................................................39
Artikel III ...............................................................................................39
  Humana glutathiontransferaser katalyserar bioaktivering av
  anticancer tiopurinprodroger ...............................................................39
Artikel IV .................................................................................................40
  Struktur-aktivitetsstudier av en hypervariable rest i My-klass GSTs i
  aktivering av tiopurinprodroger .......................................................40
Acknowledgement ....................................................................................41
References ..............................................................................................44
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Aza</td>
<td>Azathioprine</td>
</tr>
<tr>
<td>cAVTP</td>
<td>$cis$-6-(2-Acetylvinylthio)purine</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione Transferase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>MCB</td>
<td>Monochlorobimane</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi drug resistance</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>tAVTG</td>
<td>$trans$-6-(2-Acetylvinylthio)guanine</td>
</tr>
<tr>
<td>6-MP</td>
<td>6-Mercaptopurine</td>
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<tr>
<td>6-TG</td>
<td>6-Thioguanine</td>
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Introduction

The word protein comes from the Greek proteos that means first or the most important. The cell and life in general is dependent of proteins as structural components, regulators, carriers and catalysts, and each cell has to be able to synthesize a few thousand proteins for a human body to function. The difference between a skin cell and a liver cell is in principle the type of proteins they produce. Many of our characteristics depend on if and how much of different proteins that are expressed. This in turn is the consequence of the information stored in the inherited genes combined with environmental influences.

The 20 amino acid building blocks are sufficient to make proteins with an amazing number of functions as transporters of small molecules, structural proteins, transcription factors and regulatory proteins, or enzymes that enhance rates of many different types of reaction. One important group among other essential proteins contains the detoxifying enzymes. The human body is constantly exposed to noxious compounds such as aromatic amines and polycyclic aromatic hydrocarbons, but also to oxidative agents that elicit critical, even irreversible, cell injury. Sometimes this damage results in cancer. Cancer can be treated with chemotherapeutic agents that will poison and kill the cancer cell. However, the cell defense against xenobiotics in normal tissues can also be utilized by cancer cells. The concentrations of some detoxifying enzymes are known to be elevated in tumors, which may result in drug resistance.

The high detoxifying enzyme levels can be turned into an advantage by the use of prodrugs. Design of inactive prodrug to become activated by the elevated detoxifying enzymes in cancer tissue, makes it possible to avoid both drug resistance and adverse side effects that are common with anticancer drug therapy.

*Already ancient Egypt 3500 - 30 BC used chemotherapy. They used Castor oil (made from the seeds of Castor beans) and pigs' ears as a treatment. There is a lack of documentation whether the treatment worked and how.
This thesis is mainly a study of the human detoxifying enzyme superfamily of soluble glutathione transferases in their catalysis of reactions of thio-purines prodrugs with glutathione. The first prodrug, azathioprine is an immunosuppressant that has been used clinically for 40 years, and the *in vitro* investigation in paper II with 14 recombinant human cytosolic glutathione transferases helps to clarify aspects of unexpected toxicity of azathioprine observed in some individuals. In paper III two other thiopurine prodrugs, cis-acetylvinylthiopurine and trans-acetylvinylthioguanine, are investigated with the same human glutathione transferases. The results contribute to knowledge useful for deciding to whom and under what conditions the drug should optimally be administered, as glutathione transferases often are involved in chemotherapeutic resistance. Paper IV is a study of several mutants from the Mu class with the same prodrugs with the goal of clarifying structural determinants in the enzymes as well as in the substrates for efficient catalysis. The thesis also includes development of a novel fluorescent screening method to reveal active recombinant glutathione transferases directly in bacterial *E. coli* clones (paper I).
Glutathione transferases and their substrates

Glutathione transferases

A variety of biological functions have been ascribed to the glutathione transferase (GST) family of enzymes. The enzymes were first discovered in 1961 (Booth et al., 1961; Combes and Stakelum, 1961). GSTs exist in virtually every organism, indicating their important cellular role. GSTs have several functions such as cell signaling, transport and structural components (Adler et al., 1999; Chuang et al., 1999; Soltes et al., 1989). The most studied function, and perhaps one of the major characteristics of GSTs, is detoxification of xenobiotics and endogenous electrophilic compounds by catalyzing the conjugation of the electrophiles with the nucleophile glutathione (GSH). The detoxification plays a central role in the protection of cells from environmental and oxidative stress.

Classification

The mammalian soluble GSTs are divided into seven different classes, Alpha, Mu, Omega, Pi, Sigma Theta, Zeta, that occur mainly in the cytosol, and the more distantly related Kappa class containing a mitochondrial enzyme (Board et al., 1997; Board et al., 2000; Mannervik et al., 1985; Meyer et al., 1991; Meyer and Thomas, 1995; Pemble et al., 1996). The classes are named using Greek letters and division within a class is indicated by Arabic numerals (Mannervik et al., 2005). There are also microsomal GSTs called membrane associated proteins in eicosanoid and glutathione metabolism (MAPEG). They are designated MGST followed by Arabic numerals (Josephy and Mannervik, 2006). The classification is based on sequence similarities, and members of a class have more than 40% identity in their primary structures.

Tissue distribution

Soluble GSTs are widely distributed in the human body (Table 1) and some of them are more abundant than others (Johansson and Mannervik, 2001a).
Table 1. Distribution of GSTs in a few adult tissues.

<table>
<thead>
<tr>
<th>GST</th>
<th>Wide-spread</th>
<th>Brain</th>
<th>Breast</th>
<th>Colon</th>
<th>Kidney</th>
<th>Liver</th>
<th>Mitochondria</th>
<th>Placenta, gonads or adrenal glands</th>
<th>Skeletal or heart muscle</th>
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<tbody>
<tr>
<td>A1-1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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One of the most common enzymes is GST P1-1, which is found in almost every tissue except in liver. Among Mu class GSTs, GST M1-1 is a major hepatic enzyme, but is also present in many other tissues as well. Within the same class, GST M3-3 is also expressed in many tissues, while GST M4-4 and M5-5 are more restricted in occurrence. Remaining in the Mu class is GST M2-2, which is known to exist in brain as well as in both heart and skeletal muscle (Rowe et al., 1997). In Alpha class GSTs, GST A4-4 is among the more widespread enzymes. It was recently reported to exist in mitochondria, but could not be found in the cytosol of the liver (Gardner and Gallagher, 2001). GST A3-3 is primarily expressed in placenta, adrenal glands and gonads (Johansson and Mannervik, 2001b). GST A1-1 is contributing to 2-3% of the soluble liver protein (Rowe et al., 1997; van Ommen et al., 1990) and is widespread in other tissues as well (Johansson and Mannervik, 2001a). GST A2-2 is also abundant in the liver, like GSTs A1-1 and M1-1. These enzymes are also highly expressed in kidney. GST T1-1 is widely distributed with the highest concentrations in liver and kidney (Juronen et al., 1996; Sherratt et al., 1997). GST O1-1 is expressed in liver and heart, and GST O2-2 in the testis (Whitbread et al., 2005). The Kappa class enzyme GST K1-1 exists in the subcellular organelles mitochondria and peroxisomes (Morel et al., 2004). In the Zeta class, GST Z1-1 is primarily expressed in the liver, but also at lower levels in skeletal muscle and brain (Board et al., 1997). The Sigma GST is not included in the table; it is widely distributed in the human body (Kanaoka et al., 2000).
Polymorphisms

