Stockholm University

A study of Pi- and Alpha-class glutathione transferases

Characterization and protein redesign for medical applications

Aram Ismail

Doctoral Thesis in Biochemistry at Stockholm University, Sweden 2025

A study of Pi- and Alpha-class glutathione transferases

Characterization and protein redesign for medical applications

Aram Ismail

Academic dissertation for the Degree of Doctor of Philosophy in Biochemistry at Stockholm University to be publicly defended on Monday 19 May 2025 at 13.00 in C458, Kemiska övningslaboratoriet, Svante Arrhenius väg 16 C and online via Zoom, public link is available at the department website.

Abstract

Glutathione transferases (GSTs) are a family of enzymes that are key players in cellular detoxication. These enzymes catalyze the transfer of glutathione (GSH) to the electrophilic center of harmful compounds to promote their elimination.

The human Pi class (GST P1-1) is well-known for its overexpression in cancerous tissue and has been found to contribute to tumor growth and chemotherapeutic resistance. For these reasons, GST P1-1 has emerged as a promising therapeutic target to fight cancer by developing inhibitors and prodrugs (e.g. Telcyta) targeting the enzyme. GST P1-1 has also been suggested as a marker during carcinogenesis.

Apart from being cellular detoxicants some GSTs have come to develop other functions. One member of the human Alpha class, GST A3-3, plays an important role in steroid hormone biosynthesis by catalyzing the double-bond isomerization reaction of 5-androsten-3,17-dione and 5-pregnen-3,20-dione, precursors to the steroid hormones testosterone and progesterone. To date, in addition to the human enzyme, efficient ketosteroid isomerase activity has been identified in Alpha-class enzymes from equine and porcine tissues.

This thesis focuses on studying the Pi- and Alpha-class enzymes. In the first study, we characterize dog GST P1-1 and show that the enzyme shares certain class-specific similarities with the human enzyme in terms of substrate selectivity profile and inhibition profile. We also developed a thin-layer chromatography method to screen and semi-quantify Telcyta activity. In the second study, we show that the replacement of tyrosine^{109} with histidine increased the activity with the anticancer prodrug Telcyta 2.9-fold, and we also show that the mutation Q85R positively influenced the thermostability of the enzyme. In the third study, we discovered a mutant enzyme, V2 (Q40M-E41Q-A46S-Y109H-V200L), with 22-fold higher catalytic efficiency than wildtype human GST P1-1 with cumene hydroperoxide. The mutation Y109H was responsible for a 10-fold increase in catalytic efficiency. In the fourth study, we discovered that GST A3-3 from the common marmoset monkey possessed prominent ketosteroid isomerase activity, albeit significantly lower than its human and equine counterparts, it was on par with porcine GST A2-2. In the fifth study, we solved the crystal structure of equine GST A3-3 in complex with the inhibitor triethyltin bromide. The structure reveals the interaction between triethyltin bromide, GSH, and Tyr^{9} in the enzyme.

All in all, the work presented in this thesis has added to the body of knowledge on the glutathione transferases from the Pi- and Alpha-classes.

Keywords: *Glutathione transferases, GST A3-3, steroidogenesis, GST P1-1, cancer, Telcyta, enzyme inhibiton, ADEPT, protein engineering.*

Stockholm 2025
http://urn.kb.se/resolve?urn=urn:nbn:se:su:diva-240646

ISBN 978-91-8107-156-6
ISBN 978-91-8107-157-3

Department of Biochemistry and Biophysics

Stockholm University, 106 91 Stockholm

A STUDY OF PI- AND ALPHA-CLASS GLUTATHIONE TRANSFERASES

Aram Ismail

Stockholm University

A study of Pi- and Alpha-class glutathione transferases

Characterization and protein redesign for medical applications

Aram Ismail

ISBN print 978-91-8107-156-6
ISBN PDF 978-91-8107-157-3

Printed in Sweden by Universitetsservice US-AB, Stockholm 2025

Abstract

Glutathione transferases (GSTs) are a family of enzymes that are key players in cellular detoxication. These enzymes catalyze the transfer of glutathione (GSH) to the electrophilic center of harmful compounds to promote their elimination.

The human Pi class (GST P1-1) is well-known for its overexpression in cancerous tissue and has been found to contribute to tumor growth and chemotherapeutic resistance. For these reasons, GST P1-1 has emerged as a promising therapeutic target to fight cancer by developing inhibitors and prodrugs (e.g. Telcyta) targeting the enzyme. GST P1-1 has also been suggested as a marker during carcinogenesis.

Apart from being cellular detoxicants some GSTs have come to develop other functions. One member of the human Alpha class, GST A3-3, plays an important role in steroid hormone biosynthesis by catalyzing the double-bond isomerization reaction of 5-androsten-3,17-dione and 5-pregnen-3,20-dione, precursors to the steroid hormones testosterone and progesterone. To date, in addition to the human enzyme, efficient ketosteroid isomerase activity has been identified in Alpha-class enzymes from equine and porcine tissues.

This thesis focuses on studying the Pi- and Alpha-class enzymes. In the first study, we characterize dog GST P1-1 and show that the enzyme shares certain class-specific similarities with the human enzyme in terms of substrate selectivity profile and inhibition profile. We also developed a thin-layer chromatography method to screen and semi-quantify Telcyta activity. In the second study, we show that the replacement of tyrosine^{109} with histidine increased the activity with the anticancer prodrug Telcyta 2.9-fold, and we also show that the mutation Q85R positively influenced the thermostability of the enzyme. In the third study, we discovered a mutant enzyme, V2 (Q40M-E41Q-A46S-Y109H-V200L), with 22-fold higher catalytic efficiency than wildtype human GST P1-1 with cumene hydroperoxide. The mutation Y109H was responsible for a 10-fold increase in catalytic efficiency. In the fourth study, we discovered that GST A3-3 from the common marmoset monkey possessed prominent ketosteroid isomerase activity, albeit significantly lower than its human and equine counterparts, it was on par with porcine GST A2-2. In the fifth study, we

solved the crystal structure of equine GST A3-3 in complex with the inhibitor triethyltin bromide. The structure reveals the interaction between triethyltin bromide, GSH, and Tyr^{9} in the enzyme.

All in all, the work presented in this thesis has added to the body of knowledge on the glutathione transferases from the Pi- and Alpha-classes.

This thesis is based on the following publications

I. Ismail A, Lewis E, Sjödin B, Mannervik B. **Characterization of Dog Glutathione Transferase P1-1, an Enzyme Relevant to Veterinary Medicine**. *International Journal of Molecular Sciences*. 2021, 22, 4079; DOI: org/10.3390/ijms22084079

II. Ismail A, Govindarajan S, Mannervik B. **Human GST P1-1 redesigned for enhanced catalytic activity with the anticancer prodrug Telcyta and improved thermostability**. *Cancers*. 2024, *16*, 762; DOI: org/10.3390/cancers16040762

III. Ismail A, Mannervik B. **Mutations selectively evolving peroxidase activity among alternative catalytic functions of human glutathione transferase P1-1.** Antioxidants. 2024, 13, 1347; DOI: https://doi.org/10.3390/antiox13111347

IV. Ismail A, Sawmi J, Mannervik B. **Marmoset glutathione transferases with ketosteroid isomerase activity**. *Biochem. Biophys. Rep*. 2021, 27, 1-6; DOI:10.1016/j.bbrep.2021.101078

V. Škerlová J, Ismail A, Lindström H, Sjödin B, Mannervik B, Stenmark P. **Structural and functional analysis of the inhibition of equine glutathione transferase A3-3 by organotin endocrine disrupting pollutants**. *Environ. Pollut*. 2021, 268, 1-8; DOI:10.1016/j.envpol.2020.115960

My contribution to each paper

I. I supervised project student Elizabeth in the laboratory. I developed and conducted the thin-layer chromatography experiment with the anticancer prodrug Telcyta. I also wrote the corresponding section in materials and methods and reviewed the manuscript.

II. I performed all the experimental work (excluding machine learning) and prepared the first draft of the manuscript (excluding machine learning).

III. I performed all the experimental work and prepared the first draft of the manuscript.

IV. I supervised project student Julia in the laboratory and assisted in preparing the manuscript as well as reviewing and editing.

V. I performed part of the inhibition experiments and supervised project students Shanshan and Rōng for the other part of the inhibition experiments. I also prepared the corresponding figures and tables.

Additional publications not included in the thesis

VI. Shokeer A, Ismail A, Hegazy UM, Kolm RH, Mannervik B. **Mutational Analysis of the Binding of Alternative Substrates and Inhibitors to the Active Site of Human Glutathione Transferase P1–1**. *Processes*. 2020, 8, 1232; DOI: org/10.3390/pr8101232

VII. Mannervik B, Ismail A, Lindström H, Sjödin B, Ing N.H. **Glutathione Transferases as Efficient Ketosteroid Isomerases**. *Front. Mol. Biosci*. 2021, 8, 765970; DOI:10.3389/fmolb.2021.765970

VIII. Musdal Y, Ismail A, Sjödin B, Mannervik B. **Potent GST Ketosteroid Isomerase Activity Relevant to Ecdysteroidogenesis in the Malaria Vector *Anopheles gambiae***. *Biomolecules*. 2023, 13, 976; DOI: org/10.3390/biom13060976

IX. Šťastná K, Musdal Y, Ismail A, Ebihara K, Niwa R, Mannervik B. **Supreme glutathione-dependent ketosteroid isomerase in the yellow-fever transmitting mosquito Aedes aegypti**. *Biochem Biophys Res Commun*. 2024, 711, 149914; DOI: 10.1016/j.bbrc.2024.149914.

Abbreviations

GSTs	glutathione transferases
GSH	glutathione
H-Site	hydrophobic substrate binding site
G-site	glutathione binding site
CDNB	1-chloro-2,4-dinitrobenzene
ITCs	isothiocyanates
CuOOH	cumene hydroperoxide
EA	ethacrynic acid
Δ^5-AD	5-androsten-3,17-dione
Δ^5-PD	5-pregnen-3,20-dione
ADEPT	antibody-directed enzyme prodrug therapy

Table of contents

Introduction

Enzymes are nature's own catalysts, speeding up life sustaining biochemical reactions within living organisms. These reactions would take hours, days, or even months in the absence of an enzyme, but only a fraction of a second in the presence of these amazing molecular machines.

Scientific advances during the past decades have allowed enzymes to be engineered for improved or novel functions, beyond the already existing ones, making them useful targets for both industrial and medical applications.

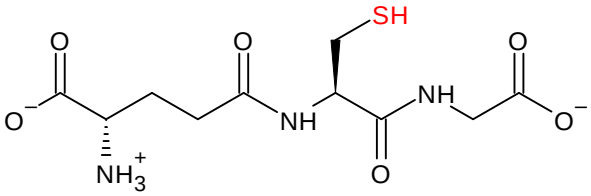
Biotransformation (detoxication)

The human body is constantly exposed to xenobiotic and endobiotic substances that can cause damage to the cells. We have protective systems to deal with such compounds. Biotransformation is a metabolic multistep process (phase I, II, and III) in which the structure of a chemical is altered through enzymatic reactions so that it becomes either active, inactive or even toxic. For the most part biotransformation results in inactive metabolites[1].

The toxic compounds subject to phase I metabolism mostly undergo oxidation reactions, making them more reactive, with the major catalyst of the reactions being the NADPH-dependent P450 enzyme family[2]. Many of the products become targets for phase II metabolism, where they are conjugated with hydrophilic cofactors such as glutathione (GSH), among others, into less toxic and more water-soluble molecules. A handful of enzymes are involved in phase II metabolism, however, conjugation with GSH is carried out by glutathione transferases (GSTs). Prior to the final step, phase III, conjugates can be further metabolized before they are transported out of the cell through the ATP-binding cassette transporters and members thereof, thereby reducing the intracellular levels of toxins. GSH-conjugates are metabolized into mercapturic acid prior to phase III elimination[3,4].

Glutathione

GSH (**Figure 1**) is a tripeptide composed of glutamic acid, cysteine, and glycine. In mammals, GSH is the most abundant non-protein thiol in the cytosol of the cell[5]. It plays an important role in the metabolism of harmful electrophilic compounds and in maintaining redox homeostasis[4] as well as modulating cell proliferation, among other functions[6]. The intracellular concentration of GSH in mammals ranges between 1–10 mM[7], with the highest concentration found in hepatocytes.



γ-glutamylcysteinylglycine

Figure 1. The structure of glutathione with the thiol group highlighted in red.

As mentioned, GSH partakes in the metabolism of toxic endogenous and exogenous electrophiles, and it does so by forming water-soluble conjugates that are less reactive, in reactions assisted by glutathione transferase.

Glutathione transferases

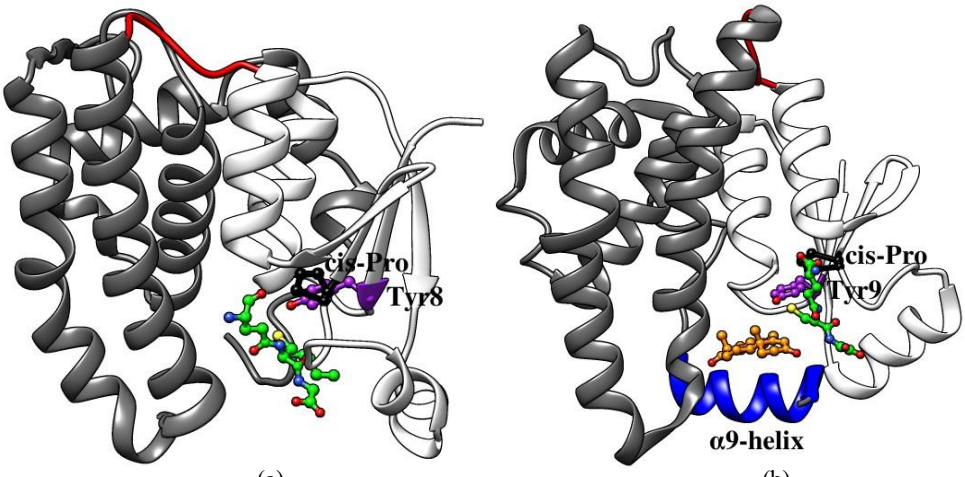
Function

GSTs can be found in almost all living organisms, and they are mostly known for their involvement in cellular phase II detoxication. These enzymes catalyze the nucleophilic attack of reduced GSH onto hydrophobic electrophilic compounds, including carcinogens, therapeutic agents, and products of oxidative metabolism[8]. The formed GSH-conjugates are more hydrophilic and can thus be eliminated from the body through the mercapturic acid pathway[9,10]. Apart from detoxication, some GSTs have evolved to partake in other cellular processes such as steroid hormone biosynthesis[11-13], cell signaling[14,15], prostaglandin biosynthesis[16,17], and storage and transport[18-21].

Structure and classification

There are seven classes of mammalian cytosolic GSTs, namely, Pi, Alpha, Mu[22], Theta[23], Omega[24], Zeta[25], and Sigma[16], and the crystal structure of each class has been solved[24,26-35]. The classes are primarily distinguished apart based on sequence similarity, and above 50% sequence identity is to be expected between the members within a class[36], and below 30% sequence identity between different classes.

All soluble GSTs are dimeric proteins composed of either two identical subunits (homodimers) or two different subunits from the same class (heterodimers)[37] with a subunit molecular mass of roughly 25 kDa. The subunit composition is reflected in the nomenclature of the enzymes, for example, GST P1-1 means that the enzyme is a member of the Pi-class and has two subunits of type 1[38]. Moreover, each subunit has an active site with two binding pockets. The binding site responsible for the binding of GSH is called the G-site, and it is located within the N-terminal domain of the protein. This domain also contains the catalytically active residue promoting GSH activation, either tyrosine, serine, or cysteine, depending on the class of GST. The binding site responsible for the binding of the hydrophobic electrophilic substrates is called the H-site[39], and it is formed between the C-terminal domain and a loop from the N-terminal domain. Topologically, the G-site is preserved among GSTs, whereas the H-site is more structurally diverse and contributes to the substrate selectivity of the various isoenzymes[8]. Moreover, all cytosolic GSTs share the same N-terminal thioredoxin-fold with a β-α-β-α-β-β-α structural motif and a C-terminal domain with a secondary structure composed of only α-helices. Another highly conserved characteristic among GSTs is the cis-Pro loop (**Figure 2, black**) which appears to be important in keeping the enzyme in a catalytically favorable pose[40]. However, although all soluble GSTs have similar protein-fold, some structural features differ. For example, the Mu class has an extra loop adjacent to the active site, Mu-loop, which results in a deeper active site pocket[29]. In contrast, Alpha- and Theta-class enzymes have an extra C-terminal alpha-helix (**Figure 2, blue**) which adds to the H-site making it smaller and more hydrophobic compared to other GSTs[30,32].



(a) **Human Pi class (P1-1)** (b) **Human Alpha class (A3-3)**

Figure 2. Crystal structures of individual subunits of Pi-class (1PGT), and Alpha-class (2VCV) GSTs with the co-crystallized ligand(s) colored in green and orange are displayed. The N-terminal and C-terminal are colored white and dark grey, respectively, while the linker region connecting the two domains is highlighted in red. The highly conserved and structurally important cis-Pro is colored in black, and the catalytically important Tyr residue in Pi-, and Alpha-class enzymes is highlighted in purple. The characteristic feature of Alpha-class GSTs, the helix α9 are shown in blue.

Mechanism of GSH activation

The pKa of the free GSH thiol group is 9.2, thus most of the GSH is in its thiol form at physiological pH. However, as GSH binds to the active site of GSTs the pKa is lowered to ~6.2–6.6[41], meaning that the ionized form is promoted thereby increasing the rate of chemical catalysis with electrophiles[4]. In the Pi, Alpha, Mu, and Sigma classes the formed thiolate anion (strong nucleophile) is stabilized by a conserved active site Tyr[8]. The corresponding residue in the Theta- and Zeta-class is a Ser residue[8,25], while the omega class possesses a Cys residue[24].

