ROLE OF GENETIC POLYMORPHISMS OF DRUG METABOLISING ENZYMES IN IDIOSYNCRATIC DRUG REACTIONS

-IN VITRO TO IN VIVO TRANSLATION-

Sanja Dragovic

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Role of genetic polymorphisms of drug metabolising enzymes in idiosyncratic drug reactions. In vitro to in vivo translation

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door

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“To enjoy the rainbow, first enjoy the rain”.

Paulo Coelho

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prof. dr. G. W. Somsen

The research described in this thesis was performed within the framework of project D3-201 “Towards novel translational safety biomarkers for adverse drug toxicity.” of the Dutch Top Institute Pharma and carried out in the Amsterdam Institute for Molecules, Medicines and Systems (AIMMS), Section of Molecular Toxicology, Department of Chemistry and Pharmaceutical Sciences, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

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Chapter 1

GENERAL INTRODUCTION

Abstract

Adverse drug reactions (ADRs) are one of the leading causes for the attrition of drug candidates in pharmaceutical industry during the drug discovery, preclinical drug development phases, and clinical use after marketing (*1*). Adverse drug reactions (ADRs) cause a broad range of clinically severe side effects including both highly predictable and dose dependent toxicities as well as relatively infrequent and idiosyncratic adverse events. Idiosyncratic drug reactions (IDRs) are normally very severe, potentially fatal, and usually detected only when the drug has been used by a large number of patients. These ADRs do not only result from increased internal drug exposure but also strongly depend on several drug- and patient-related risk factors (*2*). Excessive dose, drug accumulation and/or the formation of chemically reactive metabolites are major determinants for occurrence of (idiosyncratic) ADRs (*3*).

The main focus of the present thesis is on the elucidation of risk factors in populations for the occurrence of ADRs of selected drugs. In order to provide an overview of the various factors which may play a role in the occurrence of ADRs, this chapter describes the present knowledge on ADRs and specifically on IDRs, the drug- and patient-related risk factors, the role of metabolism and reactive metabolite formation in this type of toxicity and the strategies currently applied by pharmaceutical industry to minimize risks related to this issue during drug development programs. Specifically, the role of the polymorphic glutathione S-transferases (GSTs) in detoxification of reactive metabolites and hence risk factors for occurrence of drug toxicity will be discussed in Chapter 2. The final part of this chapter is presenting the aims and scope of the thesis.

1.1. Adverse Drug Reactions (ADRs)

1.1.1. Definitions of ADRs

The World Health Organisation (WHO) defines adverse drug reaction as "a response to a drug that is noxious and unintended and occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function". Or, in summary, an adverse drug reaction is harm directly caused by the drug at normal doses, during normal use.

ADRs are defined by Laurence (*4*) as: "A harmful or significantly unpleasant effect caused by a drug at doses intended for therapeutic effect caused by a drug at doses intended for therapeutic effect (or prophylaxis or diagnosis) which warrants reduction of dose or withdrawal of the drug and/or foretells hazard from future administration." However, these definitions exclude medication errors as a source of adverse effects, as well as reactions due to contaminants or supposedly inactive excipients in formulation.

Edwards and Aronson (*5*) proposed the following definition as the most complete one: "An appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product."

Modern drug therapy has brought significant benefits to mankind by contributing enormously in decreasing the suffering from severe diseases. It has undoubtedly made a great impact in improvement of medical treatment. However, successful drug development is often uncertain due to safety issues and toxicity which represent important causes of attrition of drug candidates during the drug discovery, preclinical and clinical drug development phases and is an important contributor to the currently poor productivity of pharmaceutical industry. Even after marketing, ADRs are a major complication as they are a considerable cause of patient morbidity and account for a significant number of patient deaths. They have become an important clinical problem and a constant concern of the public health systems. In a meta-analysis, the incidence of serious (6.7%) and fatal (0.32%) ADRs in US hospitals appeared to be extremely high and even suggested that ADRs were between the fourth and sixth commonest cause of death in the United States in 1994 (3). More recently, a study in the United Kingdom showed that 6.5% of hospital admission are due to ADRs, with a mortality rate of approximately 2% (*7*). Besides the medical impact and serious health risks for patients, ADRs also have a high socio-economic impact. It has been suggested that patients who developed ADRs during hospitalization, were hospitalised an average of 1.2–3.8 days longer than patients who did not, with a substantial increase of the healthcare and treatment costs (*8*).

ADRs represent one of the most common causes for pharmaceutical product recalls and black-box warning labels that can markedly restrict drug usage. As highlighted by Lasser *et al.*, of a total of 548 drugs approved in the period from 1975 to 1999, 45 drugs (8.2%) acquired one or more black-box warnings and 16 (2.9%) were withdrawn from the market (*9*). Examples of the drugs withdrawn from the market due to unacceptable safety profiles are shown in Table 1.

A major problem is the fact that several types of ADRs, such as cardiotoxicity and hepatotoxicity, still can not be well predicted from (pre)-clinical studies. Therefore, increased emphasis has been placed on the identification of risk factors of potential ADRs in preclinical species and humans as early as possible in the overall discovery/development process.

1.1.2. Classification of adverse drug reactions (ADRs)

ADRs can be categorised in a number of ways based on their nature and mechanism (e.g. by severity, by body systems affected, or by frequency). The classification system is dynamic and being extended over time. The most common classification of ADRs is the one that distinguishes dose-related (**type A** – augmented effects of the drug action) and non-dose-related (**type B** – bizarre reactions) adverse drug reactions. There are other groups in this system of classification but these may also be considered as subclasses or hybrids of type A and B ADRs. These are type C ADRs (chronic reactions, dose- and time-related), type D (delayed reactions, time-related), type E (withdrawal, end of use reactions) and type F (unexpected failure of therapy) (5, 12). This classification with the characteristics, some examples and the management of these ADRs is shown in Table 2.

Type A (**a**ugmented) reactions are the most commonly occurring reactions, accounting for 80 % of total ADRs. They are predictable, usually dose dependent and related to the pharmacological effect of the drug. Toxicity is due to an exaggeration of the

drug's normal effect when given at the usual dose (e.g. respiratory depression with opioids and bleeding with warfarin). They are rarely life threatening and the adverse effect is reversible by dose reduction or discontinuation of the treatment (*5*, *13*). These reactions can further be subdivided into those due to the exaggerated therapeutic response at the target site (A1 reactions) and those due to the additional ("secondary") pharmacological action (A2 reactions). Thus, for β-blockers, bradycardia and heart block are primary pharmacological adverse effects while bronchospasm is a secondary pharmacological adverse effect. The anti-inflammatory effects of nonsteroidal anti-inflammatory drugs (NSAIDs) appear to be largely attributable to inhibition of cyclooxygenase 2 (COX-2), and their gastro intestinal side-effects to inhibition of prostaglandin synthesis in the gastric mucosa, mediated via cyclooxygenase 1 (COX-1), which represents an off-target adverse effect. Many type A reactions have a pharmacokinetic basis, e.g. impaired hepatic metabolism due to a drug-drug interactions or genetic defects of drug-metabolising enzymes, leading to increased plasma concentrations at normal dose. The likelihood of developing these adverse interactions increases with the number of drugs prescribed— if five drugs are given simultaneously the chance of an adverse interaction occurring is 50% (*13*).

Type B (**b**izarre) reactions are unpredictable effects that are usually very rare and do not show classic dose-response relationships. These idiosyncratic reactions account for 5 % of all ADRs and are strongly dependent on the individual susceptibility. Idiosyncratic drug reactions (IDRs) are very serious, account for many drug-induced deaths and their exact mechanism is not known yet (*14*). They can not be predicted from the pharmacology of the drug and are often delayed. No predictable animal model is currently available so they are not observed in preclinical studies. They usually are only detected when the drug is prescribed to a large patient population. IDRs still remain a serious problem for the pharmaceutical industry. Many seem to have an immunological basis and genetic pre-disposition is likely to be an important factor, an understanding of which may help prevent such reactions in the future.

Type C (**c**hronic or **c**hemical) reactions are those that can be predicted from the chemical structure of the drug or, more commonly, a drug metabolite (*16*) and are related to both dose and time of exposure. These reactions are well-described and can be anticipated. Chemical sub-structures that can lead to toxic metabolite formation are rationalized and this concept is incorporated into drug design.

Type D (**d**elayed) reactions show a delayed response and become apparent long after the use of a medicine. Examples are carcinogenicity and teratogenicity (*16*). These types of toxicities can be precluded in pre-clinical screening assays and may therefore be prevented in humans.

Type E (**e**nd of use) reactions are associated with the treatment withdrawal of a medicine (end of dose reactions). They are uncommon and occur mostly when the treatment is stopped abruptly (*16*).

Although rare, IDRs are a major issue for drug development. Current testing is not effective in predicting their risk. Predicting which drug candidates will cause a high incidence of IDRs would significantly decrease the costs and uncertainty in drug development. For this reason main focus of this thesis is better understanding of mechanism and predicting the risk of such reactions.

Table 1. Drugs withdrawn from the market for safety reasons*

Year of withdrawal	Drug name	Therapeutic class	Reason
1975	Aminopyrine	Analgesic	Agranulocytosis
1976	Azaribine	Dermatologic (psoriasis)	Thromboembolism
1978	Phenformin	Diabetes melitus	Lactic acidosis
1980	Ticrynafen	Antihypertensive	Hepatotoxicity
1982	Benoxaprofen	Analgesic	Hepatotoxicity
1983	Zomepirac	Analgesic	Anaphylaxis
1986	Nomifensine	Antidepressant	Hemolytic anemia
1987	Suprofen	Analgesic	Flank pain syndrome
1998	Terfenadine	Antihistamine	Fatal arrhythmia
1991	Encainide	Antiarhythmic	Fatal arrhythmia
1992	Temafloxacin	Antibiotic	Hemolytic anemia Kidney failure
1993	Flosequinan	Congestive heart failure	Increased mortality
1997	Phenolphthalein	Over-the-counter laxative	Carcinogenicity
1998	Mibefradil	Antihypertensive calcium channel blocker	Drug interactions Fatal arrhythmia
1998	Bromfenac	Analgesic	Hepatotoxicity
1999	Astemizole	Antihistamine	Fatal arrhythmia
1999	Grepafloxacin	Antibiotic	Fatal arrhythmia
2000	Cisapride	Heartburn	Fatal arrhythmia
2000	Troglitazone	Antidiabetic	Hepatotoxicity
2001	Cerivastatin	Hypercholesterolemia	Rhabdomyolysis
2001	Rapacuronium	Anesthesia	Bronchospasm
2003	Levomethadyl	Opiate dependence	Fatal arrhythmia
2004	Rofecoxib	Analgesic	Hearth attack, stroke
2005	Valdecoxib	Analgesic	Skin disease
2008	Rimonabant	Cannabinoid type-1 receptor agonist	Severe depression and suicide
2009	Efalizumab	Monoclonal antibody	Progressive multifocal leukoencephalopathy
2009	Benfluorex	Anorectic and hypolipidemic agent	Risk of heart valve disease
2010	Sibutramine	Oral anorexiant	Cardiovascular events, stroke
2010	Propoxyphene	Opioid analgesic	Heart attacks, stroke

* Table adapted from (*9*–*11*).

1.2. Idiosyncratic drug reactions (IDRs)

Approximately 5 % of adverse drug reactions are idiosyncratic or type B, as described above (*7*). They refer to a group of ADRs that do not occur in most patients within the therapeutic dose range and cannot be explained by the known pharmaceutical properties of the drug (*17*). No current definition is perfect and by understanding the mechanism the term idiosyncratic would probably become obsolete. Clinical characteristics and circumstantial evidence suggest that immune-mediated toxicity is caused by reactive drug metabolites formation. Immune-mediated adverse drug reactions are thought to occur in response to drug-protein adducts that act as immunogens, driving antibody production, T-cell-mediated responses or cytotoxic immunity towards the drug in the target tissue (Figure 1). Generation of adducts requires the production of a reactive intermediates (RIs). Additionally, these reactive metabolites may induce disruption of ionic gradients and intracellular calcium stores and mitochondrial dysfunction and loss of

energy production. This impairment of cellular function can culminate in cell death. Directly cytotoxic drugs, especially those involving the liver, are usually represented by metabolic idiosyncrasy as a result from aberrant drug metabolism or clearance, leading to the accumulation of toxic metabolites and inhibition of critical cell processes. Based on a lack of fever and rash and/or lack of immediate onset on rechallenge these reactions are related to non-immune idiosyncratic reactions. Genetic or environmental factors, either alone or in combination, could be responsible for the idiosyncratic nature of these IDRs. However, there is no clear picture of what metabolic pathways might be responsible for the idiosyncratic nature of these reactions, and these characteristics are not very strong evidence against an immune-mediated mechanism.

Unlike typical toxic responses to xenobiotic agents, idiosyncratic drug reactions:

1) occur in a small fraction of people exposed to the drug,

As mentioned, they have low frequency of occurrence: less than 1 in 5000 individuals (*18*). Alternatively, drug-related hepatotoxicity is ranging from 1 in 10.000 to 1 in 100.000 patients (*19*). This explains why IDRs are not detected during clinical trials and only appear once the drug is on the market and a large population is exposed to it.

2) are typically unrelated to the drug's pharmacologic effect,

For example, idiosyncratic hepatotoxicity is most often not related to a drug's pharmacological action (*20*). Liver injury induced by NSAIDs that are nonspecific inhibitors of COX-1 and COX-2 (e.g. diclofenac, sulindac) is not associated with their mode of action. The potential to cause idiosyncratic hepatotoxicity seems to apply to entire class of NSAIDs. On the other hand, trovafloxacin has caused serious hepatotoxicity in patients, whereas levofloxacin, an antibiotic in the same fluoroquinolone class, is without this liability.

3*) demonstrate no obvious relation to dose,*

Usually IDRs are considered as not dose-dependent. However, it has been observed that IDRs are rare for drugs given at a dose of 10 mg day^{-1} or less (*21*). Most drugs involved in idiosyncratic hepatotoxicity are “high-dose” drugs, given at more than 100 mg day^{-1} (*18*). Important is that the critical exposure factor is not the administrated dose but the concentration of drug and/or drug metabolite present at the target organ; that could elicit the adverse event.

4) occur with inconsistent temporal patterns in relation to drug exposure,

There is often a delay between exposure to the drug and the onset of the IDRs (*22*). This delay can differ from drug to drug; varying from a few days, to weeks or even months depending on different types of IDRs. For example, common maculopapular rashes usually occur shortly after start of the treatment of nevirapine (*23*) while agranulocytosis more commonly occurs after one to three months of therapy of clozapine (*24*). Idiosyncratic hepatotoxicity typically occurs after a month or two of therapy, but for some drugs, such as troglitazone, the delay can be much longer and can occur after a year or more of treatment (*25*). Also, drug-induced lupus often requires a year or more before it becomes clinically evident. Reexposure usually leads to an immediate reaction, but in some cases the response is delayed and almost as long as that associated with the first adverse reaction (*26*).

Table 2. Classification and characteristics of adverse drug reactions. Table adapted from (*5*, *15*).

Type of ADR	Characteristic	Predictable	Toxicity and examples	Management
A (augmented)	Pharmacological Dose-related Common (~80%) Suggestive time relationship Variable severity, but usually mild High morbidity/low mortality Reproducible	Yes	Drug toxicity: -dysrhythmia by digoxin -nephrotoxicity by aminoglicosides Side effects: -constipation by chronic opioid use -anticholinergic effect of tricyclic antidepressants	Reduce dose or withhold Consider effects of concomitant therapy
B (bizzare)	Idiosyncratic (non related to a pharmacological action of the drug) Non-dose-related Uncommon Variable severity, proportionately more severe than type A High morbidity/high mortality Non-reproducible	No	Immunological reactions: -penicilin hypersensitivity Idiosyncratic: -acute porphyria -malignant hyperthermia -pseudoallergy (ampicillin rash)	Withhold and avoid in the future
C (chronic)	Chemical Dose-related and time-related Uncommon Related to cumulative dose Long term exposure required	Yes Can be predicted/rationalized from chemical structure	Acetaminophen hepatotoxicity Hypothalamic-pituitary-adrenal axis suppression by corticosteroids	Reduce dose or withhold; withdrawal may have to be prolonged
D (delayed)	Time-related Uncommon Usually dose-related Occurs or become apparent some time after the use of drug	Yes Available bioassays	Teratogenesis (diethylstilbestrol) Carcinogenesis Tardive dyskinesia caused by antipsychotic medication	Often intractable
E (end of use)	Withdrawal Uncommon Occur soon after withdrawal of a drug		Opiate withdrawal syndrome β-blocker (myocardial ischaemia) Clonidine (rebound hypotension)	Reintroduce and withdraw slowly
F (failure)	Unexpected failure of therapy Common May be dose related Often caused by drug interactions		Oral contraceptive: inadequate dosage, particularly with specific enzyme inducers Resistance of a micro-organism or tumor to the drug action	Increase the dose or change the drug Consider effects of concomitant therapy

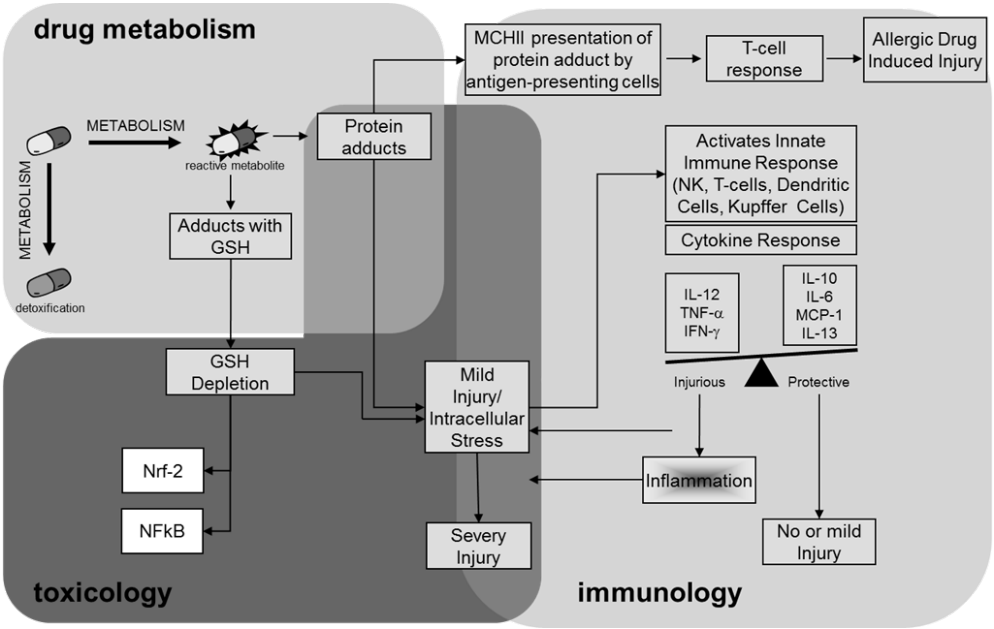


Figure 1. Mechanisms underlying idiosyncratic toxicity (TI-Pharma ADR project).

5) undergo adaptation or tolerance,

Drug that causes idiosyncratic liver failure in a small number of patients, usually causes much higher incidence (>100-fold) of increased transaminases, which are generally an indicator of liver injury (e.g. troglitazone). However, in most patients this increase in transaminases is only temporal and returns to normal despite continued treatment with the drug (*27*). Likewise, drugs that cause a lupus-like syndrome usually cause a much higher incidence of elevated antinuclear antibodies than the incidence of clinically evident autoimmunity (*28*). The mechanisms underlying adaptation is not yet discovered but appears to be common to various types of IDRs.

6) most commonly target the skin, blood elements and the liver,

The skin is probably the organ that is affected most frequently. Severe cutaneous reactions can occur, as Stevens-Johnson syndrome and toxic epidermal necrolysis. Besides this, three more common forms of idiosyncratic drug toxicity exist: anaphylactic reactions, blood dyscrasias (such as hemolytic anemia, agranulocytosis, idiosyncratic aplastic anemia) and hepatotoxicity, varying from asymptomatic increase in serum transaminases to fulminant hepatic necrosis (*29*).

7) may have a genetic basis.

Over the years, evidence has accumulated that many of IDRs depend on a combination of genetic factors. Genetic predispositions might explain the susceptibility of a small number of patients to a drug that is safe in the majority of individuals.

The genetic factors can be categorized into two broad groups: genetic factors determining pharmacokinetics (drug metabolism and transporter genes responsible for

drug disposition) and genetic factors determining pharmacodynamic responses such as genes coding for drug targets, immune response genes, cytokines, etc. (*30*). More about genetic factors will be described later under Risk factors for IDRs.

1.2.1. Theories regarding mechanisms of idiosyncratic reactions (IDRs)

Although for most drugs the exact mechanism of IDRs is not known, there are several hypotheses which are proposed to explain them (*3*, *12*, *14*, *17*, *31*). Most hypotheses have in common that the reactions have a metabolic basis involving drug metabolism polymorphisms and/or that they arise from a specific immune response to the drug or its metabolite(s) (*32*). An explanation for the major hypotheses is given here:

1. Metabolic mechanisms

The first theory involves genetic polymorphisms at the level of drug-metabolising enzymes. Drug metabolism can lead to the formation of RIs that can be toxic directly or by an immunological mechanism. A disbalance in rate of bioactivation of drug to RIs and rate of bioinactivation may lead to high degree of protein damage, resulting in toxicity. For example, individuals which are ultra-rapid metabolizers for CYP2D6 will be able to generate higher amounts of reactive intermediates if this enzyme is responsible for their formation. The enzymes involved in inactivation of these RIs, such as glutathione S-transferases (GSTs), quinone oxidoreductase (NQOI) or microsomal epoxide hydrolase (mEH), might also be deficient. The combination of these genetic factors might predispose individuals to IDRs (*33*).

2. Hapten hypothesis

Drugs are typically not immunogenic due to their low molecular weight. However, after bioactivarion to RIs and subsequent modification of proteins, the modified proteins can induce an immune response when present to the immune system (*34*).

The mechanism of penicilin-induced allergic reactions is consistent with the hapten hypothesis due to the chemical reactivity of its lactam-ring. An example of a drug that is not chemically reactive but form reactive metabolites that are acting as haptens is tienilic acid that causes hepatotoxicity. It is important to note that not all of the drugs that are metabolised to reactive metabolites are causing IDRs and covalent binding does not necessary lead to toxicity (*35*, *36*).

3. Danger hypothesis

According to the 'Danger hypothesis', covalent binding of RIs to protein as such is not sufficient to induce an immune response and cause IDRs. Simultaneous activation of the immune system by "danger signals" released from damaged or stressed cells is required. If a stress response (danger signal) is absent, immune tolerance will occur. Otherwise, cytotoxic T cells are activated, leading to up-regulation of co-stimulatory molecules and an immune response (Figure 2).

The reactive metabolites associated with drugs that cause IDRs could, besides their ability to function as haptens, cause cell damage and thereby generate a danger signal (*37*). An example is tienilic acid, known to cause idiosyncratic hepatotoxicity, for which it was shown that rats treated with one or two doses developed cellular stress in

hepatocytes (*38*). Tienilic acid actually induced changes in hepatic gene expression of the genes involved in oxidative stress, inflammation, cytotoxicity, and liver regeneration.