Polymorphism is when one gene presents several alleles in the population. This may result in qualitative or quantitative differences between individuals in the produced protein. Human cytosolic GSTs are highly polymorphic. Some polymorphisms change the catalytic activity of the polymorphic allelic variants. Interestingly, some of those alterations are linked to cancer susceptibility (Johansson and Mannervik, 2001a). In the Mu class the GSTM1 gene is missing in 50% of the Caucasian population (Seidegård et al., 1988). Individuals lacking the gene might have an increased risk to develop lung cancer (Seidegård et al., 1990). This gene has also been reported to exist in duplicate in Saudi Arabian populations (McLellan et al., 1997). GST T1-1 also has a null phenotype (Pemble et al., 1994), which has been associated with increased risk of contracting cancer (Salagovic et al., 1999). In the Alpha class, GST A1-1 and A2-2 have been characterized as polymorphic with altered expression levels (Coles and Kadlubar, 2005; Ning et al., 2004). Whether polymorphism among Alpha class GST has a consequence on human health is yet to be shown.

The structure of glutathione transferases

Cytosolic GSTs are dimers, (Figure 1) and each subunit contains a G-site, where GSH binds and which is highly conserved. In addition, a more diverse H-site exists, where the hydrophobic substrate binds. Each subunit is built of and N-terminal domain that mainly is an \(\alpha/\beta\)–structure where the G-site is (Figure 1), and a C-terminal domain, which is an \(\alpha\)-structure that holds most of the H-site.

Figure 1. GST M1-1 with glutathione bound in the G-site, one in each subunit.
Scope of glutathione transferase substrates

The diversity of electrophilic compounds that are substrates for GSTs is immense. Oxidative events give rise to organic hydroperoxides, activated alkenes, epoxides and quinones (Figure 2) (Josephy and Mannervik, 2006). These electrophiles are all natural substrates for GSTs that may cause toxicity and subsequently DNA and protein damage. In general, the substrates of GSTs are hydrophobic and their binding site is therefore named the H-site. Some of the substrates have a carbon at the electrophilic center, but some of them also have sulfur, nitrogen or oxygen. These compounds become more water-soluble after conjugation with GSH. GSTs enhance the reactivity of GSH when it becomes bound. This is effected by lowering the pKₐ value of the sulfhydryl group from 9.2, making the molecule a more reactive nucleophile (Gustafsson et al., 2001).

![Chemical structures](image)

Figure 2. Natural substrates of GSTs, 13-HPODE; 13-hydroperoxyoctadecadienoic acid.

Glutathione

Sir Fredrick Gowland Hopkins discovered GSH already in 1921 (Hopkins, 1921). GSH is a tripeptide built of γ-L-glutamyl-L-cysteinyl-glycine (figure 3) and found in almost every cell. Among other functions, GSH serves as an antioxidant, reducing agent, a free radical scavenger, a reservoir of cysteine, a regulator of calcium ion homeostasis (Estrela et al., 2006), and a link in the mitochondrial mechanism to cell death (Kroemer and Reed, 2000).
Alterations in concentration and metabolism of GSH linked to the formation of reactive oxygen species (ROS) and reactive nitrogen species are associated with several diseases such as diabetes, neurodegenerative diseases, acquired immune deficiency syndrome (AIDS), aging and cancer (Townsend et al., 2003). In mammalian cells the concentration of GSH is up to 10 mM. In fact, GSH is the most abundant low-molecular-mass thiol in all mammalian cells (Chasseaud, 1979; Josephy and Mannervik, 2006). The GSH content is apportioned such that the cytosolic-nuclear compartments will hold about 90% and the mitochondria about 10%. There is also a few percent in the endoplasmatic reticulum (Estrela et al., 2006). The aerobic respiration occurring in mitochondria is highly dependent on GSH for the prevention of oxidative damage, since there is no de novo synthesis of GSH in mitochondria. Therefore, the organelle is dependent on salvage of glutathione disulfide by glutathione reductase and on uptake of cytosolic GSH. The mitochondrial GSH concentration is preserved within physiological levels, even when cytosolic GSH is decreased, because of an active transport against a concentration gradient (Orlowski and Meister, 1970).

**Enzyme kinetics**

Kinetic constants are determined from the initial rates of reactions that an enzyme is catalyzing under steady-state conditions. The primary parameters are $k_{cat}$, the turnover number, and $K_m$, an apparent dissociation constant of enzyme bound species. The specificity constants, $k_{cat}/K_m$, is a measure of the enzyme’s catalytic efficiency. The constants give quantitative information of steps involved in the mechanism of the reaction catalyzed by the enzyme. $k_{cat}/K_m$ values are used to compare different enzymes catalytic efficiencies with the same substrate or one enzyme with different substrates (Fersht, 1999).
Cancer, drugs and resistance

Cancer
Mutation or damage of DNA occasionally result in tumor formation, but most genetic damage that occurs is repaired, or causes cell death (Jakobisiak et al., 2003). Normal cells grow and die in a precise and controlled way. In contrast, tumor formation is a genetic disease involving DNA damage, where genomic modifications cause loss of cell-cycle control and cell differentiation. Oxygen is essential to life, but is also a factor that could initiate cancer, such as ROS caused by UV radiation exposure or chemical carcinogens. Tumors can be benign or malignant and it is the malignant tumors that are cancerous. The latter grow into the surrounding tissues and give rise to metastasis. This uncontrolled cell cycle proliferation and metastatic spread needs to be inhibited.