The mechanism following GSH activation has not been fully elucidated for all classes, although computer-based studies[42,43], supported by experimental data[28,41,44-51], have given insight into the mechanism of the Pi, Alpha, and Mu classes. For the Pi and Alpha classes, it has been suggested that the proton of the GSH thiol group is transferred to the GSH glutamyl α-carboxylate (**Figure 1**) with the aid of an active site water molecule[42]. A conserved active site Tyr then stabilizes the formed thiolate anion by hydrogen bonding, preparing it for

nucleophilic attack on the electrophilic substrate[8]. On the other hand, GSH activation in the Mu class proceeds through a proton transfer from the thiol group to the imidazole nitrogen of a nearby His[107] assisted by two active site water molecules acting as a bridge[43]. Similarly to the Pi and Alpha classes, the Mu class also has a Tyr residue to stabilize the thiolate anion.

Tissue expression and polymorphism

GSTs are widely distributed in the human body, and they have been identified in almost all organs as well as erythrocytes[52-55]. However, the expression pattern and expression levels of the various isoenzymes differ from organ to organ as well as from person to person. Moreover, the enzyme levels can also be affected by diet[56,57] and exposure to toxic compounds[55,58]. In general, the human Pi class is the most widely expressed GST and has been identified in all tissues investigated, with the highest concentration detected in the brain and absent levels in the adult liver[53,59,60]. Isoenzymes from the human Alpha- and Mu-classes are the predominant GSTs in the liver and testis[53,59,60].

Genetic polymorphism has been detected in human cytosolic GSTs. Both Mu- and Theta-class GST polymorphism are associated with gene deletion resulting in the absence of the enzyme[61-64], which is a rather common occurrence among the general population[65]. Other GSTs, for example, Pi- and Alpha-classes, are more commonly associated with single nucleotide polymorphism giving rise to allelic variants with sequence variation. These variations can impact the enzyme function[66-72] in response to toxic compounds and thereby influence disease susceptibility[73-78].

Substrates

Here I will only describe the most relevant substrates for my work.

In the laboratory, a handful of synthetic substrates can be used to monitor GST activity. These substrates represent different chemical reactions that GSTs commonly catalyze, and they give information about the function of the enzyme. GST enzymes can have overlapping substrate selectivities, however, different classes of GSTs may display a stronger preference towards certain substrates in comparison to other classes. For example, some Alpha-class enzymes have high activity with cumene hydroperoxide (CuOOH)[58] or 5-androsten-3,17-dione (Δ^5-AD)[11], whereas Pi class have high activity with ethacrynic acid (EA)[68], and Mu class has high activity with 1-chloro-2,4-dinitrobenzene (CDNB)[79].

1-Chloro-2,4-dinitrobenzene (CDNB)

CDNB is a standard substrate for GSTs, meaning that almost all GSTs show activity towards this substrate[58], although poor activity has been noted with the human Theta-class (GST T1-1)[80,81]. Oftentimes, this is the first substrate to test during enzyme characterization. CDNB undergoes electrophilic aromatic substitution with GSTs, and forms the product S-2,4-dinitrophenyl-glutathione.

1-Chloro-2,4-dinitrobenzene

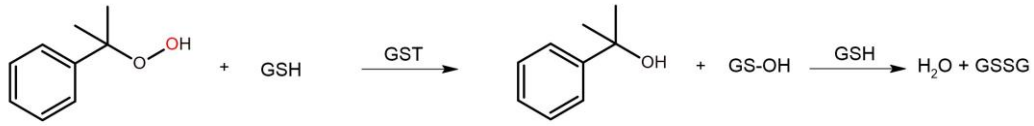
Isothiocyanates (ITCs)

Isothiocyanates are substrates for GSTs, and these organic compounds are natural hydrolysis products of glucosinolates found in cruciferous vegetables such as broccoli, cabbage, and cauliflower[82-84]. Their health benefits and protective properties against, for example, cancer come from the upregulation of GST activity[56]. ITCs undergoes nucleophilic addition reactions with GSTs to form dithiocarbamates.

Isothiocyanate

Cumene hydroperoxide (CuOOH)

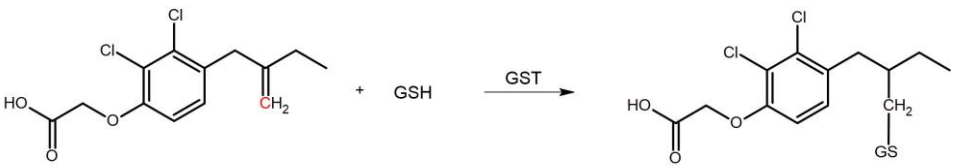
Cumene hydroperoxide is a synthetic substrate, and it functions as a surrogate to monitor if GSTs possibly exhibit physiological hydroperoxide activity. CuOOH undergoes hydroperoxide reduction reaction with GSTs[85].



Cumene hydroperoxide

Ethacrynic acid (EA)

EA is a drug with diuretic properties that is not only a substrate for GSTs but also an inhibitor[86] that has been tested clinically for cancer therapy[87,88]. EA undergoes Michael addition reaction with GSTs.



Ethacrynic acid

5-Androsten-3,17-dione (Δ^5-AD)

Δ^5-AD is physiologically converted to Δ^4-AD, which is an intermediate in the biosynthesis of testosterone[89,90]. Δ^5-AD undergoes ketosteroid double-bond isomerization with GSTs (the ability to catalyze the rearrangement of a carbon-carbon double bond, in this case going from Δ^5-AD to Δ^4-AD).

Δ^5-androsten-3,17-dione

Δ^4-androsten-3,17-dione

5-Pregnen-3,20-dione (Δ^5-PD)

Δ^5-PD is physiologically converted to Δ^4-PD, an intermediate in the biosynthesis of progesterone[89,90]. Δ^5-PD undergoes ketosteroid double-bond isomerization with GSTs (the ability to catalyze the rearrangement of a carbon-carbon double-bond, in this case going from Δ^5-PD to Δ^4-PD).

Δ^5-pregnen-3,20-dione Δ^4-pregnen-3,20-dione

Pi class

Part of the work presented in this thesis is based on the Pi class, more precisely GST P1-1, therefore the Pi class will be the focus of this section. The studies involve the GSTP1*A allele with Ile^{105} as variable position. The numbering of the residues includes the initiator methionine.

Structure

In humans, the Pi class has only one isoenzyme and that is GST P1-1 (**Figure 2a**). GST P1-1 is a dimeric protein, composed of 210 amino acids, with a subunit molecular weight of roughly 23 kDa. Although the two subunits are identical, the enzyme has been shown to not retain catalytic function as a monomer[91,92]. The tertiary structure of the enzyme has been solved[27], and the active site residues have been elucidated. The following residues belong to the G-site, and they form a network of interactions with GSH; Tyr^{8}, Arg^{14}, Trp^{39}, Lys^{45}, Gln^{52}, Leu^{53}, Ser^{66}, and Asp^{99} (Asp^{99} resides on the opposing subunit). The H-site residues interact with the hydrophobic substrate, and they constitute Tyr^{8}, Phe^{9}, Val^{11}, Arg^{14}, Ile^{105}, Tyr^{109}, Asn^{205}, and Gly^{206}. Some residues, Tyr^{8} and Arg^{14}, contribute to both sites. GST P1-1 shares the same common structural characteristics as other GSTs. However, the residues linked to the α2-loop (residues 36-52) exhibit flexibility and can adopt multiple conformations in the absence of a bound ligand whereas a single conformation of the α2-region is induced in the presence of a bound ligand[93,94]. This segment of the enzyme also holds two highly conserved residues, Cys^{48} and Tyr^{50}, and in short, mutational analysis of both these residues reveals that they are important for GSH affinity and catalytic activity[95-104]. Moreover, Tyr^{50}, which is located at the interface between the two subunits is fundamental for dimer stability[102,103].

Further structural studies on individual residues have revealed that Tyr^{109} is an important residue in the H-site and substituting Phe or Val for Tyr has been shown to have a pronounced effect on substrate selectivity and catalysis (dependent on the electrophilic substrate)[105]. Other research has investigated Arg^{14}, Lys^{45}, Gln^{52}, Gln^{65}, and Asp^{99}, with CDNB as substrate, and concluded

that Arg[14], Lys[45], Gln[52], and Gln[65] contribute to the binding of GSH. In contrast, Asp[99] appears to contribute to catalysis rather than binding[106]. Moreover, residue 105 is polymorphic in nature, and changing Ile[105] to Val, Ala, or Trp alters the substrate selectivity profile as well as the thermostability of the enzyme[68].

Polymorphism

The GST P1-1 gene is associated with genetic polymorphism, and to date, five allelic variants have been discovered. These allelic variants have changes to either position 105, 114, or 151. The identified variants are GST P1-1*A (Ile[105]/Ala[114]/Phe[151])[107], GST P1-1*B (Val[105]/Ala[114]/Phe[151])[108,109], GST P1-1*C (Val[105]/Val[114]/Phe[151])[108,109], GST P1-1*D (Ile[105]/Val[114]/Phe[151])[110], and GST P1-1*E (Val[105]/Ala[114]/Leu[151])[111]. The most common allelic variants in the general population are GST P1-1*A and *B, accounting for over 90% of the polymorphism[112]. Furthermore, the GST P1-1*B variant has been associated with an increased risk of tumor formation in certain organs upon exposure to polycyclic aromatic hydrocarbons[113] as well as an overall reduced enzyme stability compared to the *A variant[68]. The altered cancer risk is linked to differences in the substrate selectivity profile related to each variant[114].

Cancer therapy

GST P1-1 is an enzyme frequently overexpressed in many forms of human malignant tumors[115,116] and this overexpression contributes to the deactivation of chemotherapeutic agents[117-120]. Moreover, the human enzyme has also been suggested as a tumor marker for clinical settings based on the elevated serum concentrations detected during ongoing disease progression[121-124]. These findings make GST P1-1 a promising therapeutic target and were also the rationale for developing GST P1-1 inhibitors as well as GST P1-1 activated prodrugs to combat cancer.

GST P1-1 inhibitors have shown to either work as chemosensitizers, making the tumor cells more susceptible to anticancer agents[125-127], or as a myeloproliferative agent promoting apoptosis by disrupting the interaction between the GST P1-1 and Jun N-terminal kinase (JNK) complex[128-130].

Prodrugs are inactive molecules that can be converted to their active form through enzymatic activation in the target tissue. The advantage is an increased bioavailability in the target cells and reduced off-target effects because of the local release of cytotoxic metabolites. Several prodrugs have been

developed to be activated by GSTs[131,132], and one of the more promising prodrugs, activated by GST P1-1, is Telcyta.

Telcyta

Telcyta (**Figure 4**) is a glutathione analog prodrug that is designed to undergo bioactivation by GST P1-1 through a β-elimination reaction, and the selectivity towards GST P1-1 over other GSTs can be ascribed to the phenylglycine group of Telcyta (**Figure 4**)[133]. Upon activation, the drug liberates a GSH analog and a highly cytotoxic phosphorodiamidate moiety (**Figure 4**) which is believed to cross-link DNA in the same manner as an alkylating agent[134,135]. The exact mechanism of Telcyta activation is unknown, but computational studies have suggested a three-step reaction mechanism (**Figure 4**)[136]. In the first step, the carboxylate (COO^-) of Telcyta removes a proton from a nearby water molecule which in turn deprotonates the phenol group of Tyr^8 in GST P1-1. In the second step, the phenolate acts as a base and removes a proton from the scissile C-H of Telcyta. In the third step, a β-elimination takes place to release the phosphorodiamidate group. The two most common allelic variants GST P1-1*A (Ile^{105}) and *B (Val^{105}) are equally efficient in activating the prodrug[137]. Telcyta has undergone clinical trials (I, II, and III), and has shown promising antitumor activity for non-small cell lung cancer, breast cancer, as well as ovarian cancer, with a tolerable side-effect profile[138,141].

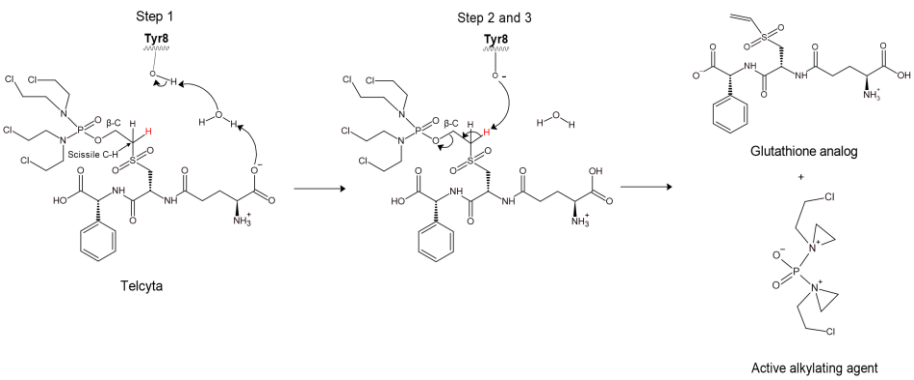


Figure 4. Telcyta activation. Once the proton (red) is abstracted, a β-elimination reaction is initiated to release the active alkylating agent. Figure taken from Paper II.

Therapeutic antibodies

Therapeutic antibodies have emerged as an important tool for the treatment of cancer. They can be customized as therapeutics to target specific antigens expressed on the surface of cancer cells, and they offer high binding specificity and affinity. Therapeutic antibodies can also be used as vectors to deliver payloads to the target tissue[142].

Antibody-directed enzyme prodrug therapy

Traditional cancer chemotherapy has a poor therapeutic index due to problems associated with drug resistance and a lack of specificity toward cancer cells. Antibody-directed enzyme prodrug therapy (ADEPT, **Figure 5**) is a promising alternative, the approach aims to selectively activate a non-toxic prodrug into a cytotoxic agent within cancerous tissue. A drug-activating enzyme accomplishes the activation and to ensure selective activation at the tumor site the enzyme is fused to and delivered by a tumor-targeting antibody[143]. However, the biggest challenge to using ADEPT clinically is the risk of triggering an immune response to the enzyme used[144-146]. One way to circumvent this is to use human-derived enzymes with drug-activating properties such as GSTs[134,147-149].

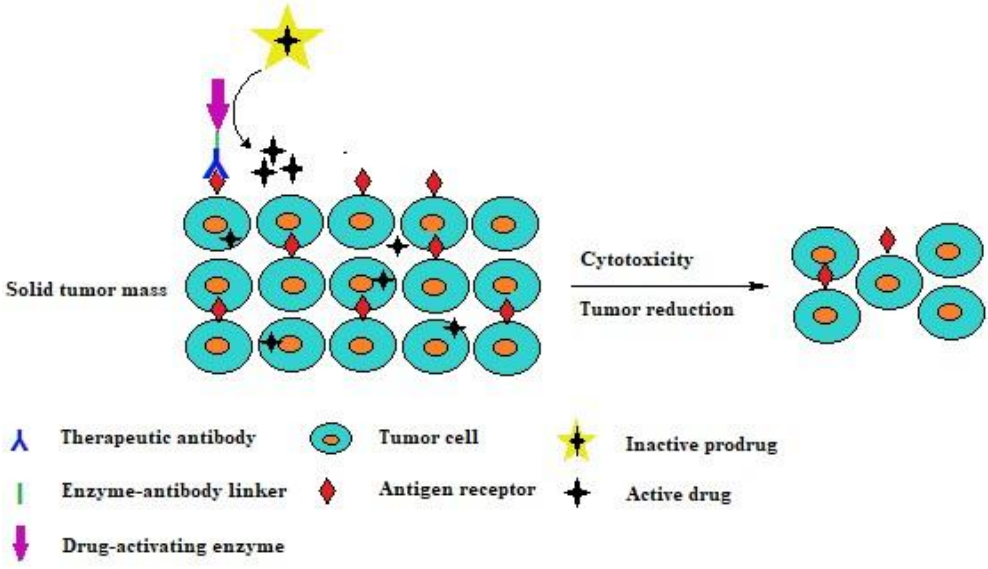


Figure 5. The concept of antibody-directed enzyme prodrug therapy (ADEPT). A drug-activating enzyme (purple) is fused to a therapeutic antibody (blue) via a linker (green). The fusion protein binds to specific antigens

(red) present on the surface of the tumor cell (teal/orange). A prodrug (yellow/black star) is administered and selectively activated into an active cytotoxic drug (black star) at the tumor tissue by the drug-activating enzyme.

Protein engineering

Protein engineering is a powerful tool to tailor enzymes for new applications. Essentially there are two approaches, directed evolution and rational design. Here I will briefly describe rational design.

Rational design

Rational design utilizes information from protein function, structure, or sequence to navigate through sequence space (all the possible sequences of a given protein) to design new enzyme variants with desirable properties. Site-specific mutagenesis is used to introduce mutations, and repeated cycles are performed until a desirable enzyme has been obtained[150]. This is also an excellent approach to determining the effect of a specific amino acid residue on protein structure and function by exchanging it, for example, for an alanine (also known as alanine scanning)[151,152].
There are plenty of examples where rational design methodology has successfully been implemented to redesign the substrate selectivity profiles of glutathione transferases. For example, GST A2-2 has been redesigned for enhanced activity with the immunosuppressant drug azathioprine[149] or transmuted into high ketosteroid isomerase activity[153], and GST M1-1 has been engineered for improved enantioselectivity towards certain epoxide-containing substrates[154].