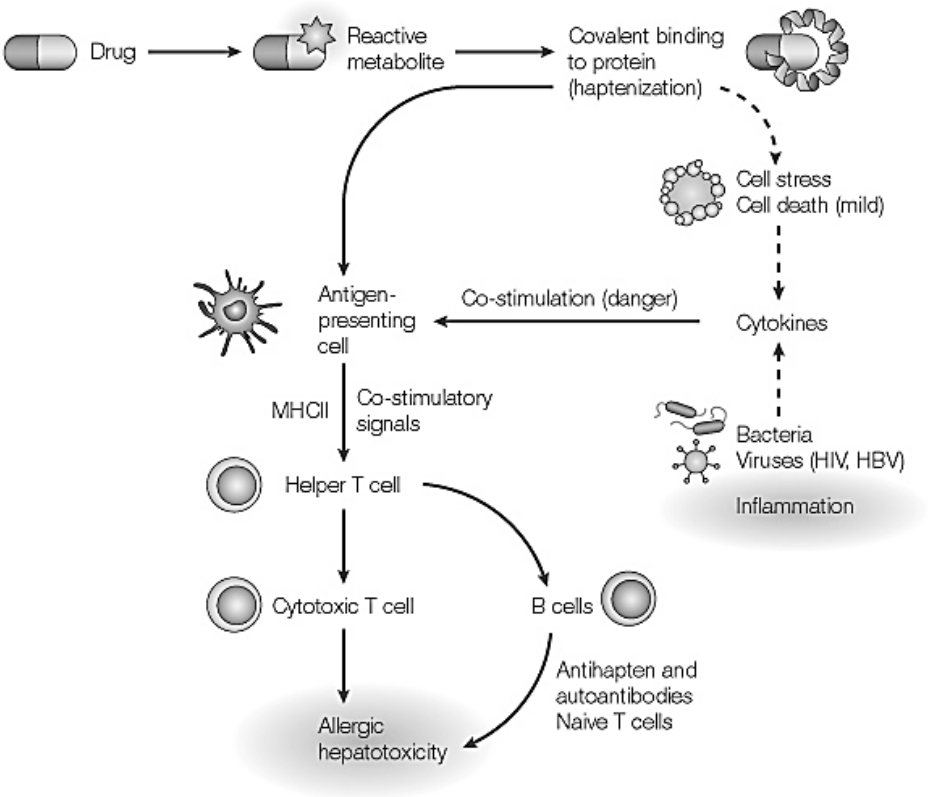


Figure 2. Danger hypothesis illustration for immune-mediated idiosyncratic hepatotoxicity. Adapted from (*39*).

4. Pharmacological interaction (PI) hypothesis

The PI theory is an alternative for the hapten hypothesis and is based on the observation that some drugs are not reactive toward proteins and still can cause immune response. According to this hypothesis some T-cells can recognize the parent drug, rather than modified peptides as proposed in the hapten hypothesis. The PI concept implies that chemically inert drugs can bind to an immune receptor in the absence of metabolism. Subsequently, this may serve as a scaffold for T-cell receptors, which upon interaction can induce an immunological reaction.

This hypothesis was merely based on the observations regarding sulfamethoxazole, but is also proposed to be applicable to lidocaine, celecoxib, mepivacain and carbamazepine (*14*, *40*).

5. Inflammagen hypothesis

This theory rationalizes the unpredictable nature of IDRs by the presence of inflammatory stress of a sufficient magnitude during drug therapy. This might temporarily lower the threshold for drug toxicity and consequently increases the susceptibility of the patient (*41*). In this situation, a drug dose that is in or even below the therapeutical range can still cause IDRs. Alternatively, the reversed mechanism is also proposed: a drug can increase the magnitude of a normally non-hazardous infection, resulting in idiosyncratic hepatotoxicity (*42*).

Combined administration of the inflammagen (lipopolysaccharide (LPS)) at non-hazardous doses and drugs related to idiosyncratic liver toxicity (i.e. diclofenac, trovafloxacin, ranitidine, chlorpromazine, amiodarone, halotane and sulindac) resulted in hepatotoxicity in rodents at doses that are otherwise non-toxic (*42*).

6. Multifactorial hypothesis

The multifactorial hypothesis is a rather general and encompassing one which is consistent with most of the other hypotheses mentioned above. This is rather a conceptual theory than a specific mechanism which assumes that IDRs result from multiple, discrete but necessary processes or conditions (*18*). Each factor has an independent probability of occurrence (Px), but all of them are required to precipitate an IDR. The product of the probability of these events is considered as the probability to develop IDRs:

Probability (IDR) = P1 x P2 x P3 x P4… x Pn

According to this hypothesis, an idiosyncratic reaction would only occur in an individual if all or significant part of critical factors occur within an appropriate and limited timeframe. These sub-processes include the exposure to a drug, physical-chemical properties of the drug (e.g. the ability to form reactive metabolites and the intrinsic risk for drug-drug interactions), genetic factors of the patient (e.g. polymorphisms in toxifying and detoxifying enzymes or transporters as well as genetic polymorphisms of the immune system, HLA genes), exposure to the environmental factors (e.g. inflammagens and metabolic inducers), diseases, and inflammation (*18*, *43*).

1.2.2. The role of drug metabolism in drug toxicity

Once entering the body, lipophilic drugs are converted enzymatically to more hydrophilic metabolite(s) to enable elimination from the body (Figure 3). This often occurs in a two-step process involving phase I reactions (oxidation, reduction, and hydrolysis) which introduce or expose polar functional groups, followed by phase II reactions which are conjugation reactions, such as glucuronidation, sulfation, acetylation and glutathione conjugation. This generally results in the formation of inactive compounds that are more water soluble and easily excreted by the kidneys. The enzymes involved are present at high levels in the liver, intestine, and kidney.

1.2.2.1. Bioactivation of drugs to reactive metabolites

Sometimes, metabolism can lead to the bioactivation of drugs and RIs can be formed (Figure 3). It is generally believed that formation of these reactive metabolite(s), rather than the parent drug itself, is responsible for the occurrence of IDRs (*44*). Both phase I and phase II metabolic enzymes can be involved in the generation of reactive species. Reactive metabolites can be broadly classified as electrophiles, oxidants, and free radicals (*45*). However, the correlation between drug bioactivation and occurrence of ADRs is not simple: not all drugs that can undergo bioactivation are associated with ADRs, and drug bioactivation is not always a mandatory step in drug toxicity (*3*).

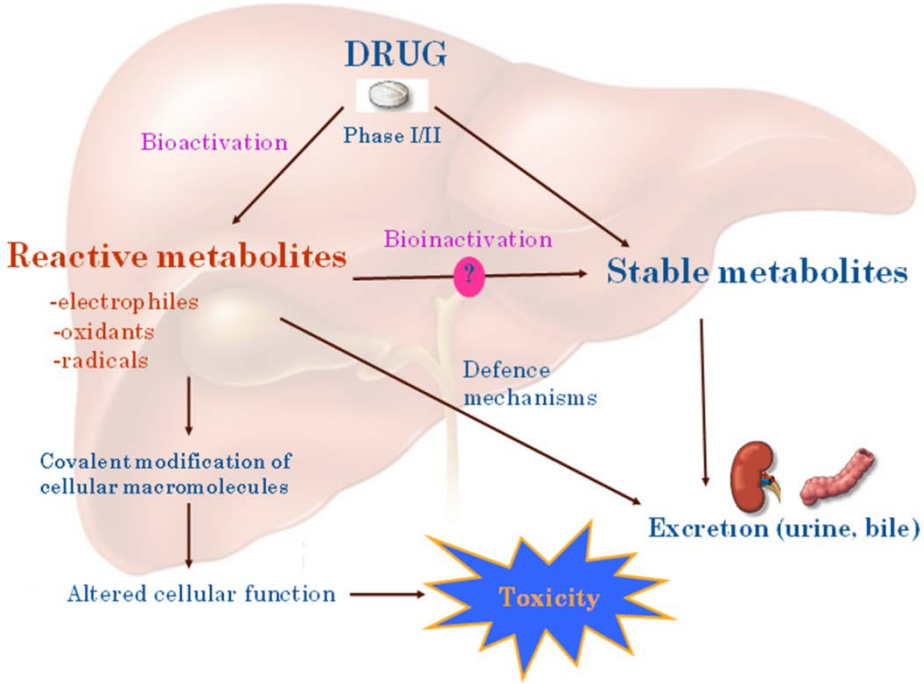


Figure 3. Relationship between drug metabolism and toxicity.
When reactive metabolites are not detoxified, interference with functional proteins, phospholipids and DNA can occur. If cellular repair is not sufficient, these interactions can result in drug toxicity.

The most important enzyme system involved in bioactivation of drugs is the family of cytochrome P450 enzymes (*46*). CYP enzymes have high concentrations in the liver but are also present in many other organs. Due to this, local bioactivation can lead to hepatotoxicity and/or organ-specific toxicity. Other oxidative enzymes can play an important role in extrahepatic drug bioactivation. For example, myeloperoxidase in white blood cells has been shown to bioactivate a wide range of drugs such as clozapine and aminopyrine. Prostaglandin H synthase may also be responsible for bioactivation in extrahepatic tissues. Phase II or conjugation enzymes (s.a. uridine glucuronosyl

transferases (UGTs)) may also be important in the bioactivation and can lead to toxicity (*47*). Many NSAIDs are bioactivated by UGT to reactive acyl glucuronides which are considered responsible for their hepatotoxicity.

The majority of reactive metabolites are electrophiles. Electrophiles are electron-deficient and can be with delocalized centers of low electron density (soft electrophiles) or with highly localized centers of low electron density (hard electrophiles) (*48*). The common reason for toxicity of electrophiles is their high reactivity towards cellular nucleophilic groups in proteins. They show reactivity towards glutathione, leading to thiol depletion, or cellular macromolecules (proteins, lipids, nucleic acids), resulting in reversible modification, irreversible adduct formation, and irreversible loss of activity. The structure and chemical nature of electrophiles are important factors determining the selectivity of their reactions with target nucleophilic macromolecules. Soft electrophiles (s.a. quinones or polarized double bounds) tend to react with endogenous soft nucleophiles, such as thiol residues in glutathione or proteins, while hard nucleophiles (s.a. epoxides or alkyl carbonium ions) tend to react with hard nucleophiles, such as the side-chain amino group of lysine residues in proteins or basic groups in DNA. Dependent on the target protein affected, covalent binding to proteins can ultimately initiate apoptosis/necrosis or activation of the host's immune system (often referred to as drug hypersensitivity or drug allergy). Chemical modification of DNA might lead to introduction of mutations that can lead to carcinogenicity (*49*).

The second class of reactive metabolites are oxidants that can oxidise cysteine thiols in GSH, leading to the GSSG formation, and/or in proteins, giving rise to protein disulfides and GSH-protein mixed disulfides. Increased oxidation of protein thiol groups has been reported in hepatocytes to play a causal role in the observed paracetamol mediated toxicity (*50*).

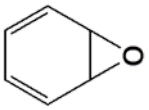
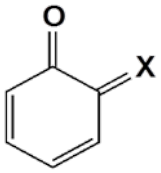
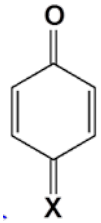
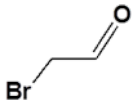
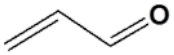
A third class of reactive metabolites, which can be linked to drug toxicity, are free radicals. Free radicals possess one or more unpaired electrons and they usually abstract a hydrogen atom from other molecules and can lead to lipid peroxidation, oxidative stress and subsequent toxicity (*51*).

1.2.2.2. Bioinactivation - detoxification of reactive metabolites

Organisms contain a large number of defence mechanisms to detoxify reactive intermediates that are formed during drug metabolism. The endogenous GSH and enzymes such as glutathione S-transferases, epoxide hydrolases, quinone reductases and alcohol/aldehyde dehydrogenases are the most important (Table 3). Catalase, glutathione peroxidases and superoxide dismutases are responsible for detoxification of reactive oxygen species (*45*). The nature and efficiency of the reactive metabolite bioinactivation is dependent on its chemical nature/reactivity, enzyme substrate-selectivity, tissue expression/localization and up-regulation of enzymes and co-factors.

Conjugation of reactive intermediates with the thiol group of the cysteine residue of GSH is one of the most important detoxifying reactions in the cell and protects macromolecules from electrophilic attack (*57*). GSH conjugates are formed either non-enzymatically or enzymatically by glutathione-S-transferases (GSTs). More detailed information on these polymorphic enzymes, their role in detoxification of reactive metabolites and correlation to ADRs will be given in Chapter 2.

Table 3. Enzymes involved in bioinactivation of reactive drug metabolites

Enzyme	Type of RIs	RI structure	Reference
Epoxide hydrolases	Epoxide		(*52*)
	Arene oxide		
Quinone reductases (NAD(P)H:Quinone Oxidoreductase 1)	Quinones Quinoids X=O, NH, CH_2		(*53*)
			
Alcohol/Aldehyde Dehydrogenases	α-halocarbonyls		(*54*)
	α,β-unsatured carbonyls		
Glutathione S-transferase	Broad range of electrophilic substrates		(*55*), (*56*)

After conjugation, GSH conjugates are efficiently catabolised to the corresponding cysteine-conjugates by γ-glutamyltranspeptidases (cleaves the glutamate residue) and cysteinyl-glycine dipeptidases that are present in liver and kidneys. After acetylation of the cysteine moiety via cysteine-S-conjugate N-acetyl transferase, the corresponding mercapturic acids are formed which are actively excreted in urine (*55*). This is a reversible reaction, and mercapturic acids can be deacetylated again by N-deacetylases. Other metabolic pathways of cysteine-S-conjugates also exist and some of the products are toxic.β-elimination of cysteine S-conjugates can result in the formation of thiol compounds, which can be very reactive themselves or which may rearrange to form other highly reactive intermediates (s.a. thioketene, thiirane, and thioacylhalids). These metabolic pathways are shown in Figure 4 together with corresponding enzymes

(*55*). Thioether conjugates are useful biomarkers for a reflection of internal exposure to electrophilic chemicals (*58*). Measurement of GSH conjugation-related metabolites in human urine can contribute considerably to understanding of the molecular mechanisms underlying bioactivation processes.

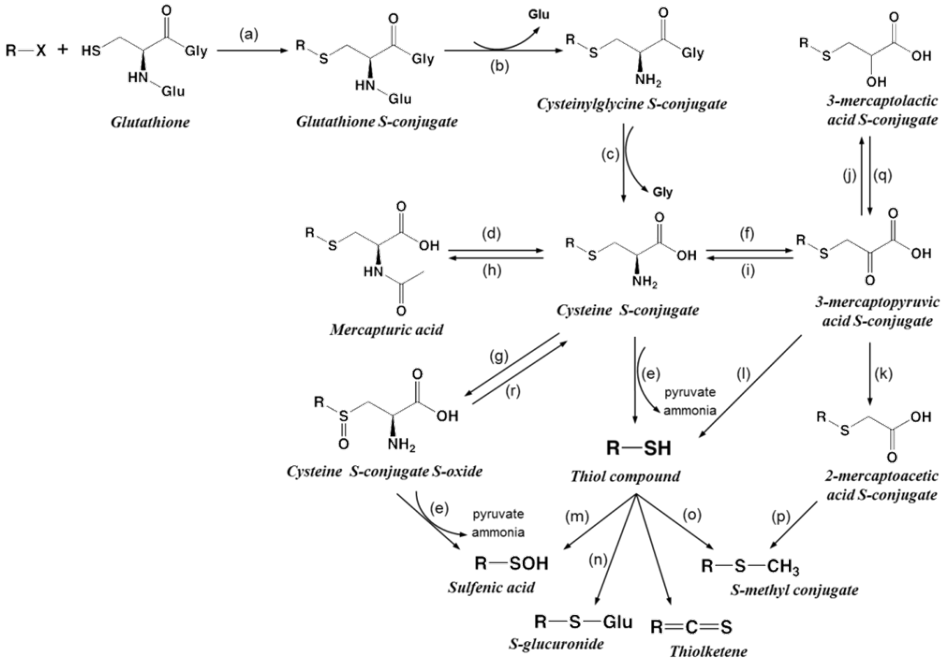


Figure 4. Formation and possible metabolic pathways of glutathione S-conjugates to mercapturic acids and other, partially protein-reactive glutathione-mediated metabolites (thiol, thioketene). Adapted from (*55*).

Steps are catalyzed by: (a) glutathione S-transferase; (b) γ-glutamyltranspeptidase; (c) dipeptidases: cysteineglycine dipeptidase and aminopeptidase M; (d) cysteine conjugate N-acetyltransferase; (e) cysteine conjugate β-lyase; (f) cysteine conjugate transaminase and L-amino acid oxidase; (g) cysteine conjugate S-oxidase; (h) N-deacetylase; (i) transaminases; (j) 3-mercaptopyruvic acid S-conjugate reductase; (k) decarboxylase; (l) enzyme not yet characterized; (m) S-oxygenase; (n) uridine diphosphate-glucuronyl transferase; (o) S-methyl transferase; (p) decarboxylase; (q) 3-mercaptolactic acid S-conjugate oxidase; (r) sulfoxide reductase.

1.2.3. Risk factors for occurrence of IDRs

Idiosyncratic toxicity is one of the least understood issues in toxicology. Understanding of the individual risk factors is necessary. These toxicities depend on several drug- and patient- related risk factors that are unique to individual (Figure 5). Drug-related risk factors include metabolism, bioactivation and covalent binding, and inhibition of key cell functions while patient-related risk factors include underlying disease, age, gender, comedications, nutritional status, activation of the innate immune

system, physical activity, and genetic predispositions (*2*). IDRs occur only when several risk factors converge.

1.2.3.1. Exposure to drugs with idiosyncratic toxicity

IDRs do not show a clear dose dependency. Some patients with high doses have no toxicity whereas other patients have severe toxicity at a low dose. However, some drugs have markedly higher potential to cause either dose dependent toxicity or IDRs than other drugs, and overall this is unrelated to overt pharmacological activity or clinical efficacy. Examples include neuroleptics (rank order of IDR risk clozapine > olanzapine, quetiapine); anxiolytics (IDR risk alpidem > zolpidem), antidiabetics (IDR risk carutamide > tolbutamide) and volatile anesthetics (rank order halothane > enflurane > isoflurane > desflurane) (*59*).

Drug concentration in the plasma and/or target organ that are important for the critical exposure could be affected by genetic factors (e.g. level of metabolic enzymes) and environmental factors (e.g. concomitant food and drug intake).

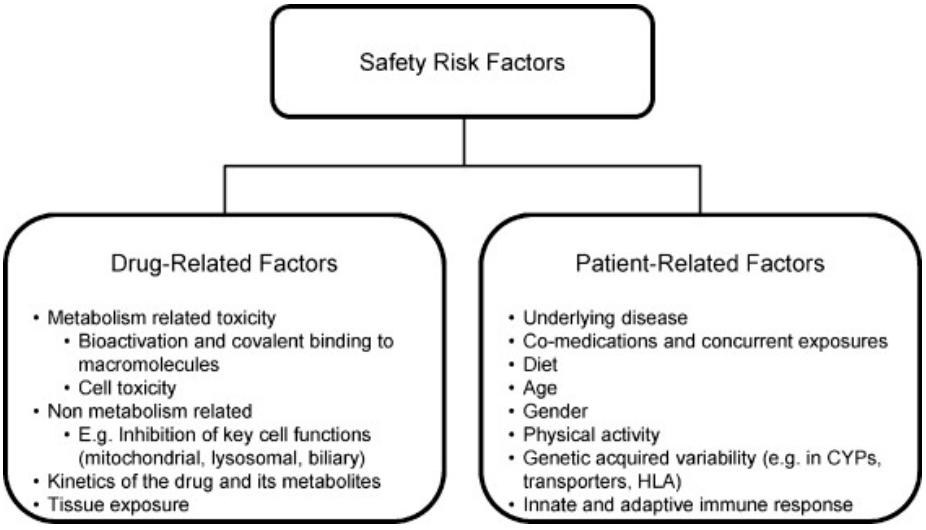


Figure 5. Risk factors for occurrence of IDRs can be drug- and patient-dependent.
In either case, they can be apparent in the phenotype (e.g. histopathology in animal models or obesity in the patient) or occult (e.g. drug-adduct formation in animal models that does not present a phenotype, or a genetic predisposition in the patient). Adapted from (*60*).

1.2.3.2. Chemistry of the drug and its bioactivation

Although more relevant for type C ADRs, idiosyncratic toxicity is also considered to be caused by the presence of reactive functional groups of the drug, or most likely its

metabolite (*61*). Definition of chemical properties which may predispose to idiosyncratic drug toxicity would help preventing the marketing of high risk drugs (*18*).

Formation of reactive metabolites and covalent binding of reactive metabolites to proteins is common property of drugs causing IDRs. The major enzymes involved in reactive/toxic metabolite generation are P450s (*62*, *63*). The use of toxicophores within new therapeutic agents should be limited as an approach to minimize drug-induced toxicity (*64*).

The result of covalent binding of RIs of drugs to proteins may be inhibition of critical cell processes introducing antigenicity. For example, valproate can cause fatal liver toxicity in rare cases due to the inhibition of mitochondrial function which is caused by the reactive 4-en- and 2,4-diene-valproate metabolites (*65*).

1.2.3.3. Environmental and patient-related risk factors

Pre-existing diseases (e.g. hepatitis, diabetes) are considered risk factors for developing IDRs. Patients with HIV on antiretroviral drug therapy have an increased risk for idiosyncratic hepatotoxicity when they are co-infected with chronic hepatitis B virus and C virus (*66*, *67*). Another established relationship between pre-existing disease and IDRs is the induction of severe hepatic injury and encephalopathy by salicylates in patients with Reye's syndrome (*68*).

Immune activation has also a significant role in IDRs development. Internal exposure to endotoxin or LPS during drug treatment can produce liver toxicity in animals treated with otherwise tolerated levels of drugs (*42*). For example, a nontoxic dose of ranitidine, diclofenac, and sulindac developed liver toxicity in rats treated with a non-hepatotoxic dose of LPS (*69*–*71*). Ketoconazole and clozapine exhibited synergistic toxicity with LPS in both mice and human liver slices (*72*, *73*).

A large number of drugs which cause IDRs have clinically significant interactions with co-administered drugs and drug-drug interactions are therefore widely studied (*74*). As CYP3A4 is the major enzyme involved in drug metabolism, inhibition or activation of this enzyme is often the cause of drug-drug interactions (*75*). Also, nutritional supplements and herbal remedies are important and these, together with co-administrated drugs should be taken into consideration when examining case studies for potential ADRs.

Diet can influence on development of IDRs in two ways: drug-food interactions and overall nutritional status. For example, CYP3A4 activity is inhibited by furanocoumarins in grapefruit juice and can cause a large increase in exposure to drugs that are metabolised by this enzyme (*76*). On the other hand, it has been shown that fasting and malnutrition are enhancing the toxicity of APAP (type C ADR), possibly by depletion of hepatic glutathione (*77*).

In general, the susceptibility to IDRs is age and gender dependent. For a number of the drugs, such as flucloxacillin, it is described that adults have an increased risk for hepatic injury (*78*). This might be due to decreased clearance, reduced hepatic blood flow, and drug-drug interactions as the average number of taken drugs increases with age. Young children appear to be more susceptible to valproate-linked IDRs due to age-

dependent differences in drug metabolism enzymes that are involved in formation of toxic metabolite (*79*).

1.2.3.4. Genetic factors

Genetic factors also play an important role in individual susceptibility to IDRs. Identification of the predisposing genotypes sensitive to IDRs may improve drug therapy by facilitating pre-screening of carriers for specific genetic biomarkers. This might help avoiding that individuals predisposed to IDRs are administered with such drugs.

Polymorphism or mutations in drug metabolizing enzyme and drug transporter genes which leads to unusual drug accumulation in the target organ are common mechanisms underlying severe ADRs (*80*, *81*). As P450s are the major phase I drug-metabolising enzymes, the influence of genetic polymorphisms of CYP2C9, CYP2C19, and CYP2D6 is well studied. These genetically determined P450s are involved in the metabolism of 20–30% of clinically used drugs (*80*, *82–84*). Patients with CYP2C9*2 and CYP2C9*3 genetic variations, which encode for less active CYP2C9, are at an increased risk of bleeding with warfarin anticoagulant treatment (*80*). Some other examples are given in Table 4.

Genetic polymorphisms of several phase II enzymes are also important and lead to serious effects if doses are not adjusted based on the patient genotype (*80*). For example, mutations in the gene encoding thiopurine S-methyltransferase (TPMP) predispose patients that are on the treatment with thiopurine drugs to severe hematologic toxicity. Also, patients homozygous for the UGT1A1*28 allele with reduced UGT1A1 activity, are at increased risk of diarrhoea and/or leukopenia with irinotecan anticancer therapy. Polymorphism of the N-acetyltransferase 2 (NAT2) gene differentiates slow acetylators as being more susceptible to toxicity associated with aniline-based drugs such as isoniazid, sulfamethoxazole, dapsone and procainamide (*86–88*). Polymorphisms of GSTs and their consequences will be discussed in more details in Chapter 2.