Anticancer drugs
The key to destroy cancer cells by chemotherapy is to interfere with DNA synthesis or mitosis. There are many different mechanisms and targets, among others are inhibition of enzymes of nucleic acid metabolism, or proteins implicated in the cell replication (Gatti and Zunino, 2005). Alkylating agents where among the first chemotherapeutic agents developed. They act directly on DNA, causing DNA strands to cross-link and the result is DNA breaks. Chemotherapeutic antibiotics bind to DNA and make the DNA uncoil thereby prevent transcription and the subsequent protein production. Antimetabolites replaces the natural bases in DNA and will also lead to inhibited cell growth and cell death.

Drug resistance
The cell defense against cancer comprises many different components. The DNA-repair system that corrects mistakes and damage in DNA. The ATP-binding cassette (ABC) transporter proteins have an important roll in guard-
ing tissues from a vast array of toxicants. For example, they work in the blood-brain barrier that protects the brain from chemical insults. The ABC proteins include among others P-glycoprotein, multidrug resistance protein 1 (MRP1), MRP2, and the breast cancer resistance protein (BCRP) (Leslie et al., 2005). The ABC proteins are expressed in tissues such as lung, liver and kidney, and are therefore able to influence absorption, metabolism and elimination of xenobiotics. The cancer cell can also utilize the protection against the treatment of chemotherapeutic agents, thereby causing multi drug resistance (MDR).

Glutathione transferase and drug resistance

GSTs have been implicated in the development of resistance toward chemotherapeutic agents by catalyzing their conversion to GSH-drug conjugates. The resistance could be caused by GSTs known to be elevated in tumors such as GST P1-1 (Mannervik et al., 1987). It is also known that repeated exposure to chemotherapeutic agents can lead to higher expression levels of GSTs in tumor cells and eventually to development of MDR (Hayes and Pulford, 1995). The induction is suggested to be an adaptive response mechanism, since some of the inducing compounds are substrates for GSTs.

Glutathione and drug resistance

The functions of GSH, involving the protection of the cell and DNA against free radicals and carcinogens, also include disarming of chemotherapeutic agents. This is one of the reasons why tumors show anti cancer drug resistance. It is common that the GSH levels increase in cancer cells compared to normal tissue levels and contribute to drug resistance (Estrela et al., 2006). Increased levels of GSH has also been found in cell lines resistant to alkylating agents (Tew, 1994).

Glutathione depletion in cancer therapy

A deficiency of GSH puts the cell at risk for oxidative damage. Therefore, cancer cells with low levels of GSH are more sensitive to gamma irradiation than normal cells (Meister, 1991) or to chemotherapeutic agents. By reducing GSH concentrations in cancer cells, tumors would be easier to eliminate. So far, are GSH depleting agents nonspecific like L-buthionine-sulfoximine (BSO), an inhibitor of \(\gamma\)-glutamylcysteine synthetase (Meister, 1991).
Anticancer drugs as prodrugs

A goal in the use of an anticancer prodrug is to adjust the dose for the individual phenotype and minimize adverse side effects in normal tissues and maximize the cytotoxic effect in the tumor. Prodrugs are inactive molecules that need to be chemically transformed to exert their pharmacological effects. The requirement of activation can be used to advantage in combination with known elevated GSH levels and GST expression in cancer cells. Prodrugs can be designed that specifically target such overexpressing tumors. Tumor-activated agents have the potential to mediate selective cytotoxicity and thereby possibly avoid adverse effects like anemia, nausea and hair loss, which often accompany chemotherapy. Prodrugs for GST activation have been developed in the last 10 years (Izbicka et al., 1997; Morgan et al., 1998; Satyam et al., 1996). For example TER286 is a latent drug activated by GSTs P1-1 and A1-1 to produce a nitrogen mustard alkylating agent.

Importance of *in vitro* studies

The concept of toxicity is not unambiguous, since the effect is dose-dependent and varies with the individual. New cytostatic drug candidates are first evaluated with human tumor cell cultures but cell lines used in research are known to differ in the expression of GSTs (Castro et al., 1990; Tew et al., 1996) and similar diversity characterizes human tumors treated clinically. The *in vitro* studies of drugs with isolated enzymes are therefore important contributions to the overall knowledge of the metabolic fate of the substrates.
Present investigation

The papers of this thesis describe studies of multiple soluble human GSTs and their relevance to anticancer and immunosuppressive drugs. GSTs are implied in both the cellular defense of normal tissues against cancer, and in the multidrug resistance (MDR) of many tumors caused by their intrinsic and drug induced overexpression. GSTs are also used as activators of prodrugs. We have developed a screening method for identifying active mutated GST variants, as well as assays for investigation of the catalytic efficiencies with novel substrates and the formation of their products. We have studied almost all soluble human GSTs with new thiopurine prodrugs and another thiopurine clinically used for immunosuppression for over 4 decades. The investigated GSTs included 14 enzymes from seven classes including Alpha, Kappa, Mu, Omega, Pi, Theta and Zeta. We have also investigated the importance of a hyper-variable active-site residue in the activation of two thiopurine prodrugs by Mu class GSTs.

Paper I

Screening for recombinant glutathione transferases active with monochlorobimane

GSTs have been used in the search for enzymes with novel properties and catalytic specificities and they have proved to be good scaffolds for redesign (Babbitt, 2000; Broo et al., 2002; Nilsson et al., 2000; Pettersson et al., 2002). Screening of libraries is often one of the limitations in the experimental search for interesting mutants with altered efficiencies or substrate specificities. In paper I a screening method was developed for identification of catalytically active enzymes in combinatorial cDNA libraries of mutated GST derivatives expressed in E. coli. The method can be used directly on bacterial colonies grown on agar plates, where the active clones when sprayed with the fluorogenic substrate monochlorobimane (MCB) will fluoresce (Figure 4).
Figure 4. Photograph of bacterial colonies growing on an agar plate. The clones where sprayed with MCB and fluoresce under long wavelength UV-light if they contain active GSTs. Two fluorescent clones are marked with arrows.

The fluorescence of the adduct GS-bimane is based on the conjugation of MCB with GSH (Figure 5). Halogenated bimane molecules are essentially nonfluorescent, and they have been used to quantify GSH in cells (Rice et al., 1986) and to measure GST activity (Hulbert and Yakubu, 1983). The background reaction of the conjugation of endogenous bacterial GSH with MCB is low, and therefore the fluorescent clones containing enzyme that catalyzes the reaction can easily be picked out among the non-active clones under long wavelength UV-light.

![Monochlorobimane and GS-bimane](image)

Figure 5. Structure of monochlorobimane and adduct after conjugation with GSH.