Machine-learning

Computer-assisted redesign of proteins has become a more common utility in recent years to efficiently explore sequence space. The initial substitutions are scattered across the “functional space” to maximize the information obtained to build a sequence-function model (all information is taken into account, including unimproved and negative sequences). In the second round of mutagenesis, the model proposes new variants to screen based on the initial information[155-156]. Other computer-based protein redesign uses, for example, fractional factorial design methodology to reduce the number of protein variants that must be mapped to improve protein function. Here, sequence information is utilized to identify and score feasible substitutions. Multiple gene variants are designed, and substitutions are systematically incorporated, and the same

substitution appears in more than one variant. This way, the individual contribution of each substitution can be calculated and used to construct new variants with improved function for the second round of mutagenesis[157-160].

Alpha class

Part of the work presented in this thesis is based on Alpha-class enzymes, in particular GST A3-3, and therefore the Alpha-class will be the focus of this section.

Alpha-class GSTs and ketosteroid isomerase activity

The primary function of Alpha-class GSTs, like other GSTs, is metabolism of toxic compounds. High efficiency has been noted with substrates like alkenals, and hydroperoxides[161-163]. Early work on rat and human enzymes revealed that GSTs also exhibit ketosteroid isomerase activity, although modest[164]. However, it was not until the discovery of human GST A3-3 as an efficient catalyst of double-bond isomerization of the steroid substrates, Δ^5-AD and Δ^5-PD, that it was suggested that a distinct physiological function (biosynthesis of steroid hormones), other than detoxication, possibly had emerged for this enzyme[11].

The production of testosterone and progesterone in the human steroidogenic pathway proceeds through the double-bond isomerization of Δ^5-AD to Δ^4-AD and Δ^5-PD to Δ^4-PD. Prior to the identification of GST A3-3, 3β-hydroxysteroid dehydrogenase was the only known enzyme to catalyze this reaction physiologically[165]. The three-dimensional structure of human GST A3-3 in complex with Δ^4-AD can be seen in **Figure 2b**.

Following the identification of the prominent ketosteroid isomerase activity of the human GST A3-3[11], another human Alpha-class enzyme, GST A1-1, was also identified as an active ketosteroid isomerase, although to a significantly lesser extent than human GST A3-3[11]. The search for corresponding GSTs in other mammals has continued. Currently, two additional Alpha-class enzymes have been recognized as efficient ketosteroid isomerases, GST A2-2 from pig[166] and GST A3-3 from horse tissues[13], with the equine enzyme being classified as the most efficient ketosteroid isomerase known in mammals.

Although some Alpha-class enzymes share very high sequence similarity, significant differences in catalytic efficiency with steroid substrates have been observed. For example, human GST A2-2 and GST A3-3 share above 80% sequence identity, despite this, a 5000-fold difference in ketosteroid isomerase activity has been noted between the two enzymes[153,167]. Mutagenesis studies have revealed that changes in a few active site residues (10, 12, 111, 208, and 216) are responsible for the observed difference, with position 111 having the biggest negative impact due to steric effects caused by the larger Phe occurring in GST A2-2 compared to the smaller Leu in GST A3-3[167].

Catalytic mechanism

A three-step reaction mechanism with two rearrangement steps has been proposed for the isomerization reaction of Δ^5-AD by GST A3-3[168]. In the first step activated GSH (GS^-) serves as a base and deprotonates the C4 atom (**Figure 6**). The resulting delocalized electrons are spread between the O3 atom and the C6 atom. The negative charge allocated to the O3 oxygen atom of the enolate is stabilized by an ionic hydrogen bond with the GSH-Gly mainchain amide (**Figure 6**). Prior to the second step a conformational rearrangement of the Tyr^9 sidechain takes place (**Figure 6**). In the second step the C6 atom abstracts the proton from the Tyr^9 sidechain hydroxyl, generating Δ^4-AD, the product of the isomerization reaction (**Figure 6**). Prior to the third step the Tyr^9 sidechain undergoes structural rearrangement to directly interact with the GSH thiol (**Figure 6**). In the third step Tyr^9 abstracts the proton from the GSH thiol (**Figure 6**).

In the described mechanism GSH serves two functions: (1) the GSH thiol together with Tyr^9 catalyzes the proton transfer from the C4 atom of Δ^5-AD to the C6 atom of Δ^5-AD; (2) the GSH mainchain amide stabilizes the negative charge formed on the O3 atom of the enolate intermediate.

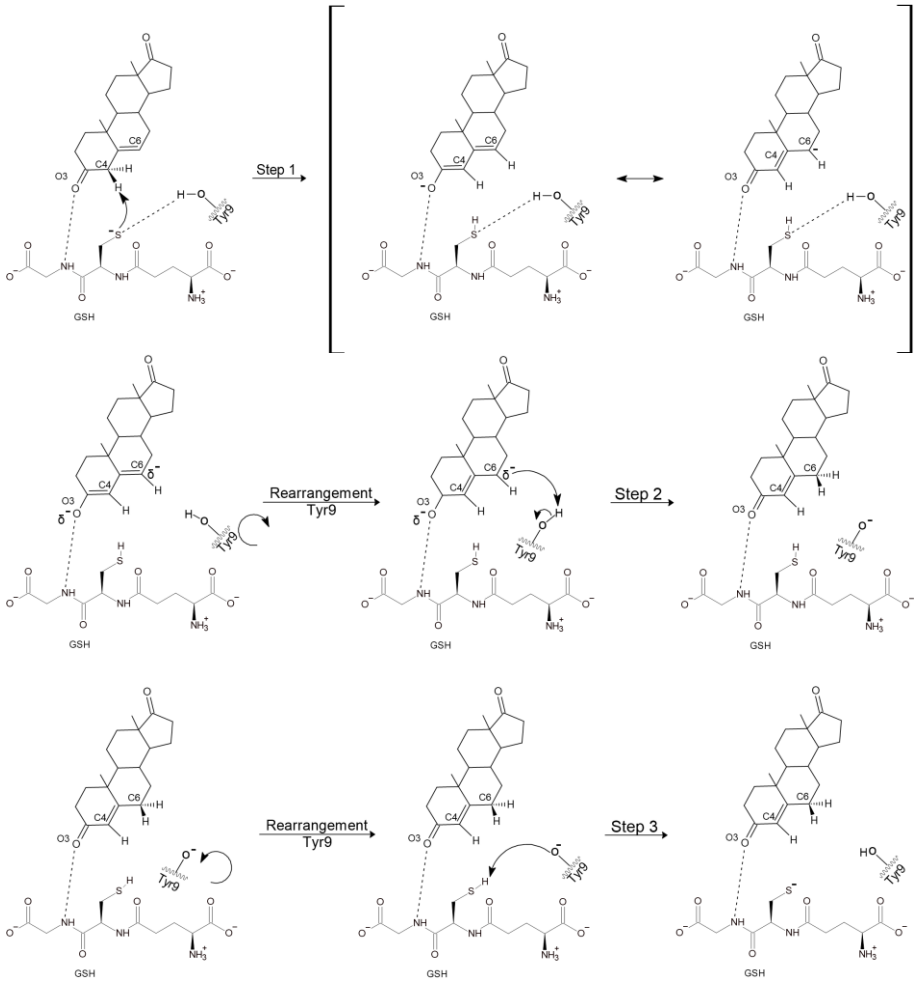


Figure 6. The proposed ketosteroid isomerase mechanism of Δ^5-AD catalyzed by GST A3-3. The mechanism involves three catalytic steps and two Tyr^9 rearrangement steps. Figure based on reference 168.

Tissue distribution

The two most active ketosteroid isomerases known in mammals, equine and human GST A3-3, have both been identified in high levels in steroidogenic tissue such as adrenal gland, testis, and ovary[11,13].

Experimental model organism

Model organisms are non-human species that can be used to study, for example, biological processes, human diseases, effects and adverse effects of drug candidates[169,170]. Hence, model organisms are important tools in research to better understand these complex physiological events. In this thesis two Alpha-class GSTs from the common marmoset monkey were subject for investigation for steroidogenic function, on the basis that the organism could serve as an alternative experimental model animal to the larger mammals, pig and horse, in which highly active GST steroid isomerases had previously been identified[13,166].

Methods

Here I will describe the thin-layer chromatography method that was developed and used to screen and semi-quantify Telcyta activity in papers I and II.

Telcyta activity assay

Telcyta is cleaved into a nitrogen mustard-like agent and a glutathione-like moiety upon activation (**Figure 4**). The active alkylating agent in Telcyta lacks a chromophore thus making the compound undetectable by UV-light. On the contrary, the glutathione-like moiety of the molecule contains a UV-detectable chromophore. However, in a spectrophotometric assay no difference would emerge between the parent compound and the glutathione-like product as they carry the same chromophore. However, the size and polarity difference between the parent compound and glutathione-analog product offers the opportunity for chromatographic separation. The original Telcyta activity assay was developed in 1994 by Lyttle and coworkers and was based on HPLC with UV-detection[134]. As a cost-efficient alternative we developed a thin-layer chromatography method to assay Telcyta activity.

A stock solution of 20 mM Telcyta was prepared in DMSO and then diluted to 6 mM in 0.1 M sodium phosphate buffer pH 7.4 (buffer A). 0.5 mg/ml enzyme stock solution was prepared in buffer A. Telcyta and enzyme were mixed in a 1:1 ratio to give a final concentration of 0.25 mg/ml and 3 mM, respectively. A background reaction sample (no enzyme) was also prepared. All samples were incubated in a water-bath at 37 °C for a total of 17.5 min. Sampling occurred every 3.5 min, starting from time-point 0, and 1 µl of reaction mixture was withdrawn and applied to a silica coated thin-layer chromatography plate. Each plate was then allowed to develop inside the chromatography chamber with a mobile phase consisting of ethyl acetate, acetic acid, methanol, and Milli-Q water (10:3:3:2 ratio). Following chromatography, the plates were dried at 50 °C for 10 min. The analytes on the plates were visualized by ninhydrin staining (0.5% w/v in methanol) at 37 °C for 10 min. Each

plate was photographed, and ImageJ was used to quantify the size and intensity of each spot representing the remaining substrate (starting material) and product at each time point. The background reaction was subtracted from the enzymatic reaction at each time point. Individual graphs were constructed for starting material and product by plotting time vs peak area (derived from intensity and size of spot stains). The slope of the initial linear part of the progress curve was used as a measure of catalytic activity.

A schematic overview of the experimental setup can be seen in **Figure 7**.

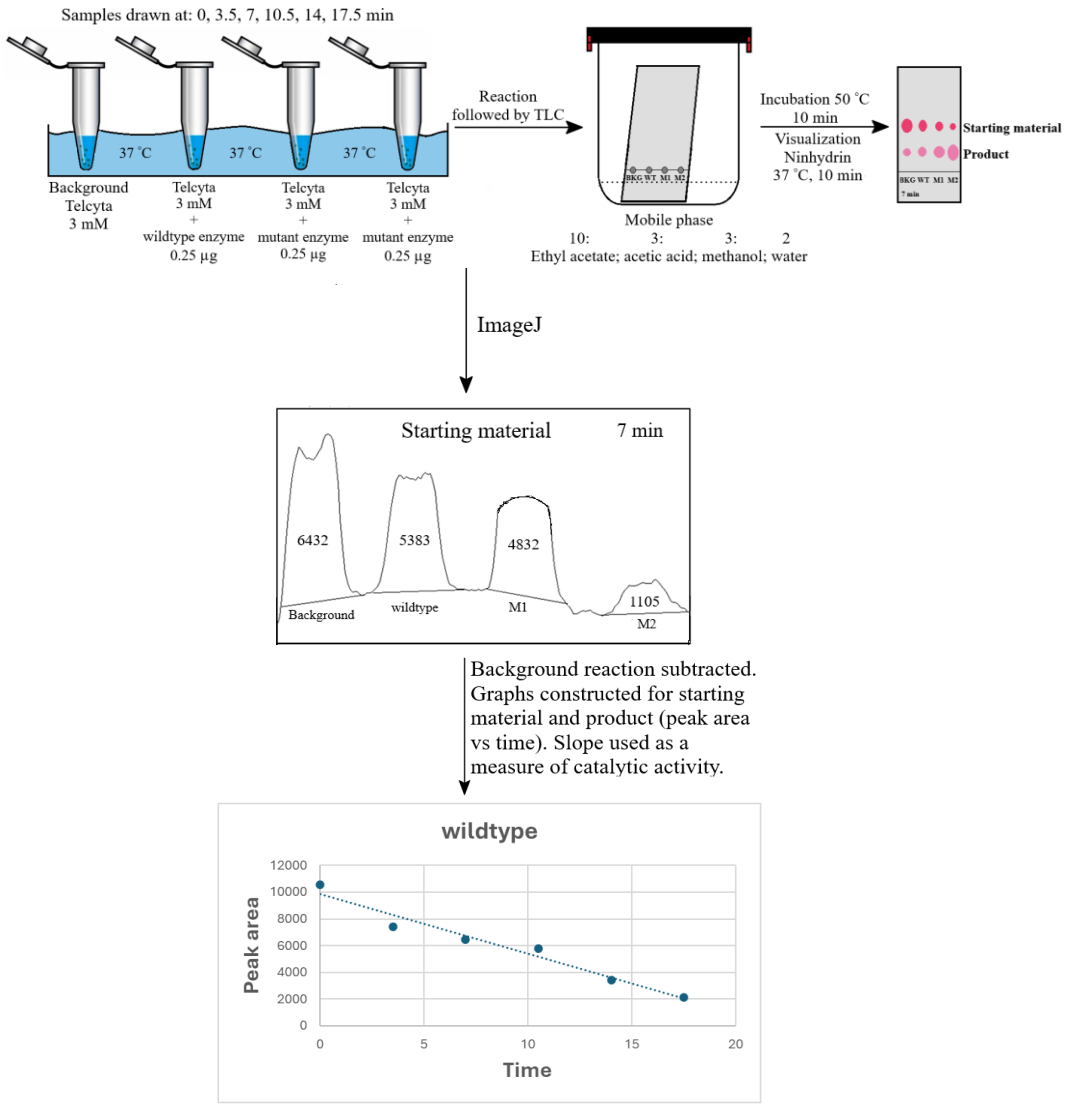


Figure 7. Schematic illustration of the Telcyta assay.

Summary of results

This thesis focuses on studying the glutathione transferase Pi- and Alpha-class enzymes. The Pi-class enzyme has medical relevance related to cancer therapy, while the Alpha-class enzyme is involved in steroid hormone biosynthesis.

Paper I: Characterization of dog glutathione transferase P1-1, an enzyme relevant to veterinary medicine

Aim

This paper aimed to characterize dog GST P1-1 to investigate its potential as a therapeutic target for cancer therapy, similar to its human counterpart.

Results

Dog GST P1-1 was successfully purified and then tested spectrophotometrically with multiple alternative substrates (**Table 1**). The human enzyme has been added for comparison (**Table 1**). Examining the specific activities of the two enzymes, the canine enzyme appears to have an overall reduction in the kinetic profile compared to the human enzyme. For example, the canine enzyme showed an almost 5-fold lesser CDNB activity, roughly a 19-fold drop in allyl and propyl ITC activity, as well as an approximately 10-fold lower activity with cyclohexyl and phenethyl ITCs, although the activity with benzyl isothiocyanate was only 1.5-fold lower (**Table 1**).

For the Pi, Alpha, and Mu classes the substrates AD, CuOOH, and EA are class distinguishing, and characteristic for a Pi-class enzyme is low activity with AD and CuOOH, with relatively high EA activity[22,171]. The two enzymes showed no significant difference with respect to AD and EA activity (**Table 1**), while the canine enzyme displayed a 2-fold elevated activity with CuOOH, however, this activity was still within the expected range for a Pi-class enzyme[22,171].

The sequence identity between the two enzymes reaches 87.1%, corresponding to 25 variable positions, with most of the differences occurring in residues outside the active site. The only active site residue to differ between the two enzymes is located at position 105, in which the canine enzyme has an Ala in contrast to Ile found in the human enzyme. Part of the observed discrepancy in activity can most likely be ascribed to this Ala. Mutagenesis studies on the human enzyme, $Ile^{105} \rightarrow Ala^{105}$, has shown that this mutation causes a 50% loss in CDNB activity, 9-fold decrease in phenethyl ITC activity, and more than a 3-fold increase in EA activity[172]. Furthermore, this also suggests that the non-active site residues play a role in the activity differences noted between the two enzymes.

Table 1. Comparison of specific activities of dog GST P1-1 and human GST P1-1 with alternative substrates.

Substrate	Specific activity (µmol/min/mg)	
	Dog GST P1-1	Human GST P1-1/Ile105[1]
1-Chloro-2,4-dinitrobenzene (CDNB)	23.0 ± 1.3	106
Cumene hydroperoxide (CuOOH)	0.071 ± 0.013	0.03
Phenethyl isothiocyanate (PEITC)	6.37 ± 1.03	60
Benzyl isothiocyanate (BITC)	39.0 ± 5.2	63
Allyl isothiocyanate (AITC)	1.94 ± 0.60	38
Propyl isothiocyanate (PITC)	2.55 ± 0.20	49
Cyclohexyl isothiocyanate (cHITC)	1.27 ± 0.08	11
Ethacrynic acid (EA)	2.05 ± 0.13	2.0
Δ^5-androsten-3,17-dione (Δ^5-AD)	0.014 ± 0.005	0.01

[1]Data from[68,173]. N/A = no activity.

In the next step, we screened the enzyme with various inhibitors. Five inhibitors known to inhibit GSTs[22] were tested with dog GST P1-1 and benzyl isothiocyanate was used as the substrate due to its high activity with dog GST P1-1 (**Table 1**). The results suggest that these inhibitors are just as potent for the dog enzyme as they are for the human enzyme (micromolar range) (**Table 2**), in some cases even more, except for triethyltin bromide which was 14-fold less potent with the dog enzyme compared to human enzyme, approaching the millimolar range. The most potent inhibitor was found to be Cibacron Blue with an IC_{50} value of 43 ± 7 nM (**Table 2**).