There is increased evidence that drug induced hypersensitivity reactions are determined by genetic polymorphism of human lymphocyte antigen (HLA). There are reports of a relationship between adverse reactions and HLA polymorphisms, particularly immune-mediated hypersensitivity reactions in the skin upon exposure to abacavir and carbamazepine (*30*). Abacavir-induced hypersensitivity reactions are strongly associated (odds ratio 960) with the HLA-B*5701 genotype (Figure 6), and to a lesser degree, a haplotypic Hsp70-Hom variant (*89*). A strong association (odds ratio 895) between HLA-B*1502 and carbamazepine-induced toxic epidermal necrolysis in Han Chinese is described (*90*) although the same association was not observed in a European population (*91*). Also, an association exist between HLA-B*5801 and allopurinol-induced toxic epidermal necrolysis (*92*).

Drugs can also cause changes in gene expression by epigenetic mechanisms that do not involve changes in the DNA sequence. Epigenetic effects, methylation of DNA and histone deacetylation, may be responsible for the occurrence of IDRs (*93*).

Table 4. Relationship between polymorphic drug metabolizing enzymes and certain ADRs

Enzyme	Drug	Adverse reaction
Phase I (P450) enzymes		
CYP1A2	Typical antipsychotic	Tardive dyskinesia
CYP2B6	Methadone	Higher post-mortem concentrations
	Efavirenz	Neuropsychological toxicity
CYP2C9	Warfarin	Haemorrhage
	Tolbutamide	Hypoglycaemia
	Phenytoin	Phenytoin toxicity
	NSAIDs	Gastrointestinal bleedings
	S-acenocoumarol	Serious bleeding events
CYP2C19	Mephenytoin	Neurotoxicity
	Diazepam	Prolonged sedation
	Clopidogrel	Increased risk of bleeding
CYP2D6	Antiarrhythmic	Arrhythmias
	β-Blockers	Bradycardia
	Tricyclic antidepressant	Confusion
	Opioids	Dependence
	Metoclopramide	Acute dystonic reaction
	Codeine	Respiratory depression
		Fatal morphine toxicity in neonate
	Tramadol	Respiratory depression
	Phenformin	Lactic acidosis
	Perhexiline	Hepatotoxicity
CYP3A4	Anti-leukemic agents	Treatment-related leukaemia
CYP3A5	Tacrolimus	Nephrotoxicity in renal transplant recipients
Phase II enzymes		
Plasma butyrylcholinesterase	Succinylcholine	Prolonged apnoea
N-acetyltransferase	Sulfonamides	Hypersensitivity
	Amonafide	Myelotoxicity
	Procainamide, hydralazine, isoniazid	System lupus erythematosus
Thiopurine methyltransferase	6-Mercaptopurine, azathioprine	Myelotoxicity, treatment-related second tumours
Dihydropyrimidine dehydrogenase	5-Fluorouracil	Myelotoxicity
UDP glucuronosyl transferase 1A1	Irinotecan	Diarrhoea, myelosuppresion

Table adapted from (*81*, *84*, *85*).

1.3. Methods for the generation and detection of RIs

Despite all the research, drug-induced toxicities remain a serious problem in drug therapy and special attention is needed for safety assessment at the drug discovery stage. Although much of the safety-related attrition occurs in the course of preclinical safety evaluation, some adverse events fail to manifest in animals. Current hypotheses based on retrospective studies suggest that metabolic activation of drugs and formation of RMs is an initial step in many drug-induced adverse events, such as direct damage to target organs and immune-mediated toxicity. It is still not possible to accurately predict the potential for toxicity of a compound that has been shown to undergo metabolic activation. A combination of *in vitro* and/or *in vivo* methods is used in many pharmaceutical companies to profile novel drug candidates for their potential to form reactive intermediates and to assess their potential to cause ADRs. Predicting ADRs remains

challenging but these combined approaches may identify drug candidates showing unacceptable safety profiles, as shown in Figure 7 (*3*, *95*).

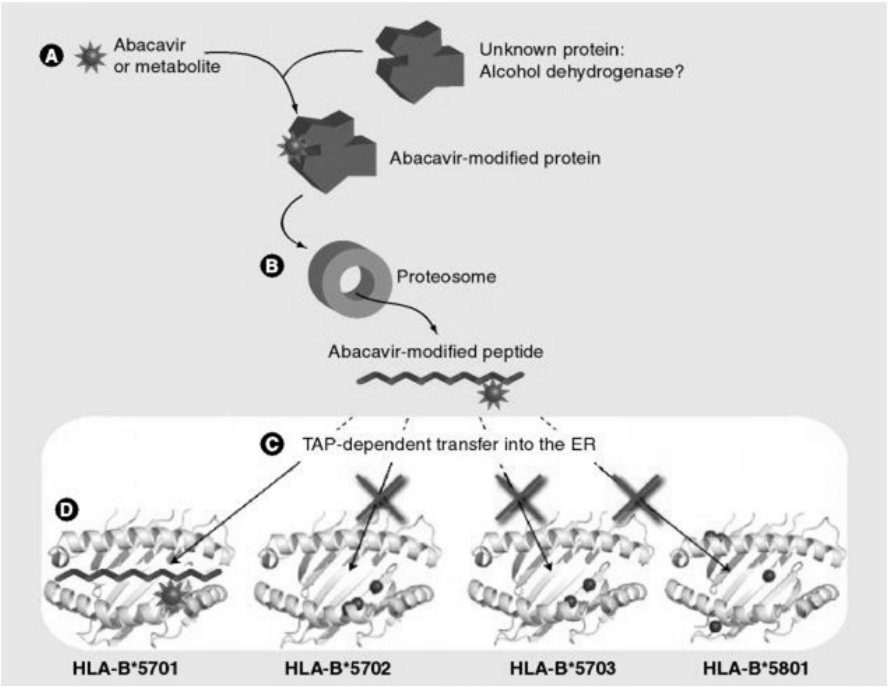


Figure 6. Abacavir-induced hypersensitivity
HLA-B*5701 restricted presentation of an immunogenic ligand. Adapted from (*94*).

These decision schemes depend on the phase of drug research and the available input information. There is no universal scheme and individual research projects have to develop their own decision criteria on how to link experiments that are carried out to study reactive metabolites and toxicity outcomes in humans. In early research during drug design and optimisation, the 'avoidance strategy' is applied and medicinal chemistry efforts are such that compound design is avoiding RI formation while still maintaining acceptable pharmacological properties (Figure 7a). Anticipated daily dose and the urgency of the clinical need for a new treatment are also considered in this stage. In late preclinical or early clinical research, covalent binding (CB) are used for decision-making, for example if the extensive clinical safety database is needed (Figure 7b). Total dose, indication and existing preclinical toxicology data are taken into consideration in this decision scheme. In late preclinical or clinical phases, weight-of-evidence approaches where multiple data and end points that have some relationship to clinical safety (in vitro CB data, metabolic routes, animal tissue distribution data, in vitro safety data, etc.) are considered simultaneously, together with data on RI formation. The total sum of these data is considered in decision-making.

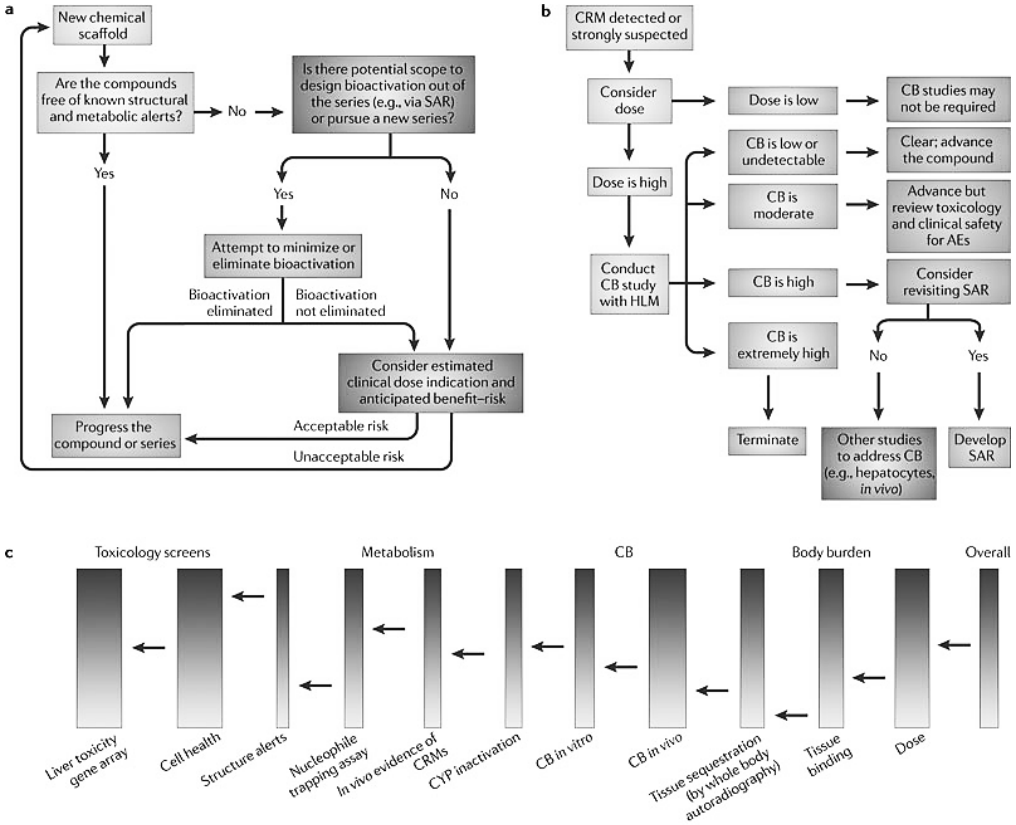


Figure 7. Decision schemes for handling bioactivation information in various stages of drug research: a) RM data in early research during drug design and optimization, b) CB data in late preclinical or early clinical research, and c) Weight-of-evidence approaches in late preclinical or clinical phases of research consider multiple data end points together with data on RM formation. Adapted from (*3*).
*AE: adverse events; CB: covalent binding; CRM: chemically reactive metabolite; RM: reactive metabolite, SAR: structure–activity relationship.

Methods for screening drug candidates for RI generation, identification of problematic compounds, and steps for minimizing the potential of bioactivation are developed in pharmaceutical companies. LC–MS/MS techniques are playing a dominant role in the detection, identification and quantification of reactive metabolites for both, *in vitro* and *in vivo* assessment during the drug discovery and development processes (Table 5) and will be discussed further in more details.

1.3.1. In vitro assessment of reactive metabolites formation

Since formation of reactive metabolites play a crucial role in many types of drug toxicity, their quantification is an important factor in drug development. Because of their instability and reactive nature, and the relatively small quantities produced during

metabolism, sensitive and specific approaches are required for their detection. The methods for the detection of RIs formation and testing the interaction potential with cellular macromolecules can be categorized into two types: (1) trapping methods using unlabelled drugs and nucleophilic trapping agents such as GSH and (2) covalent binding methods using radiolabeled drugs (*96*).

1.3.1.1. Trapping experiments for the detection of electrophilic metabolites

The most common strategy for the detection of electrophilic reactive metabolites is the use of a trapping agent, most commonly GSH, followed by liquid chromatography-mass spectrometry (LC-MS). Nuclear magnetic resonance (NMR) analysis subsequently can reveal structural information of the bound electrophile which gives information on the bioactivating pathway involved (*96*). The advantage of trapping with GSH is its applicability in high throughput screening (HTS). However, it should always be considered that the information obtained is mainly qualitative and not necessarily predictive for toxicity on its own. This is because these studies in fact show the binding of reactive metabolites to a major detoxification element (GSH) rather than critical proteins. Nevertheless, trapping studies with GSH do provide qualitative insight in the production of reactive electrophilic metabolites, and allows structural optimization of drug candidates to minimize this problem.

General trapping experiments consist of three components: the formation of the reactive metabolites, the trapping of electrophiles and the subsequent analytical procedures.

1. Bioactivation (formation of reactive intermediates)

For the *in vitro* generation of the electrophilic metabolites, incubations with human or rat liver microsomes are commonly performed in the presence of the cytochrome P450 co-factor NADPH with or without a regenerating system. Human liver microsomes (HLM) still account for the most popular *in vitro* model, providing an affordable way to give a good indication of the CYP and UGT metabolic profile (*98*). The activity of HLMs can vary substantially between individuals. This is solved by the application of commercially available pooled microsomes resulting in a representative enzyme activity (*99*). Individual human liver microsomes can also be used to screen for the inter-individual variability in the biotransformation of a drug. It is also possible to identify the critical CYPs involved in the biotransformation of the drug using individual HLMs by correlating the enzyme activity of a particular CYP, using a bank of human donors, to the metabolism of the drug. Although the most popular, liver microsomes do not completely represent *in vivo* situation. Results cannot be used for quantitative estimations of *in vivo* human biotransformation, because CYPs and UGTs are present in the microsomal fraction but there is no competition with other enzymes (e.g. NAT2, GST, and ST).

To study the contribution of a single metabolic enzyme to the bioactivation pathway of the compound under investigation and production of reactive electrophiles, recombinant human CYPs and UGTs in supersomes are used. All common human CYPs,

co-expressed with NADPH-cytochrome P450 reductase and optionally cytochrome b5, and UGTs are offered in supersomes. The different genotypes of several CYP isozymes (e.g. CYP2C9*1, CYP2C9*2, and CYP2C9*3) are also commercially available which allows studies on the influence of different polymorphisms on the drug bioactivation.

Table 5. Role of LC–MS/MS techniques in the detection, characterization and quantification of reactive metabolites in drug discovery and development.

Investigation of bioactivation of drug candidates	LC–MS/MS approach
Screening for *in vitro* reactive metabolites in early discovery	Screening and characterization of reactive metabolites in trapping experiments LC–MS
Quantification of *in vitro* reactive metabolites from early to late discovery	Quantification of reactive metabolites in trapping experiments LC–MS/UV LC–MS LC–MS/fluorescence detection LC–MS/radiodetection
In vivo evaluation of bioactivation in toxicology species at the preclinical development stage	Non-radiolabeled drug disposition study Analysis of GSH adducts in bile by LC–MS/UV Screening and quantification of mercapturic acids in urine by LC–MS Monitoring other biomarkers of reactive metabolites by LC–MS Radiolabeled ADME study Analysis of small thiol-adducts and biomarkers by LC–MS/radiodetection
In vivo evaluation of bioactivation in humans at the clinical development stage	Radiolabeled ADME study Analysis of small thiol-adducts by LC–MS/radiodetection Non-radiolabeled clinical study Monitoring of mercapturic acids in urine and other biomarkers by LC–MS

Table adapted from (*97*).

Considering the fact that non-microsomal enzymes can also participate in bioactivation, different *in vitro* models exist that represent the *in vivo* situation better or support the activity of non-CYP enzymes, e.g. liver cytosol, liver S-9 fractions, hepatocytes, different liver derived cell lines, liver slices, isolated perfused livers, neutrophils (*98*, *100*). The liver cytosolic fraction contains the soluble phase II enzymes, e.g., N-acetyl transferases, glutathione S-transferases, and sulfotransferases, while the liver S9 fraction contains both microsomal and cytosolic fractions, which provides a more complete representation of the metabolic profile, as they contain both phase I and phase II activity. However, the overall enzyme activity is lower in the S9 fraction compared to microsomes or cytosol, which may leave some metabolites unnoticed (*98*).

Systems such as fresh or cryopreserved hepatocytes, primary cell cultures, liver slices and whole, perfused livers are well-established models used for investigative work. Most cell lines derived from hepatoma lose expression of the major phase I metabolizing enzymes, thus limiting the *in vitro* identification of metabolism-mediated drug toxicity. CYP450-transfected cell lines have also been shown to metabolize specific substrates and generate metabolite profiles qualitatively similar to those produced by isolated human microsome and supersome preparations. Lately, the incubation of liver slices in nutrient-enriched media offers a powerful tool to study biotransformation *in vitro* (*72*). One of the advantages of liver slices over hepatocytes is that all the different cell types are present,

including their metabolizing enzymes, in their natural tissue-matrix configuration. Although an isolated perfused liver represent the best the *in vivo* situation, this model is not widely used. There are no human livers available for such studies and animal livers are not always the correct model for human drug biotransformation. This is a useful model only in cases in which bile secretion is of importance or when validation of other *in vitro* methods is required.

An alternative for the use of human enzyme systems are bioengineered mutants of the highly active bacterial enzyme cytochrome P450 BM3 (CYP102A1) from Bacillus megaterium. Over the past years, increasing attention has focused on developing bacterial P450 variants with human-like metabolic activity to overcome limiting features of human P450s, such as low catalytic activity, instability, membrane-bound structure. Advantages of the bacterial P450 in comparison to human P450 are their stability, higher catalytic activity and their ability to be engineered towards the production of human-relevant metabolites (*101*). Recently, several P450 BM3 mutants were obtained by a combination of random- and site-directed mutagenesis, and used to metabolize drugs (*102*, *103*). It is also shown that the use of this drug-metabolizing mutants can be advantageous for drug bioactivation because of the generation of high quantities of human relevant reactive electrophilic drug metabolites (*104*, *105*). This concept will be further used in this thesis.

Electrochemistry has also been used to generate electrophilic reactive metabolites in the early stage of drug development process. This purely instrumental technique, with a simple set-up, enables generating reactive metabolite(s) in a "cleaner" medium, which prevents the further laborious isolation and purification steps in case of protein binding experiments. The potency of electrochemistry in trapping experiments is well established for clozapine, diclofenac, troglitazone by Madsen et al. (*106*–*108*). However, although electrochemistry in some cases has been shown to produce human-relevant oxidation products, it cannot mimic all P450-dependent oxygenation reactions, such as hydroxylation of unactivated sp^3 CH-groups, which are frequently occurring and synthetically challenging (*109*). Moreover, the regioselectivity of drug oxidation is often governed by the topology of the active site of P450s (or other enzymes), rather than the oxidation energies of the different positions, that limits practical application of this approach due to low physiological relevance of electrochemically generated metabolites.

2. Electrophile trapping

For the detection of electrophilic metabolites, a nucleophilic trapping agent has to be included in the incubations containing the drug of interest with the bioactivating system. If the conjugate formed of the reactive metabolite and the trapping agent is sufficiently stable, it is detectable in subsequent NMR or LC-MS analysis (Figure 8). A variety of trapping agents are used.

Physiologically relevant tripeptide glutathione (GSH) is the most frequently used trapping agent. GSH reacts via the thiol group in the cysteine with soft electrophiles like α,β-unsaturated ketones, quinones and quinone imines, as well as with medium hard electrophiles like epoxides, aryl halides and nitrenium ions (*63*). Reactions between reactive intermediates and GSH can be chemical or catalysed by glutathione S-transferases. The corresponding GSH adducts are typically analyzed and characterized by

liquid-chromatography mass spectrometry (LC-MS). GSH adducts show a characteristic fragmentation pattern consisting of losses of 75 Da (glycine) and/or 129 Da (γ-glutamate) corresponding to the loss of the peptidic side chains of GSH and this has been exploited to develop sensitive and selective MS-based methodologies for the screening of GSH adducts; such as neutral loss (NL), precursor ion (PI) and multiple reaction monitoring (MRM) scanning techniques (*110*–*112*). Reported limitations according trapping experiments with GSH are the low capacity to trap hard electrophiles and the instability of some GSH conjugates (*3*). Moreover, the analysis of GSH conjugates with LC-MS/MS has a limited selectivity since the characteristic neutral loss of 129 Da is also observed for some endogenous compounds that are present in biological matrices (*96*).

To improve the selectivity and reliability and to make detection of electrophiles easier and more sensitive, several GSH analogues have been developed, including fluorescent-labelled analogues, radiolabeled analogues and stable isotope labelled GSH. An overview of the different glutathione-based trapping agents and methods are depicted in Table 6 (*114*–*121*). Unfortunately, these analogues are also exclusively reactive toward soft and medium hard electrophiles.

Next to GSH, N-acetyl cysteine (NAC) can be used as alternative trapping agent. This adduct are also applicable as standards for analysis of *in vivo* samples (e.g. urine) since GSH adducts are *in vivo* degraded, ultimately resulting in mercapturic acids. Another advantage is that NAC conjugates may have the potential to generate more fragments from cleavages of the drug moiety providing critical information on structures of the reactive metabolites (*122*). However, the trapping might be less efficient, especially for conjugations of which the formation is catalyzed by GSTs (*63*).

All these trapping agents react efficiently with soft and medium hard electrophiles. The trapping of hard electrophiles, like iminium ions, ketons and aldehydes requires hard nucleophilic trapping reagents (*118*). The cyanide anion (CN-) is often used for the trapping of iminium ions, while amines (methoxylamine and semicarbazide) are used for the trapping of ketons and aldehydes. Cyanide adducts also show a typical fragmentation pattern allowing the development of sensitive and selective MS methodologies such as neutral loss scanning (27 Da) (*63*, *123*). Also, the sensitivity and specificity of the trapping experiments is enhanced when stable isotope labelling ($^{13}C^{15}N^-$) is applied (*63*, *123*).

Bifunctional trapping agents that combine the properties of hard and soft nucleophilic trapping agents have been developed to capture both hard and soft electrophilic metabolites simultaneously. γ-Glutamyl-cysteine-lysine (γ-GSK) contains both a soft nucleophilic moiety (cysteine) and a hard nucleophilic residue (lysine) and the obtained results, using furans as model compounds, suggested efficient trapping of both soft and hard reactive metabolites simultaneously and thus, these two types of electrophiles can be simultaneously analyzed by neutral loss MS scan, which is a significant improvement compared to the GSH trapping approach (*124*).

3. Analytical procedure/strategies (methods) to detect formed conjugates

The last step in the trapping experiments is the detection of the formed conjugates. Structural information can be obtained by the combination of LC-MS/MS and NMR.

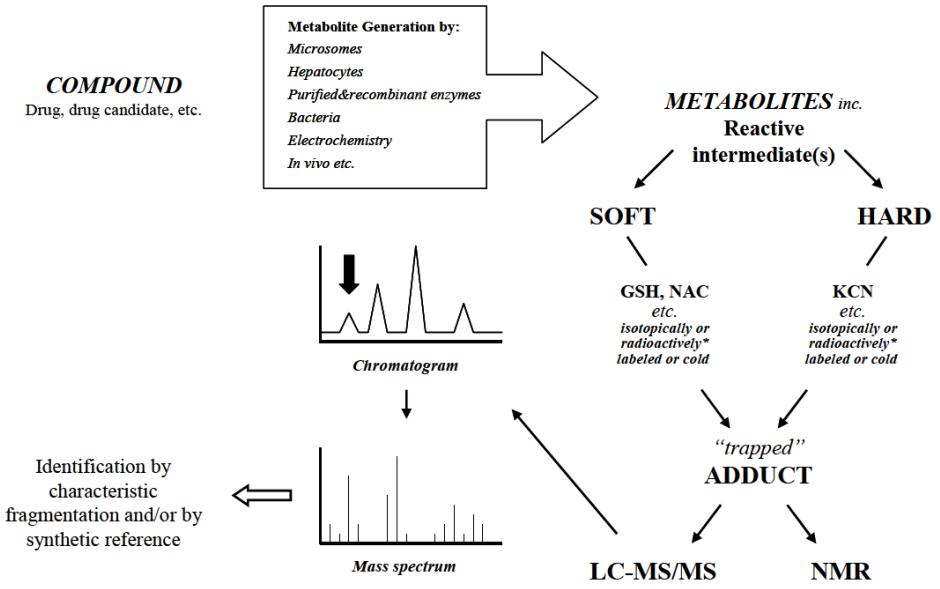


Figure 8. Graphic representation of generation and characterization of RIs in trapping experiments. *Radioactively-labelled parent compound or trapping agent are applied in in vivo studies, and determination of trapped adduct(s) is subjected to other techniquessuch as scintillation counting. Adapted from (*113*).

Because of its high sensitivity, LC-MS is the most often used analytical method. As mentioned, the most common MS/MS mode for screening of GSH conjugates is the neutral loss scan of 129 in combination with positive ionization. However, some conjugates like aliphatic and benzylic thioether conjugates may escape detection by 129 Da constant neutral loss scanning (*111*, *125*). As an alternative approach, negative ionization-precursor ion scan for the collision induced anion with an m/z ratio of 272 (GSH minus the H_2S moiety) is used. All GSH adducts of acetaminophen and diclofenac were detected with the neutral loss approach, with the additional of previously unidentified adduct for troglitazone. However, the positive ionization-neutral loss approach provides more structural information (*126*).