The screening method was tested with GSTs A1-1, A2-2, A3-3, A4-4, M1-1, M2-2, M3-3, M4-4, M5-5, P1-1, T1-1, and T2-2. Along with the screening method we also showed that bacteria expressing GSTs could be analyzed by flow cytometry. The conjugation of MCB and GSH was catalyzed by 11 out of 12 tested GST enzymes. GST T1-1 and as well as an inactive P1 monomer (Abdalla et al., 2002), which are not active with MCB, were used as negative controls. The P1 monomer was used in discrimination tests mixed with the active GST P1-1 in different ratios. The method showed a sensitivity to detect 1 active clone among 1,000 inactive ones. It is therefore possible to screen through several thousands of variants in a few minutes. The method was tested with a library constructed by DNA shuffling of GSTs T1-1 and T2-2 (Broo et al., 2002). Both fluorescent and non-fluorescent clones were picked and investigated for activity. Two substrates for GST T2-2 were used, MCB and 1-menaphthyl sulfate (MS) with which GST T1-1 is not
active. 100% of the fluorescent clones were active with MCB as expected and 70% of them where active with MS, while none of the non-fluorescent clones were active with MS. The lack of perfect correlation between MCB and MS activities is in agreement with altered substrates specificities ratios among the members in the GST T1/T2 library reported by Broo et al., 2002.

We conclude that this method can easily be used to screen libraries generated from most cytosolic GSTs in the search for proteins with altered functions and structures.

Paper II

Divergent activities of human glutathione transferases in the bioactivation of azathioprine

Azathioprine (Aza) is a prodrug that has been used clinically since the 1960s, and the bioactivation of Aza was expected to be catalyzed by GSTs but had not been investigated with isolated human GSTs. Today Aza is mainly used for immunosuppression in connection with organ transplantations and in the treatment of inflammatory diseases. The bioactivation of Aza with GSH produces its active metabolite 6-mercaptopurine (6-MP) (Figure 6), and the catalytic effects of 14 recombinant human cytosolic GSTs were investigated in paper II.

![Figure 6. The thiopurine 6-mercaptopurine.](image)

In the metabolism of Aza (Figure 7), to eventually become the antimetabolites thioguanine nucleotides (TGN), there are several competing pathways. One of them involves the enzyme thiopurine methyl transferase (TPMT) that, by methylation, inactivates adducts at two different steps. 6-MP and 6-thioguanosine 5-monophosphate are methylated to 6-methyl mercaptapurine (6-MMP) and 6-methyl mercaptapurine ribonucleotide (6-MMPR), respectively. TPMT is known to be polymorphic and some genotypes lead to low enzyme activity (Weinshilboum, 2001). Individual differences of TPMT
expression can shift the balance between the inactivated methylated molecules and the active antimetabolites TGNs. This has been considered as the reason for the idiosyncratic drug reactions observed in connection with Aza treatment, and some clinics therefore genotype their patients before the administration of Aza (Cara et al., 2004).

Figure 7. Metabolic pathways following the reaction of Aza with GSH catalyzed by GSTs. Metabolic pathways following the reaction of Aza with GSH catalyzed by GSTs. XO, xanthine oxidase catalyzes the formation of 6-TU, thiouric acid, TPMT, thiorpurine methyltransferase catalyzes the inactivation by methylation to 6-MMP, 6-methylmercaptopurine, 6-MMPR, 6-methylmercaptopurinoribonucleoside, HPRT, hypoxanthineguanine phosphoribosyltransferase catalyses the first step to 6-TGN, 6-thioguanine nucleotides

Our study of the 14 human soluble GSTs showed that seven of them had measurable activities, and three of the enzymes were distinguished as the best catalysts of the conjugation of Aza with GSH. Steady-state kinetics were investigated for these variants. All three GSTs, A2-2, M1-1 and A1-1, ranked after their catalytic efficiencies with Aza, are highly expressed in the liver. We established that the products of the reaction of Aza and GSH were 6-MP and glutathionyl-imidazole (Figure 7) with LC/LC/MS.
Table 2. Relative contributions to the bioactivation of azathioprine

<table>
<thead>
<tr>
<th>GST</th>
<th>Small intestine</th>
<th>Erythrocytes</th>
<th>Liver</th>
<th>Apparent rate constant in liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg</td>
<td>µg</td>
<td>µg</td>
<td>min⁻¹</td>
</tr>
<tr>
<td>A1-1</td>
<td>3 - 10</td>
<td>0</td>
<td>3 - 10</td>
<td>0.24 – 0.80</td>
</tr>
<tr>
<td>A2-2</td>
<td>1 - 2</td>
<td>0</td>
<td>1 - 7</td>
<td>0.16 – 0.55</td>
</tr>
<tr>
<td>M1-1</td>
<td>?</td>
<td>0</td>
<td>9</td>
<td>0.37</td>
</tr>
<tr>
<td>Nonenzymatic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.008</td>
</tr>
</tbody>
</table>

GST A2-2, with a $k_{cat}/K_m$ of 1.17 mM⁻¹ s⁻¹, was the most effective catalyst with Aza. This enzyme usually has similar or lower activity than GST A1-1, but with this substrate the reverse is true. GST A1-1, with a $k_{cat}/K_m$ of 0.53 mM⁻¹ s⁻¹, is one of the most abundant enzymes in the liver. It has been reported to represent 1 % of the hepatic cytosolic proteins (Coles and Kadlubar, 2005). To estimate the enzyme’s contributions to the bioactivation of Aza in the liver, the first-order rate constants were calculated for the GST-catalyzed reactions (Table 2). They are based on the $k_{cat}/K_m$ values multiplied by the GST expression levels reported by Coles and Kadlubar (2005). Due to the difference in expression levels of GST A1-1 and A2-2, their contributions to the bioactivation of Aza in the liver even up (Table 2). GST M1-1 with a $k_{cat}/K_m$ of 0.48 mM⁻¹ s⁻¹, is also one of the major hepatic enzymes in the liver, and we conclude that most of the bioactivation of Aza catalyzed by GSTs is carried out in the liver. The calculations showed that the GST-catalyzed contributions are approximately 2 orders of magnitude more effective than the uncatalyzed reaction. In the small intestine, the relative rates would be similar to those in liver, but in the erythrocytes the GSTs probably make a negligible contribution (Table 2).

As GSTs are known be polymorphic, with both null alleles and duplicated genes for GST M1-1 (McLellan et al., 1997; Seidegård et al., 1988). We
conclude that individual differences in GST expression can be behind unexpected cytotoxic effects. Therefore, GST genotypes could be worth investigating before Aza administration.