Table 2. Effect of enzyme inhibitors on dog GSTP1-1. Human enzymes have been added for comparison.

Inhibitor	IC_{50} (µM) Dog GST P1-1	IC_{50} (µM) Human GST P1-1
Ethacrynic acid	4.38 ± 1.96	3.3[a]
Triethyltin bromide	84 ± 2	6[b]
Triphenyltin chloride	3.11 ± 0.38	> 10[b]
Tributyltin acetate	0.85 ± 0.13	4[b]
Cibacron Blue	0.043 ± 0.007	0.5[b]

[a]Data from[174]
[b]Data from[175]

Paper II: Human GST P1-1 redesigned for enhanced catalytic activity with the anticancer prodrug Telcyta and improved thermostability

Aim

This paper aimed to enhance the activity of human GST P1-1 with the anticancer prodrug Telcyta as a first step towards a functional antibody-directed enzyme prodrug therapy.

Results

Due to the limited screening efficiency for Telcyta activity, we initially used a rational design approach, and sites for mutagenesis were selected based on a previously published model structure of Telcyta docked to the active site of human GST P1-1[136]. For the first round of mutagenesis, we identified two sites, Tyr^{8} and Tyr^{109}, based on the proximity to the Telcyta activation site (the scissile CH_2 bond). For amino acid substitution, we opted for histidine, glutamic acid, or aspartic acid as the deprotonated form of the respective sidechain could promote a proton-abstraction reaction[37] via an alternative functional group, either by assisting Tyr^{8} or through an alternative reaction trajectory.

All mutants were assayed with Telcyta by the thin-layer chromatography method we developed in **paper II**. We found that a single-point mutation (Tyr → His) in position 109 increased the catalytic activity 2.9-fold compared to the wildtype enzyme (**Table 3**). Furthermore, both mutations in position 8, Tyr → His/Glu, had a severe negative impact, displaying activities similar to the non-enzymatic reaction. Further rounds of mutagenesis with the rational design methodology yielded no mutant with activity exceeding that of Y109H.

Table 3. Catalytic activity of human GST P1-1 mutants with the prodrug Telcyta relative to the wildtype human GST P1-1. The absolute value of Telcyta is unknown.

	Activity relative to wildtype GSTP1-1
Variants	**Telcyta (fold)**
Human P1-1	1
Human P1-1 variant Y109H	2.9 ± 0.6
Human P1-1 variant V6 (Q85R-C102S-S106T-Y109H-V200L)	3.1 ± 0.9

To explore larger segments of sequence space we continued our redesign of GST P1-1 with a machine-learning approach[157] and Y109H served as the par-

ent. We constructed variants with multiple systematically introduced mutations and each mutation appeared in more than one variant. This way, the individual contribution of each mutation can be mapped with the minimum number of experiments. Evolutionarily non-conserved sites and all the naturally occurring variability within each site were identified. Based on certain pre-selected parameters[159] position and substitutions were scored and selected for mutagenesis. None of the tested variants from the machine-learning libraries significantly improved the activity above that of Y109H with Telcyta.

A procedure like ADEPT requires the drug-activating enzyme to be stable long enough to exert a biological effect, and introducing mutations in the enzyme can cause a decrease in thermostability[176]. Based on this, the next step of the study was to investigate the thermostability of all variants with activity on par with Y109H by measuring the residual activity with the substrate CDNB under standard conditions. The substitution Y109H was found to drastically decrease the half-life of the wildtype enzyme from 9.1 to 2.4 min. The introduction of 4 simultaneous mutations to Y109H increased the half-life of the mutant V6 (Q85R-C102S-S106T-Y109H-V200L) from 2.4 to 10.9 min, while also maintaining the activity **(Table 3)**. Even though the half-life was restored close to that of the wildtype GST P1-1, the wildtype enzyme appears to be more stable over time (**Figure 8**). The second-order decay observed for wildtype GST P1-1 is in accord with previous research[177]. Computer modeling suggested that the mutation Q85R positively influenced the thermostability. The mutant Q85R-Y109H was constructed, and it was shown to improve the half-life from 2.4 to 5.6 min.

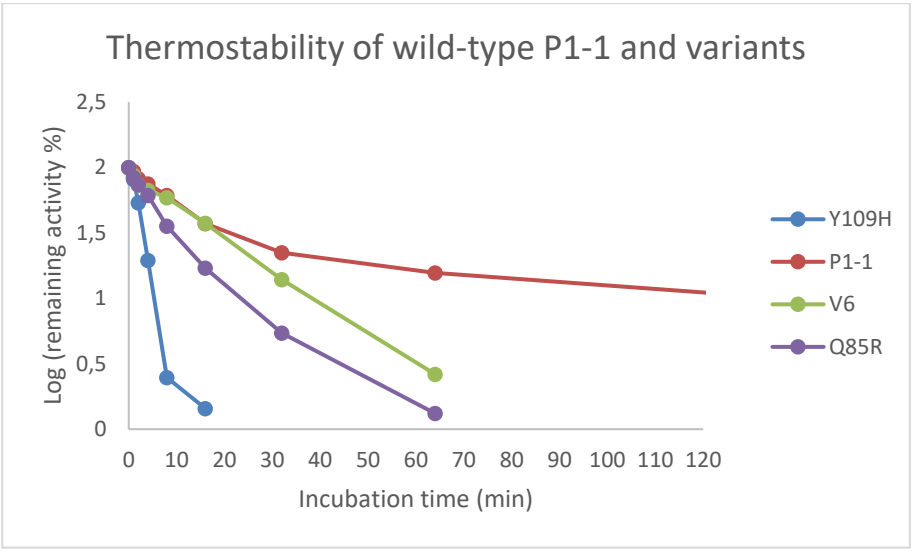


Figure 8. Thermal stability test of GSTP1-1 variants. Enzymes were incubated in 0.1 M sodium phosphate buffer pH 6.5 at 50 °C, and each enzyme was incubated at 0.20 mg/ml. Sampling occurred at different time points (0, 2, 4, 8, 16, 32, 64, and 128 min), and the residual activity was measured with CDNB under standard conditions. Figure modified from Paper II.

Paper III: Mutations selectively evolving peroxidase activity among alternative catalytic functions of human glutathione transferase P1-1

Aim

This paper aimed to screen the mutant libraries of human GST P1-1 from **paper II** with four alternative substrates. The rationale being that small changes to the enzyme's primary structure could have a large impact on the enzyme's substrate selectivity profile.

Results

Physiological organic hydroperoxides (ROOH) can be detoxified into corresponding alcohol and water by glutathione peroxidase. In the laboratory, cumene hydroperoxide can serve as a surrogate to monitor the enzyme's hydroperoxidase activity (**Figure 9**).

$$ROOH + 2\ GSH \longrightarrow ROH + H_2O + GSSG$$

Glutathione peroxidases can be subdivided into selenocysteine-containing enzymes and non-selenium-dependent enzymes. GSTs belong to the latter category, and within the mammalian cytosolic GSTs the Alpha-class members are in general the most active peroxidases[58], while the Pi-class has modest activity with cumene hydroperoxide[178].

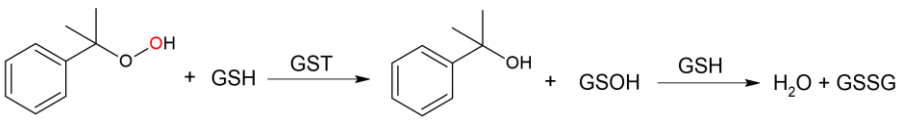


Figure 9. The reaction between cumene hydroperoxide and GSH catalyzed by GSTs.

The screening procedure led to the discovery of two mutants, R1 (Y109H) and V2 (Q40M-E41Q-A46S-Y109H-V200L), that were more prominent than the others with respect to CuOOH activity (**Table 4**). Y109H was a first-generation single-point mutant, while V2 was a second-generation multi-point mutant based on the Y109H substitution. The specific activity of Y109H and V2 with CuOOH was 16.3- and 30-fold, respectively, higher than that of wildtype GST P1-1 (**Table 4**). Moreover, a 5-fold decrease in PEITC and CDNB activity was observed. Lastly, the EA activity was almost unaffected by the Y109H substitution (**Table 4**).

Table 4. Specific activities of wildtype human GST P1-1, R1 (Y109H), and the Y109H based mutant V2 with alternative substrates. The substrate selectivity profile of Human GST P1-1 has been added for comparison[122,172].

		Specific activity μmol min^{-1} mg^{-1}			
P1-1 Variant	***Substrate***	**CDNB**	**PEITC**	**CuOOH**	**EA**
Human GST P1-1		106 ± 4	60 ± 4	0.030 ± 0.001	2.0 ± 0.1
R1 (Y109H)		20.9 ± 0.7	13.4 ± 0.1	0.49 ± 0.03	1.7 ± 0.1
V2 (Q40M-E41Q-A46S-Y109H-V200L)		19.2 ± 0.7	11.8 ± 0.5	0.9 ± 0.2	1.66 ± 0.08

The Michaelis-Menten parameters were characterized for the respective mutants. Compared to the wildtype enzyme, Y109H had a 34-fold higher k_{cat}, and a 3-fold higher K_m, resulting in a 10-fold increase in the catalytic efficiency (k_{cat}/K_m) (**Table 5**). The mutant V2 had a 57-fold higher k_{cat} and a 2.6-fold higher K_m compared to the wildtype enzyme, resulting in a 22-fold higher catalytic efficiency (**Table 5**). The enhanced activities thus mainly stem from elevated k_{cat} values. Moreover, V2 had a 2.2-fold higher catalytic efficiency than Y109H (**Table 5**).

Table 5. Steady-state kinetic parameters for wildtype GST P1-1 and two mutant enzymes with the most active substrate CuOOH. The concentration of CuOOH ranged from 0.09-3 mM, while the concentration of GSH was kept constant at 1 mM.

Substrate	***Kinetic parameters***	***P1-1 variant***		
CuOOH		*Wildtype*	*Y109H*	*V2*
	k_{cat} (s^{-1})	0.039 ± 0.005	1.3 ± 0.4	2.2 ± 0.4
	K_m (mM)	1.25 ± 0.25	4.3 ± 1.6	3.3 ± 0.9
	k_{cat}/K_m (mM^{-1} s^{-1})	0.031 ± 0.005	0.31 ± 0.10	0.68 ± 0.16

Paper IV: Marmoset glutathione transferases with ketosteroid isomerase activity

Aim

Human GST A3-3 play an important role in steroid hormone biosynthesis, inhibition of the enzyme can therefore be of pharmacological value. The common marmoset monkey could serve as an experimental model animal to test novel drugs for medical conditions related to overproduction of steroid hormones. Based on this we characterized GST A3-3 and GST A1-1 from the common marmoset monkey in search for steroid isomerase activity.

Results

Both enzymes were successfully purified and tested with Δ^5-AD and Δ^5-PD (**Table 6**).

Table 6. Specific activities of marmoset GST A3-3 and marmoset GST A1-1 with Δ^5-AD and Δ^5-PD. The activities of other highly active steroid isomerases from the Alpha class of other mammals have been added for comparison.

	Specific activity (μmol min^{-1} mg^{-1})				
Substrate	Marmoset GST A3-3	Marmoset GST A1-1	Human GST A3-3[a]	Equine GST A3-3[b]	Porcine GST A2-2[c]
Δ^5-AD	62.1 ± 1.8	1.96 ± 0.08	197 ± 15	194 ± 10	53 ± 2
Δ^5-PD	4.57 ± 0.27	0.22 ± 0.02	37 ± 2	92.2 ± 9.5	1.9 ± 0.1

a[11],b[13],c[166]

The specific activity of marmoset GST A3-3 with Δ^5-AD and Δ^5-PD was 62.1 and 4.57 μmol min^{-1} mg^{-1}, respectively, which is roughly 30- and 20-fold higher than that of marmoset GST A1-1, respectively (**Table 6**). Moreover, the Δ^5-AD activity of marmoset GST A3-3 was roughly 3-fold lower than its human and equine counterparts (**Table 6**), however, the activity was still on par with that of porcine GST A2-2 (**Table 6**). Regarding the Δ^5-PD activity, marmoset GST A3-3 had an 8- and 20-fold lower activity than human GST A3-3 and equine GST A3-3, respectively, but was more than 2-fold higher than porcine GST A2-2 (**Table 6**).

In the next step the Michaelis-Menten kinetic parameters for both enzymes with Δ^5-AD were determined (**Table 7**). Δ^5-AD was selected as the substrate due to being more active with both enzymes compared to Δ^5-PD, and that it could be used at 10 times higher concentration than Δ^5-PD.

Table 7. Steady-state kinetic parameters for the ketosteroid isomerization of Δ^5-AD. Human and equine GST A3-3 as well as porcine GST A2-2 have been added for comparison[11,13,166].

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Marmoset GST A3-3	0.374 ± 0.087	261 ± 49	698
Marmoset GST A1-1	0.303 ± 0.034	6.89 ± 0.79	22.7
Human GST A3-3	0.024 ± 0.024	204 ± 22	8600 ± 800
Equine GST A3-3	0.0137 ± 0.0015	219.2 ± 7.9	16000 ± 1900
Porcine GST A2-2	0.025 ± 0.002	20 ± 1.5	800 ± 125

The k_{cat} values for GST A3-3 from marmoset, equine, and human are all very similar, around 200 s^{-1} (**Table 7**). The differences observed in catalytic efficiency (k_{cat}/K_m) between the enzymes mainly stem from differences in K_m (**Table 7**). The catalytic efficiency of marmoset GST A3-3 with Δ^5-AD was 12.3- and 22.9-fold lower than human and equine GST A3-3, respectively, in contrast to the 31-fold elevated catalytic efficiency noted in comparison to marmoset GST A1-1. Moreover, marmoset GST A3-3 was almost equally efficient as porcine GST A2-2 in catalyzing the isomerization reaction with Δ^5-AD.

The glutathione binding site is conserved among all these Alpha-class enzymes, while the H-site shows slight variations between the enzymes. The H-site is formed by 13 residues in position 10, 12, 14, 15, 107, 108, 110, 111, 208, 213, 216, 220, and 222[35,179]. Mutational analysis on selected individual residues in the active site of the highly active steroid isomerase human GST A3-3 to the corresponding residues found in the active site of the not so active steroid isomerase human GST A2-2 has revealed that position 10, 12, 111, 208, and 216 are important for efficient steroid isomerase activity[167], and differences in aminoacids at these positions are primarily the reason why human GST A3-3 is a far more active steroid isomerase (5000-fold) than human GST A2-2[167]. This is not to say that other active site or non-active site residues are not of equal importance. However, the most detrimental effect was observed when Leu in position 111 in human GST A3-3 was substituted for Phe as in human GST A2-2, leaving the enzyme with only 4% of the catalytic efficiency of the wildtype GST A3-3[167]. The corresponding aminoacid difference in position 111 as in human GST A3-3 and GST A2-2 is also seen between marmoset GST A1-1 and GST A3-3 in which marmoset GST A1-1 carries the bulkier Phe. Homology modeling of marmoset GST A1-1 and GST A3-3 (based on the crystal structure of human GST A3-3 with Δ^5-AD bound to the active site) suggests that the low catalytic efficiency of marmoset GST A1-1 is due to steric effects caused by the larger ring of Phe^{111} which prevents proper binding of the steroid substrate (**Figure 10**).

Porcine GST A2-2 and marmoset GST A3-3 only differ in two H-site positions, 12 (Gly for pig and Ala for marmoset) and 213 (Leu for pig and Val for marmoset), which appears to not have any significant effect on the Δ^5-AD activity in this particular case. The same variation in position 213 can also be seen between the highly active steroid isomerases, human (Leu) and equine (Val) GST A3-3.

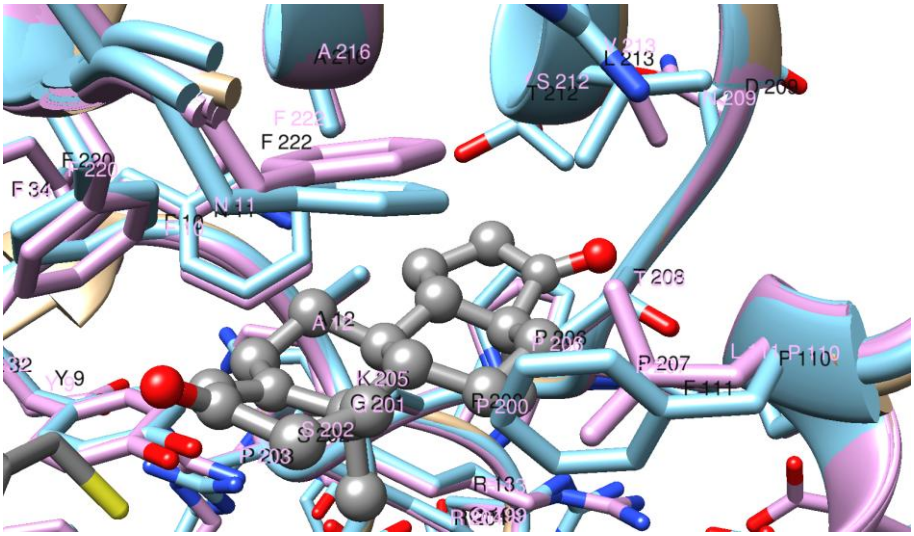


Figure 10. Active-site models of marmoset GST A1-1 (light blue) and GST A3-3 (pink) based on the 3D-structure of human GST A3-3 in complex with Δ^4-androsten-3,17-dione (colored by element in grey, shown as ball and stick). The thiol group of GSH (yellow) and the catalytically important Tyr^9 are shown in the bottom left corner. Figure taken from Paper IV.