Even though LC-MS/MS is the most widely used approach for the analysis of drug metabolism, in most situations the entire molecular structure can not be unravelled. In these situations the additional use of NMR is required. NMR is less sensitive and requires high purity of the sample and high quantities of the metabolite (at least 1 μg and even 1 mg for high quality spectra). However, recent improvements of NMR sensitivity, NMR magnets have become stronger (today ca. 600–800 MHz is standard), made NMR a useful tool for the analysis of drug metabolites as absolute amounts of sample required for analysis have become smaller (ca. 100 mM in biofluids or tissue extracts (*127*).

Application of LC–NMR hyphenated analytical system mitigates sample isolation and purification required when using NMR alone (*128*).

Covalent binding to critical proteins is often considered the first step in the process ultimately leading to toxicity. For this reason it is important to screen compounds for their capability to form protein adducts. Trapping experiments and protein binding analysis are often complementary and together provide valuable information (*96*). However, it is still difficult to define what level of covalent binding could be considered as safe, and which level might cause toxicity (*63*). Based on data from a wide range of toxicants, a threshold of 50 pmol/mg microsomal protein was proposed previously (*63*). However, several other considerations, like the daily drug dose or concentration in blood and liver, should be considered for the final decision of how much covalent binding is permissible.

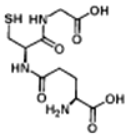
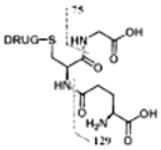
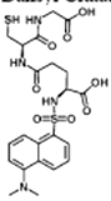
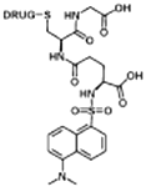
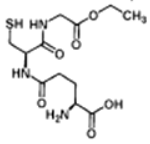
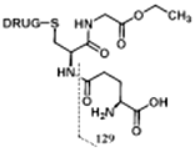
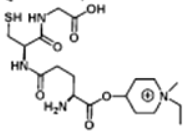
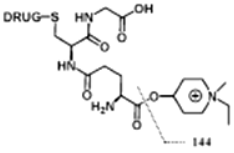
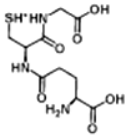
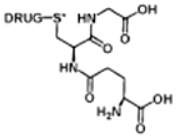
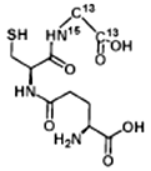
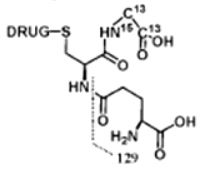
1.3.1.2. Covalent binding analysis and detection of protein adducts

Recent studies examining P450-mediated covalent binding of 18 drugs (nine hepatotoxins and nine non-hepatotoxins) to liver microsomes, S-9, and/or hepatocytes showed no correlation between extent of covalent binding and toxicity (*35*, *36*). A number of examples that display a high degree of protein covalent modification, yet are not associated with a significant incidence of toxicity. This phenomenon is evident with the acetaminophen regioisomer, 3'-hydroxyacetanilide (AMAP), which forms GSH- and protein-reactive metabolites (*129*). However, AMAP does not exhibit the hepatotoxicity *in vivo* in mice observed with acetaminophen although level of covalent binding was comparable (*130*). Ex vivo, in mouse, rat and human precision-cut liver slices incubations, a marked species differences in APAP and AMAP toxicity were observed. It was shown that AMAP is toxic in rat and human liver slices and cannot be considered as non-toxic isomer of APAP (*131*).

Despite this, *in vitro* studies with radiolabeled drugs remain the golden standard to quantify covalent binding of reactive metabolites. The production of radiolabeled analogues, of which ^{3}H and ^{14}C are the most commonly used, is expensive and time consuming, and is usually only available in the late stages of drug development. Covalent binding studies, in contrast to trapping experiments, provide quantitative information about the metabolic activation. They do not offer structural information regarding the metabolites (*96*).

Nevertheless, it is important for the mechanistic understanding of the toxicity to identify the protein targets that are covalently modified by reactive metabolites (*117*). Mass spectrometry of intact protein or tryptic digests can reveal the presence of adducts on known proteins (*132*). The Hanzlik group uses isotopic variants of "model" toxic compounds, such as bromobenzene, thioacetamide and thiobenzamide, as probes of metabolism and covalent binding to proteins in vivo, in isolated hepatocytes and in enzymatic systems in vitro. Adducted proteins are localised on 2D gels by autoradiography and identified, along with their adducts, by modern methods of proteomic analysis based on HPLC-MS/MS. A large database of more than 300 proteins targeted by diverse reactive metabolites and this information is publicly available (*133*).

Table 6. Glutathione-based trapping agents and screening methods for RIs

Trapping agent	Trapped Drug-conjugate	Detection	Characteristics	References
Glutathione 		MS	+ Standard methodology + Structural information - Sensitivity and selectivity dependent on MS instrument	(*111*, *112*)
Dansyl Glutathione 		FLD MS	+ Quantitative + Fluorescence detection + Not MS-dependent + Structural information	(*114*)
Glutathione Ethyl Esther 		MS	+ Extraction possible + Increased sensitivity + Structural information - Prolonged retention time	(*115*)
Quaternary Ammonium Glutathione 		MS	+ Fixed positive charge + Semi-quantitative + Structural information	(*116*, *117*)
Tritiated ^{3}H-Glutathione 		Radioactivity MS	+ Quantitative + Structural information - Radioactivity - Need HPLC separation from non-reacted [3H]GSH	(*118*)
Mixture Glutathione and stable isotope-labeled Glutathione 		MS	+ Unique isotopic MS signature + Unambiguous identification of GSH adducts + Structural information - Radioactivity	(*119*)

Examples of glutathione-based trapping agents and methods used to identify and characterize *in vitro*-generated "soft" reactive metabolites of drugs. Advantages (+) and disadvantages (-) of the methodologies are mentioned as well.

The sensitivity of MS might not be optimal to identify adducts in a method based on complete proteins (*134*). Moreover, no structural information about the covalent binding is obtained. For this purpose characterisation of peptide adducts is required (*117*). These experiments provide structural insight of specific haptens that are possibly

involved in the underlying mechanisms, as specific targets for adduct formation might discriminate between individual toxicities. In our laboratory, Boerma et al. used highly active P450 BM3 M11his enzyme for bioactivation of acetaminophen, clozapine and troglitazone in the presence of the model target protein human GSTP1-1. After digestion of the model protein, adducted and non-adducted GST peptides were analyzed by LCMS/MS. It was shown that P450 BM3 mutants with high capacity to activate drugs into relevant RMs can be employed to produce protein adducts to study the nucleophilic selectivity of highly reactive electrophiles (*135*).

Different strategies have also been applied for the investigation and/or detection of drug protein adducts *in vivo* (*149*, *150*). First, the isolation of drug-protein adducts from the tissue using chromatographic and/or electrophoretic techniques is required. Direct (semi)-quantitative analysis of protein adducts can be performed using radioisotope-based assays (when the drug is radio-labeled) or by immunological techniques (after raising antibodies against drug-protein adduct in animals) (*151*). Further digestion of proteins by proteases, with subsequent isolation and purification steps of the peptides, may facilitate adduct analysis. Adduct and/or drug moiety detection can be performed using different techniques but usually involves LC-MS/MS analysis. For example, on-line approach for the detection of covalent adducts to the cysteine-34 residue of human serum albumin has been described measuring NAPQI (the reactive metabolite of acetaminophen) and CDNB adducted albumin (*152*).

1.3.1.3. Mechanism-based CYP inhibition

Sometimes, ultra-reactive metabolites are so reactive that they do not escape its site of formation. They covalently bind to the enzyme leading to mechanism-based inhibition as metabolism precludes enzyme inactivation (*136*, *137*). Inhibition of CYP-mediated drug metabolism is one of the causes of drug-drug interactions (DDIs) and can result in serious clinical consequences. Some fatal adverse reactions of drugs are mediated by metabolism-dependent DDIs, which have been responsible for the withdrawal of some drugs from the market. Co-administration of the calcium-channel blocker and potent CYP3A4 inactivator mibefradil and simvastatin in patients with hypertension has been associated with increased cases of myopathy including rhabdomyolysis. The mechanism-based inactivation of the CYP3A4-catalyzed metabolism of simvastatin by mibefradil results in elevated plasma concentrations of the statin that leads to myopathy or rhabdomyolysis (*138*, *139*). Given the potential for such life-threatening drug-drug interactions, the manufacturer of mibefradil announced a voluntary withdrawal of the drug from the market worldwide.

CYP inhibition is now being investigated earlier in the drug discovery process to avoid costly failures in drug development. Screening for mechanism-based inhibition of a CYP isoform relies on the detection of changes in inhibitory activity with preincubation of the test compound in the presence or absence of NADPH in liver microsomes or recombinant CYPs followed by the addition of probe substrates. The metabolism of the probe substrate is measured and it was suggested by Berry and Zhao (*140*) that metabolism-dependent inhibitors show at least a 1.5-fold shift in IC50 after a 30 min preincubation.

Also, the influence of specific CYP isozymes in the formation of reactive metabolites can be studied by HLMs inhibition studies in the presence of isoform specific inhibitors. Table 7 lists the various probe substrates and inhibitors for the different CYP isozymes.

Table 7. Recommended *in vitro* probe substrates and inhibitors for CYP isoenzymes.

	Substrates		Inhibitors	
Isoform	'Preferred'	'Acceptable'	'Preferred'	'Acceptable'
CYP1A2	Ethoxyresorufin Phenacetin	Caffeine Theophylline Acetanilide Methoxyresorufin	Furafylline	α-naphthoflavone
CYP2A6	Coumarin			Coumarin
CYP2B6	S-Mephenytoin	Bupropion		Sertraline
CYP2C8	Paclitaxel		`glitazones'	
CYP2C9	S-Warfarin	Tolbutamide Diclofenac	Sulphaphenazole	
CYP2C19	S-Mephenytoin Omeprazole			Ticlopidine Nootkatone
CYP2D6	Bufuralol Dextromethorphan	Metoprolol Debrisoquine Codeine	Quinidine	
CYP2E1	Chlorzoxazone	4-nitrophenol Lauric acid		4-methyl pyrazole
CYP3A4	Midazolam Testosterone	Nifedipine Felodipine Cyclosporin Terfenadine Erythromycin Simvastatin	Ketoconazole Troleandomycin	Cyclosporin

Table adapted from (*141*).

1.3.2. In vivo assessment of reactive metabolites formation

Although *in vitro* screening tools can assess the potential of novel compounds to be bioactivated to RIs, *in vivo* data on covalent binding of drug metabolites to GSH and/or proteins are relevant for risk assessment. While GSH adducts and/or their decomposition products, s.a. cysteine conjugates and mercapturic acids measured *in vivo* represent short-term exposure to reactive chemicals, protein adducts to albumin or hemoglobin better reflect chronic exposure to electrophiles (*142*).

LC–MS techniques also play an essential role in the analysis of RIs formed *in vivo* in ADME studies in toxicology species and humans from late drug discovery to clinical development (Table 5). These results allow the confirmation of bioactivation pathways observed in liver microsomal incubations, the determination of reactive metabolites mediated by non-microsomal enzymes or multiorgan biotransformation reactions, which

are usually not detected by *in vitro* screening methods (*143*), and the assessment of contribution of bioactivation to the total drug clearance in humans and toxicology species.

To assess *in vivo* bioactivation of a given drug candidate, detection and structural identification of GSH adducts in bile samples has been shown using LC–MS and/or NMR after dosing a compound to bile duct cannulated rats. 3H-labeled compounds are used for quantitative determination of RIs by the radioactivity analyses of GSH adducts and protein covalent binding in liver tissues (*144*). To assess the exposure levels of reactive metabolites *in vivo* in toxicology species and humans, quantitative analysis of mercapturic acids of a given drug are performed. Mercapturic acids are important biomarkers and characterization of urinary mercapturic acid derivatives represents a classic approach for investigation of the metabolic activation of xenobiotics *in vivo*. The detection of mercapturic acid derivatives of diclofenac, felbamate and nevirapine in the urine of humans treated with these drugs has been described (*145–147*). For screening for unknown reactive drug metabolites formed *in vivo*, a method using constant neutral loss of 129Da in the negative ion mode combined with product ion scans (positive and negative mode) has been applied to detection of mercapturic acids in human urine (*148*).

1.4. Aims and scope of the thesis

A large proportion of the ADRs are considered to result from the formation of chemically reactive metabolites generated in humans. These metabolites can react with nucleophilic residues in proteins and other macromolecules, which may lead to ADRs or IDRs. Although the role of bioactivation to RIs has been reasonably well established and accepted, and methodologies like reactive metabolite trapping and covalent binding continue to develop in an attempt to detect the occurrence of bioactivation, the challenge remains to predict the likelihood for idiosyncratic toxicities. The identification of risk factors which determine which patients are susceptible for the occurrence of ADRs of certain drugs is necessary. Using a translational approach, from the molecule level to the patient, this TI-Pharma project aims to identify biomarkers and to develop tools for the early prediction of adverse drug reactions. The ability to identify adverse effects in an early stage of drug development will prevent much discomfort in patients and economic loss.

Conjugation of reactive intermediates to GSH is an important detoxification mechanism that can be spontaneous or mediated by glutathione S-transferases (GSTs). Although the formation of GSH conjugates has been demonstrated in many *in vitro* studies for the drugs causing IDRs, the role of GSTs in catalysing GSH conjugation of reactive drug metabolites *in vivo* has remained relatively unexplored. An increasing number of GST genes are recognised as being polymorphic. Interestingly, genetic polymorphisms of GSTs have been associated with an increased susceptibility of several forms of cancers, alcoholic liver disease, and toxic hepatitis caused by chemicals and drugs. Several clinical studies have demonstrated an increased susceptibility to idiosyncratic drug-induced liver injury by the combined GSTM1-T1 double-null genotype. However, further proofs on the role of the GSTs and their polymorphic isoenzymes in the detoxification of reactive metabolites formed by bioactivation of drugs, in both, *in vitro* and clinical studies, are needed.

The main aim of this thesis was better understanding of the underlying mechanisms of the IDRs and identification of possible risk factors for individual susceptibility. Major goal of the thesis was to investigate the role of human GSTs in inactivation of electrophilic drug metabolites generated in *in vitro* studies using human liver microsomes and a drug-metabolizing bacterial P450 BM3 mutants as bioactivation systems. The second aim of the thesis was to investigate the effects of genetically polymorphic enzymes involved in bioactivation of drugs (specifically clozapine) and detoxification of reactive intermediates formed *in vitro* as well as *in vivo* by analysing urine samples of the patients on drug treatment.

This chapter, **Chapter 1**, contains an introduction to ADRs and IDRs. The role of drug metabolism and formation of reactive metabolites, risk factors for occurrence of IDRs, and the strategies for the risk assessment for the novel drug candidates are described. An overview of the literature regarding polymorphic GSTs, their role in the detoxification of reactive metabolites and correlation of these polymorphisms with the occurrence of IDRs will be given in **Chapter 2**.

Chapter 3 focuses on the role of human GSTs in the inactivation of reactive metabolites of clozapine formed by human and rat liver microsomes and a drug-metabolizing bacterial P450 BM3 mutant, P450 102A1M11H, as bioactivation systems. We compared the ability of four recombinant human GSTs (hGSTA1-1, hGSTM1-1, hGSTP1-1, and hGSTT1-1) to catalyze the GSH conjugation of formed reactive metabolites by comparison of GST-catalysed conjugation reaction with non-enzymatic conjugation reaction. Similar approaches were used to investigate the effects of human GSTs on the GSH conjugate formation of diclofenac as described in **Chapter 4.**

Several studies suggested variations among individuals in regards to enzyme activity due to hGSTP1-1 polymorphisms. In **Chapter 5,** we investigated if Ile105Val and Ala114Val mutations, resulting in four allelic variants of hGSTP1-1, affecting the ability of these enzymes to inactivate the reactive drug metabolites of paracetamol, clozapine, and diclofenac formed by bioactivation *in vitro* by human liver microsomes and drug metabolizing P450 BM3 mutants.

Chapter 6 describes the application of Cytochrome P450 BM3 mutants for clozapine bioactivation and structural characterization of clozapine GSH conjugates. By screening of a library of BM3 mutants, a mutant was selected for the generation of large amounts of clozapine reactive metabolite to enable structural elucidation of all major human relevant GSH conjugates by NMR.

In **Chapter 7,** involvement of individual human CYPs in the bioactivation and formation of clozapine reactive intermediates was investigated. Clozapine was investigated using fourteen commercially available recombinant human P450s and formation of reactive intermediates was quantified by using GSH and cyanide as nucleophilic trapping agents. We also studied the effect of specific inhibitors of P450 isoenzymes in human liver microsomes, as well as bioactivation of clozapine in human liver microsomes from 100 different individuals.

Chapter 8 describes the variability in clozapine metabolic profile and bioactivation in human liver slices incubations and patients' urine samples. Metabolites of the bioactivation pathway of clozapine, urinary thioethers, reference standards were produced starting from corresponding GSH conjugates using enzymatic and simple

chemical synthesis. The metabolic profile of identified conjugates was comapared within individuals, both clozapine patients and liver donors, taking into account genotyping results for polymorphic GSTs. A preliminary association study was performed to investigate the role of genetic polymorphism of four hGST as risk factor for CLZ-induced agranulocytosis.

In the last part of the thesis, **Chapter 9**, an overall summary will be given, including general conclusions and perspectives for future work.

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Chapter 2

POLYMORPHIC HUMAN GLUTATHIONE S-TRANSFERASES AS A RISK FACTOR FOR IDIOSYNCRATIC ADVERSE DRUG EVENTS: REVIEW

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Review in preparation.

Abstract

Bioactivation of drugs to chemically reactive metabolites is often recognized as a risk factor involved in the pathogenesis of idiosyncratic drug reactions (IDRs). Glutathione S-transferases (GSTs) are considered to be the major phase II enzymes involved in the detoxification of electrophilic xenobiotics by catalyzing conjugation reactions to glutathione (GSH). An increasing number of studies have shown an association between genetic polymorphisms (especially deletions) of human GST genes and susceptibility to drug-induced idiosyncratic toxicity. For example, several clinical studies demonstrated an increased susceptibility to idiosyncratic drug-induced liver injury due to the combined GSTM1-T1 double-null genotype. *In vivo* animal studies have been described using knockout mice as a model for studying GST polymorphisms. *In vitro* studies have also been performed to investigate the role of GSTs in the inactivation of electrophilic drug metabolites. This review presents an overview of currently known genetic polymorphisms of GSTs, a summary of the studies on the role of GST polymorphisms in the detoxification of reactive drug metabolites and the implication of genetic polymorphisms for drug toxicities. The review substantiates still insufficiently proven importance of GST polymorphisms as a risk factor for the occurrence of idiosyncratic toxicity.

1. Introduction

Most drug-metabolizing enzymes are known to display genetic polymorphism (*1*, *2*). Mutations in the genes for drug-metabolizing enzymes may encode for enzyme variants with higher or lower activity. Furthermore gene deletions or introduction of premature stop codons can lead to the total or partial absence of the activity of related proteins. These genetic polymorphisms give rise to distinct subgroups in the population that differ in their ability to metabolize drugs (*3*). Many of the variations were only first identified by the occurrence of adverse reactions after normal doses of drugs to patients. In drug development, therefore, it is important to consider and predict the effect of genetic polymorphisms on the efficacy and safety of electrophilic candidate drugs and/or reactive metabolites (*4*). It has been hypothesized that idiosyncratic toxicity of drugs and other chemicals may be the consequence of genetic polymorphisms at the level of drug metabolism and the immune system.

Glutathione S-transferases (GSTs) are known to be major detoxifying phase II enzymes in many organisms. GSTs catalyze the conjugation of glutathione (GSH) to electrophilic substrates in order to inactivate them and facilitate their extraction from the body, thereby preventing reactions to biomacromolecules which might lead to toxicity (*5*, *6*). In addition, they can reduce hydroperoxides to the corresponding alcohol and oxidized GSH, i.e. GSSG, which plays an important role in cellular protection against oxidative stress (*7*). An increasing number of GST genes are being recognized as polymorphic. In humans, marked inter-individual differences exist in the expression of class alpha, mu, pi and theta GSTs (*8*, *9*). Genetic polymorphisms of GSTs have been associated with an increased susceptibility of several forms of cancers, alcoholic liver disease, and hepatitis caused by chemicals and drugs (*10*–*14*).

Here, we will review the potential impact of GST polymorphisms on the occurrence of adverse events and, more specifically, the role of human GSTs in the

inactivation of chemically reactive drug metabolites. Several clinical studies suggested an increased susceptibility to idiosyncratic drug-induced liver injury due to the combined GSTM1-T1 double-null genotype (*10*, *12*). Furthermore, knock-out and humanized animal studies provided additional information about consequences of GST polymorphisms for susceptibility to drug toxicity (*15*). Previously, only few *in vitro* studies have been performed to investigate the role of GSTs in the inactivation of electrophilic drug metabolites (*16*–*19*). Lately, however, the role of human GSTs in the GSH conjugation of reactive drug metabolites was more extensively studied using P450s as bioactivation systems (*20*, *21*). Although still poorly understood, some of these findings clearly point to a role of these enzymes as a general protecting mechanism against different forms of toxicities and to potentially useful strategies to eliminate the latent risks of reactive metabolites in drug development (*4*). Genetically determined reduction in the ability to detoxify electrophilic reactive metabolites, which is expected among individuals with GST null genotypes, might play a role in determining or predicting the risk for ADRs related to some drugs.

2. Glutathione S-transferases

In nature, there are three major families of widely expressed proteins that display glutathione S-transferase activity. Two families comprise soluble enzymes that are not strongly related to each other, designated as cytosolic and mitochondrial GSTs, Figure 1(a) and 1(b) (*22*, *23*). The third family is composed by the "membrane-associated proteins in eicosanoid and glutathione" metabolism (MAPEG enzymes), which comprise microsomal GSTs (*24*). There are certain similarities in the structural protein folding between cytosolic and mitochondrial GSTs (*22*), but no structural similarities to the MAPEG enzymes (*25*).

The soluble GST enzymes of mammalian species are well characterized and have been divided into eight different classes: alpha, mu, pi, sigma, theta, omega, zeta, and kappa according to their amino acid sequence similarity, substrate specificity and primary and tertiary structures. The first seven are classified as cytosolic in mammalian species, whereas the kappa-class GSTs are expressed in the mitochondria and peroxisomes (*11*, *22*, *26*). Cytosolic GSTs are the major family and together with mitochondrial may play animportant role in defense against chemical and oxidative stress (5). Other classes of cytosolic GSTs are recognized in non-mammalian species, designated as beta, delta, epsilon, lambda, phi, tau and the "U" class (*7*, *27*).

The most abundant GST enzymes belong to the cytosolic families alpha, mu and pi. These cytosolic classes are encoded by genes which are assembled in clusters and are dimeric enzymes that contain two subunits of 22-25 kDa (*28*, *29*). The expression pattern of GSTs is distinct in different organs. An overview of the tissue distribution of human GSTs is shown in Table 1. The expression pattern differs also between fetal and adult tissues. In embryonic and early fetal development GSTs are present in high amounts. GSTP1 is the most important GST isoform at these stages (*30*). Alpha-class GSTs are expressed during both periods, while GSTP1 expression levels reduce strongly at the end of the prenatal period. Furthermore, GST expression is also influenced by sex and age.