Paper III

Human glutathione transferases catalyzing the bioactivation of anticancer thiopurine prodrugs

The antimetabolites 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) (Figure 8) are widely used cytotoxic agents. The Nobel Prize winners Gertrude Elion and George Hitchings originally synthesized 6-MP and 6-TG by replacing the oxygen with sulfur at carbon 6 of the natural purines hypoxanthine and guanine, respectively. The thiopurines caused inhibition of DNA synthesis, and 6-MP was found useful in the treatment of acute lymphoblastic leukemia. 6-MP and 6-TG are prodrugs and their active metabolite is thioguanine nucleotide (6-TGN) (Figure 8). 6-(2-Acetylvinylthio)purine (cAVTP) and 6-(2-acetylvinylthio)guanine (tAVTG) (Figure 8) are prodrugs of 6-MP and 6-TG, respectively. cAVTP and tAVTG contain a novel butenone moiety that allows them to react selectively with sulfhydryl nucleophiles to release 6-MP and 6-TG, respectively (Gunnarsdöttrir et al., 2002). The activation of these prodrugs catalyzed by GSTs was the topic of the study in paper III.
The study of the bioactivation of cAVTP and tAVTG revealed that the process consisted of two consecutive reactions. It was discovered by a decrease followed by an increase in the UV absorbance when GST activities were measured spectrophotometrically with cAVTP (Figure 9).

The consecutive reactions were made evident by fitting the monitored curves by a bi-phasic-exponential rate equation that is characteristic for consecutive reactions. In the reaction with tAVTG, a transient adduct (GS-tAVTG) was detected with HPLC. In Figure 10 we show the proposed consecutive reaction with cAVTP (A) where GST catalyzes the addition of GSH to the activated double bond of the S-substituent of the prodrug (Figure 8).

\[ A_{\text{tot}} = A_0 e^{-k_1 t} + A_1 e^{k_2 t} + C \]

The release of the parental drug and the GS-butenone (B) appears to be uncatalyzed by GSTs. This is the rate-limiting step in the bioactivation of cAVTP and tAVTG when the reaction is catalyzed by GSTs. The calculated
rate constants displayed an increasing $k_1$ and a decreasing $k_2$ with increased enzyme concentration (Table 3).

Table 3. Rate constants of consecutive reactions catalyzed by GST M1-1

<table>
<thead>
<tr>
<th>GST</th>
<th>320 nm $k_1$</th>
<th>320 nm $k_2$</th>
<th>350 nm $k_1$</th>
<th>350 nm $k_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1-1</td>
<td>$(0.9 \pm 0.38) \times 10^{-2}$</td>
<td>$(0.9 \pm 0.35) \times 10^{-2}$</td>
<td>$(1.0 \pm 0.36) \times 10^{-2}$</td>
<td>$(0.9 \pm 0.39) \times 10^{-2}$</td>
</tr>
<tr>
<td>4</td>
<td>$(1.4 \pm 0.03) \times 10^{-2}$</td>
<td>$(0.6 \pm 0.01) \times 10^{-2}$</td>
<td>$(1.5 \pm 0.07) \times 10^{-2}$</td>
<td>$(0.7 \pm 0.07) \times 10^{-2}$</td>
</tr>
<tr>
<td>8</td>
<td>$(3.8 \pm 0.03) \times 10^{-2}$</td>
<td>$(0.4 \pm 0.00) \times 10^{-2}$</td>
<td>$(4.5 \pm 0.05) \times 10^{-2}$</td>
<td>$(0.3 \pm 0.01) \times 10^{-2}$</td>
</tr>
<tr>
<td>40</td>
<td>$(7.3 \pm 0.01) \times 10^{-2}$</td>
<td>$(0.4 \pm 0.00) \times 10^{-2}$</td>
<td>$(9.1 \pm 0.08) \times 10^{-2}$</td>
<td>$(0.3 \pm 0.01) \times 10^{-2}$</td>
</tr>
</tbody>
</table>

13 recombinant human GSTs from seven classes were monitored in vitro for catalytic activity with the inactive α,β-unsaturated substituted prodrugs and GSH. Eight of the 13 GSTs were kinetically investigated under steady-state conditions. Three enzymes had particularly notable catalytic efficiencies GST A4-4, M1-1 and M2-2 (Table 4). GST A4-4 was the most effective enzyme with tAVTG of them all, with a specificity constant ($k_{cat}/K_m$) of 130 mM$^{-1}$ s$^{-1}$. GST M1-1 was second with 115 and 52 mM$^{-1}$ s$^{-1}$ for cAVTP and tAVTG, respectively. GST M2-2 was in third place with 22 mM$^{-1}$ s$^{-1}$ for cAVTP.

Table 4 - $k_{cat}/K_m$ values and specific activities of GSTs catalyzing thiopurine prodrugs in reaction with GSH.

<table>
<thead>
<tr>
<th>GST</th>
<th>CAVTP</th>
<th>tAVTG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}/K_m$</td>
<td>mM$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>A1-1</td>
<td>2.2 ± 0.1</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>A2-2</td>
<td>0.5 ± 0.03</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>A3-3</td>
<td>1.3 ± 0.1</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>A4-4</td>
<td>3.7 ± 0.3</td>
<td>129.9 ± 5.6</td>
</tr>
<tr>
<td>M1-1</td>
<td><strong>114.6 ± 4.8</strong></td>
<td><strong>51.8 ± 3.1</strong></td>
</tr>
<tr>
<td>M2-2</td>
<td><strong>22.4 ± 1.2</strong></td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>M4-4</td>
<td>3.0 ± 0.1</td>
<td>15.8 ± 1.0</td>
</tr>
<tr>
<td>M5-5</td>
<td>7.3 ± 0.3</td>
<td>14.8 ± 0.6</td>
</tr>
</tbody>
</table>
The knowledge of the efficiencies above can be utilized for optimizing the administration of the prodrugs in tumor tissues expressing these enzymes (Table 4), and the differential effects of the members cover variations in GST expression profiles. For example, individuals lacking the GSTM1 gene (Seidegård et al., 1988), which in some populations is absent in up to 50% of the individuals, tAVTG could be used. The latter prodrug is activated by GST A4-4, which is widely expressed in human tissues (Johansson and Mannervik, 2001a). It should be noted that GST P1-1 did not activate cAVTP or tAVTG especially well. For GST P1-1, known to be expressed in tumor tissue, anticancer prodrugs have been developed (Morgan et al., 1998). cAVTP and tAVTG could be candidates for treatment of cancers in prostate, liver and breast that do not express GST P1-1 (Lin and Nelson, 2003).