Paper V: Structural and functional analysis of the inhibition of equine glutathione transferase A3-3 by organotin endocrine disrupting pollutants

Aim

This paper aimed to characterize the interaction between GSH and the highly toxic and endocrine disrupting organotin compounds by solving the crystal structure of equine GST A3-3 in complex with one of the organotin compounds.

Results

Equine GST A3-3 was successfully purified, and inhibition studies were carried out with three organotin compounds, namely, triethyltin bromide, triphenyltin chloride, and tributyltin acetate (**Table 8**). The results show that all three compounds were potent inhibitors of EcaGST A3-3, with the most prominent being tributyltin acetate (**Table 8**).

Table 8. The IC_{50}-values for three organotin compounds with EcaGST A3-3.

Inhibitor	IC_{50} (µM)
Triethyltin bromide	9.6 ± 2.1
Triphenyltin chloride	3.4 ± 0.4
Tributyltin acetate	0.28 ± 0.02

Further kinetic studies on the inhibition mechanism showed that triethyltin bromide was a strictly competitive inhibitor with an inhibition constant (K_i) of 4.0 µM (**Figure 11**).

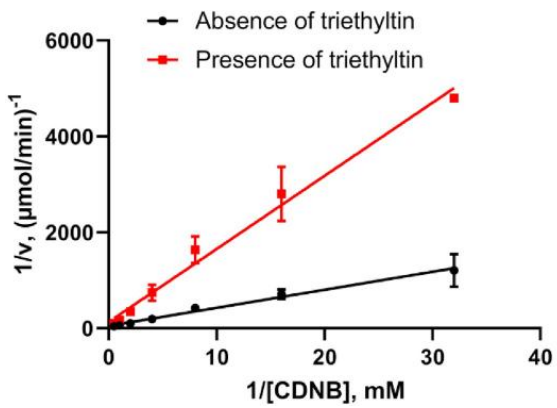


Figure 11. Lineweaver-Burk plot showing the competitive inhibition of triethyltin with equine GST A3-3. Figure modified from Paper V.

The most soluble inhibitor of the three, triethyltin bromide, was selected for crystallization. The crystal structure of equine GST A3-3 in complex with

GSH and the inhibitor triethyltin was solved at a resolution of 2.2 Å. The structure reveals that GSH forms a conjugate with triethyltin bromide via the tin atom. Moreover, the tin atom also forms a bond with the oxygen atom of the hydroxyl group of Tyr^{9} in the active site of the enzyme (**Figure 12**). This type of direct binding between the three participating atoms is also likely responsible for the potent inhibition observed with the organotin compounds.

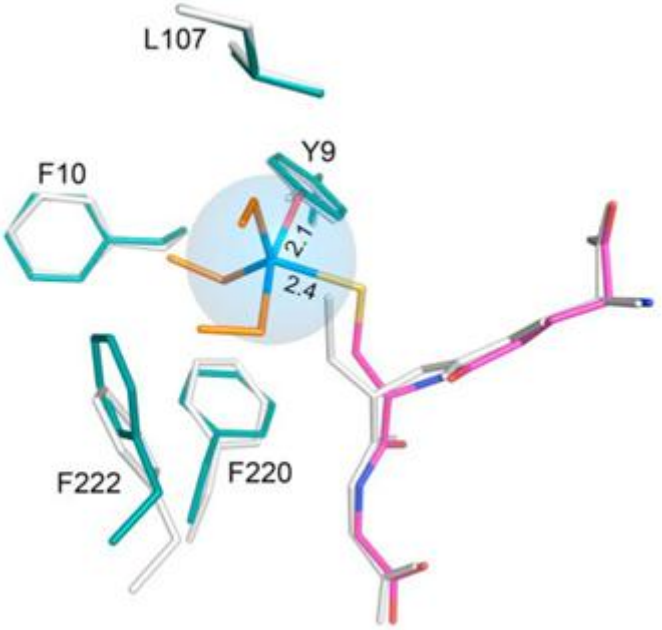


Figure 12. Triethyltin (orange with the tin atom shown in blue) conjugated with glutathione (light purple with the sulphur atom marked in yellow) in the active site of equine GST A3-3. Residues interacting with triethyltin are marked in cyan. The lengths in Å between the Sn-O and Sn-S bonds are 2.1 and 2.4 Å, respectively. The equine GST A3-3 structure in complex with glutathione (light grey) is shown for comparison. Figure modified from Paper V.

Conclusions and future perspectives

The contribution of this thesis can be summarized in a few key points.

Conclusions

In **paper I** we show that dog GST P1-1 shares certain class-specific similarities to the human enzyme in terms of substrate specificity and inhibition profiles. Our findings are not only of veterinary clinical relevance to possibly develop GST P1-1 targeted anticancer therapies in dogs but may also be beneficial for translational research as well.
We also developed a thin-layer chromatography procedure to screen and semi-quantify Telcyta activity. Our procedure can serve as a cost-efficient alternative to HPLC.

In **paper II** we constructed a GST P1-1 mutant with enhanced activity (Y109H) with the anticancer prodrug Telcyta and improved thermostability (Q85R). Our findings have therapeutic potential as a first step to develop an antibody-directed enzyme prodrug therapy application based on Telcyta.

In **paper III** we discovered two GST P1-1 mutants, Y109H and V2 (Q40M-E41Q-A46S-Y109H-V200L) with elevated cumene hydroperoxide activity compared to the wildtype enzyme. From an evolutionary perspective, we demonstrate how an important physiological function such as peroxidase activity can emerge from a low activity variant with only a few mutations.

In **paper IV** we show that marmoset GST A3-3 possess notable ketosteroid isomerase activity with Δ^5-AD and to lesser extent with Δ^5-PD. The physiological function of the enzyme needs to be investigated; however, the common marmoset monkey could possibly be used as an experimental model animal for conditions related to steroid hormone overproduction.

In **paper V** we solved the crystal structure of equine GST A3-3 in complex with triethyltin and GSH. We show that the thiolate group of GSH forms a covalent bond with triethyltin, which in turn forms a covalent bond to the oxygen atom of Tyr^9 in equine GST A3-3. This is the first crystal structure of a GST with an organotin compound bound to the active site. Our findings help

to better understand the inhibitory mechanism of competitive organotin compounds.

Future perspectives

Glutathione transferases are highly diversified enzymes, their function range from physiological to biomedical applications like ADEPT.

Although much progress has been made in the field of cancer over the past decades, the prospect of curing certain types of cancers remains poor compared to others. More efficient treatment modalities with higher selectivity, lower cytotoxicity to normal tissue, and potential to circumvent drug resistance are required. One therapeutic strategy that has emerged to fulfill such criteria is ADEPT[180]. Despite promising preclinical trials, the only ADEPT system to progress to clinical trials involves the bacterial enzyme carboxypeptidase G2 (CPG2)[180]. However, the main limitation for CPG2 and other drug activating enzymes are related to issues of immunogenic character.

Our engineered human GST P1-1 with enhanced Telcyta activation has a high probability of being nonimmunogenic. The continued research includes construction of a fusion protein between the mutant enzyme and a therapeutic antibody with affinity for epitopes on the surface of cancer cells. Of importance to investigate is whether the fusion protein retains catalytic function and antigen binding capability as well as the tumor-killing effects of the prodrug combined with the engineered GST construct compared to the wildtype enzyme.

Human GST A3-3 is an enzyme involved in the physiological catalysis of Δ^5-AD to Δ^4-AD and Δ^5-PD to Δ^4-PD, the final steps in the biochemical pathways leading to production of testosterone and progesterone, respectively[11]. Blocking the expression or activity of the enzyme causes a significant reduction in hormone biosynthesis, meaning that the enzyme has potential as a therapeutic target related to conditions caused by excessive steroid hormone production[181]. Model organisms provide a convenient way to assess the physiological role of the enzyme and to test the effects of drug candidates. Before the common marmoset monkey can be used as a model organism for endocrine related disorders, the observed ketosteroid isomerase activity has to be elucidated in a physiological context, possibly by disrupting the marmoset GST A3-3 gene or inhibiting the enzyme and measure serum hormone levels. The expression levels in the common steroidogenic tissues, placenta, ovary, and adrenal gland remains to be investigated as well.

Populärvetenskaplig sammanfattning på svenska

Enzymer finns i alla levande organismer, och utgör grunden för allt liv. De är biologiska katalysatorer som påskyndar kemiska processer i kroppen genom att sänka den energi som krävs för att reaktionen ska fortskrida. Utan katalysatorer så skulle reaktionerna ta timmar, dagar, eller till och med månader, men bara bråkdelen av en sekund i närvaro av enzym. Enzymer deltar i allt från energiproduktion till programmerad celldöd.

Glutationtransferaser

Glutationtransferaser (GSTs) är endogena avgiftningsenzymer vars huvuduppgift är att skydda cellerna från toxiska substanser (inklusive läkemedel) genom konjugering med peptiden glutation (GSH). Det bildade konjugatet blir därmed mer vattenlösligt och kan då lättare utsöndras från kroppen. Alla cytosoliska GSTs från däggdjur är dimera proteiner med ett aktivt säte per subenhet. Det aktiva sätet har i sin tur två bindningsfickor, den ena fickan för inbindning av GSH (G-säte) och den andra fickan för inbindning av hydrofoba elektrofila substrat (H-säte). Vidare finns det sju klasser av cytosoliska GSTs i däggdjur, däribland, Alfa, Mu, Omega, Pi, Sigma, Theta, och Zeta. Denna mångfald bland enzymerna tillsammans med dess diversifierade substratselektivitet gör GSTs till utmärkta måltavlor för att med hjälp av ingenjörskonst modifiera substratselektiviteten hos enskilda enzymer mot förbättrade eller nya substratselektiviteter.

Pi-Klassen och cancer

I människor består Pi-klassen av ett enzym, GST P1-1, och detta enzym har visat sig vara överuttryckt i en del cancertumörer relativt frisk vävnad, vilket är en bidragande faktor till den läkemedelsresistens som cancercellerna uppvisar. Dessa faktum har man utnyttjat för att utveckla hämmare mot GST P1-1 eller GST P1-1 aktiverade prodroger som tillvägagångssätt för att bekämpa cancer. En av dessa prodroger är Telcyta och läkemedlet har visat sig ha en

positiv effekt i kliniska tester, dock är det begränsat till de fall där cancercellerna har höga nivåer av inneboende GST P1-1. För att kunna aktualisera en behandlingsmetod så krävs dels ett enzym som mer effektivt kan aktivera Telcyta, och dels ett sätt att selektivt leverera enzymet till tumörcellerna. Det första problemet kan lösas genom att göra förändringar i enzymets nukleotidsekvens tills det att man fått ett förbättrat enzym. Det andra problemet kan lösas genom att koppla enzymet till en terapeutisk antikropp med affinitet för epitoper på ytan av cancercellerna.

Alfa-Klassen och biosyntes av steroidhormoner

Frånsett den naturliga avgiftningsfunktion som GSTs besitter så har vissa enzymer utvecklat alternativa funktioner. Ett enzym tillhörande Alfa-klassen från människa, GST A3-3, är av väsentlig betydelse för biosyntes av könshormoner genom att katalysera isomeriseringsreaktionen av 5-adrostendion och 5-pregnendion, prekursorer till testosteron och progesteron. Förutom det humana enzymet så har hög steroidisomeras aktivitet identifierats i Alfa-klass enzymer från häst och gris.

Hämning av GST A3-3 har visat sig reducera hormonproduktion, vilket innebär att enzymet är av medicinsk betydelse för potentiell behandling av endokrina avvikelser i form av överproduktion av hormoner. Inom läkemedelsutveckling är djurförsök alltid ett måste och de vanligaste modellorganismerna, mus och råtta, innehar inte ett särskilt aktivt steroidisomeras enzym och är därför inte lämpade. Större djur såsom gris och häst kan vara svåra att experimentera på och är därför inte heller optimala för tester av nya läkemedel, att hitta lämpliga modellorganismer är därför av stor betydelse.

I denna avhandling presenteras en rad studier som är avsedda att öka förståelsen för Pi-och Alfa-klassenzymerna.

Studie I

I denna studie karaktäriserade vi GST P1-1 från hund för att undersöka dess potential som terapeutisk måltavla för cancerterapi, likt dess humana motsvarighet. Våra resultat visade att hund GST P1-1 delar vissa klasspecifika likheter med det humana enzymet med avseende på substratspecificitet samt inhiberingsprofil. Våra fynd är inte bara av veterinärmedicinsk relevans för att gagna utvecklingen av GST P1-1 baserad cancerterapi i hundar utan även också av betydelse för translationell forskning.
Vi utvecklade även en metod baserad på tunnskiktskromatografi för screening och semi-kvantifiering av Telcyta-aktivitet. Vår metod kan fungera som ett kostnadseffektivt alternativ till högupplöst vätskekromatografi.

Studie II
I denna studie förbättrade vi både aktiviteten av humant GST P1-1 med anticancer prodrogen Telcyta och enzymets termostabiliteten genom att konstruera och screena mutant-bibliotek. Våra resultat visade att en punktmutation i position 109 där histidin ersatt tyrosin förbättrade aktiviteten 2.9-faldigt samtidigt som stabiliteten av enzymet minskade. Enzymets stabilitet kunde återställas till vildtypsenzymets nivå med bibehållen katalytisk förmåga genom att introducera fyra mutationer samtidigt. Mutationen Q85R visade sig ha en positiv inverkan på den ökade termostabiliteten. Våra resultat är av terapeutisk betydelse som ett första steg i utvecklingen av en Telcyta-baserad behandling i form av ”antibody-directed enzyme prodrug therapy”.

Studie III
I denna studie undersökte vi aktiviteten av mutant-bibliotek från den tredje studien med fyra alternativa substrat. Anledningen var att små förändringar i enzymets primärstruktur kan ha stor inverkan på enzymets substratselektivitetsprofil. Vi identifierade två mutanter, Y109H samt V2 (Q40M-E41Q-A46S-Y109H-V200L), med 10- respektive 23-faldig förhöjd katalytisk effektivitet med kumenväteperoxid jämfört med vildtypsenzymet. Från ett evolutionärt perspektiv så visar våra resultat hur en viktig fysiologisk funktion såsom peroxidasaktivitet uppkommer från en enzymvariant med låg aktivitet med enbart ett fåtal mutationer.

Studie IV
I denna studie undersökte vi steroidisomerasaktiviteten hos GST A3-3 från marmosettapa genom att mäta enzymaktiviteten med bland annat 5-androstendion och 5-pregnendion. Våra resultat visade att marmoset GST A3-3 hade en relativ hög aktivitet med 5-androstendion och även om den katalytiska effektiviteten var 12- respektive 23-faldigt lägre än det humana och ekvina (häst) enzymerna så var det ändå i linje med den katalytiska effektiviteten noterat hos gris. Det krävs emellertid mer forskning för att utreda den fysiologiska funktionen kopplat till den höga steroidisomeras aktivitet hos enzymet. Våra fynd visar att marmosettapa möjligtvis kan användas som modellorganism för endokrina sjukdomstillstånd relaterat till överproduktion av till exempel steroidhormoner.

Studie V
I denna studie undersökte vi interaktionen mellan GSH, organiska tennföreningar, och ekvint GST A3-3 genom att lösa kristallstrukturen av enzymet i komplex med GSH och trietyltenn. Våra resultat visade att trietyltenn hade en strikt kompetitiv inhiberingsmekanism med enzymet. Vi såg även att svavelatomen i GSH binder till tennatomen i trietyltenn, och att tennatomen i sin tur bildar en kovalent bindning till enzymet via Tyr^{9}, vilket är en bidragen faktor till den potenta inhibering som trietyltenn uppvisar med enzymet. Detta är den

första kristallstrukturen av ett GST tillsammans med en organisk tennförening bundet till det aktiva sätet. Våra fynd ökar förståelsen för inhiberingsmekanismen associerat med kompetitiva organiska tennföreningar.

Sammantaget har arbetet som presenteras i denna avhandling bidragit till en ökad förståelse för glutation transferaser inom Pi- och Alfa-klassen.

Acknowledgments

First, to my supervisor Bengt Mannervik, thank you for all these years and for the opportunity to do a PhD in your group. I am very thankful for all the guidance, knowledge, and ideas you have shared with me; you are a great person, and this thesis would not have been completed without you. I would also like to thank you and Anne-Charlotte for all the invitations to Gräsö.
I would also like to thank Birgitta for all the help in the laboratory and all the conversations, you are a kind person and a great colleague. Thanks to Helena for introducing me to the acetylcholine esterase lab and for conversations in the office. Also, thanks to Yaman for all the good times in the lab and fun conversations. I would also like to take the opportunity to thank past members of the Mannervik group whom I have had the pleasure to meet: Aslam, Urvi, Usama, and Abeer. Last but not least, Jana, thank you for the fruitful collaboration resulting in the fifth publication within this thesis.

Thanks to all project students (Zhenzhu, Peshang, Shanshan, Rōng, Julia, Libby, Jessica, Sadid, and Katarina) who have worked very hard to contribute to our research, and whom I had the privilege to supervise in the laboratory.

I would also like to thank Sridhar Govindarajan and Claes Gustafsson, our main collaborators from the company ATUM, for all the clones you have supplied us with over the years and for the kind invitation to visit your company.

Finally, thanks to Andreas Barth for reading my thesis, also thanks to my co-supervisor Pål Stenmark, my evaluator Martin Högbom, all other group leaders, administration staff, and PhD students at DBB.