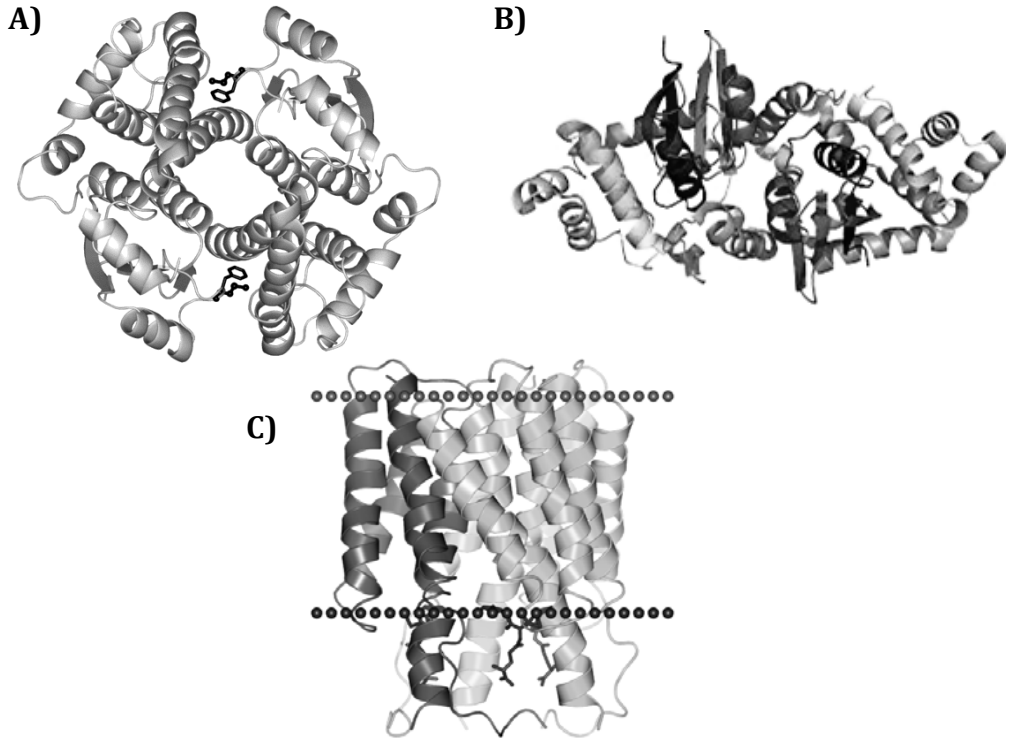

Figure 1. Ribbon diagrams of (a) cytosolic, (b) mitochondrial, and (c) microsomal GST dimers.

2.1. Functions of GSTs in cellular processes

GSTs are known to bind a wide variety of ligands. Next to catalyzing the conjugation of GSH to toxic electrophilic centers of endogenous and exogenous compounds and formation of the corresponding GSH conjugates, Figure 2, GSTs exert various other functions (*31*, *32*). They also function as intracellular transport proteins for hydrophobic endogenous and exogenous ligands. GSTs exert peroxidase and steroid isomerase activities. Recently it was shown that GSTP1-1 can inhibit the c-Jun N-terminal kinase, an important defensive line against H_2O_2-induced cell death (*7*).

2.1.1. *Detoxification of xenobiotics through the mercapturic acid pathway*

GSTs major role constitutes phase II drug-metabolism where they contribute to the detoxification of a wide variety of electrophilic foreign compounds, such as epoxides, alkyl- and aryl-halides, isothiocyanates, *α,β*-unsaturated carbonyls and quinones (*39*). These reactions usually follow phase I drug-metabolism reactions, mostly catalyzed by cytochrome P450, that introduce electrophilic centers. These electrophiles can be trapped by the endogenous nucleophiles, e.g. reduced GSH, in reaction that can be both spontaneous and/or catalyzed by GSTs (Figure 2I).

Table 1. Human GSTs and distribution in the tissues*

GST family	Class	Protein	Organ
Cytosolic	Alpha	GSTA1	testis ~ liver >> kidney ~ adrenal > pancreas
		GSTA2	liver ~ testis ~ pancreas > kidney > adrenal > brain
		GSTA3	placenta
		GSTA4	small intestine ~ spleen > liver ~ kidney > brain
Cytosolic	Mu	GSTM1	liver > testis > brain > adrenal ~ kidney > lung
		GSTM2	brain ~ skeletal muscle ~ testis > heart > kidney
		GSTM3	testis >> brain ~ small intestine > skeletal muscle
		GSTM4	brain, heart, skeletal muscle
		GSTM5	brain, heart, lung, testis
Cytosolic	Pi	GSTP1	brain > heart ~ lung ~ testis > kidney ~ pancreas
Cytosolic	Sigma	GSTS1	fetal liver, bone marrow
Cytosolic	Theta	GSTT1	kidney ~ liver > small intestine > brain ~ prostate
		GSTT2	liver
Cytosolic	Zeta	GSTZ1	fetal liver, skeletal muscle
Cytosolic	Omega	GSTO1	liver ~ heart ~ skeletal muscle > pancreas >kidney
Mitochondrial	Kappa	GSTK1	liver (mitochondria)
Microsomal	MAPEG	MGST-I	liver ~ pancreas > prostate > colon ~ kidney > brain
		MGST-I-like I	testis > prostate > small intestine ~ colon
		MGST-II	liver ~ skeletal muscle ~ small intestine > testis
		MGSTIII	heart > skeletal muscle ~ adrenal gland, thyroid
		LTC_4S	platelets ~ lung > liver
		FLAP	lung ~ spleen ~ thymus >> small intestine

*Data based on (*33*–*38*).

The catalytic activity of GSTs is based on the fact that it can bind both hydrophobic electrophiles and GSH, and on the ability of the enzyme to lower the pKa of the sulfhydryl (-SH) group of GSH from 9.0 to ± 6.5 *(39)*. The thiolate anion (GS^-), in the binding site for GSH, has strongly increased nucleophilicity which leads to spontaneous reaction with electrophilic xenobiotics, bound at a nearby site (*40*). Using X-ray crystallography, it has been elucidated that a tyrosine or serine residue at their active site controls the stabilization of the thiolate anion (GS-) through hydrogen bonds (*41*, *42*). While the GSH-binding site exhibits a high specificity for GSH, the substrate-binding site displays a broad specificity toward hydrophobic compounds resulting in a wide range of substrates.

The formed GSH conjugate is removed from the cell drug transporters, e.g. multi-drug resistance associated proteins. The GSH conjugates are then further catabolised by cleavage of the glutamate and glycine residue, as shown in Figure 3, to corresponding cysteine conjugates. Cysteine conjugates can be excreted in urine as mercapturic acid after acetylation. Dependent on the structure of the electrophile, cysteine conjugate might be cleaved to thiol by *β*-lyase, the latter being further transformed to methylthio-conjugates and related sulphur-containing metabolites (*5*, *6*). The excretion of the sulfur-metabolites has been proposed as a tool to assess extends of human exposure to electrophilic drugs and/or metabolites conjugating to GSH (*43*, *44*).

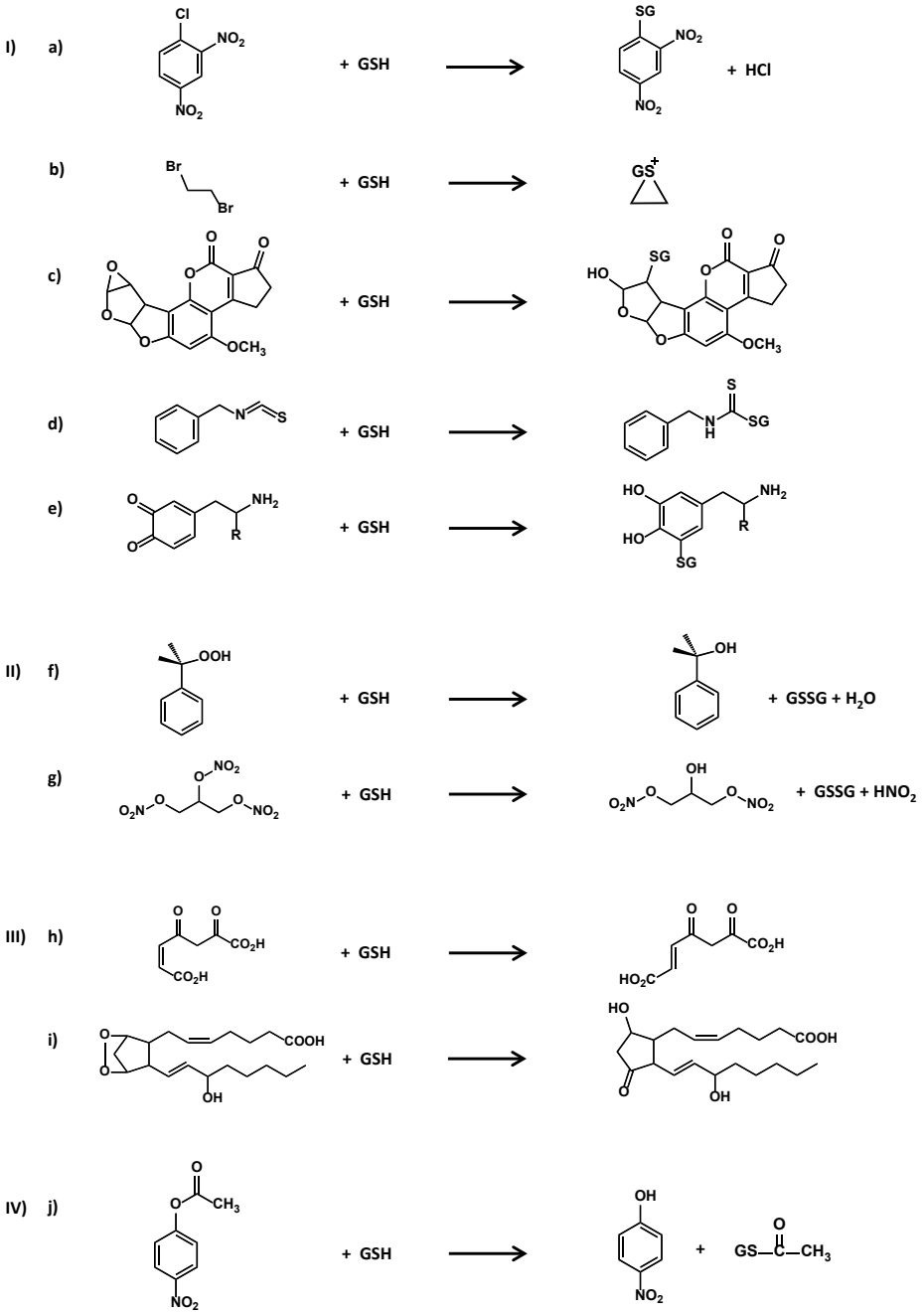

Figure 2. Examples of GST catalyzed reactions: I) conjugation, II) reduction, III) isomerization, and IV) thiolysis. The substrates are: (a) CDNB, (b) dibromoethane, (c) aflatoxin B1-8,9-epoxide, (d) benzylisothiocyanate, (e) an o-quinone model, (f) CuOOH, (g) trinitroglycerin, (h) maleylacetoacetate, and (i) PGH2 (conversion to PGD2 is depicted), and (j) 4-nitrophenyl acetate. Adapted from (*5*).

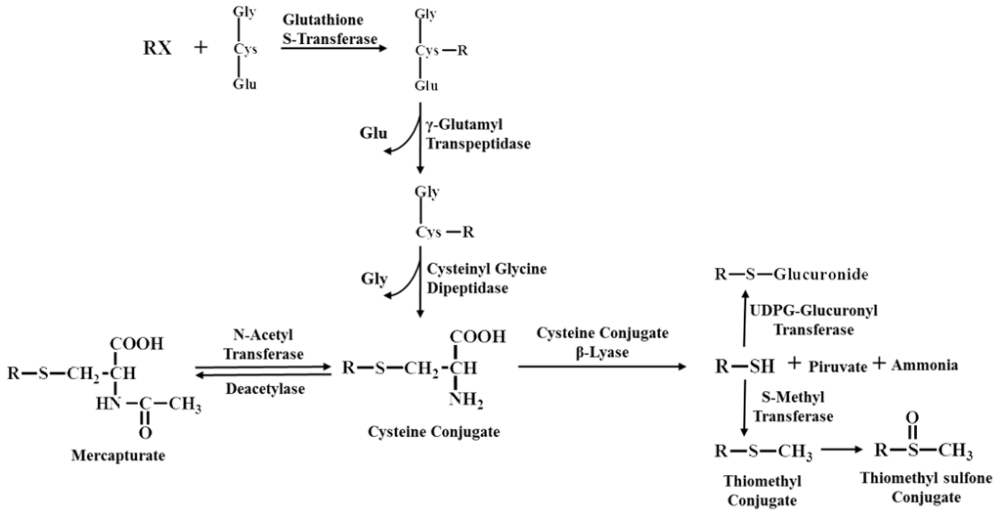

Figure 3. Detoxification of xenobiotics and metabolism of glutathione conjugates through the mercapturic acid pathway.

2.1.2. Bioactivation of xenobiotics by GSTs

Besides detoxification, GSH conjugation also may play an important role in the production of cytotoxic, genotoxic or mutagenic metabolites from xenobiotics. Formed GSH conjugates can be chemically unstable or act as a direct alkylating agents. Several GSH-dependent bioactivation reactions have been described and examples are given in Table 2:

1) GSH conjugates of vicinal dihaloakanes can form electrophilic sulphur mustard. For example, the conjugation of dichloromethane results in a highly unstable S-monochloromethylglutathione, which is able to bind covalently to DNA (*6*, *45*, *46*). 1,2-dihaloethanes are also activated by initial GSH conjugation by forming an episulfonium intermediate formed by an intramolecular halogen-substitution. By modifying DNA these are leading to genotoxicity (*46*). Some alkenes, like butadiene and isoprene, first need activation by CYPs into an electrophile before their mutagenic potential is further increased by GST catalyzed formation of GSH conjugates (*47*);
2) β-lyase mediated bioactivation of cysteine S-conjugates and generation of reactive thioketenes, thionoacylhalides, thiiranes, and thiolactones in the kidney, which are involved in the selective nephrotoxicity of haloalkenes, such as hexachlorobutadien, tetrafluoroethylen, tetrachloroethene *(48, 60)*;
3) some xenobiotics are initially detoxified by GST-catalysed conjugation but undergoes spontaneous reversal of the initial conjugate to regenerate the toxic xenobiotic. For example, isothiocyanates are reversibly conjugated by GST with GSH to thiocarbamates which spontaneously degrade to their isothiocyanates that can be taken up again by the cell and reconjugated with GSH. This can lead

finally to depletion of intracellular GSH and binding of isothiocyanates to proteins, which can result in cell death *(49)*;

4) release of toxic agents from organic precursors, like thiocyanates and nitrosoguanidines; Although under normal conditions the toxification ability of GSTs is described as undesirable, it can be beneficial in cancer chemotherapy in case tumor cells overexpress GST enzymes (*61*). TER286 (TLK286) is a cytotoxic drug, which is activated by GSTs through a *β*-elimination reaction to yield an active analogue of cyclophosphamide, a nitrogen mustard, which then spontaneously yields aziridinium ring moieties that can alkylate DNA, Figure 4 (*50*, *51*). The prodrugs JS-K and PABA/NO are another examples which generates cytolytic nitric oxide upon metabolism by GST, Figure 4 (*52*).

2.1.3. Binding of non-substrate ligands

Several GSTs act as non-enzymatic binding proteins, ligandins, by interacting with hydrophobic compounds like bilirubin, steroid hormones, bile acids, fatty acids and thyroid hormones at a site different from the catalytic site (*62*–*65*). It has been proposed that this function prevents excessive accumulation of these compounds at the membrane or within the cell (*66*).

2.1.4. Metabolism of endogenous compounds by GSTs

It has been also proposed that several GST isoforms have certain metabolic functions in the synthesis of biologically important endogenous molecules (Table 2). Thus GSTs have a function in the biosynthesis of specific prostaglandins (PGs) and leukotrienes (LTs), which are mediators of inflammation and hypersensitivity. For example, MAPEG transferases are involved in synthesis of PGE_2 (*24*), $PGF_{2\alpha}$ (*53*), and LTC_4 (*57*). Certain cytosolic GSTs are also involved in prostanoid biosynthesis (Table 2). Several alpha-class GSTs catalyze reduction of PGH2 to $PGF_{2\alpha}$ (*54*, *69*). Mu-class GSTs, M2-2 and M3-3 display activity towards PGE2 synthesis in human brain, (*70*). The sigma-class GSTs has shown to catalyze the isomerization reactions of PGF_2 to PGD_2 (*55*). In addition to synthesis, class alpha-, mu- and pi-GSTs also catalyse the conjugation of PGA2 and PGJ2 with GSH and these conjugates are then further eliminated from the cell by the MRP transporter (*56*).

GSTs are also involved in synthesis of steroid hormones. Alpha-class GSTs, present in steroidogenic tissues, exhibit ketosteroid isomerase activity, catalyzing the conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids, which is one of the steps in steroid synthesis (*58*, *71*).

The zeta-class GSTs is also involved in the degradation of phenylalanine and tyrosine by catalyzing the last step in their catabolism, cis-trans isomerization of maleylacetoacetate to fumarylacetoacetate (*59*).

Table 2. Bioactivation of xenobiotics and metabolism of endogenous compounds by GSTs

(Endogenous)substrates	***Metabolites and reactive intermediates***	***Biological effects***	***Enzyme***	***Reference***
Bioactivation of foreign compounds by GSTs				
Dihaloalkanes: Dichloromethane, Dibromoethane and 1,2-dihaloethanes	S-haloalkylglutathione and formaldehyde Episulphonium anion	Carcinogenic, genotoxic, DNA modifications	GSTT1-1	(*46*)
Butadiene and Isporene after bioactivation with P450s	GSH conjugate of formed diepoxide	Mutagenic	GSTT1-1	(*47*)
Haloalkenes: Hexachlorobutadiene, Tetrachloroethene	Thioketenes, thionoacylhalides, thiiranes, thiolactones	Nephrotoxic, nephrocarcinogenic	MAPEG/β-lyase	(*48*)
Isothiocyanates (allyl-, benzyl-, phenethyl-) Isocyanates (methyl-)	Thiocarbamates	Intracellular GSH deplition; can lead to cell death		(*49*)
TER 286 prodrug	Nitrogen mustard	Cytotoxic: treatment of malignant desease	GSTs in cancer cells	(*50*), (*51*)
PABA/NO prodrug	Cytolitic nitric oxide			(*52*)
Metabolism of endogenous compounds by GSTs				
Arachidonic acid	Metabolism of eicosanoides (leukotrienes (LT) and prostaglandins (PG))	Physiological messengers, inflamation process	MAPEG and cytosolic GSTs	
PGH_2	PGE_2	Inflamation process	MAPEG GSTA3-3	(*24*)
PGH_2	$PGF_{2\alpha}$	Inflamation process	GSTA1-1 GSTA2-2 MAPEG	(*53*), (*54*)
PGH_2	PGD_2	Signaling molecules	GSTS1-1 GSTA2-2	(*55*)
PGA2 PGJ2	Corresponding GSH conjugates	Half-life regulation of prostaglandins	GSTA1-1 GSTM1-1 GSTP1-1	(*56*)
LTA4	LTC4	Inflamation process	MAPEG	(*57*)
Cholesterol	Steroid hormones biosynthesis	Hormone-related issues	GSTA3-3 GSTA1-1	(*58*)
Phenylalanine	Acetoacetate and fumaric acid	Catabolism of phenylalanin and tyrosine	GSTZ	(*59*)

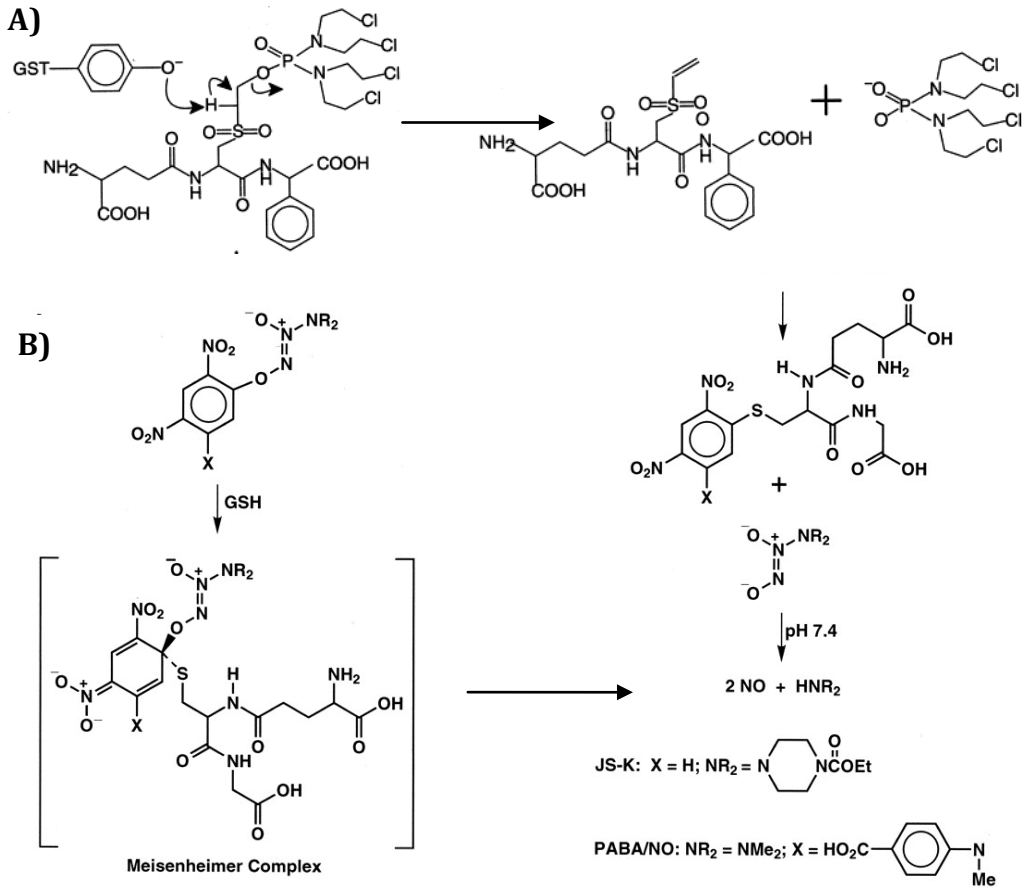

Figure 4. Activation of prodrugs TER286 (A) and PABA/NO (B) through the GST-catalyzed formation of a GSH-conjugate. Adapted from (50, 52).

2.1.5. Protection against oxidative stress by GSTs

During oxidative stress, reactive oxygen species are formed, such as $O_2^{\bullet-}$, H_2O_2, and •OH, which cause damage to membrane lipids, proteins and DNA, which in turn give rise to mutagenic and cytotoxic degradation products (*72*). Protection against these by-products of oxidative stress is provided by enzymatic systems like aldehyde dehydrogenase, alcohol dehydrogenase, and selenium-dependent glutathione peroxidase. Many GST isoenzymes, both soluble and MAPEG, have selenium-independent peroxidase activity using GSH as reductive cofactor (*73*). They catalyze the reduction of organic hydroperoxides to their corresponding alcohols and formation of oxidized glutathione (GSSG). GSTs are capable to reduce hydroperoxides of phospholipids, fatty acids, and DNA and can catalyse inactivation of electrophilic downstream products originating from lipid

peroxidation or protein oxidation, such as reactive aldehydes, 4-hydroxynonenal and acrolein (*74*, *75*).

2.1.6. Modulation of signaling pathways by GSTs

GSTs are involved in the regulation of signaling pathways in the cells by protein-protein interactions with important signaling tyrosine kinases, which are involved in the control of stress response, inflammation, cellular differentiation, proliferation and apoptosis (*28*, *29*, *76*). It is shown that GST-pi sequesters c-Jun N-terminal kinase (JNK)/stress-activated protein kinases (SAPKs) under normal conditions. In response to oxidative stress, GST-pi dissociates from JNK, which can lead to the initiation of apoptosis. GST-pi also controls apoptosis signaling pathways by interaction with TNF receptor associated factor 2 (TRAF2) (*29*, *77*). Isoenzymes of alpha- and mu-classes are also suggested to inhibit the activation/ phosphorylation of c-Jun by JNK by binding the JNK-Jun complexes, although less efficient than GST-pi class (*29*). Theta-class of GSTs are implicated in the inhibition of pro-apoptotic function of Bax (*78*), while omega-class GSTs interact with the calcium channel ryanodine receptors in skeletal and cardiac sarcoplasmic reticulum, indicating a protective role in mammalian cells from radiation damage and Ca^{2+} dependent apoptosis in cardiac muscle (*79*).

3. Polymorphic variants of glutathione-S transferases

Most human GSTs are known to display genetic polymorphisms. Generally single-nucleotide polymorphisms (SNPs) occur and for some enzymes gene deletions. The substitutions of the amino acid residues may cause changes in the substrate-binding site of GSTs resulting in lack or alteration of enzymatic activity toward several substrates (2, *5*, *80*, *81*). These GSTs, and in particular those lacking enzyme activity due to gene deletion (e.g. GSTM1*O, GSTT1*O), may be associated with increased sensitivity to toxic compounds. The most extensively studied GST polymorphisms occur in GST classes mu, theta and pi. Polymorphisms also affect the activity of GSTs from alpha, omega, kappa and zeta-classes, but the functional and toxicological implications of these polymorphisms are less well studied (*9*). Little is known about polymorphisms in MAPEG genes (*82*, *83*). An overview of the soluble GST polymorphisms is given in Table 3.