The study also demonstrated the possibility of forming more than one product of GSH. Two isomeric monoconjugates and one bis-glutathionyl-butenone were found following a reaction catalyzed by GSTs (Figure 11). The reaction products of cAVTP and GSH, incubated with liver cytosol, were investigated with LC/MS and MS/MS. The monoconjugates cis- and trans-glutathionyl-butenone were detected as two HPLC peaks with different migration times and identical masses. The glutathionyl-butenone moiety of a monoconjugate still keeps the Michael acceptor feature of a reactive enone, and there was evidence for the generation of an adduct with two glutathionyl groups, named bis-GS-butenone (Figure 11). There is no indication that bis-GS-butenone production is catalyzed GSTs, but nevertheless it is possible that formation of the bis-glutathionyl adduct could contribute to GSH depletion.

We conclude that the results of the divergent catalytic efficiency of the GSTs with cAVTP and tAVTG, contribute to useful knowledge for deciding to whom and under what conditions the drug should optimally be administered, as glutathione transferases often are involved in chemotherapeutic resistance.
Paper IV

Importance of a hypervariable active-site residue in human Mu class glutathione transferases catalyzing the bioactivation of chemotherapeutic thiopurine prodrugs

In paper IV we investigated the importance of a hypervariable residue in the human Mu class by determining the catalytic efficiencies of GST M1-1 and GST M2-2 and six mutated variants of them with the two anticancer thiopurine prodrugs cis-6-(2-acetylvinylthio)purine (cAVTP) and trans-6-(2-acetylvinylthio)guanine (tAVTG) (Figure 12).

Figure 12. Ball and stick structures of cAVTP and tAVTG, respectively. The colors designate: blue, nitrogen; yellow, sulfur; red, oxygen; gray and white are carbon and hydrogen, respectively. (Courtesy of Sanela Kurtovic.)

GST M1-1 and M2-2 both belong to the Mu class and consist of 217 amino acids. They differ in 34 amino acids and only a few of those are localized to the active site. In spite of their high resemblance they differ in substrate selectivity, although they also overlap in their specificity as several GSTs do. For example GST M1-1 is particularly efficient with epoxides compared to GST M2-2, and GST M2-2 a better catalyst with other substrates such as ortho-quinone aminochrome compared to GST M1-1 (Ivarsson et al., 2003).

The mutants investigated differ from the corresponding wild-type GSTs only in position 210 in the active site. This position was identified among others in an evolutionary hypervariable rate analysis with GST M2-2. Hypervariable positions are under positive selection and could contribute to functional variations. The position proved to be important and demonstrated particular functional potential when it was mutated (Ivarsson et al., 2003). Substitutions affected both the catalytic efficiency and the substrate specificities. Residue 210 in GST M2-2 has been substituted with all amino acids and with several amino acids in GST M1-1. We have chosen the most active mutants reported with alternative substrates (Ivarsson and Mannervik, 2005; Norrgård et al., 2006) for investigations with cAVTP and tAVTG.
The investigated enzymes were wild-type GST M1-1 and two mutated variants with alanine and threonine, designated M1Ala and M1Gly, respectively. The wild type of GST M2-2 and four mutated variants in position 210 of alanine, glycine, proline, and serine (designated M2Ala, M2Gly, M2Pro, and M2Ser, respectively) were investigated. The $k_{\text{cat}}/K_m$ values (Table 5) determined for GSTs M1-1 and M2-2 showed similarities with values obtained with the mutants in having higher catalytic efficiency with cAVTP than with tAVTG. The exceptions are wild-type GSTs M4-4 and M5-5 that also were compared and contain a Thr and Gly, respectively in position 210 (Table 5).

**Table 5** - $k_{\text{cat}}/K_m$ based on initial rates GST M1-1 wild type (wt) has a serine and GST M2-2 wt has a threonine in the mutated position.

<table>
<thead>
<tr>
<th>GST</th>
<th>$k_{\text{cat}}/K_m$</th>
<th>cAVTP</th>
<th>tAVTG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Variant</td>
<td>mM$^{-1}$ s$^{-1}$</td>
<td>mM$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>M1Ser (wt)</td>
<td>M1Ser</td>
<td>114.6 ± 4.8</td>
<td>51.8 ± 3.1</td>
</tr>
<tr>
<td>M1Thr (wt)</td>
<td>M1Thr</td>
<td>3.2 ± 0.4</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>M2Thr (wt)</td>
<td>M2Thr</td>
<td>22.4 ± 1.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>M2Ala</td>
<td>M2Ala</td>
<td>49.6 ± 4.7</td>
<td>7.9 ± 0.3</td>
</tr>
<tr>
<td>M2Gly</td>
<td>M2Gly</td>
<td>25.1 ± 1.3</td>
<td>9.7 ± 0.4</td>
</tr>
<tr>
<td>M2Pro</td>
<td>M2Pro</td>
<td>12.0 ± 0.3</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>M2Ser</td>
<td>M2Ser</td>
<td>87.0 ± 1.8</td>
<td>20.3 ± 1.0</td>
</tr>
<tr>
<td>M4Thr (wt)</td>
<td>M4Thr</td>
<td>3.0 ± 0.1</td>
<td>15.8 ± 1.0</td>
</tr>
<tr>
<td>M5Gly (wt)</td>
<td>M5Gly</td>
<td>7.3 ± 0.3</td>
<td>14.8 ± 0.6</td>
</tr>
</tbody>
</table>
We demonstrate that the catalytic activity of GST M1-1 with cAVTP or tAVTG is successively diminished when wild-type Ser-210 is mutated into Ala followed by Thr. GST M2-2 on the other hand, becomes more efficient when mutating the wild-type Thr-210 into Ala and Ser (Table 5, Figure 13). The results suggest that the hydroxyl group of Ser in position 210 stabilizes the transition state of the GST-catalyzed reaction with both substrates. GST M2-2 variants containing glycine and proline in position 210 were found in the middle. The low efficiencies of GSTs containing Thr in position 210 is probably due to steric hindrance caused by the β-methyl group of the side chain.

Figure 13. Cluster analysis of catalytic efficiencies for GSTs M1-1 and M2-2 and their mutants with cAVTP and tAVTG prodrugs. The blacks dot marks each cluster’s centroid. The data are in log format for easier overview.

By comparing the specificity constants using multivariate analysis the activities with cAVTP and tAVTG were demonstrated to change in parallel, and a correlation analysis showed a linear relationship (correlation coefficient 0.86) with a slope of 0.36 ± 0.08 with a zero intercept at the origin. This means that the efficiency of the studied enzymes on average is three times higher with cAVTP than with tAVTG. When the higher efficiency with cAVTP is compared with the second-order rate constants for the reaction with GSH, which are 0.06 and 0.17 mM⁻¹·min⁻¹ for cAVTP and tAVTG, respectively, the higher chemical reactivity is not reflected in the $k_{cat}/K_m$ values. The cluster analysis based on Euclidian distances also showed that
three cluster were formed. The Ser variants in one group, Ala and Gly variants in the second and Thr and Pro variants in the third and that their centroids lie in a straight line from the origin (Figure 13).