References

1. Almazroo OA, Miah MK, Venkataramanan R. Drug metabolism in the liver. *Clin. Liver. Dis.* **2017**, 21, 1–20; DOI: 10.1016/j.cld.2016.08.001
2. Meyer UA. Overview of enzymes of drug metabolism. *J. Pharmacokinet. Biopharm.* **1996**, 24, 449–459; DOI: 10.1007/BF02353473
3. Shanu-Wilson J, Evans L, Wrigley S, Steele J, Atherton J, Boer J. Biotransformation: Impact and application of metabolism in drug discovery. *ACS. Med. Chem. Lett.* **2020**, 11, 2087–2107; DOI: 10.1021/acsmedchemlett.0c00202
4. Josephy PD & Mannervik B. Molecular Toxicology; 2nd Ed.; Oxford University Press, New York, USA, **2006**.
5. Lu J, Holmgren A. The thioredoxin antioxidant system. *Free Radic. Biol. Med.* **2014**, 66, 75–8; DOI: 10.1016/j.freeradbiomed.2013.07.036
6. Lu SC. Glutathione synthesis. *Biochim. Biophys. Acta.* **2013**, 1830, 3143–3153; DOI: 10.1016/j.bbagen.2012.09.008
7. Meister A. Glutathione metabolism and its selective modification. *J. Biol. Chem.* **1988**, 263, 17205–17208.
8. Armstrong RN. Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chem. Res. Toxicol.* **1997**, 10, 2–18; DOI: 10.1021/tx960072x
9. Hanna PE, Anders MW. The mercapturic acid pathway. *Crit. Rev. Toxicol.* **2019**, 49, 819–929; DOI: 10.1080/10408444.2019.1692191
10. Ramsay EE, Dilda PJ. Glutathione S-conjugates as prodrugs to target drug-resistant tumors. *Front. Pharmacol.* **2014**, 5, 181; DOI: 10.3389/fphar.2014.00181
11. Johansson AS, Mannervik B. Human glutathione transferase A3-3, a highly efficient catalyst of double-bond isomerization in the biosynthetic pathway of steroid hormones. *J. Biol. Chem.* **2001**, 276, 33061–33065; DOI: 10.1074/jbc.M104539200.
12. Pettersson PL, Mannervik B. The role of glutathione in the isomerization of delta 5-androstene-3,17-dione catalyzed by human glutathione transferase A1-1. *J. Biol. Chem.* **2001**, 276, 11698–11704; DOI: 10.1074/jbc.M009146200
13. Lindström H, Peer SM, Ing NH, Mannervik B. Characterization of equine GST A3-3 as a steroid isomerase. *J. Steroid Biochem. Mo.l Biol.* **2018**, 178, 117–126; DOI: 10.1016/j.jsbmb.2017.11.011
14. Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, Pincus MR, Sardana M, Henderson CJ, Wolf CR, Davis RJ, Ronai Z. Regulation of JNK signaling by GSTp. *EMBO J.* ***1999***, 18, 1321–34; DOI: 10.1093/emboj/18.5.1321. PMID: 10064598; PMCID: PMC1171222.
15. Wang T, Arifoglu P, Ronai Z, Tew KD. Glutathione S-transferase P1-1 (GSTP1-1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the C terminus. *J. Biol. Chem.* **2001**, 276, 20999–21003; DOI: 10.1074/jbc.M101355200
16. Meyer DJ, Thomas M. Characterization of rat spleen prostaglandin H D-isomerase as a sigma-class GSH transferase. *Biochem. J.* **1995**, 311, 739–742; DOI: 10.1042/bj3110739
17. Kanaoka Y, Ago H, Inagaki E, Nanayama T, Miyano M, Kikuno R, Fujii Y, Eguchi N, Toh H, Urade Y, Hayaishi O. Cloning and crystal structure of hematopoietic

prostaglandin D synthase. *Cell.* **1997**, 90, 1085–95. DOI: 10.1016/s0092-8674(00)80374-8. Erratum in: *Cell*, **1999**, 96, 449; PMID: 9323136.
18. Habig WH, Pabst MJ, Fleischner G, Gatmaitan Z, Arias IM, Jakoby WB. The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. *Proc. Natl. Acad. Sci. U S A*. **1974**, 71, 3879–3882; DOI: 10.1073/pnas.71.10.3879
19. Lok HC, Rahmanto YS, Hawkins CL, Kalinowski DS, Morrow CS, Townsend AJ, Ponka P, Richardson DR. Nitric oxide storage and transport in cells are mediated by glutathione S-transferase P1-1 and multidrug resistance protein 1 via dinitrosyl iron complexes. *J. Biol. Chem*. **2012**, 287, 607–618; DOI: 10.1074/jbc.M111.310987
20. Lo Bello M, Nuccetelli M, Caccuri AM, Stella L, Parker MW, Rossjohn J, McKinstry WJ, Mozzi AF, Federici G, Polizio F, Pedersen JZ, Ricci G. Human glutathione transferase P1-1 and nitric oxide carriers; a new role for an old enzyme. *J. Biol. Chem*. **2001**, 276, 42138–45; DOI: 10.1074/jbc.M102344200
21. Labrou NE, Papageorgiou AC, Pavli O, Flemetakis E. Plant GSTome: structure and functional role in xenome network and plant stress response. *Curr. Opin. Biotechnol.* **2015**, 32, 186–194; DOI: 10.1016/j.copbio.2014.12.024.
22. Mannervik B, Alin P, Guthenberg C, Jensson H, Tahir MK, Warholm M, Jörnvall H. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. *Proc. Natl. Acad. Sci. U S A*. **1985**, 82, 7202–6; DOI: 10.1073/pnas.82.21.7202
23. Meyer DJ, Coles B, Pemble SE, Gilmore KS, Fraser GM, Ketterer B. Theta, a new class of glutathione transferases purified from rat and man. *Biochem. J.* **1991**, 274, 409–414; DOI: 10.1042/bj2740409
24. Board PG, Coggan M, Chelvanayagam G, Easteal S, Jermiin LS, Schulte GK, Danley DE, Hoth LR, Griffor MC, Kamath AV, Rosner MH, Chrunyk BA, Perregaux DE, Gabel CA, Geoghegan KF, Pandit J. Identification, characterization, and crystal structure of the Omega class glutathione transferases. *J. Biol. Chem.* **2000**, 275, 24798–806; DOI: 10.1074/jbc.M001706200
25. Board PG, Baker RT, Chelvanayagam G, Jermiin LS. Zeta, a novel class of glutathione transferases in a range of species from plants to humans. *Biochem. J.* **1997**, 328, 929–935; DOI: 10.1042/bj3280929
26. Bruns CM, Hubatsch I, Ridderström M, Mannervik B, Tainer JA. Human glutathione transferase A4-4 crystal structures and mutagenesis reveal the basis of high catalytic efficiency with toxic lipid peroxidation products. *J. Mol. Biol.* **1999**, 288, 427–39; DOI: 10.1006/jmbi.1999.2697
27. Reinemer P, Dirr HW, Ladenstein R, Huber R, Lo Bello M, Federici G, Parker MW. Three-dimensional structure of class pi glutathione S-transferase from human placenta in complex with S-hexylglutathione at 2.8 A resolution. *J. Mol. Biol*. **1992**, 227, 214–26; DOI: 10.1016/0022-2836(92)90692-d
28. Raghunathan S, Chandross RJ, Kretsinger RH, Allison TJ, Penington CJ, Rule GS. Crystal structure of human class mu glutathione transferase GSTM2-2. Effects of lattice packing on conformational heterogeneity. *J. Mol. Biol*. **1994**, 238, 815–32; DOI: 10.1006/jmbi.1994.1336
29. Ji X, Zhang P, Armstrong RN, Gilliland GL. The three-dimensional structure of a glutathione S-transferase from the mu gene class. Structural analysis of the binary complex of isoenzyme 3-3 and glutathione at 2.2-A resolution. *Biochemistry*. **1992**, 31, 10169–10184; DOI: 10.1021/bi00157a004
30. Sinning I, Kleywegt GJ, Cowan SW, Reinemer P, Dirr HW, Huber R, Gilliland GL, Armstrong RN, Ji X, Board PG. Structure determination and refinement of human alpha class glutathione transferase A1-1, and a comparison with the Mu and Pi class enzymes. *J. Mol. Biol*. **1993**, 232, 192–212; DOI: 10.1006/jmbi.1993.1376
31. Patskovsky YV, Patskovska LN, Listowsky I. An asparagine-phenylalanine substitution accounts for catalytic differences between hGSTM3-3 and other human class

mu glutathione S-transferases. *Biochemistry.* **1999**, 38, 16187–16194; DOI: 10.1021/bi991714t
32. Rossjohn J, McKinstry WJ, Oakley AJ, Verger D, Flanagan J, Chelvanayagam G, Tan KL, Board PG, Parker MW. Human theta class glutathione transferase: the crystal structure reveals a sulfate-binding pocket within a buried active site. *Structure*. **1998**, 6, 309–22; DOI: 10.1016/s0969-2126(98)00034-3
33. Polekhina G, Board PG, Blackburn AC, Parker MW. Crystal structure of maleylacetoacetate isomerase/glutathione transferase zeta reveals the molecular basis for its remarkable catalytic promiscuity. *Biochemistry.* **2001**, 40, 1567–76; DOI: 10.1021/bi002249z
34. Tars K, Larsson AK, Shokeer A, Olin B, Mannervik B, Kleywegt GJ. Structural basis of the suppressed catalytic activity of wild-type human glutathione transferase T1-1 compared to its W234R mutant. *J. Mol. Biol.* **2006**, 355, 96–105; DOI: 10.1016/j.jmb.2005.10.049
35. Gu Y, Guo J, Pal A, et al. Crystal structure of human glutathione S-transferase A3-3 and mechanistic implications for its high steroid isomerase activity. *Biochemistry.* **2004**, 43, 15673–15679; DOI: 10.1021/bi048757g
36. Mannervik B, Awasthi YC, Board PG, Hayes JD, Di Ilio C, Ketterer B, Listowsky I, Morgenstern R, Muramatsu M, Pearson WR. Nomenclature for human glutathione transferases. *Biochem. J.* **1992**, 282, 305–6; DOI: 10.1042/bj2820305
37. Mannervik B, Jensson H. Binary combinations of four protein subunits with different catalytic specificities explain the relationship between six basic glutathione S-transferases in rat liver cytosol. *J. Biol. Chem.* **1982**, 257, 9909–9912.
38. Mannervik B, Board PG, Hayes JD, Listowsky I, Pearson WR. Nomenclature for mammalian soluble glutathione transferases. *Methods Enzymol.* **2005**, 401, 1–8; DOI: 10.1016/S0076-6879(05)01001-3
39. Mannervik B, Guthenberg C, Jakobson I, Warholm M. In Conjugation Reactions in Drug Biotransformation; Aitio, A., Ed;. pp. 101–110. Elsevier North-Holland, Amsterdam. **1978**.
40. Allocati N, Casalone E, Masulli M, Ceccarelli I, Carletti E, Parker MW, Di Ilio C. Functional analysis of the evolutionarily conserved proline 53 residue in Proteus mirabilis glutathione transferase B1-1. *FEBS Lett*. **1999**, 445, 347–50; DOI: 10.1016/s0014-5793(99)00147-7
41. Caccuri AM, Antonini G, Board PG, Parker MW, Nicotra M, Lo Bello M, Federici G, Ricci G. Proton release on binding of glutathione to alpha, Mu and Delta class glutathione transferases. *Biochem. J.* **1999**, 344, 419–25.
42. Dourado DF, Fernandes PA, Mannervik B, Ramos MJ. Glutathione transferase: new model for glutathione activation. *Chemistry.* **2008**, 14, 9591–9598; DOI:10.1002/chem.200800946
43. Dourado DF, Fernandes PA, Ramos MJ. Glutathione transferase classes alpha, pi, and mu: GSH activation mechanism. *J. Phys. Chem. B*. **2010**, 114, 12972–80. DOI: 10.1021/jp1053875.
44. Oakley AJ, Lo Bello M, Battistoni A, Ricci G, Rossjohn J, Villar HO, Parker MW. The structures of human glutathione transferase P1-1 in complex with glutathione and various inhibitors at high resolution. *J. Mol. Biol.* **1997**, 274, 84–100; DOI: 10.1006/jmbi.1997.1364
45. Widersten M, Björnestedt R, Mannervik B. Involvement of the carboxyl groups of glutathione in the catalytic mechanism of human glutathione transferase A1-1. *Biochemistry*. **1996**, 35, 7731–42. DOI: 10.1021/bi9601619.
46. Adang AE, Brussee J, van der Gen A, Mulder GJ. The glutathione-binding site in glutathione S-transferases. Investigation of the cysteinyl, glycyl and gamma-glutamyl domains. *Biochem. J.* **1990**, 269, 47–54. DOI: 10.1042/bj2690047.
47. Gustafsson A, Pettersson PL, Grehn L, Jemth P, Mannervik B. Role of the glutamyl alpha-carboxylate of the substrate glutathione in the catalytic mechanism of human

glutathione transferase A1-1. *Biochemistry*. **2001**, 40, 15835–45. DOI: 10.1021/bi010429i. PMID: 11747461.
48. Adang AE, Meyer DJ, Brussee J, Van der Gen A, Ketterer B, Mulder GJ. Interaction of rat glutathione S-transferases 7-7 and 8-8 with gamma-glutamyl- or glycyl-modified glutathione analogues. *Biochem. J.* **1989**, 264, 759–64. DOI: 10.1042/bj2640759.
49. Dirr H, Reinemer P, Huber R. X-ray crystal structures of cytosolic glutathione S-transferases. Implications for protein architecture, substrate recognition and catalytic function. *Eur. J. Biochem*. **1994**, 220, 645–61. DOI: 10.1111/j.1432-1033.1994.tb18666.x.
50. Párraga A, García-Sáez I, Walsh SB, Mantle TJ, Coll M. The three-dimensional structure of a class-Pi glutathione S-transferase complexed with glutathione: the active-site hydration provides insights into the reaction mechanism. *Biochem. J.* **1998**, 333, 811–6. DOI: 10.1042/bj3330811.
51. Caccuri AM, Lo Bello M, Nuccetelli M, Nicotra M, Rossi P, Antonini G, Federici G, Ricci G. Proton release upon glutathione binding to glutathione transferase P1-1: kinetic analysis of a multistep glutathione binding process. *Biochemistry.* **1998**, 37, 3028-34. DOI: 10.1021/bi971903g.
52. Sherratt PJ, Pulford DJ, Harrison DJ, Green T, Hayes JD. Evidence that human class Theta glutathione S-transferase T1-1 can catalyse the activation of dichloromethane, a liver and lung carcinogen in the mouse. Comparison of the tissue distribution of GST T1-1 with that of classes Alpha, Mu and Pi GST in human. *Biochem. J.* **1997**, 326, 837–846; DOI:10.1042/bj3260837
53. Rowe JD, Nieves E, Listowsky I. Subunit diversity and tissue distribution of human glutathione S-transferases: interpretations based on electrospray ionization-MS and peptide sequence-specific antisera. *Biochem. J.* **1997**, 325, 481–486; DOI: 10.1042/bj3250481
54. Landi S. Mammalian class theta GST and differential susceptibility to carcinogens: a review. *Mutat. Res*. **2000**, 463, 247–283; DOI: 10.1016/s1383-5742(00)00050-8
55. Eaton DL, Bammler TK. Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol. Sci.* **1999**, 49, 156–164; DOI: 10.1093/toxsci/49.2.156
56. Zhang Y, Talalay P. Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. *Cancer Res.* **1994**, 54, 7 Suppl, 1976s–1981s.
57. Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc. Natl. Acad. Sci. U S A.* **1997**, 94, 10367–72; DOI: 10.1073/pnas.94.19.10367
58. Kurtovic S, Shokeer A, Mannervik B. Emergence of novel enzyme quasi-species depends on the substrate matrix. *J Mol Biol*. **2008**, 382, 136–153; DOI: 10.1016/j.jmb.2008.07.003
59. Van Ommen B, Bogaards JJ, Peters WH, Blaauboer B, van Bladeren PJ. Quantification of human hepatic glutathione S-transferases. *Biochem. J.* **1990**, 269, 609–613; DOI: 10.1042/bj2690609
60. Strange RC, Faulder CG, Davis BA, Hume R, Brown JA, Cotton W, Hopkinson DA. The human glutathione S-transferases: studies on the tissue distribution and genetic variation of the GST1, GST2 and GST3 isozymes. *Ann. Hum. Genet*. **1984**, 48, 11–20; DOI: 10.1111/j.1469-1809.1984.tb00829.x
61. Pemble S, Schroeder KR, Spencer SR, Meyer DJ, Hallier E, Bolt HM, Ketterer B, Taylor JB. Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. *Biochem. J.* **1994**, 300, 271–6; DOI: 10.1042/bj3000271
62. Zhao Y, Marotta M, Eichler EE, Eng C, Tanaka H. Linkage disequilibrium between two high-frequency deletion polymorphisms: implications for association studies involving the glutathione-S transferase (GST) genes. *PLoS Genet.* **2009**, 5, e1000472; DOI: 10.1371/journal.pgen.1000472