3.1. GST-alpha class polymorphisms

The alpha-class genes are localized at chromosome 6p12 which encodes five functional genes, GSTA1-A5, and seven pseudogenes (*84*). GSTA1, GSTA2 and GSTA4 are widely expressed in human tissues, with highest concentration in the liver (*5*). GSTA3 is rarely expressed, selectively in steroidogenic tissue, while GSTA5 protein is normally undetectable.

Five SNPs are described for GSTA1. One is a silent base substitution in the exon 5 and the other four are localised on the proximal promoter (*106*). Two allelic variants, GSTA1*A and GSTA1*B, contain these four linked base substitutions, at positions -631G or T, -567T, -69C and -52G for GSTA1*A and -631G, -567G, -69T, -52A for GSTA1*B (*84*, *85*). The main effect of these polymorphisms is the low hepatic expression of GSTA1-1 in

individuals homozygous for GSTA1*B (*5*, *37*). Specifically, the -52 substitution is considered to be responsible for decreased promoter activity in GSTA1*B (*28*). Also, glutathionylation of anti-cancer agents like the nitrogen mustard analogues, chloroambucil, mephalen and thiotepa is less effective with GSTA1*B (*107*, *108*). Consequently, patients with GSTA1*A/*A had a significantly higher rate of elimination of busulfan than those with heterozygous genotype (*109*).

The GSTA2 locus consists of five polymorphic variants, GSTA2*A-E, with different SNPs reported at amino acid positions 110, 112, 196 and 210 (Table 3). The catalytic activity for three of the polymorphic variants does not seem to differ from wild-type. Only GSTA2*E containing Pro110Ser mutation was shown to have lower activity towards several substrates, e.g. 4-vinylpyridine, and cumene-, t-butyl- and arachidonic acid hydroperoxides. However, k_{cat}/K_m for CDNB were similar for all four variants (*37*, *86*, *110*).

An Ile71Leu substitution in GSTA3 was identified as polymorphism with low gene frequency exclusively in African populations (*87*). The GSH-conjugating activity of the leucine containing isoform was significantly reduced in a range of reactions due to a diminished affinity for GSH. This could be implicated in diseases caused by oxidative stress in steroidogenic tissue, where GSTA3-3 is selectively expressed. Polymorphisms in GSTA3 may also affect steroidogenesis. Although, the delta(5)-androsten-3,17-dione isomerase activity of GSTA3-3 was not affected, diminished stability of the L71 isoform could indirectly affect testosterone and progesterone synthesis in individuals carrying this allele.

3.2. GST-mu class polymorphisms

The genes of the mu class of GSTs (GSTM1-M5) genes are located at chromosome 1p13.3. GSTM1 was found to have four polymorphic alleles, GSTM1*A, GSTM1*B, a null allele GSTM1*0 (GSTM1 null), and gene duplication GSTM1*1x2.

The SNP at position 534 (G>C) results in the Lys173Arg substitution, resulting into the alleles GSTM1*A and GSTM1*B (*28*, *90*, *111*). The catalytic efficiencies of the enzymes encoded by these alleles appears to be similar (*11*, *90*).

The GSTM1 null allele is suggested to result from unequal crossing of loci of GSTM1 and GSTM2, due to the presence of two almost identical 4.2-kb regions flanking the GSTM1 gene (*112*). Approximately half of the Caucasian population, 67% of Australian, and 22% of Nigerian are homozygous deleted for this allele and, therefore, fail to express the protein (*113*, *114*). The GSTM1*0 genotype was associated with an increased risk of developing some types of lung cancers, in particular adenocarcinomas (*115*, *116*) and squamous cell carcinomas (*117*, *118*). Also, a significant association with the risk of developing bladder cancer (*119*, *120*), adenocarcinoma of the stomach and colon (*121*, *122*), and pituitary adenomas (*123*) was demonstrated while some studies, on the other hand, did not show association (*121*, *124*).

Table 3. Overview of polymorphisms in human GSTs*

Class	Allele	Alterations in gene or in nucleotides	Protein or amino acid affected	Phenotype or genetic consequences	References
Alpha	GSTA1*A	-631T/G, -567T, -69C, -52G	"Reference" protein levels	WT allele	(*84*, *85*)
	GSTA1*B	-631G, -567G, -69T, -52A	Low protein levels in liver	Lower catalytic activity	(*84*, *85*)
	GSTA2*A	328C, 335G, 588G, 629A	110P, 112S, 196K, 210E	Unchanged	(*86*)
	GSTA2*B	328C, 335G, 588G, 629C	110P, 112S, 196K, 210A	Unchanged	(*86*)
	GSTA2*C	328C, 335C, 588G, 629A	110P, 112T, 196K, 210E	Unchanged	(*86*)
	GSTA2*D	328C, 335G, 588T, 629C	110P, 112S, 196N, 210A	Unchanged	(*86*)
	GSTA2*E	328T, 335G, 588G, 629A	110S, 112S, 196K, 210E	Decreased catalytic activity	(*86*)
	GSTA3*A		71I	WT allele	(*87*)
	GSTA3*B		71L	Deminished activity and stability	(*87*)
Mu	GSTM1*0	Gene deletion	Gene deletion in intron 6	No protein/activity	(*88*)
	GSTM1*A	519G	173K	WT allele	(*89*)
	GSTM1*B	519C	173N	WT allele	(*89*)
	GSTM1*1x2	Gene duplication	Overexpression to WT	Ultrarapid activity	(*90*)
	GSTM3*A	WT, reference intron 6	WT, 147G, 224V	WT allele	(*91*)
	GSTM3*B	3 base deletion in intron 6	Protein unchanged	Increased expression to WT	(*91*)
	GSTM3*C	439G, 670A	147G, 224I	Highest catalytic activity	(*92*)
	GSTM3*D	439T, 670G	147W, 224V	Changed catalytic activity	(*92*)
	GSTM3*E	439T, 670A	147W, 224I	Lowest catalytic activity	(*92*)
	GSTM4*A	WT gene, 2517T	"Reference" protein level	WT allele	(*93*)
	GSTM4*B	2517C change in intron 6	Protein unchanged	Not determined	(*93*)
Pi	GSTP1*A	313A, 341C, 555C	105I, 114A, 185S	WT allele	(*81*), (*94*)
	GSTP1*B	313G, 341C, 555T	105V, 114A, 185S	Substrate dependent activity change	(*81*), (*94*)
	GSTP1*C	313A, 341T, 555T	105V, 114V, 185S	Substrate dependent activity change	(*81*), (*94*)
	GSTP1*D	313A, 341T	105I, 114V	Substrate dependent activity change	(*81*), (*94*)
Sigma	GSTS1*A	IVS2 +11 A	"Reference" protein level	No functional change	(*5*)
	GSTS1*B	IVS2 +11 C	Protein unchanged	No functional change	(*5*)
Theta	GSTT1*0	Gene deletion	Gene deletion	No protein/activity	(*5*)
	GSTT1*A	310A	104T	WT allele	(*95*)
	GSTT1*B	310C	104P	Decreased catalytic activity	(*95*)
	GSTT2*A	2732G	139M	No functional change	(*96*)
	GSTT2*B	2732A	139I	No functional change	(*96*)
	GSTT2P	3255T	196 Stop	Inactive	(*96*)
Zeta	GSTZ1*A	94A, 124A, 245C	32K, 42R, 82T	WT allele	(*97*), (*98*)
	GSTZ1*B	94A, 124G, 245C	32K, 42G, 82T	25% with DCA, 400% with FCA	(*97*), (*98*)
	GSTZ1*C	94G, 124G, 245C	32E, 42G, 82T	25% with DCA, 400% with FCA	(*97*), (*98*)
	GSTZ1*D	94G, 124G, 245T	32E, 42G, 82M	Lower conjugation activity	(*99*)
Omega	GSTO1*A	419C, 464-IVS4+1 AAG	140A, 155E	WT allele	(*100*), (*101*)
	GSTO1*B	419C, 464-IVS4+1deleted	140A, 155 deleted	Unstable protein, higher activity	(*100*), (*101*)
	GSTO1*C	419A, 464-IVS4+1 AAG	140D, 155E	No functional change	(*100*), (*101*)
	GSTO1*D	419A, 464-IVS4+1 deleted	140D, 155 deleted	Unstable protein	(*100*), (*101*)
	GSTO2*A	424A	142N	WT allele	(*100*), (*101*)
	GSTO2*B	424G	142D	No functional change	(*100*), (*101*)
Kappa	GSTK1*A	-1308G, -1332G	WT	WT allele	(*102*), (*103*)
	GSTK1*B	-1308T, -1332 G	Altered transcription	Changed expression level	(*102*), (*103*)
MAPEG	MGST1*A	598T (noncoding 3')	WT	WT allele	(*83*)
	MGST1*B	598G (noncoding 3')	Unchanged	Not determined	(*83*)
	LTC_4S*A	444A (promoter)	WT	WT allele	(*104*)
	LTC_4S*B	444C (promoter)	Increase in protein levels	Possible increased responsivness to	(*104*)
	FLAP*A	no *Hind*III site in intron II	WT	aspirin	(*105*)
	FLAP*B	T→C forming *Hind*III site	Unchanged	WT allele Not determined	(*105*)

*Table adapted from (*5, 8*). WT – wild-type.

GSTM1*1x2 is a variant, resulting from a duplication of the GSTM1 gene. This allelic variant was identified in a population in Saudi Arabia, with 3% of the population. This population shows ultrarapid GSTM1 activity that might result in an enhanced defensive effect against some carcinogens. However, the consequences of multiple GSTM1 gene as a safety factor for the related diseases needs to be determined (*90*).

Polymorphisms have also been identified for the GSTM3, and GSTM4 genes (*5*, *28*, *91*, *125*). The GSTM3 locus contains two alleles, A and B. Compared to the wild-type, GSTM3*A allelic variant, GSTM3*B contains a three base deletion in intron 6, showing a frequency of 0.16 in the Caucasian population (*91*). This difference creates a recognition site for the YYI transcription factor in GSTM3*B, leading to increased expression of GSTM3*B (*11*). It was shown that GSTM3*A occur more frequently in patients with multiple cutaneous basal cell carcinoma and is associated with an increased risk for laryngeal squamous cell carcinoma, whereas GSTM3*B was putatively protective (*126*, *127*). Additionally, several SNPs in GSTM3 have been identified. These are the rare Gln174Trp substitution detected only in Southern Chinese subjects and the more commonly present Val224Ile substitution (*92*). These two polymorphisms form four distinct haplotypes (Table 3). GSTM*C isoform showed highest and GSTM*E the lowest activity for the range of tested substrates, e.g. CDNB, CuOOH, ethacrinic acid. Two other SNPs were discovered after GSTM3 promoter sequencing: A/C and A/G SNPs, 63 and 783 bp upstream of the codon 1 start site, respectively (*28*, *128*). The GSTM3 −63C allele is associated with increased expression of GSTM3, while there is no association between A−783G SNP and GSTM3 expression. For the GSTM4 gene, an SNP at position 2517 (2517T>C) was identified in intron 6, for which it is shown to be associated with increased lung cancer risk (*93*).

3.3. GST-pi class polymorphisms

A single gene, located on chromosome 11, encodes the human GSTP1 variants. There are two SNPs identified for GSTP1, characterized by nucleotide transition 313A>G in exon 5 and 341C>T in exon 6, resulting in amino acid substitutions Ile105Ala and Ala114Val, respectively (*5*, *28*, *81*, *94*). These amino acids appear to occur within the active site of the pi-class GST enzyme and the two SNPs lead to four functional allelic variants, identified as wild-type GSTP1*A (105Ile, 114Ala), GSTP1*B (105Val, 114Ala), GSTP1*C (105Val, 114Val), and GSTP1*D (105Ile, 114Val). The Ile105 allele is predominant, occurring in 60–80% of individuals, with homozygote wild type constituting approximately 50% of the population in Caucasians, 60–70% in Asians, but somewhat less than that in African Americans. The variant GSTP*B homozygote occurs at less than 10% except in African Americans, where it was found at a frequency of 19%. Data available for the codon 114 polymorphism indicate that this variant allele is less frequent than is the codon 105 variant (*9*).

The substitution of isoleucine for the less bulkier and more hydrophobic valine residue at position 105 results in substrate-dependent variations of the enzyme activity toward electrophilic compounds (*5*, *28*). The variant with 105Val exhibit higher efficiency in the conjugation reaction with polycyclic aromatic hydrocarbon diol-epoxides than the variant with 105Ile (*81*, *129*), while it showed lower activity in GSH-conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) (*129*, *130*). On the other hand, functional consequences of alanine to valine substitution at position 114, which is not located close

to the catalytic site, are not known, though it is suggested that they may augment the effect of the I105V polymorphism, e.g. cisplatin (*131*).

Differences in chemotherapeutic response and cancer susceptibility for a wide variety of tumors including ovarian, breast, colon, pancreas and lymphoma are associated with the GSTP1 genotype (*132*). Patients with GSTP1*B allele had a diminished capacity to detoxify platinum-based anti-cancer agents, which makes them favorable for response rates due to the role of GSTP1 in cisplatin resistance via formation of platinum–GSH conjugates (*133*). Also, patients having the Val105 polymorphism, GSTP1*B and GSTP1*C, had a better response to platinum-based chemotherapy and survived longer in acute and chronic myeloid leukemias, glioma, multiple myeloma, Hodgkin's lymphoma, and cancers of the bladder, colorectum, esophagus, stomach, testicles and many other cancers (*134*–*138*).

3.4. GST-theta class polymorphisms

Genes for both members of the theta class of GSTs are located on chromosome 22q11.2. The GSTT1 has two functional alleles, GSTT1*A and GSTT1*B, and a null allele, GSTT1*0. The difference between the two functional alleles is caused by an SNP at position 310 (310A>C) in exon 3 of GSTT1, where the GSTT1*A contains a threonine residue and GSTT1*B has a proline residue at amino acid position 104 (Thr104Pro). This aminoacid substitution caused the decrease in catalytic activity of GSTT1*B compared to GSTT1*A, when methyl chloride was used as a substrate (*95*). The null polymorphism is likely to result of the unequal crossover of two highly homologous regions flanking the GSTT1 gene, thereby deleting a 54 kb fragment containing the complete GSTT1 gene (*112*, *139*). Individuals with a homozygous GSTT1 null genotype do not express any enzyme, with the result that the GSH conjugation of dichloromethane and ethylene oxide is not catalyzed by blood samples from this population (*140*). The gene frequency of GSTT1 null genotype differs significantly between different races and ethnic groups. This genotype is less frequent in the Caucasian population (13-31%) and Mexican Americans (11%) compared to the GSTM1 null genotype, but is comparable to the GSTM1 null genotype frequency in African Americans (22%) and Asian populations (generally 40-60%) (*5*, *28*, *112*). GSTT1*0 genotype has been associated with an increased risk of several types of cancers, like the head, neck and oral cavity cancer, bladder cancer, meningioma, acute myeloid leukemia and squamous cell carcinoma (*141*, *142*). The GSTT1 gene status has been correlated with a response to chemotherapy. Higher response to chemotherapy and a longer relapse-free survival was observed with patients having GSTT1*0 in breast and ovarian cancer, while, conversely, in follicular non-Hodgkin's Lymphoma a significantly worse event-free survival was observed with this patients (*143*, *144*). Possible involvement in metabolism/detoxifying of the anticancer agents used in the therapy still needs to be addressed. GSH concentration which might depend on GST activity is important for cisplatin binding and that way reducing availability of platinum-based therapy and/or inactivation of other anticancer drugs, like cyclophosphamide and Adriamycin (*108*).

The GSTT2 gene also contains two functional polymorphic variants, and a pseudogene (GSTT2P) (*96*). The two functional alleles GSTT2*A and GSTT2*B differ by a single nucleotide substitution 2732G>A in exon 4, resulting in the substitution Met139Ile. The enzyme activity, however, does not seem to be affected (*8*, *11*, *145*). In addition, SNPs

in the GSTT2 promoter region resulted in different GSTT2 enzyme expression levels (-537G>A, -277T>C, -158G>A, and -129T>C) (*146*, *147*). It has been shown that these SNPs are associated with colorectal cancer risk (*147*). The pseudogene originates from two nucleotide transitions at positions 841 (841G>T) in intron 2, that could result in defective splicing, and 3255 (3255T>C) in exon 5 resulting in the substitution of 196Arg by a stop signal.

3.5. GST-zeta class polymorphisms

Identified polymorphisms of the GSTZ1 gene are three functional SNPs resulting in amino acid substitutions Glu32Lys, Gly42Arg and Thr82Met (Table 3). Different combinations of these SNPs determine four allelic variants GSTZ1*A (32Lys, 42Arg, 82Thr), GSTZ1*B (32Lys, 42Gly, 82Thr), GSTZ1*C (32Glu, 42Gly, 82Thr), and GSTZ1*D (32Glu, 42Gly, 82Met) (*8*, *97*, *98*). In Caucasian population (n = 141) all three alleles were found, with frequencies of 0.09, 0.28 and 0.63 for GSTZ1*A, GSTZ1*B, and GSTZ1*C, respectively (*97*). The catalytic efficiency for GSH conjugation of dichloroacetic acid was higher for GSTZ1*A compared to the other variants and correlates with the presence of Arg at position 42 (*98*, *99*). GSTZ1*D, on the other hand, showed a reduced catalytic activity due to a high sensitivity to substrate inhibition (*99*, *148*). GSTZ1-deficient mice which were used as a model to provide insights in metabolic deficiencies, showed an elevated urinary excretion of fumarylacetoacetate with occurring renal injury following phenylalanine and tyrosine overload (*149*).

3.6. GST-omega class polymorphisms

Three human GST-omega class genes have been identified: two actively transcribed, GSTO1 and GSTO2 lying on chromosome 10q25.1, and a reverse-transcribed pseudogene, GSTO3p on chromosome 3 (*101*). Several polymorphisms have been reported in the coding and noncoding regions of the GSTO-class genes with four gene polymorphisms identified in many ethnic groups: GSTO1*A140D, GSTO1*E155del, GSTO1*E208K, and GSTO2*N142D (*100*, *150*, *151*). GSTO1*A140D and GSTO2*N142D are two common polymorphisms, present at different frequencies in populations worldwide (*151*). The GSTO1*D140 allele shows higher frequencies in European populations (34-37%), than observed in Africans (4-21%) and Asians (15-20%) while for GSTO2*N142D similar allele frequencies were observed in Europeans (38-39%) and Asians (22-33%) and higher for African origin population (67-86%). On the other hand, GSTO1*E155del and GSTO1*E208K mutation frequencies never exceeded 10%, with no significant differences among populations (*151*).

Two functional polymorphisms of GSTO1 have been identified: SNP resulting in an A140D amino acid substitution and one that alters a splice junction and causes the deletion of E155 (Table 3). The most frequent variant, GSTO1*C (140Asp, 155Glu), showed no functional changes towards GSH conjugation (CDNB conjugation). Specific activities that are not associated with other human GSTs were also characterised using representative substrates. Glutathione-dependent thioltransferase activity, measured towards hydroxyethyl disulfide (HEDS, was shown to be decreased. Glutathione-dependent reduction of dehydroascorbate, which is the rate-limiting reaction in the biotransformation of inorganic arsenic, was measured towards monomethylarsonic acid

(MMA(V)) and was similar to that of wild-type (*152*). Deletion of residue E155 produces an unstable protein (GSTO1*B) which, in contrast, showed an increased enzymatic activity towards both CDNB and HEDS (*101*). Additionally, E155 deletion variant is linked to K208 allele, while E208K substitution on its own had no functional effect (*100*, *151*). GSTO1*B has been shown to be a risk factor for Alzheimer's Disease (AD) (*100*). Underlying mechanisms could be explained by three hypothesis based on decreased GSTO1-1 activities: the antioxidant activity of GSTO1-1 may protect brain tissue against oxidative stress; regulation of interleukin-1β activation which might modulate inflammation in AD; involvement in the arsenic biotransformation pathway might cause modulation of arsenic neurotoxicity.

Several more non-synonimous SNPs have been described for GSTO1 (*150*). Polymorphisms resulting in Cys32Tyr and Ala236Val amino acid substitutions were observed only in Caucasian- and Mexican-American subjects, respectively, whereas SNPs that resulted in Ala140Asp and Glu208Lys changes were present in all four investigated American populations. A significant decrease in enzyme expression was observed with Cys32Tyr substitution, where substituted cysteine is in the active-site and responsible for the reduction reactions catalyzed by GSTO1 (*150*, *153*). For Val236 allozyme expression was significantly increased, while expressions of the other proteins, Asp140 and Lys208, were similar to those for the wild type. For the Thr217Asn substitution, no significant difference was observed in GSH conjugation activity (CDNB), thioltransferase activity (HED) was decreased to 40% while for MMA(V) reductase activity this variant exhibited similar kinetics in comparison to wild type (*152*).

The GSTO2 locus also appears to be highly polymorphic, with high number of reported SNPs (*150*). Although most of the SNPs are located in the noncoding sequence, four missense variants have been characterized in the coding region. The Asn142Asp substitution (GSTO2*B) is the most common polymorphism found in all populations and seems not to be associated with functional changes (*100*, *153*). Val41Ile and Leu158Ile changes were observed only in African- and Caucasian-American subjects, respectively, while a Cys130Tyr polymorphism change in amino acid sequence was present in African- and Han Chinese American population (*150*). Expression levels of Tyr130 and Ile158 variants were strikingly reduced, while for the Ile41 and Asp142 allozymes levels were at approximately 80% of the wild type.

3.7. GST-kappa class polymorphisms

For the GSTK1 gene, located at the 7q34 chromosome, no variation has been observed in the coding sequence. However, two SNPs were identified in the 5' non-coding region of GSTK1, located at 1308 bp and 1032 bp upstream from the transcription start site, that appear to alter GSTK1 transcription (*103*, *154*). The 1308 bp SNP was found in a Chinese population and involves a -1038G>T transition, while the SNP at -1032 (-1032G>C transition) was only found in an African population (*103*). The genetic variance in the 5' flanking region affects the regulation of GSTK1 gene expression and was recently found to be associated with better insulin secretion and fat deposition (*103*, *154*). These observations provide the first functional insight into genetic factors that regulate hGSTK1 expression which might associate with these metabolic diseases.

3.8. Polymorphisms of MAPEG members

Several of the genes of the MAPEG members have been reported to show genetic polymorphisms (*83*, *104*, *105*). A number of SNPs in MGST1 have been reported in healthy Japanese volunteers (*82*). It has been also shown that MGST1 polymorphisms may be risk factors for colorectal cancer risk among Han Chinese (*155*). 25 variants in MGST3 have been reported in Pima Indians based on identified SNPs (*156*). However, confirmation of true alleles these SNPs reflect, and their biological consequences, still requires evaluation in larger populations and in other ethnic groups as no evidence was found for association with type 2 diabetes mellitus in selected diabetic and non-diabetic Pima Indians.

Promoter polymorphisms have been reported in the LTC4S gene, −1072G/A, and −444A/C, and these appear to influence lung function (*157*) and to be associated with sensitivity to aspirin (*104*). In the FLAP gene, a number of SNPs have been shown in the population of Iceland (*158*). A four-SNP haplotype was found to be associated with increased risk for myocardial infarction and stroke, and this was attributed to increased production of LTB4 (*158*).

4. GST polymorphisms and adverse drug reactions (ADRs)

By catalyzing the conjugation of electrophilic moieties to GSH, GSTs play a crucial role in the detoxification of reactive drug metabolites and other xenobiotics by preventing their binding to cellular proteins and modulating the by-products of oxidative stress (*5*). In this regard, genetically determined deficiencies in GSTs and by inference variability in the detoxification of reactive drug metabolites could lead to differences in the individual vulnerability towards IDRs. Individuals with GST null genotypes might be at higher risk for toxicity due to a lower detoxification capacity and a higher exposure to the reactive metabolites.