Modeling of the solution structures of cAVTP and tAVTG indicates differences in their ground state where the acetyl group of the side chain in cAVTP is not in plane with the carbon-carbon double bond (Figure 1). In tAVTG the carbonyl and the carbon-carbon are coplanar. The differences in ground state may be the reason why there are higher activities with cAVTP. tAVTG is more extended why the molecule might need more space in the active site than cAVTP. This could be the explanation with M2Ala, M2Gly, and M2Pro but not with M2Ser (Table 5).

$k_{cat}/K_m$ values can be translated into a measure of differences in the Gibbs free energies of transition state stabilization (Fersht, 1999). The values of the different catalytic efficiencies were used in the equation $\Delta G^\dagger = -RT \ln(k_{cat}/K_m)^A/(k_{cat}/K_m)^B$ where A and B denote different enzymes. The $\Delta G^\dagger$ values were 9.0 and 5.6 kJ mol$^{-1}$ comparing GSTs M1Ser (wt) with M1Thr for cAVTP and tAVTG, respectively. Corresponding values for GSTs M2Thr (wt) with M2Ser were –3.4 and –7.6 kJ mol$^{-1}$ where the negative signs correspond to enhanced transition state stabilization and higher efficiency with Ser in position 210.

We conclude that the effects of the mutations were qualitatively parallel for the alternative substrates, but vary significantly in magnitude. In addition that steric hindrance may interfere with transition state stabilization. Finally that from the evolutionary perspective the data show that a point mutation can alternatively enhance or attenuate the activity with a particular substrate and illustrate the functional plasticity of GSTs.
Summary

In paper I a screening method was developed for identification of catalytically active enzymes in combinatorial cDNA libraries of mutated glutathione transferase (GST) derivatives expressed in E. coli. The method is based on spraying monochlorobimane (MCB) directly over bacterial colonies growing on agar. The substrate MCB become fluorescent under long wavelength UV light, when the bacterial colony contains active GSTs that catalyze the conjugation with endogenous glutathione (GSH). Fluorescent colonies can be picked and cultured for activity measurement in lysate the next day. Eleven out of twelve GSTs investigated where active with MCB. This method can easily be used to screen libraries generated from most cytosolic GSTs in the search for proteins with altered functions and structures. In paper II the immunosuppressant azathioprine (Aza), a thiopurine and prodrug that has been used clinically for 40 years was investigated with 14 GSTs. Three enzymes showed prominent catalytic activities with Aza and all of them are highly expressed in the liver. We estimated the contribution of the three enzymes GSTs A1-1, A2-2 and M1-1 bioactivation of Aza in the liver and concluded that it was about 2 orders of magnitude more effective than the uncatalyzed reaction. GST bioactivation of Aza could to clarify aspects of idiosyncratic reactions observed in some individuals. In paper III two other thiopurine prodrugs, cis-acetylvinylthiopurine (cAVTP) and trans-acetylvinylthioguanine (tAVTG), were investigated with the same human glutathione transferases. The results displayed diverse catalytic activities with the 13 GSTs investigated. cAVTP and tAVTG were more chemically reactive with GSTs than Aza. A mechanism of consecutive reactions was proposed. The studies contribute to knowledge useful for deciding to whom and under what conditions the drug should optimally be administered. Paper IV was a study of the same prodrugs as in paper III with several mutants from the Mu class characterized by a point mutation of a hypervariable residue. We conclude that the effects of the mutations were qualitatively parallel for cAVTP and tAVTG, but they vary significantly in magnitude; steric hindrance may interfere with transition-state stabilization, and From the evolutionary perspective the data show that a point mutation can alternatively enhance or attenuate the activity with a particular substrate and illustrate the functional plasticity of GSTs.
Summary in Swedish

Min forskning är en studie av ett stort antal lösliga humana glutationtransferseraser (GSTs) och deras betydelse för cytostatika och immunosuppresiva läkemedel. GSTs är inblandade i både den normala cellens försvar mot cancer och i läkemedelsresistens i tumorer. Resistens som orsakas av överproduktion av GSTs kan uppstå både naturligt och till följd av av cytostatikabehandling. GSTs kan användas som aktivator av pro-läkemedel.

Vi har utvecklat en gallringsmetod för att identifiera aktiva muterade GSTs men även utvecklat system för mätning av enzymernas effektivitet med de studerade läkemedlen samt identifiering av de produkter som bildas. Vi har studerat reaktionerna med nästan alla kända lösliga humana GSTs med två nya cytostatikkandidater och ett läkemedel som sedan 1960-talet har använts kliniskt för att dämpa immunförsvaret bland annat för att undvika avstötning efter organtransplantering. Vi har också tittat på hur mutationen av en hypervariabel aminosyra påverkar de katalytiska aktiviteterna med de två nya cytostatikkandidaterna.

Glutationtransferaser

Glutationtransferaser (GSTs) är en familj av enzymer som finns i stort sett alla organismer och har många uppgifter som strukturella proteiner, transport av små molekyler eller del av steroidmetabolismen. En av GSTs funktioner som har studerats i hög grad är deras katalys av avgiftningsreaktioner av olika mutations- och cancerframkallande ämnen. Giftiga ämnen får vi in i kroppen när vi andas och äter. Andra ämnen blir skadliga när olika metaboliska reaktioner sker i kroppen. När de giftiga ämnenas konjugerar med en antioxidant glutation (GSH) blir de mer vattenlösliga och därmed lättare att utsöndra med urinen. Medlemmarna i GST-familjen katalyserar konjugeringreaktioner mellan GSH och olika gifter vilket (gör att reaktionen går mycket fortare) minimerar skador på och i cellen.