63. Xu S, Wang Y, Roe B, Pearson WR. Characterization of the human class Mu glutathione S-transferase gene cluster and the GSTM1 deletion. *J. Biol. Chem.* **1998**, 273, 3517–3527; DOI:10.1074/jbc.273.6.3517
64. Seidegård J, Vorachek WR, Pero RW, Pearson WR. Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due to a gene deletion. *Proc. Natl. Acad. Sci. U S A.* **1988**, 85, 7293–7297; DOI:10.1073/pnas.85.19.7293
65. Johansson A-S, Mannervik B. Interindividual variability of glutathione transferase expression. In Interindividual variability in human drug metabolism; G. M. Pacifici and O. Pelkonen, eds;. Taylor & Francis, London, pp. 460–519. **2001**.
66. Löf A, Johanson G, Rannug A, Warholm M. Glutathione transferase T1 phenotype affects the toxicokinetics of inhaled methyl chloride in human volunteers. *Pharmacogenetics.* **2000**, 10, 645–653; DOI:10.1097/00008571-200010000-00007
67. Watson MA, Stewart RK, Smith GB, Massey TE, Bell DA. Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. *Carcinogenesis.* **1998**, 19, 275–280; DOI:10.1093/carcin/19.2.275
68. Johansson AS, Stenberg G, Widersten M, Mannervik B. Structure-activity relationships and thermal stability of human glutathione transferase P1-1 governed by the H-site residue 105. *J. Mol. Biol.* **1998**, 278, 687–698; DOI: 10.1006/jmbi.1998.1708
69. Tetlow N, Board PG. Functional polymorphism of human glutathione transferase A2. *Pharmacogenetics.* **2004**, 14, 111–116; DOI: 10.1097/00008571-200402000-00005
70. Sundberg K, Johansson AS, Stenberg G, Widersten M, Seidel A, Mannervik B, Jernström B. Differences in the catalytic efficiencies of allelic variants of glutathione transferase P1-1 towards carcinogenic diol epoxides of polycyclic aromatic hydrocarbons. *Carcinogenesis*. **1998**, 19, 433–6; DOI: 10.1093/carcin/19.3.433
71. Zhang W, Modén O, Mannervik B. Differences among allelic variants of human glutathione transferase A2-2 in the activation of azathioprine. *Chem. Biol. Interact.* **2010**, 186, 110–117; DOI: 10.1016/j.cbi.2010.04.028
72. Hu X, O'Donnell R, Srivastava SK, Xia H, Zimniak P, Nanduri B, Bleicher RJ, Awasthi S, Awasthi YC, Ji X, Singh SV. Active site architecture of polymorphic forms of human glutathione S-transferase P1-1 accounts for their enantioselectivity and disparate activity in the glutathione conjugation of 7beta,8alpha-dihydroxy-9alpha,10alpha-ox y-7,8,9,10-tetrahydrobenzo(a)pyrene. *Biochem. Biophys. Res. Commun*. **1997**, 235, 424–8; DOI: 10.1006/bbrc.1997.6777
73. Coles BF, Kadlubar FF. Human alpha class glutathione S-transferases: genetic polymorphism, expression, and susceptibility to disease. *Methods Enzymol.* **2005**, 401, 9–42; DOI: 10.1016/S0076-6879(05)01002-5
74. Klusek J, Nasierowska-Guttmejer A, Kowalik A, Wawrzycka I, Lewitowicz P, Chrapek M, Głuszek S. *GSTM1*, *GSTT1*, and *GSTP1* polymorphisms and colorectal cancer risk in Polish nonsmokers. *Oncotarget*. **2018**, 9, 21224–21230; DOI: 10.18632/oncotarget.25031
75. Brockmöller J, Kerb R, Drakoulis N, Staffeldt B, Roots I. Glutathione S-transferase M1 and its variants A and B as host factors of bladder cancer susceptibility: a case-control study. *Cancer Res*. **1994**, 54, 4103–4111.
76. Rebbeck TR. Molecular epidemiology of the human glutathione S-transferase genotypes GSTM1 and GSTT1 in cancer susceptibility. *Cancer Epidemiol. Biomarkers Prev.* **1997**, 6, 733–743.
77. Whitbread AK, Mellick GD, Silburn PA, Le Couteur DG, Board PG. Glutathione transferase Omega class polymorphisms in Parkinson disease. *Neurology.* **2004**, 62, 1910–1911; DOI: 10.1212/01.wnl.0000125282.09308.b1

78. Piirilä P, Wikman H, Luukkonen R, Kääriä K, Rosenberg C, Nordman H, Norppa H, Vainio H, Hirvonen A. Glutathione S-transferase genotypes and allergic responses to diisocyanate exposure. *Pharmacogenetics*. **2001**, 11, 437–45; DOI: 10.1097/00008571-200107000-00007
79. Norrgård MA, Ivarsson Y, Tars K, Mannervik B. Alternative mutations of a positively selected residue elicit gain or loss of functionalities in enzyme evolution. *Proc. Natl. Acad. Sci. U S A.* **2006**, 103, 4876–4881; DOI: 10.1073/pnas.0600849103
80. Jemth P, Mannervik B. Kinetic characterization of recombinant human glutathione transferase T1-1, a polymorphic detoxication enzyme. *Arch. Biochem. Biophys.* **1997**, 348, 247–54; DOI: 10.1006/abbi.1997.0357.
81. Shokeer A, Larsson AK, Mannervik B. Residue 234 in glutathione transferase T1-1 plays a pivotal role in the catalytic activity and the selectivity against alternative substrates. *Biochem. J.* **2005**, 388, 387–92; DOI: 10.1042/BJ20042064.
82. Conaway CC, Getahun SM, Liebes LL, Pusateri DJ, Topham DK, Botero-Omary M, Chung FL. Disposition of glucosinolates and sulforaphane in humans after ingestion of steamed and fresh broccoli. *Nutr. Cancer*. **2000**, 38, 168–78; DOI: 10.1207/S15327914NC382_5. Erratum in: *Nutr. Cancer*, 2001, 41, 196. PMID: 11525594.
83. Kolm RH, Danielson UH, Zhang Y, Talalay P, Mannervik B. Isothiocyanates as substrates for human glutathione transferases: structure-activity studies. *Biochem. J.* **1995**, 311, 453–9; DOI: 10.1042/bj3110453.
84. Meyer DJ, Crease DJ, Ketterer B. Forward and reverse catalysis and product sequestration by human glutathione S-transferases in the reaction of GSH with dietary aralkyl isothiocyanates. *Biochem. J.* **1995**, 306, 565–9; DOI: 10.1042/bj3060565.
85. Prohaska JR. The glutathione peroxidase activity of glutathione S-transferases. *Biochim. Biophys. Acta.* **1980**, 611, 87–98; DOI: 10.1016/0005-2744(80)90045-5.
86. Ploemen JH, van Ommen B, Bogaards JJ, van Bladeren PJ. Ethacrynic acid and its glutathione conjugate as inhibitors of glutathione S-transferases. *Xenobiotica*. **1993**, 23, 913–923; DOI: 10.3109/00498259309059418
87. O'Dwyer PJ, LaCreta F, Nash S, Tinsley PW, Schilder R, Clapper ML, Tew KD, Panting L, Litwin S, Comis RL. Phase I study of thiotepa in combination with the glutathione transferase inhibitor ethacrynic acid. *Cancer Res*. **1991**, 51, 6059–65.
88. Petrini M, Conte A, Caracciolo F, Sabbatini A, Grassi B, Ronca G. Reversing of chlorambucil resistance by ethacrynic acid in a B-CLL patient. *Br. J. Haematol.* **1993**, 85, 409–410; DOI: 10.1111/j.1365-2141.1993.tb03187.x
89. Montgomery R, Conway TW, Spector AA, Chappell D. Biochemistry: A Case-oriented Approach; 6th Ed.; Mosby Year Book, Inc, St. Louis, MO, pp. 587–618, **1996**.
90. Samuels LT, Helmreich ML, Lasater MB, Reich H. An enzyme in endocrine tissues which oxidizes 5-3 hydroxy steroids to delta 5-3 unsaturated ketones. *Science.* **1951**, 113, 490–1. DOI: 10.1126/science.113.2939.490.
91. Abdalla AM, Bruns CM, Tainer JA, Mannervik B, Stenberg G. Design of a monomeric human glutathione transferase GSTP1, a structurally stable but catalytically inactive protein. *Protein Eng*. **2002**, 15, 827–834; DOI: 10.1093/protein/15.10.827
92. Fabrini R, De Luca A, Stella L, Mei G, Orioni B, Ciccone S, Federici G, Lo Bello M, Ricci G. Monomer-dimer equilibrium in glutathione transferases: a critical reexamination. *Biochemistry*. **2009**, 48, 10473–82; DOI: 10.1021/bi901238t
93. Stella L, Caccuri AM, Rosato N, Nicotra M, Lo Bello M, De Matteis F, Mazzetti AP, Federici G, Ricci G. Flexibility of helix 2 in the human glutathione transferase P1-1. time-resolved fluorescence spectroscopy. *J. Biol. Chem*. **1998**, 273, 23267–73; DOI: 10.1074/jbc.273.36.23267.
94. Hitchens TK, Mannervik B, Rule GS. Disorder-to-order transition of the active site of human class Pi glutathione transferase, GST P1-1. *Biochemistry*. **2001**, 40, 11660–11669; DOI: 10.1021/bi010909+

95. Ricci G, Lo Bello M, Caccurri AM, Pastore A, Nuccetelli M, Parker MW, Federici G. Site-directed mutagenesis of human glutathione transferase P1-1. Mutation of Cys-47 induces a positive cooperativity in glutathione transferase P1-1. *J. Biol. Chem*. **1995**, 270, 1243–8; DOI: 10.1074/jbc.270.3.1243.
96. Lo Bello M, Petruzzelli R, De Stefano E, Tenedini C, Barra D, Federici G. Identification of a highly reactive sulphydryl group in human placental glutathione transferase by a site-directed fluorescent reagent. *FEBS Lett.* **1990**, 263, 389–91; DOI: 10.1016/0014-5793(90)81421-j
97. Tamai K, Shen HX, Tsuchida S, Hatayama I, Satoh K, Yasui A, Oikawa A, Sato K. Role of cysteine residues in the activity of rat glutathione transferase P (7-7): elucidation by oligonucleotide site-directed mutagenesis. *Biochem. Biophys. Res. Commun*. **1991**, 179, 790–7; DOI: 10.1016/0006-291x(91)91886-h
98. Caccuri AM, Petruzzelli R, Polizio F, Federici G, Desideri A. Inhibition of glutathione transferase pi from human placenta by 1-chloro-2,4-dinitrobenzene occurs because of covalent reaction with cysteine 47. *Arch. Biochem. Biophys*. **1992**, 297, 119–22; DOI: 10.1016/0003-9861(92)90648-g.
99. Nishihira J, Ishibashi T, Sakai M, Nishi S, Kumazaki T, Hatanaka Y, Tsuda S, Hikichi K. Characterization of cysteine residues of glutathione S-transferase P: evidence for steric hindrance of substrate binding by a bulky adduct to cysteine 47. *Biochem. Biophys. Res. Commun*. **1992**, 188, 424–32; DOI: 10.1016/0006-291x(92)92402-j
100. van Iersel ML, Ploemen JP, Lo Bello M, Federici G, van Bladeren PJ. Interactions of alpha, beta-unsaturated aldehydes and ketones with human glutathione S-transferase P1-1. *Chem. Biol. Interact*. **1997**, 108, 67–78; DOI: 10.1016/s0009-2797(97)00096-3
101. Oakley AJ, Lo Bello M, Ricci G, Federici G, Parker MW. Evidence for an induced-fit mechanism operating in pi class glutathione transferases. *Biochemistry*. **1998**, 37, 9912–7; DOI: 10.1021/bi980323w
102. Stenberg G, Abdalla AM, Mannervik B. Tyrosine 50 at the subunit interface of dimeric human glutathione transferase P1-1 is a structural key residue for modulating protein stability and catalytic function. *Biochem. Biophys. Res. Commun*. **2000**, 271, 59–63; DOI: 10.1006/bbrc.2000.2579
103. Hegazy UM, Mannervik B, Stenberg G. Functional role of the lock and key motif at the subunit interface of glutathione transferase p1-1. *J. Biol. Chem*. **2004**, 279, 9586–96; DOI: 10.1074/jbc.M312320200
104. Hegazy UM, Tars K, Hellman U, Mannervik B. Modulating catalytic activity by unnatural amino acid residues in a GSH-binding loop of GST P1-1. *J. Mol. Biol*. **2008**, 376, 811–826; DOI: 10.1016/j.jmb.2007.12.013
105. Lo Bello M, Oakley AJ, Battistoni A, Mazzetti AP, Nuccetelli M, Mazzarese G, Rossjohn J, Parker MW, Ricci G. Multifunctional role of Tyr 108 in the catalytic mechanism of human glutathione transferase P1-1. Crystallographic and kinetic studies on the Y108F mutant enzyme. *Biochemistry*. **1997**, 36, 6207–17; DOI: 10.1021/bi962813z.
106. Widersten M, Kolm RH, Björnestedt R, Mannervik B. Contribution of five amino acid residues in the glutathione-binding site to the function of human glutathione transferase P1-1. *Biochem. J*. **1992**, 285, 377–81; DOI: 10.1042/bj2850377. Erratum in: *Biochem. J*. **1992**, 287, 1023.
107. Kano T, Sakai M, Muramatsu M. Structure and expression of a human class pi glutathione S-transferase messenger RNA. *Cancer Res. **1987***, 47, 5626–5630.
108. Ali-Osman F, Akande O, Antoun G, Mao JX, Buolamwini J. Molecular cloning, characterization, and expression in Escherichia coli of full-length cDNAs of three human glutathione S-transferase Pi gene variants. Evidence for differential catalytic activity of the encoded proteins. *J. Biol. Chem.* **1997**, 272, 10004–10012; DOI: 10.1074/jbc.272.15.10004

109. Board PG, Webb GC, Coggan M. Isolation of a cDNA clone and localization of the human glutathione S-transferase 3 genes to chromosome bands 11q13 and 12q13-14. *Ann. Hum. Genet*. **1989**, 53, 205–213; DOI: 10.1111/j.1469-1809.1989.tb01786.x
110. Watson MA, Stewart RK, Smith GB, Massey TE, Bell DA. Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. *Carcinogenesis.* **1998**, 19, 275–280; DOI:10.1093/carcin/19.2.275
111. Lin HJ, Johansson AS, Stenberg G, Materi AM, Park JM, Dai A, Zhou H, Gim JS, Kau IH, Hardy SI, Parker MW, Mannervik B. Naturally occurring Phe151Leu substitution near a conserved folding module lowers stability of glutathione transferase P1-1. *Biochim. Biophys. Acta*. **2003**, 1649, 16–23; DOI: 10.1016/s1570-9639(03)00149-3
112. Sharma A, Pandey A, Sharma S, Chatterjee I, Mehrotra R, Sehgal A, Sharma JK. Genetic polymorphism of glutathione S-transferase P1 (GSTP1) in Delhi population and comparison with other global populations. *Meta Gene*. **2014**, 2, 134–42; DOI: 10.1016/j.mgene.2013.12.003
113. Harries LW, Stubbins MJ, Forman D, Howard GC, Wolf CR. Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. *Carcinogenesis.* **1997**, 18, 641–644; DOI: 10.1093/carcin/18.4.641
114. Allan JM, Wild CP, Rollinson S, Willett EV, Moorman AV, Dovey GJ, Roddam PL, Roman E, Cartwright RA, Morgan GJ. Polymorphism in glutathione S-transferase P1 is associated with susceptibility to chemotherapy-induced leukemia. *Proc. Natl. Acad. Sci. U S A.* **2001**, 98, 11592–7; DOI: 10.1073/pnas.191211198. Erratum in: *Proc. Natl. Acad. Sci. U S A*. **2001**, 98, 15394.
115. Mannervik B, Castro VM, Danielson UH, Tahir MK, Hansson J, Ringborg U. Expression of class Pi glutathione transferase in human malignant melanoma cells. *Carcinogenesis*. **1987**, 12, 1929–32; DOI: 10.1093/carcin/8.12.1929
116. Howie AF, Forrester LM, Glancey MJ, Schlager JJ, Powis G, Beckett GJ, Hayes JD, Wolf CR. Glutathione S-transferase and glutathione peroxidase expression in normal and tumour human tissues. *Carcinogenesis*. **1990**, 11, 451–8; DOI: 10.1093/carcin/11.3.451
117. Goto S, Kamada K, Soh Y, Ihara Y, Kondo T. Significance of nuclear glutathione S-transferase pi in resistance to anti-cancer drugs. *Jpn. J. Cancer. Res*. **2002**, 93, 1047–1056; DOI: 10.1111/j.1349-7006.2002.tb02482.x
118. Dong SC, Sha HH, Xu XY, Hu TM, Lou R, Li H, Wu JZ, Dan C, Feng J. Glutathione *S*-transferase π: a potential role in antitumor therapy. *Drug Des. Devel. Ther*. **2018**, 12, 3535–3547; DOI: 10.2147/DDDT.S169833
119. Sawers L, Ferguson MJ, Ihrig BR, et al. Glutathione S-transferase P1 (GSTP1) directly influences platinum drug chemosensitivity in ovarian tumour cell lines. *Br. J. Cancer.* **2014**, 111, 1150–1158; DOI: 10.1038/bjc.2014.386
120. Black SM, Beggs JD, Hayes JD, Bartoszek A, Muramatsu M, Sakai M, Wolf CR. Expression of human glutathione S-transferases in Saccharomyces cerevisiae confers resistance to the anticancer drugs adriamycin and chlorambucil. *Biochem. J*. **1990**, 268, 309–15; DOI: 10.1042/bj2680309
121. Hirata S, Odajima T, Kohama G, Ishigaki S, Niitsu Y. Significance of glutathione S-transferase-pi as a tumor marker in patients with oral cancer. *Cancer*. **1992**, 70, 2381–7; DOI: 10.1002/1097-0142(19921115)70:10<2381::aid-cncr2820701002>3.0.co;2-9
122. Niitsu Y, Takahashi Y, Saito T, Hirata Y, Arisato N, Maruyama H, Kohgo Y, Listowsky I. Serum glutathione-S-transferase-pi as a tumor marker for gastrointestinal malignancies. *Cancer*. **1989**, 63, 317–23; DOI: 10.1002/1097-0142(19890115)63:2<317::aid-cncr2820630219>3.0.co;2-p