Genetic polymorphisms of GSTs has been associated with an increased susceptibility to several forms of cancers (*145*, *159*, *160*), alcoholic liver disease (*161*), and chronic hepatitis C virus infection (*162*). In this way, the prominent role of these enzymes as a detoxification system in humans is well supported. An increasing number of studies is being performed to determine whether GSTs play a role in determining susceptibility to ADRs. For several drugs, GSTM1 and T1 null alleles have been associated with enhanced risk of hepatotoxicity (*10*, *163*, *164*). Several independent studies in animal models supported a role of GSTs in the prevention of chemically-induced hepatotoxicity (*15*, *165*). *In vitro* studies were also performed to evaluate the ability of polymorphic GSTs to detoxify electrophilic drug metabolites (*16*, *20*, *21*). Below, the current status on the studies that are underlying the role of GSTs as a general detoxification mechanism of reactive drug metabolites is reviewed. Studies that are considering functional polymorphisms of GSTs as potential genetic markers for the occurrence of IDRs are also comprised.

4.1. GST polymorphisms as a risk factor for ADRs in human association case-control studies

Drug metabolism and formation of electrophilic reactive intermediates in the liver are considered to play an important role into drug-induced liver injury (DILI) (*4*). Genetic variation studies in DILI are usually focused on genes involved in drug metabolism, as polymorphisms in these genes might increase the formation or decrease the detoxification rates of reactive drug metabolites (*166*). Lucena *et al*. (*10*) investigated the role of GSTM1 and GSTT1 null genotypes in susceptibility to DILI in 154 Caucasian (Spanish) patients and a control group of 250 sex and age-matched healthy individuals administering a wide range of drugs. Individuals with a double-null genotype of GSTM1/T1 had a significantly higher risk for DILI compared with positive GSTM1 and/or GSTT1 genotype individuals (Odds Ratio (OR) = 2.70; P=0.003) (Table 4). Similar relationships were observed for DILI caused by antibacterials (n=44; OR=3.52; P=0.002) and non-steroidal anti-inflammatory drugs (n=19; OR=5.61 P=0.001). Patients with amoxicillin-clavulanate hepatotoxicity had a 2.81-fold increased risk (P=0.037). The effect of the double-null GSTM1-T1 genotype was consistent in cases of diclofenac- and nimesulide-induced hepatotoxicity among patients receiving NSAIDs (Table 4). These results suggested that the GSTM1 and GSTT1 double-null genotype is possibly associated with the DILI susceptibility for several drugs. However, low number of cases, e.g. only 4 cases of diclofenac- and 5 cases nimesulide-induced hepatotoxicity were included. Therefore, additional studies are required to further support the protective role of hGSTs against drug-induced hepatotoxicity.

An endogenous antioxidant deficiency, leading to idiosyncratic liver damage may occur as a consequence of cytosolic oxidant stress generated from drug metabolism or from oxidant stress directly generated in mitochondria. However, after re-analyzing the data, the investigators found no significant association between null genotypes and DILI from agents (e.g. cardiovascular therapy, endocrine therapy) other than anti-bacterials and non-steroidal anti-inflammatory drugs (n= 92; OR= 1.97; P <0.005) (data not shown, personal communication by Lucena) (*167*). Based on these results and the wide variety of mechanisms involved in hepatotoxicity, it is clear that the GST-genotypes do not explain all DILI cases, and that multifactorial and multigenic processes are involved in the DILI, including those involved in cellular signaling, adaptation, regeneration/repair processes and immunological components.

It was shown recently that the double-null GSTM1 and GSTT1 genotypes was associated with troglitazone-induced plasma transaminase increases (*164*) and might be an important risk factor for troglitazone-induced hepatotoxicity. A genotype analysis by Watanabe et al. (*164*) showed that 40% of the patients with hepatotoxicity (n=25) have a double-null GSTM1 and GSTT1 genotype vs. 15% of the patient control group (n= 85) (OR = 3.7; 95% CI: 1.354-10.066, P = 0.008). Therefore a deficiency in GST-dependent detoxication of reactive troglitazone metabolites (Figure 5) seems to be a risk for hepatotoxicity. However, since 15% of the control group, which was treated with troglitazone without developing toxicity, also possessed the same genotype, Satoh *et al.* (*168*) hypothesized that other mechanisms, such as immune-mediated reactions, should also contribute to the hepatotoxicity. The involvement of immune reactions supported by high correlation of HLA gene polymorphism to the idiosyncratic drug-induced adverse reactions including hepatotoxicity was implied to be concerned in the toxic mechanism of

troglitazone by a study of Ikeda *et al.* (*169*). Moreover, the double positive GSTM1 and GSTT1 genotype was found in 3 % of cases suggesting that the double null genotype combination is not solely responsible for the troglitazone-induced hepatotoxicity and that polymorphisms of unknown genes could also be involved (*169*).

Table 4. *GSTM1* and *GSTT1* genotype distribution in DILI patients and in healthy controls.*

	GSTM1 Genotype, n (%)		GSTT1 Genotype, n (%)		Nr. of active genotypes, n (%)		
	Null	Active	Null	Active	Two	One	None
Patients (154)	86 (55.8)	68 (44.2)	45 (29.2)	109 (70.8)	51 (33.1)	75 (48.7)	28 (18.2)
Controls (250)	113 (45.2)	137 (54.8)	58 (23.2)	192 (76.8)	97 (38.8)	134 (53.6)	19 (7.6)
Statistics							
OR (95% CI)	1.53 (1.02-2.30)		1.37 (0.87-2.15)		0.78 (0.51–1.19)	0.82 (1.02–0.63)	2.70 (1.45–5.03)
P value	0.085		0.394		0.544	0.730	0.003
Anti-infectives for systemic use							
Antibacterials (n = 44)							
Amoxicillin-clavulanate (n = 32)					12	14	6
Macrolides (n = 4)					1	3	0
Quinolones (n = 3)					1	0	2
Other (n = 5)					1	2	2
Drugs for treatment of tuberculosis (n = 5)					1	4	0
NSAIDs							
Acetylsalicylic acid (n = 1)					0	0	1
Diclofenac (n = 4)					0	2	2
Ibuprofen (n = 5)					3	2	0
Indomethacin (n = 1)					1	0	0
Naproxen (n = 1)					0	1	0
Nimesulide (n = 5)					1	1	3
Ketorolac (n = 1)					0	1	0
Rofecoxib (n = 1)					1	0	0
Central nervous system							
Antiepileptics (n = 4)					0	3	1
Anxiolytics (n = 6)					1	4	1
Antidepressants (n = 6)					1	4	1
Other (n = 8)					3	5	0
Cardiovascular system							
ACE inhibitors + ARAII (n = 6)					1	3	2
Serum lipid reducing agents (n = 10)					2	6	2
Other (n = 1)					1	0	0
Drugs for peptic ulcer (n = 8)					3	5	0
Antineoplastic agents, immunosupressive agents, and endocrine therapy							
Asparaginase (n = 1)					1	0	0
Azathioprine (n = 4)					3	0	1
Leflunomide (n = 2)					0	2	0
Flutamide (n = 5)					4	1	0
Herbal plants (n = 4)					1	3	0
Other (n = 21)					8	9	4

*Table adapted from (*10*).

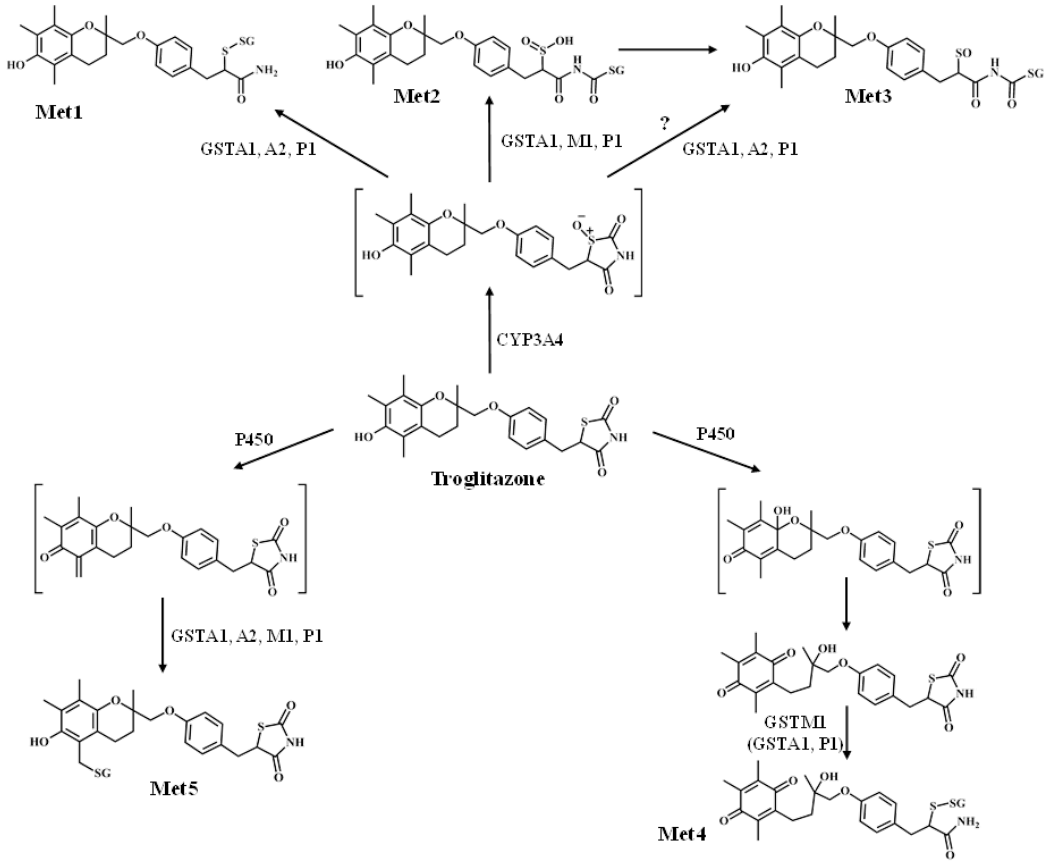

Figure 5. Bioactivation (P450s) and detoxification (GSTs) pathways of troglitazone. Detoxification of troglitazone reactive metabolites (shown in brackets) through GST-mediated GSH conjugation is shown. Adapted from (*21*).

GSTM1 null, GSTT1 null and the double-null GSTM1 and GSTT1 genotype combinations seem also to be associated with the susceptibility for tacrine-induced hepatotoxicity (*170*). Tacrine is bioactivated to a reactive quinonemethide (*171*), as shown in Figure 6, GSTs might catalyze GSH conjugation of this metabolite so that it can be safely excreted from the body. Green *et al.* (*172*) observed that there was no significant difference in the frequency of GSTM1 null genotype in patients with tacrine transaminitis (n= 33) when compared to patients (n= 37) treated with tacrine who did not develop hepatic toxicity (OR= 1.1; 95% CI: 0.4-3.1). These results indicated that the GSTM1 status alone cannot be used clinically to predict individual susceptibility to tacrine transaminitis. Becquemont and Simon first suggested that GSTT1 null genotype may be a risk factor for tacrine-induced hepatotoxicity, although for the combined GSTM1-T1 null genotype no increased individual susceptibility was found (*170*). Later, the same group reported that neither GSTM1 nor GSTT1 alone could predict individual susceptibility to tacrine hepatotoxicity (*12*). However, the combined GSTM1-T1 null genotype was observed in 18

patients (13%; 95% CI from 7% to 18%) of whom 13 had an elevated plasma alanine aminotransferase (ALT), i.e. at least three times the upper limit of normal during the study period. It was concluded that the association of the GSTM1-T1 null genotype was an independent risk factor, which increases the susceptibility to tacrine hepatotoxicity.

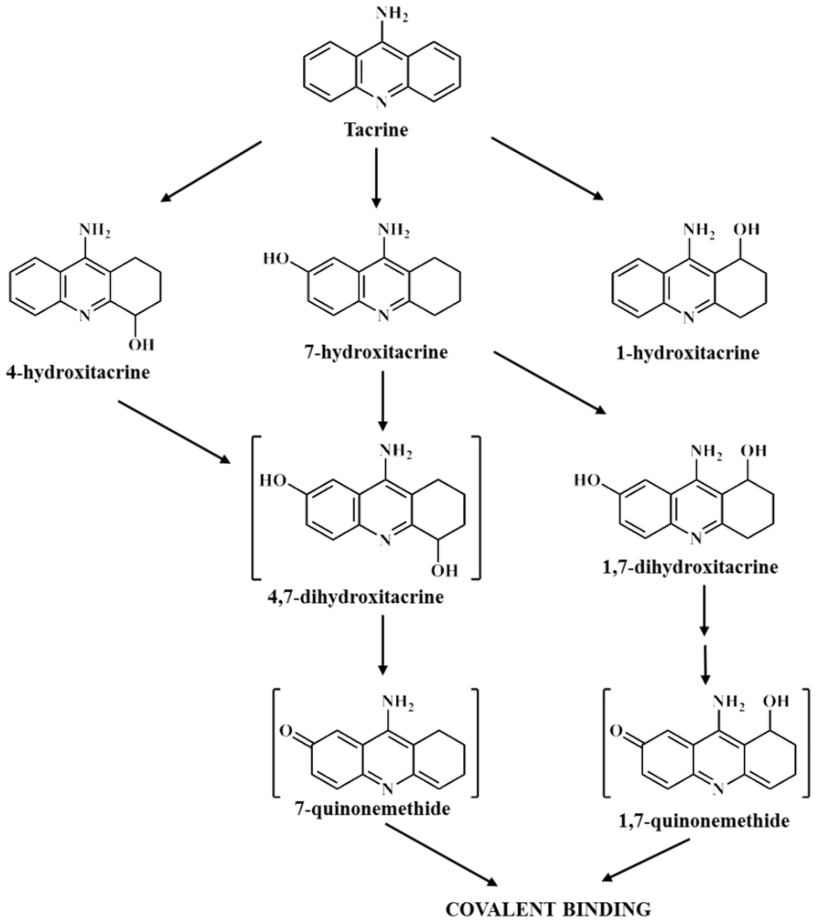

Figure 6. Bioactivation of tacrine and formation of reactive quinonemethides. Adapted from (*171*).

Buchard *et al.* investigated whether GSTP1, GSTT1 and GSTM1 polymorphisms reflect risk factors in APAP-poisoned patients by investigating the relation with the prothrombin time (PT), which is a sensitive marker for survival (*173*). A borderline association between a high PT level and a genetic profile with a GSTT1 homozygous deletion compared to two functional copies of the gene (P=0.05) was found. However, no association between GST genotype and ALT was demonstrated. This indicated that the frequency of a homozygous GSTP1*C in the APAP-poisoned patients was significantly

lower (P= 0.047) than in the control group, indicating that the GSTP1*C genotype may reduce the risk of being APAP poisoning.

For anti-tuberculosis drugs (ATD), many contradictory results are reported (*14*, *175–177*). The value of GSTM1 and GSTT1 null genotypes as genetic predictors of ATD-induced hepatotoxicity is, therefore, still unclear. The first-line ATD isoniazid, rifampicin and pyrazinamide are commonly associated with hepatotoxicity (*178*), although the exact molecular mechanism and involvment of GSTs are not clear (*179*). Isoniazid metabolism and formation of reactive metabolites, hydrazine, is considered to damage cellular macromolecules and lead to toxicity in the liver, as presented in Figure 7 (*180*). Oxidative stress was also shown to be involved in occurrence of isoniazid-induced hepatotoxicity (*181*, *182*). Reduced glutathione levels and reduced GST activity of glutathione-S transferase, catalase and superoxide dismutase after isoniazid or hydrazine administration to rats indicated this *(181)*. Besides GSTs, the N-acetyltransferase slow acetylator, without the NAT2*4 allele (*183*, *184*), and the cytochrome P450 2E1 homozygous wild type (*185*, *186*) have all been reported as risk factors ATD-induced hepatotoxicity. The mechanisms of rifampicin- and pyrazinamide- are unknown and there is no evidence yet for the presence of toxic metabolites *(180)*.

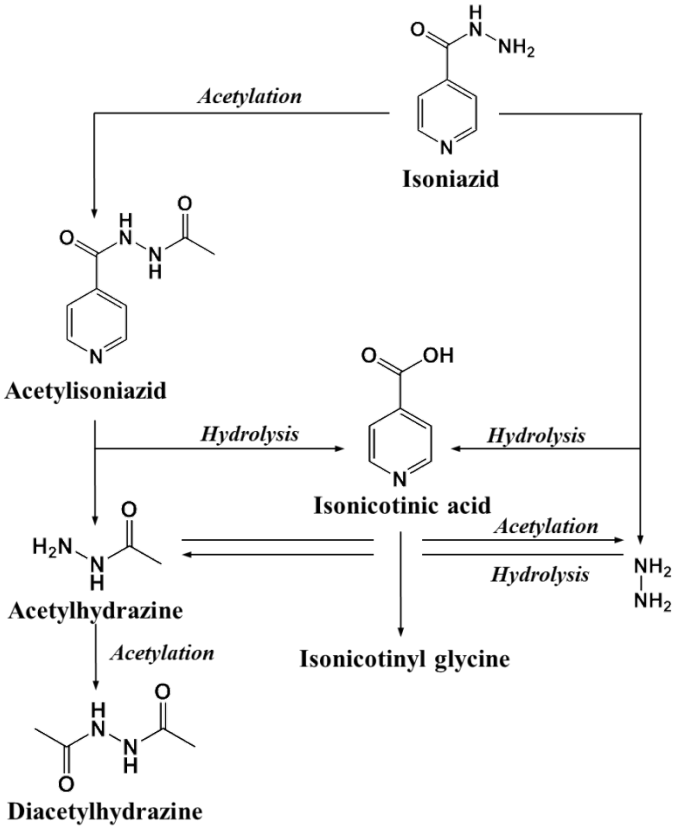

Figure 7. Izoniazid metabolism. Adapted from *(180)*.

In an Indian population, Roy *et al.* (Table 5), showed that a GSTM1 null genotype predisposes individuals to ATD-induced hepatotoxicity with active tuberculosis (*177*). This polymorphic genotype was present in 52% of the patients, compared to 24% in the control group. Although a different frequency was observed for the GSTT1 null genotype between cases (15%) and controls (3%), this was not statistically significant (P>0.05) due to the small sample size.

Huang *et al.* (*14*) observed similar results in a case-control study in a Taiwanese population, in which the role of genetic polymorphisms of GSTM1 and GSTT1 with several hepatotoxic drugs including anti-tuberculosis, antibiotics and NSAIDs were studied. Subjects carrying the GSTM1 null genotype had an increased risk only for ATD–liver injury (Table 5). A GSTT1 null genotype did not show a different frequency between case and control patients.

Table 5. *GSTM1* and *GSTT1* genotype association with ATD-induced hepatotoxicity

Study	Genotypes	Controls		Cases		Odds ratio (95% CI)	*P* value
		n	%	n	%		
Roy et al.	*GSTM1* null	8	24	17	52	2.13 (1.25-3.50)	<0.05
(*177*)	*GSTT1* null	1	3	5	15	not significant	>0.05
Huang et al.	*GSTM1* null	29	46	42	67	2.23 (1.07-4.67)	0.033
(*14*)	*GSTT1* null	25	40	24	38	not significant	-
Sun *et al.*	*GSTM1* null	37	39	59	61	2.62 (1.45-4.75)	0.001
(*187*)	*GSTT1* null	26	27	29	30	1.18 (0.61-2.29)	0.62
Leiro et al.	*GSTM1* null	12	34	25	41.7	0.73 (0.31-1.73)	0.47
(*176*)	*GSTT1* null	17	49	12	26.7	2.60 (1.08-6.24)	0.03
	GSTM1/T1 null	7	20	6	10	2.25 (0.70-7.32)	0.17
Chatterjee et al.	*GSTM1* null	49	49	25	49.0	1.00 (0.51-1.97)	1
(*175*)	*GSTT1* null	3	3	3	5.9	2.02 (0.39-10.39)	0.41
	*GSTM1/T1*null	11	11	3	5.9	0.51 (0.13-1.90	0.39

These data from these two studies were incorporated in a meta-analysis by Sun *et al.* (*187*) to increase the strength of the postulated genetic associations between the NAT2, CYP2E1, GSTM1 and GSTT1 polymorphisms and susceptibility to ATD-induced toxicity. A GSTM1 null genotype showed to be related to an increased risk of ATD-induced toxicity in tuberculosis patients, while there was no significant evidence for the GSTT1 null genotype. The statistically non-significant results regarding the homozygous GSTT1 polymorphism and ATD-induced hepatotoxicity in the individual studies may be due to the small sample size and low frequency of patients. It was concluded that re-evaluation and confirmation are still needed in a large-scale population study, and attempts to evaluate gene-to-gene and gene-to environment interactions on risk of ATD-induced liver injury should be encouraged in the future.

In contrary, a case-control study by Leiro *et al.* (*176*) reported that patients (n=35) with active tuberculosis carrying homozygous GSTT1 null genotype had a significant association and might be a risk factor for ATD-induced hepatotoxicity in Caucasians, while no significant association between the GSTM1 null genotype and ATD-induced hepatotoxicity compared to controls (n=60) was observed (Table 5).

Chatterjee *et al.* (*175*) performed a larger prospective case-control study in an Indian population into possible associations between GSTM1 and GSTT1 null genotypes

with ATD-induced hepatotoxicity. Results showed that both GSTM1 and GSTT1 null genotypes individually, nor double-null genotypes were associated with ATD-induced hepatotoxicity in the Indian population (Table 5). These results are not in agreement with two previous studies in Asian populations (Taiwanese and Indian) and this could be attributed to various factors. The criteria for case and controls were not identical. Although on the same ATD therapy, the therapeutic regimen, dosage and disease severity could have been confounding factors in the studies. Asian populations show a high ethnic diversity. The genotype frequencies of GSTM1 and GSTT1 null in the normal Indian population are in the ranges (20–79%) and (8–22%), respectively (*188*, *189*) and variation in sample studies and small sample studies could influence conclusions.

4.2. GST polymorphism as a risk factor for idiosyncratic toxicity in in vivo animal studies

4.2.1. Glutathione S-transferase knockout and transgenic mice as a model for toxicity

Genetically modified animals have been used to examine the role of individual drug-metabolizing enzymes *in vivo* in the toxicity by xenobiotics. Cyp1a1, Cyp1a2, Cyp1b1, and Cyp2e1 knockout (null) mice were produced and used to examine *in vivo* metabolism, toxicity, and carcinogenesis (*190*). For example, double-null CYP2E1 and CYP1A2 mice showed the protection against APAP toxicity probably due to greatly diminished production of the toxic electrophile, NAPQI *(191)*. On the other hand, the CYP1A2-null mouse was used to investigate the in-vivo contribution of CYP1A2 to clozapine pharmacokinetics and pharmacodynamics showing that CYP1A2 is the major determinant of clozapine clearance, mainly via the demethylation and has a negligible contribution to the N-oxidation *(192)*. Cyp3a knockout mice showed increased sensitivity to the anticancer drug docetaxel, detoxified by CYP3a metabolism *(193)*. Transgenic Cyp3a–/– mice expressing human CYP3A4 were used in the same study to determine the relative importance of intestinal versus hepatic Cyp3a in first-pass metabolism. This showed that expression of CYP3A4 in the intestine dramatically decreased absorption of docetaxel into the bloodstream, while hepatic expression aided systemic docetaxel clearance.

Similar to the CYP knock-in and knockout mice, also several lines of knockout mice for cytosolic GSTs have been established and their phenotypes have been characterized (Table 6). This enabled to examine the contribution of the GST isoforms *in vivo* (*15*).

Gsta3 null mice were used for testing the relationship between sensitivity to aflatoxin (AFB1) carcinogenesis and the level of this GST subunit *(194)*. P450 metabolism of AFB1 forms reactive epoxide intermediates, which bind to DNA and induce mutations. AFB1-8,9-epoxide is inactivated by GST-catalyzed conjugation to GSH (Figure 8). Gsta3 is critical for protecting mice from AFB1 toxicity based on the enhanced sensitivity in newborn mice and in adult mice after liver injury that is closely related to a lower level of GST activity in the liver *(195)*. This is also supported by the mGSTA3 protection against 8,9-epoxide when transfected into hamster cells *(196)*. Ilic *et al.* showed that Gsta3 null mice are much more sensitive to acute cytotoxic and genotoxic effects of AFB1 confirming that Gsta3 has an important role in the wild type mice *(194)*.