Cancer

Normalt delar sig och växer cellerna på ett ordnat och kontrollerat sätt. Cancer inträffar när cellerna börjar växa snabbt och okontrollerat, vilket ofta resulterar i en tumör. Alla tumörer är inte elakartade tumörer (maligna) som
kan invadera omgivande vävnad men dom som är maligna kan sprida sina cancerceller vidare i kroppen. Då uppstår dottertumörer, metastaser som i sin tur kan sprida sig och växa in i omkringliggande vävnad. För att stoppa den okontrollerade celldelningen behandlar man ibland med cytostatika (cellgifter)

Pro-läkemedel


Enzymkinetik

Det går att mäta initialhastigheter för ett enzym s katalys av en viss reaktion. Hastigheterna anpassas till en modell som heter Michaelis-Menten ekvation vilken ger vissa kinetiska konstanter såsom $k_{cat}$ som talar om hur fort ett enzym kan omvandla substratmolekyler till produkt. $K_m$ är en annan konstant som kan beräknas utifrån modellen och den kan vara ett mått på hur subtratet binder till enzymet. Ett enzym s specificitetskonstant $k_{cat}/K_m$ för ett visst subtrat kan därmed också bestämmas. Denna beskriver hur effektivt och specifikt enzymet är att omvandla just det här subtratet till produkt. Specificitetskonstanten kan sedan jämföras med andra enzym för samma substrat eller för att jämföra olika substrat för ett och samma enzym.

Biverkningar och resistens

Biverkningar beror på att cellen har blivit förgiftad. I behandling av en tumör med cytostatika är det en önskad effekt, men när övrig normal vävnad också påverkas så kan det leda till illamående och hårafvall. Resistens av läkemedel kan bero på hur mycket av ett protein eller hur ett protein ser ut som är involverat i läkemedelsmetabolismen. Protein som produceras i mycket högre eller lägre koncentrationer än vad som är normalt kan göra att en substans ansamlas och orsakar toxicitet det vill säga biverkningar. Avgiftningsenzyme utgör en del av cellens försvar motgifter. Om dessa avgiftningsenzyme
finns i högre koncentrationer kan det bryta ner läkemedlet som då inte har någon verkan, det vill säga cellen uppvisar resistens mot detta läkemedel.

Glutationtransferaser i läkemedelsmetabolism
Generna för två medlemmar i GST familjen, GST M1-1 och GST T1-1 saknas hos ungefär 50% respektive 30% bland européer. Det innebär att dessa individer inte har de enzymerna. Ett annat enzym, GST P1-1 är känt att finnas i onormalt höga koncentrationer i vissa tumörtyper. I båda fallen kan det påverka läkemedelsmetabolism både positivt och negativt. GST P1-1 kan både medverka till att ett läkemedels verkan försvinner det vill säga till resistens. Men det finns också prodroger som syntetiserats för att använda de förhöjda GST P1-1 koncentrationerna genom att läkemedelsmolekylen aktiveras av enzymerna. Följden av individuella skillnader i om eller hur mycket GSTs som produceras kan vara en bakomliggande orsak till toxicitet och resistens. Men de individuella skillnaderna kan via pro-läkemedel även användas för att undvika oönskad toxicitet och resistens.
**Min forskning**

**Artikel I**

**Gallring av rekombinanta glutationtransferaser (GSTs) (aktiva med monoklorobiman)**


**Applicering**

Evolutionära enzymstudier kan inbegripa bibliotek av muterade enzym med slumpvisa punktmutationer eller av sekvenser som satts samman slumpvis. Biblioteken kan bestå av flera miljoner mutantvarianter. Känsligheten i ovan nämnda sektionsmetod gör att mycket tid kan sparar i jämförelse med att plocka kloner i blindt. Att direkt mäta GST-aktivitet på lysatet kan ge en uppfattning om mutantens egenskaper beroende på korrelationen mellan MCB och den önskade substratspecificiteten för mutanten. Av de GSTs som undersöktes med metoden var alla utom en aktiv med MCB. Metoden kan därför även användas i enklare subklonning av GST, t ex vid rationell enzymdesign eller vid ett byte av plasmid.
Artikel II

Divergenta aktiviteter av humana glutationtransferaser i dess bioaktivering av azatioprin


Vår studie av GSTs och Aza visar att den farmakologiska aktiveringen av Aza är ungefär 100 gånger mer effektiv i närvaro av GSTs än när enzymen inte finns med i reaktionen. Vi har därför dragit slutsatsen att graden av GST-uttryck kan bidra till biverkningar av Aza och att det därför kan vara värt att undersöka patientens genotyp innan Azabehandling sätts in.

Artikel III

Humana glutathiontransferaser katalyserar bioaktiveringen av anticancer tiopurinprodroger

13 lösliga humana GSTs undersökes med två pro-läkemedelkandidater för cancerbehandling. Kandidaterna 6-(2-acetylvinyltio)purin (cAVTP) och 6-(2-acetylvinyltio)guanin (tAVTG) hade mätbar aktivitet med åtta respektive nio av enzymerna som som karaktäriserades. Resultatet visade varierande katalytiska aktiviteter. cAVTP och tAVTP visade sig vara mer kemiskt reaktiva med GSTs än Aza. Vi föreslår en mekanism för konsekutiva reaktioner:
Förslaget är att först steget av reaktionen (addition av GSH) katalyseras av GSTs men att frisläppandet av den aktiva substansen 6-MP och 6-tioguanin (6-TG) inte är det. Studien bidrar till kunskap som är värdefull för att veta under vilka förhållanden som pro-läkemedlet skall distribueras för att verka optimalt. De beror på i vilken vävnad cancern finns och vilket enzym som uttrycks där. Individuella skillnader i enzymproduktion kan bidra till hur pro-läkemedlet aktiveras.

Artikel IV

Struktur-aktivitetsstudier av en hypervariable rest i My-klass GSTs i aktiveringen av tiopurinprodroger

Här studerades samma prodroger som i artikel III med enzymer och mutanter från myklassen. Det visade sig att GST M1-1 hade en hög specificitetskonstant både med cAVTP och tAVTG. GST M2-2 hade en relativt hög specificitetskonstant med cAVTP men en 22 gånger lägre aktivitet med tAVTP. Detta gav bra förutsättningar för att studera förändring i subsstratspecificitet med GST M1-1 och M2-2 enzymer som punktmuterats i DNA sekvensen. Mutationen är en enkelmutation av aminosyra nr 210 i enzymet. Den aminosyran har tidigare rapporterats vara variabel i enzymets evolution. Det visade sig att en enda mutation i enzymet kan resultera i stora förändringar i substratspecificiteten.

Vi sammanfattar därför att effekten av punktmutaionerna är parallellt jämförbara för både cAVTP- och tAVTG-aktiviteten, men att de skiljer sig åt i storlek. Mutanter med en aminosyra som tar mer plats gav sämre aktivitet för båda molekylerna så vi antar att utrymmet är av betydelse. Från det evolutionära perspektivet visar våra data att en enda mutation kan öka eller minska aktiviteten för ett substrat och det visar GST enzymernas funktionella plasticitet.
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