123. Hida T, Kuwabara M, Ariyoshi Y, Takahashi T, Sugiura T, Hosoda K, Niitsu Y, Ueda R. Serum glutathione S-transferase-pi level as a tumor marker for non-small cell lung cancer. Potential predictive value in chemotherapeutic response. *Cancer*. **1994**, 73, 1377–82; DOI: 10.1002/1097-0142(19940301)73:5<1377::aid-cncr2820730511>3.0.co;2-n
124. Tsuchida S, Kimura J, Hayakari M, Ishikawa T. Rinsho Byori. [Usefulness of glutathione S-transferase as a tumor marker]. *Rinsho. Byori*. **1997**, 45, 1125–1132.
125. Tew KD, Bomber AM, Hoffman SJ. Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug resistant and sensitive cell lines. *Cancer Res.* **1988**, 48, 3622–3625.
126. Hall A, Robson CN, Hickson ID, Harris AL, Proctor SJ, Cattan AR. Possible role of inhibition of glutathione S-transferase in the partial reversal of chlorambucil resistance by indomethacin in a Chinese hamster ovary cell line. *Cancer Res*. **1989**, 49, 6265–6268.
127. Ford JM, Hait WN, Matlin SA, Benz CC. Modulation of resistance to alkylating agents in cancer cell by gossypol enantiomers. *Cancer Lett.* **1991**, 56, 85–94; DOI: 10.1016/0304-3835(91)90198-q
128. Ruscoe JE, Rosario LA, Wang T, Gaté L, Arifoglu P, Wolf CR, Henderson CJ, Ronai Z, Tew KD. Pharmacologic or genetic manipulation of glutathione S-transferase P1-1 (GSTpi) influences cell proliferation pathways. *J. Pharmacol. Exp. Ther.* **2001**, 298, 339–45.
129. Raza A, Galili N, Callander N, Ochoa L, Piro L, Emanuel P, Williams S, Burris H 3rd, Faderl S, Estrov Z, Curtin P, Larson RA, Keck JG, Jones M, Meng L, Brown GL. Phase 1-2a multicenter dose-escalation study of ezatiostat hydrochloride liposomes for injection (Telintra, TLK199), a novel glutathione analog prodrug in patients with myelodysplastic syndrome. *J. Hematol. Oncol*. **2009**, 2, 20; DOI: 10.1186/1756-8722-2-20
130. Raza A, Galili N, Smith S, Godwin J, Lancet J, Melchert M, Jones M, Keck JG, Meng L, Brown GL, List A. Phase 1 multicenter dose-escalation study of ezatiostat hydrochloride (TLK199 tablets), a novel glutathione analog prodrug, in patients with myelodysplastic syndrome. *Blood*. **2009**, 113, 6533–40; DOI: 10.1182/blood-2009-01-176032
131. Allocati N, Masulli M, Di Ilio C, Federici L. Glutathione transferases: substrates, inihibitors and pro-drugs in cancer and neurodegenerative diseases. *Oncogenesis*. **2018**, 7(1), 8; DOI: 10.1038/s41389-017-0025-3
132. Ruzza P, Calderan A. Glutathione Transferase (GST)-Activated Prodrugs. *Pharmaceutics*. **2013**, 5, 220–231; DOI: 10.3390/pharmaceutics5020220
133. Lyttle MH, Hocker MD, Hui HC, Caldwell CG, Aaron DT, Engqvist-Goldstein A, Flatgaard JE, Bauer KE. Isozyme-specific glutathione-S-transferase inhibitors: design and synthesis. *J. Med. Chem*. **1994**, 37, 189–94; DOI: 10.1021/jm00027a024
134. Lyttle MH, Satyam A, Hocker MD, Bauer KE, Caldwell CG, Hui HC, Morgan AS, Mergia A, Kauvar LM. Glutathione-S-transferase activates novel alkylating agents. *J. Med. Chem*. **1994**, 37, 1501–7; DOI: 10.1021/jm00036a016.
135. Morgan AS, Sanderson PE, Borch RF, Tew KD, Niitsu Y, Takayama T, Von Hoff DD, Izbicka E, Mangold G, Paul C, Broberg U, Mannervik B, Henner WD, Kauvar LM. Tumor efficacy and bone marrow-sparing properties of TER286, a cytotoxin activated by glutathione S-transferase. *Cancer Res*. **1998**, 58, 2568–75.
136. Dourado DF, Fernandes PA, Ramos MJ, Mannervik B. Mechanism of glutathione transferase P1-1-catalyzed activation of the prodrug canfosfamide (TLK286, TELCYTA). *Biochemistry*. **2013**, 52, 8069–78; DOI: 10.1021/bi4005705
137. Cai D, Chew J, Wray J, Kumar V, Danko S, Sambucetti L, Gomez R, Keck JG. GST P1-1 polymorphisms do not affect the rate of TELCYTA™ (TLK286) prodrug activation. *Cancer Res.* **2004**, 64 (Suppl. S7), 883.
138. Rosen LS, Brown J, Laxa B, Boulos L, Reiswig L, Henner WD, Lum RT, Schow SR, Maack CA, Keck JG, Mascavage JC, Dombroski JA, Gomez RF, Brown GL.

Phase I study of TLK286 (glutathione S-transferase P1-1 activated glutathione analogue) in advanced refractory solid malignancies. *Clin. Cancer Res*. **2003**, 9, 1628–38.
139. Rosen LS, Laxa B, Boulos L, Wiggins L, Keck JG, Jameson AJ, Parra R, Patel K, Brown GL. Phase 1 study of TLK286 (Telcyta) administered weekly in advanced malignancies. *Clin. Cancer Res*. **2004**, 10, 3689–98; DOI: 10.1158/1078-0432.CCR-03-0687
140. Kavanagh JJ, Gershenson DM, Choi H, Lewis L, Patel K, Brown GL, Garcia A, Spriggs DR. Multi-institutional phase 2 study of TLK286 (TELCYTA, a glutathione S-transferase P1-1 activated glutathione analog prodrug) in patients with platinum and paclitaxel refractory or resistant ovarian cancer. *Int. J. Gynecol. Cancer*. **2005**, 15, 593–600; DOI: 10.1111/j.1525-1438.2005.00114.x
141. Vergote I, Finkler N, del Campo J, Lohr A, Hunter J, Matei D, Kavanagh J, Vermorken JB, Meng L, Jones M, Brown G, Kaye S; ASSIST-1 Study Group. Phase 3 randomised study of canfosfamide (Telcyta, TLK286) versus pegylated liposomal doxorubicin or topotecan as third-line therapy in patients with platinum-refractory or -resistant ovarian cancer. *Eur. J. Cancer*. **2009**, 45, 2324–32; DOI: 10.1016/j.ejca.2009.05.016
142. Conilh L, Sadilkova L, Viricel W, Dumontet C. Payload diversification: a key step in the development of antibody-drug conjugates. *J. Hematol. Oncol.* **2023**, 16, 3; DOI: 10.1186/s13045-022-01397-y
143. Bagshawe KD, Springer CJ, Searle F, Antoniw P, Sharma SK, Melton RG, Sherwood RF. A cytotoxic agent can be generated selectively at cancer sites. *Br. J. Cancer*. **1988**, 58, 700–3; DOI: 10.1038/bjc.1988.293
144. Napier MP, Sharma SK, Springer CJ, Bagshawe KD, Green AJ, Martin J, Stribbling SM, Cushen N, O'Malley D, Begent RH. Antibody-directed enzyme prodrug therapy: efficacy and mechanism of action in colorectal carcinoma. *Clin. Cancer Res*. **2000**, 6, 765–72.
145. Bagshawe KD, Sharma SK, Springer C, Antoniw P. Antibody directed enzyme prodrug therapy: a pilot scale clinical trial. *Tumor Targeting.* **1995**, 1, 17–29.
146. Martin J, Stribbling SM, Poon GK, Begent RH, Napier M, Sharma SK, Springer CJ. Antibody-directed enzyme prodrug therapy: pharmacokinetics and plasma levels of prodrug and drug in a phase I clinical trial. *Cancer Chemother. Pharmacol*. **1997**, 40, 189–201; DOI: 10.1007/s002800050646
147. Findlay VJ, Townsend DM, Saavedra JE, Buzard GS, Citro ML, Keefer LK, Ji X, Tew KD. Tumor cell responses to a novel glutathione S-transferase-activated nitric oxide-releasing prodrug. *Mol. Pharmacol.* **2004**, 65, 1070–9; DOI: 10.1124/mol.65.5.1070.
148. Shami PJ, Saavedra JE, Wang LY, Bonifant CL, Diwan BA, Singh SV, Gu Y, Fox SD, Buzard GS, Citro ML, Waterhouse DJ, Davies KM, Ji X, Keefer LK. JS-K, a glutathione/glutathione S-transferase-activated nitric oxide donor of the diazeniumdiolate class with potent antineoplastic activity. *Mol. Cancer Ther*. **2003**, 2, 409–17.
149. Zhang W, Modén O, Tars K, Mannervik B. Structure-based redesign of GST A2-2 for enhanced catalytic efficiency with azathioprine. *Chem. Biol*. **2012**, 19, 414–421; DOI: 10.1016/j.chembiol.2012.01.021
150. Song Z, Zhang Q, Wu W, Pu Z, Yu H. Rational design of enzyme activity and enantioselectivity. *Front. Bioeng. Biotechnol*. **2023**, 11, 1129149; DOI:10.3389/fbioe.2023.1129149
151. Morrison KL, Weiss GA. Combinatorial alanine-scanning. *Curr. Opin. Chem. Biol.* **2001**, 5, 302–307; DOI: 10.1016/s1367-5931(00)00206-4
152. Cunningham BC, Wells JA. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science*, **1989**, 244, 1081–1085.

153. Pettersson PL, Johansson AS, Mannervik B. Transmutation of human glutathione transferase A2-2 with peroxidase activity into an efficient steroid isomerase. *J. Biol. Chem.* **2002**, 277, 30019–30022; DOI: 10.1074/jbc.M204485200
154. Ivarsson Y, Norrgård MA, Hellman U, Mannervik B. Engineering the enantioselectivity of glutathione transferase by combined active-site mutations and chemical modifications. *Biochim. Biophys. Acta*. **2007**, 1770, 1374–1381; DOI: 10.1016/j.bbagen.2007.06.002
155. Yang KK, Wu Z, Arnold FH. Machine-learning-guided directed evolution for protein engineering. *Nat. Methods*. **2019**, 16, 687–694; DOI: 10.1038/s41592-019-0496-6
156. Notin P, Rollins N, Gal Y, Sander C, Marks D. Machine learning for functional protein design. *Nat. Biotechnol*. **2024**, 42, 216–228; DOI: 10.1038/s41587-024-02127-0
157. Govindarajan S, Mannervik B, Silverman AJ, Wright K, Regitsky D, Hegazy U, Purcell JT, Welch M, Minshull J, Gustafsson C. Mapping of amino acid substitutions conferring herbicide resistance in wheat glutathione transferase. *ACS Synth. Biol.* **2015**, *4*, 221–227; DOI: 10.1021/sb500242x
158. Welch M, Govindarajan S, Ness JE, Villalobos A, Gurney A, Minshull J, Gustafsson C. Design parameters to control synthetic gene expression in Escherichia coli. *PLoS One*. **2009**, 4, e7002; DOI: 10.1371/journal.pone.0007002
159. Liao J, Warmuth MK, Govindarajan S, Ness JE, Wang RP, Gustafsson C, Minshull J. Engineering proteinase K using machine learning and synthetic genes. *BMC Biotechnol*. **2007**, 7, 16; DOI: 10.1186/1472-6750-7-16
160. Musdal Y, Govindarajan S, Mannervik B. Exploring sequence-function space of a poplar glutathione transferase using designed information-rich gene variants. *Protein Eng. Des. Sel.* **2017**, 30, 543–549; DOI: 10.1093/protein/gzx045.
161. Hubatsch I, Ridderström M, Mannervik B. Human glutathione transferase A4-4: an alpha class enzyme with high catalytic efficiency in the conjugation of 4-hydroxynonenal and other genotoxic products of lipid peroxidation. *Biochem. J.* **1998**, 330, 175–9; DOI: 10.1042/bj3300175.
162. Zhao T, Singhal SS, Piper JT, Cheng J, Pandya U, Clark-Wronski J, Awasthi S, Awasthi YC. The role of human glutathione S-transferases hGSTA1-1 and hGSTA2-2 in protection against oxidative stress. *Arch. Biochem. Biophys*. **1999**, 367, 216–24; DOI: 10.1006/abbi.1999.1277.
163. Kurtovic S, Shokeer A, Mannervik B. Emergence of novel enzyme quasi-species depends on the substrate matrix. *J. Mol. Biol.* **2008**, 382, 136–153.
164. Benson AM, Talalay P, Keen JH, Jakoby WB. Relationship between the soluble glutathione-dependent delta 5-3-ketosteroid isomerase and the glutathione S-transferases of the liver. *Proc. Natl. Acad. Sci. U S A*. **1977**, 74, 158–62; DOI: 10.1073/pnas.74.1.158.
165. Talalay P, Wang VS. Enzymic isomerization of delta5-3-ketosteroids. *Biochim. Biophys. Acta*. **1955**, 18, 300–1; DOI: 10.1016/0006-3002(55)90079-2.
166. Fedulova N, Raffalli-Mathieu F, Mannervik B. Porcine glutathione transferase Alpha 2-2 is a human GST A3-3 analogue that catalyses steroid double-bond isomerization. *Biochem. J*. **2010**, 431, 159–67; DOI: 10.1042/BJ20100839.
167. Johansson AS, Mannervik B. Active-site residues governing high steroid isomerase activity in human glutathione transferase A3-3. *J. Biol. Chem*. **2002**, 277, 16648–54; DOI: 10.1074/jbc.M201062200. Epub 2002 Feb 28.
168. Dourado DF, Fernandes PA, Mannervik B, Ramos MJ. Isomerization of Δ5-androstene-3,17-dione into Δ4-androstene-3,17-dione catalyzed by human glutathione transferase A3-3: a computational study identifies a dual role for glutathione. *J. Phys. Chem. A*. **2014**, 118, 5790–800; DOI: 10.1021/jp410810q. Epub 2014 Apr 28.
169. Nainu F, Jota Baptista C, Faustino-Rocha A, Oliveira PA. Editorial: Model organisms in experimental pharmacology and drug discovery 2022. *Front. Pharmacol.* **2023**, 14, 1143934; DOI: 10.3389/fphar.2023.1143934.

170. Bertile F, Matallana-Surget S, Tholey A, Cristobal S, Armengaud J. Diversifying the concept of model organisms in the age of -omics. *Commun. Biol.* **2023**, 6, 1062; https://doi.org/10.1038/s42003-023-05458-x
171. Mannervik B, and Danielson UH. Glutathione transferases- structure and catalytic activity. *CRC Crit. Rev. Biochem. Mol. Biol.* **1988**, 23, 283-337; DOI: 10.3109/10409238809088226.
172. Johansson A-S, Stenberg G, Widersten M, Mannervik B. Structure-activity relationship and thermal stability of human glutathione transferase P1-1 governed by the H-site residue 105. *J. Mol. Biol*. **1998**, 278, 687–698; DOI: 10.1006/jmbi.1998.1708.
173. Mannervik B, Guthenberg C, Jensson H, Tahir MK, Warholm M, Ålin P. Species and Tissue Differences in the Occurrence of Multiple Forms of Rat and Human Glutathione Transferases. In: Paton W, Mitchell JF, Turner P. editors. *Proceedings of the IUPHAR 9th International Congress of Pharmacology.* Volume 3. Macmillan Press; London, UK. pp. 225–230. **1984**.
174. Ploemen JH, van Ommen B, van Bladeren PJ. Inhibition of rat and human glutathione S-transferase isoenzymes by ethacrynic acid and its glutathione conjugate. *Biochem Pharmacol*. **1990**, 40, 1631–1635; DOI: 10.1016/0006-2952(90)90465-w
175. Tahir MK, Guthenberg C, Mannervik B. Inhibitors for distinction of three types of human glutathione transferase. *FEBS Lett*. **1985**, 181, 249–252; DOI: 10.1016/0014-5793(85)80269-6
176. Tokuriki N, Stricher F, Schymkowitz J, Serrano L, Tawfik DS. The Stability Effects of Protein Mutations Appear to be Universally Distributed. *J. Mol. Biol*. **2007**, 369, 1318–1332; DOI: 10.1016/j.jmb.2007.03.069
177. Aceto A, Caccuri AM, Sacchetta P, Bucciarelli T, Dragani B, Rosato N, Federici G, Di Ilio C. Dissociation and unfolding of Pi-class glutathione transferase. Evidence for a monomeric inactive intermediate. *Biochem. J*. **1992**, 285, 241–245; DOI: 10.1042/bj2850241
178. Nuccetelli M, Mazzetti AP, Rossjohn J, Parker MW, Board P, Caccuri AM, Federici G, Ricci G, Lo Bello M. Shifting substrate specificity of human glutathione transferase (from class Pi to class alpha) by a single point mutation. *Biochem. Biophys. Res. Commun.* **1998**, 252, 184–189; DOI: 10.1006/bbrc.1998.9575.
179. Tars K, Olin B, Mannervik B. Structural basis for featuring of steroid isomerase activity in alpha class glutathione transferases. *J. Mol. Biol*. **2010**, 397, 332–40; DOI: 10.1016/j.jmb.2010.01.023. Epub 2010 Jan 18. PMID: 20083122.
180. Sharma SK, Bagshawe KD. Translating antibody directed enzyme prodrug therapy (ADEPT) and prospects for combination. *Expert Opin. Biol. Ther*. **2017**, 17, 1–13; DOI: 10.1080/14712598.2017.1247802. Epub 2016 Oct 24.
181. Raffalli-Mathieu F, Orre C, Stridsberg M, Hansson Edalat M, Mannervik B. Targeting human glutathione transferase A3-3 attenuates progesterone production in human steroidogenic cells. *Biochem. J*. **2008**, 414, 103–9. DOI: 10.1042/BJ20080397.