Table 6. Summary of GST knockout mice and studied phenotype changes

GST deletion	Phenotype changes	Reference
Gsta3	↑ AFB1-DNA adducts	*(194)*
Gsta4	↑ sensitivity to paraquat ↑ sensitivity to CCl_4 ↑ protein carboxylation, mitochondrial disfunction, and ROS	*(197, 198)*
Gstm1	↓ activity towards DCNB development of methaemoglobinaemia ↑deficit in social behaviours ↓ APAP hepatotoxicity	*(199-201)*
Gsto1	Marginal change in arsenic sensitivity	*(202)*
Gstp1/p2	↑ skin tumorigenesis ↑ lung tumorigenesis ↑ colon tumorigenesis ↓ APAP hepatotoxicity; altered JNK regulation ↑ myeloproliferation ↑ spontaneous tumours ↑ cisplatin nephrotoxicity ↓ protein S-glutathionylation ↑ cyclophosphamide-induced bladder toxicity ↑ MPTP sensitivity of dopaminergic neurons	*(174, 203-206)*
Gsts1	↓ allergic reactivity ↑ severity and duration of delayed-type hypersensitivity reaction	*(207)*
Gstt1	↓ activity toward GSTT substrates	*(208)*
Gstz1	↑ accumulation of tyrosine metabolites; dietary phenylanine lethal ↑ oxidative stress; enlarged liver, kidneys and spenic atrophy; dietary phenylalanine lethal	*(149, 209)*

The Gsta4 null mice were used to investigate the role of this isoenzyme in the metabolism of 4-hydroxynon-2-enal (4-HNE), a lipid peroxidation (LPO) product that is a strong electrophile forming covalent adducts with proteins and, to a lesser extent, nucleic acids and phospholipids. The detoxification of 4-HNE is mainly, although not entirely, via conjugation to GSH and catalyzed by GSTs. Although many GSTs are capable to catalyse 4-HNE reaction to GSH, specialized isoforms, exemplified by the murine mGSTA4-4, are carring out this function (e.g. specialised members of the Alpha class in mammals, Sigma and Delta in invertebrates and Pi in nematodes). The Gsta4 null mouse showed a reduced ability to conjugate 4-HNE, and had an increased steady-state level of this aldehyde in tissues *(197)*. As hepatotoxicity of carbon tetrachloride (CCl_4) has been suggested to be due to the generation of free radicals leading to membrane LPO, Dwivedi *et al.* performed studies to compare the hepatotoxicity of CCl_4 in GSTA4-4 null (−/−) and wild type (+/+) mice. CCl_4-mediated hepatotoxicity in mGSTA4-4 knockout mice was accelerated by the initiation of rapid LPO leading to a marked increase in intracellular 4-HNE concentration. Also, it was observed that Gsta4-4 plays a significant protective role only during the early stages of this toxic insult *(198)*.

Gstm1-null mice were used to investigate role of the GSTM1 gene in toxicological responses to 1,2-dichloro-4-nitrobenzene (DCNB). Gstm1-null mice showed markedly lower ability for GSH conjugation, only 6.1 to 21.0% of the wild-type control to DCNB and 26.0 to 78.6% of the wild-type control to 1-chloro-2,4-dinitrobenzene (CDNB) in liver and kidney cytosols. A single oral administration of DCNB to Gstm1-null mice resulted in larger AUC (5.1–5.3 times, versus the wild-type control) and higher Cmax (2.1–2.2 times,

versus the wild-type control) for the plasma concentration of DCNB, and smaller AUC (9.4–17.9%) and lower Cmax (9.7–15.6%) for the plasma concentration of M0, a GSH-related metabolite of DCNB, methylsulfone-N-acetyl metabolite. These results are suggesting that Gstm1-null mice is a good GSTM1 deficiency model for ADME/Tox studies *(199)*. Further investigations showed that Gstm1-null mice are more susceptible to DCNB toxicity, as reflected by increased methemoglobinemia, after single dose administration *(201)*. However, in repeated-dose studies of DCNB, the higher predisposition to methemoglobinemia was attenuated by adaptive responses.

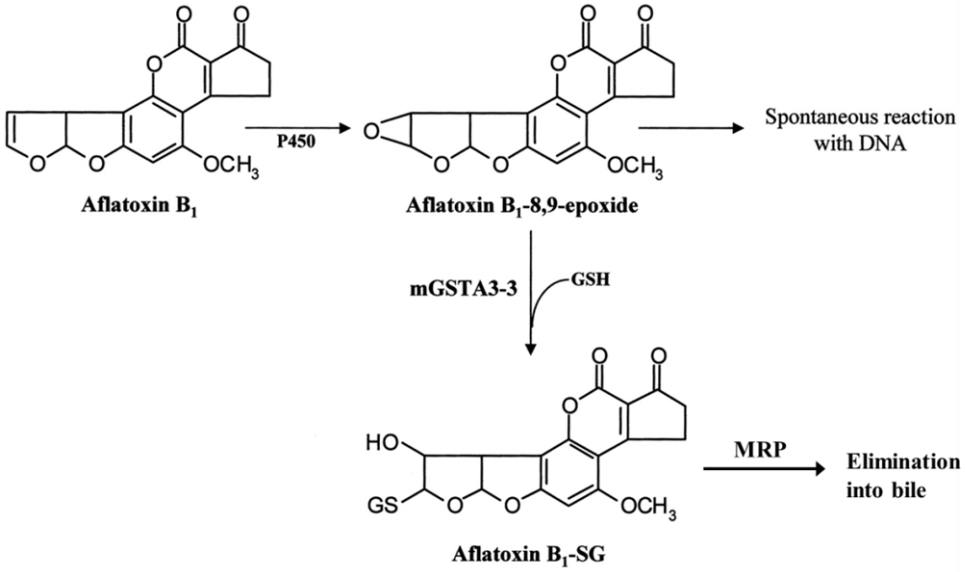

Figure 8. Bioactivation of aflatoxin B1 by Cytochrome P450 to genotoxic 8,9-epoxide. Mouse GSTA3-3 catalyzes detoxification of AFB1-8,9-epoxide to GSH. Adapted from *(210)*.

On the other hand, Gstm1- and Gstp1/p2-null mice showed protection against APAP toxicity *(174, 200)*. Bioactivation of APAP, as shown in Figure 9, by cytochrome P450s to its reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), which can deplete GSH and bind covalently to proteins, which can lead to life threatening hepatotoxicity after overdosing *(211)*. Detoxification of NAPQI by conjugation with GSH proceeds via non-enzymatic and enzymatic reactions catalyzed by GSTs (*17*). Unexpectedly, both Gstp1/p2-null and Gstm1-null mice were resistant to APAP-induced hepatotoxicity. Similar amounts of NAPQI covalent binding to liver proteins and APAP-GSH conjugate concentration in bile were observed in both wild-type and Gstp1/p2-null mice, which suggested that Gstp1/p2 does not contribute to the GSH-conjugation of APAP but plays a novel and important role in APAP-induced hepatotoxicity (*174*). Although the exact mechanism is not elucidated, an increased constitutive c-jun N-terminal kinase (JNK) activity seems to cause the resistance in Gstp1/p2-null mice *(205)*. Hepatic protein

expression profiles of Gstp-null and wild-type mice, using a proteomic approach, showed that there was no enhanced expression of other GST isoforms in the null mice compared with the wild-type *(212)*. Moreover, some other proteins, members of the thiol specific antioxidant family of proteins, were expressed at a higher level, which could reflect the role of GSTP in cell signaling. Marked decrease in total GSH was also observed in both, wild-type and Gstm1-null mice, after administration of APAP, which means that the exposure to NAPQI was similar. This suggested that GSTM1 is also not involved in the enzymatic conjugation of NAPQI by GSH *in vivo*, consistent with the report of Gstp1/p2-null mice (*174*). Measuring phosphorylation of c-JNK, which mediates the signal of APAP-induced hepatocyte necrosis, implied that Gstm1-null mice are resistant to APAP-induced hepatotoxicity due to suppressed phosphorylation of JNK as a main mechanism, although other mechanism, e.g. GSTM1 acting as an upstream factor that induces JNK activation through phosphorylation of glycogen synthase kinase-3β (GSK-3β) and mitogen-activated protein kinase kinase 4 (MKK4), cannot be ruled out. Overall this indicated a novel function of GSTM1 as a signal-modulating factor in APAP-induced hepatotoxicity. It needs to be investigated if the resistance to APAP-induced hepatotoxicity in Gstm1-null mice and role of GSTM1 as a signal modulating factor and not only as a conjugation enzyme is inconsistent with the occurrence of DILI in humans with GSTM1-null genotype as not all patients with GSTM1-null genotype are predisposed to DILI (*13*, *177*).

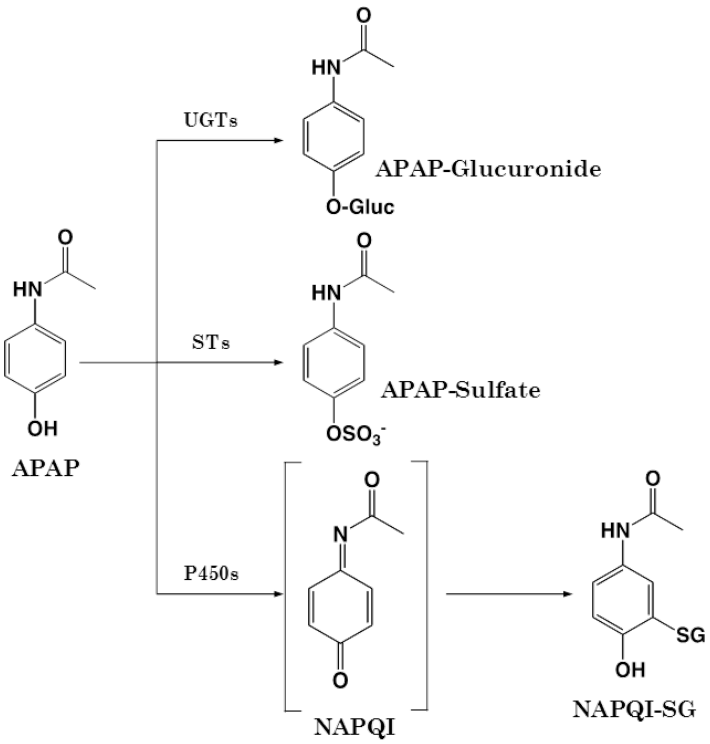

Figure 9. Metabolic pathways of acetaminophen in humans after administration of clinically used doses. Adapted from *(218)*.
UGTs - UDP-glucuronosyltransferases; STs – sulfotransferases.

To better understand the role(s) of GSTP in carcinogenesis and drug resistance, as mentioned Gstp1/p2 null mice was developed in which the entire GSTP gene locus was disrupted (212). Mice characterization using aspecific substrate CDNB for hepatic cytosolic activity showed little change between wild-type and null mice, while there was no activity measured in null mice towards ethacrynic acid, a marker substrate for GST pi *(212)*. Several studies were performed to investigate the role of GSTP in tumorigenesis using Gstp1/p2 null mice *(213 - 216)*. For example, increased numbers of lung adenoma were found in Gstp-null mice after exposure to tabaco related carcinogens (benzo[a]pyrene (BaP), 3-methylcholanthrene (3-MC)) and urethane (8.3-, 4.3-, and 8.7-fold increase for BaP, 3-MC, and urethane, respectively) *(215)*. It was also shown that GSTP protects against cyclophosphamide-induced bladder toxicity by detoxification of acrolein, the major urotoxic cyclophosphamide metabolite *(206)*. Cyclophosphamide-induced bladder ulcerations were more numerous and more severe in Gstp-null mice.

It was also tested if Gstm1- and Gstt1-null mice can be used as a human relevant model by measuring the activities of hepatic GSTs and comparing the results with those of GSTM1- and GSTT1-null genotypes in humans *(207, 217)*. Fujimoto *et al.* showed that GST activity toward 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP), DCM, and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in liver and kidney cytosols is significantly lower in Gstt1-null mice than in wild-type controls *(207)*. Similar, the null genotypes of GSTM1/Gstm1 and GSTT1/Gstt1 significantly decreased GST activities in both human and mice liver towards p-nitrobenzyl chloride (NBC) and dichloromethane (DCM), respectively *(217)*. These studies showed that comparison of hepatic GST-activities between humans and mice using genotype information might be valuable in using Gst-null mice as potentially relevant models for humans.

4.3. Role of polymorphic GSTs in in vitro detoxification of reactive drug metabolites

As summarized in section 4.1, several clinical studies showed statistically significant associations of GST polymorphisms with the occurrence of ADRs. However, so far, relativelly few *in vitro* studies have confirmed the role of GSTs in the inactivation of the electrophilic metabolites of the drugs (*16–21*).

It was shown that several rat and human GSTs catalyze the reaction between GSH and synthetical NAPQI, the reactive metabolite of acetaminophen (*17*). Based on the product formed, both GSH-conjugation and the reduction of NAPQI back to APAP were catalyzed to a certain extent. Using stop-flow kinetics, the rate of APAP-SG for spontaneous and enzymatically catalyzed conjugation was measured. GST-isoenzymes 1-1, 2-2, and 7-7 were particularly active towards APAP-SG formation, while GST 4-4 was not active (Table 7). The ratio APAP-SG/APAP increased in the presence of GSTs in order 7-7 >> 3-3 > 4-4 > 2-2 > 1-1. The reaction products of NAPQI with GSH were also measured in the presence of cysteine to test the ability of GST-isoenzymes to protect other nucleofiles against modification. The results obtained for rat isoenzymes were matching stop-flow kinetics data. Human GSTs were also evaluated this way and conjugation activities were in order $\pi > \delta > \mu$, while GST δ catalysed reduction as well (Table 8).

Table 7: The effects of rat GSTs on the conjugation of reactive drug intermediates

Substrate		Conjugate						Ref.
APAP			V_{max} ($\mu molmg^{-1}s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)	Product formation (μM)	(*17*)
	None	APAP-SG	-	-	-	-	7.2± 0.2	
	1-1	APAP-SG	1.8 ± 0.5	27 ± 10	90 ± 30	3 x 10^6	7.0 ± 0.2	
	2-2	APAP-SG	0.74 ± 0.06	1.3 ± 0.4	37 ± 3	3 x 10^7	7.6 ± 0.2	
	3-3	APAP-SG	0.10 ± 0.01	7 ± 1	5.1 ± 0.4	8 x 10^5	8.4 ± 0.2	
	4-4	APAP-SG	-	-	-	-	7.8 ± 0.2	
	7-7	APAP-SG	3.3 ± 0.3	7 ± 1	165 ± 13	2 x 10^7	9.8 ± 0.2	
Valproic acid			Product formation (nmol/mg protein)					(*18*)
	Boiled cytosol	5-GS-(*E*)-2-ene VPA-NACA	Trace					
		5-GS-(*E*)-3-ene VPA-NACA	8.8 ± 0.5					
	Cytosol, untreated	5-GS-(*E*)-2-ene VPA-NACA	82.1 ± 10.9					
		5-GS-(*E*)-3-ene VPA-NACA	121.3 ± 10.6					
	Boiled mitoplast	5-GS-(*E*)-2-ene VPA-NACA	Trace					
		5-GS-(*E*)-3-ene VPA-NACA	4.9 ± 0.3					
	Mitoplast, untreated	5-GS-(*E*)-2-ene VPA-NACA	1.0 ± 0.3					
		5-GS-(*E*)-3-ene VPA-NACA	8.3 ± 0.6					
	Partially purified GST	5-GS-(*E*)-2-ene VPA-NACA	1358.6 ± 126.5					
		5-GS-(*E*)-3-ene VPA-NACA	2746.8 ± 50.6					

Anticonvulsant valproic acid (VPA) endured a serious drawback in use due to a rare but fatal liver toxicity *(219)*. Formation of reactive (E)-2,4-diene VPA by mitochondrial β-oxidation and/or microsomal P450-catalyzed dehydrogenation is suggested to be responsible for toxicity, Figure 10. As support for this mechanism, the N-acetylcysteine conjugate of (E)-2,4-diene VPA was found in the urine of VPA-treated patients, with 3-4 times higher levels in patients that developed hepatotoxicity *(216)*. The conjugated double bonds of (E)-2,4-diene VPA are activated through formation of the corresponding CoA thioester to react with GSH *(220, 221).* This is also proven by the need to use N-acetylcysteamine thioester of (E)-2,4-diene VPA, which is structural mimic of corresponding CoA thioester. GSH conjugates were not formed in reaction involving GST, GSH, and (E)-2,4-diene VPA (*18*). The role of GSTs in GSH conjugation of (E)-2,4-diene VPA was studied using rat subcellular fractions as the source for GSTs (Table 7). In presence of GSTs, besides increased amounts of conjugation products, an additional GSH conjugate of valproic acid, 5-GS-2-ene VPA was found *in vivo* in bile of rats treated with valproic acid but not in non-enzymatic GSH-conjugation to its reactive diene metabolite, where only 5-GS-3-ene VPA was detected (*18*).

Although being an effective therapy for refractory epilepsy, the use of felbamate has been limited due to the reports of hepatotoxicity and aplastic anemia. Formation of the reactive metabolite, 2-phenylpropenal, is considered to play a role in observed toxicities. It has been shown that detoxification of 2-phenylpropenal by GSH occurs *in vivo* by identification of the corresponding mercapturates in the urine of both rats and

patients *(222)*. Dieckhaus *et al.* investigated the role of GST in the detoxification of 2-phenylpropenal using isolated hGSTs (*16*). The rate of GSH conjugation to 2-phenylpropenal was increased in the presence of hGSTA1-1, hGSTM1-1, and hGSTP1-1 isoforms. The half-life of 2-phenylpropenal and other kinetic data showed that 2-phenylpropenal is a substrate for all three isoforms tested, being best catalyzed by hGSTM1-1, followed by hGSTP1-1, and then hGSTA1-1 (Table 8). In GSH-depleted patients, the role of GST may become increasingly important and any GST polymorphisms resulting in a loss of activity may further promote felbamate toxicity. It was aslo shown that 2-phenylpropenal inhibits reversibly GSTP1-1 and irreversibly GSTM1-1. The irreversible inhibition of GSTM1-1 may be important in understanding the toxicities associated with felbamate as GSTM1-1 represents a potential target for 2-phenylpropenal haptenization in vivo, which may in turn mediate the observed idiosyncratic reactions.

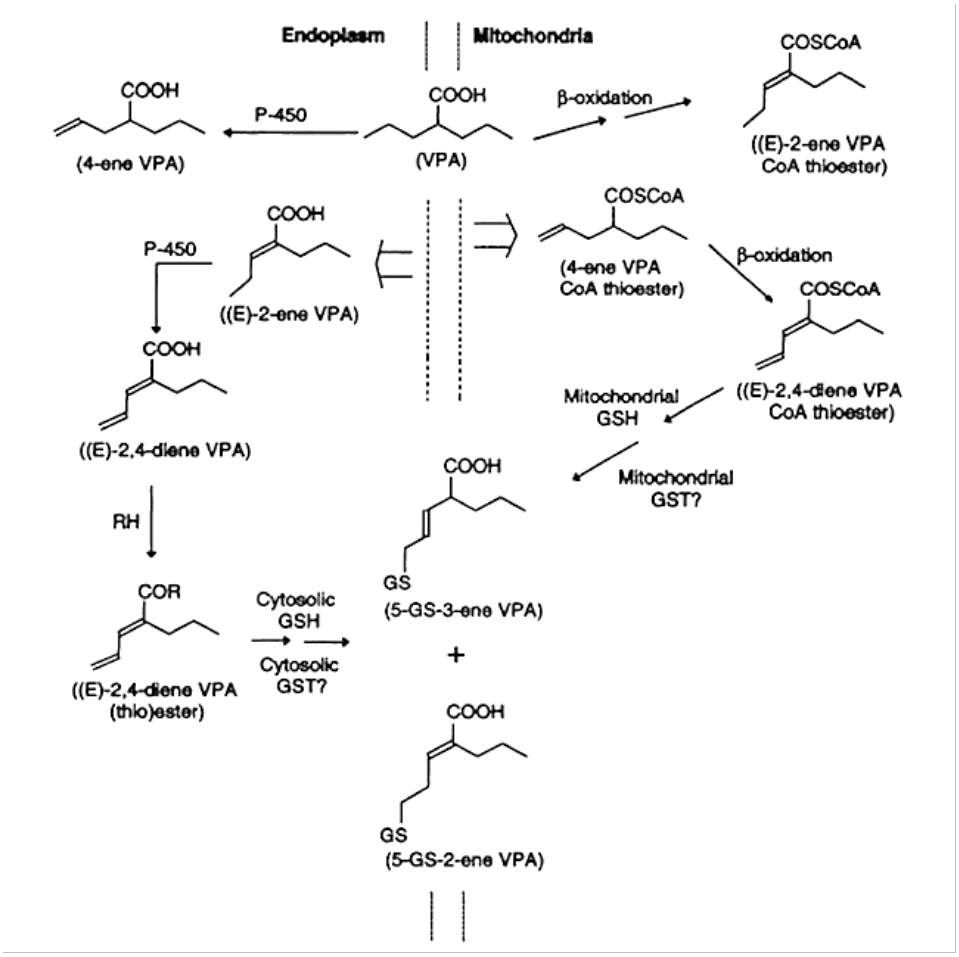

Figure 10. Bioactivation of valproic acid to (E)-2,4-diene VPA and formation of its GSH conjugates (*18*).

Table 8: The effects of human glutathione S-transferases on the conjugation of drug reactive intermediates

Substrate	Conjugate		No GST	hGSTA1-1	hGSTM1-1	hGSTP1-1	hGSTT1-1	Ref.
APAP	APAP	Product formation	3.4 ± 0.07	3.4 ± 0.2	2.2 ± 0.1	1.3 ± 0.03	-	(*17*)
	APAP-SG	(μM)	3.5 ± 0.07	5.3 ± 0.3	5.5 ± 0.3	7.4 ± 0.2	-	
	APAP-Cys		3.7 ± 0.08	1.7 ± 0.08	1.9 ± 0.09	1.3 ± 0.03	-	
Felbemate		$T_{1/2}$ (h)	37.8±6.8	26.4±2.4	11.3±1.2	17.7±0.4	-	(*16*)
		K_m (μM)	-	217±107	153±57	55±10	-	
		k_{cat}/K_m ($min^{-1}\mu M^{-1}$)	-	0.042±0.005	0.275±0.035	0.164±0.005	-	
Zileuton		$T_{1/2}$ (h)	6.4±0.4	2.6±0.1	0.53±0.02	0.32±0.04	-	(*19*)
		K_m (μM)	-	-	48.3±3.7	89.9±0.8	-	
		k_{cat}/K_m ($min^{-1}\mu M^{-1}$)	-	-	0.46±0.16	0.36±0.12	-	
Troglitazone	Met1	Relative peak area (%)	100	170*/170*	140	100	165*	(*21*)
	Met2		100	200*/105	280*	95	170*	
	Met3		100	150*/115*	100	90*	140*	
	Met4		100	120/120	185*	100	135*	
	Met5		100	220*/175*	165*	115	180*	
Clozapine	Total	(%)	100 ± 0.8	152 ± 3.5	274 ± 2.5	368 ± 1.5	Not active	(*20*)
	CG-1	Relative amounts	95.3 ± 0.7	77.8 ± 2.0	79.8 ± 0.5	17.5 ± 0.1	Not active	
	CG-3	(%)	4.7 ± 0.1	2.9 ± 0.1	1.5 ± 0.1	1.1 ± 0.0	Not active	
	CG-4		nd	nd	6.5 ± 0.2	0.2 ± 0.1	Not active	
	CG-5		nd	0.8 ± 0.1	nd	31.6 ± 0.1	Not active	
	CG-6		nd	18.5 ± 0.1	12.2 ± 0.1	49.6 ± 0.1	Not active	
Diclofenac	Total	Absolute amounts	2.5±0.2 (104.3±0.4)	3.4±1.3	15.9±2.3	21.7±2.6	125.8±4.0	*(225)*
	M2	(μM)	0.45±0.2 (11.27±1.0)	0.34±0.04	2.39±0.2	13.32±2.1	13.63±3.0	
	M5		1.12±0.2 (63.01±5.8)	1.91±0.3	11.96±1.2	1.39±0.6	74.48±2.0	
	M6		0.64±0.1 (7.41±0.2)	0.64±0.2	0.74±0.2	1.28±0.1	8.78±0.2	
	M7		n.d. (17.0±0.8)	n.d.	n.d.	n.d.	22.1±1.1	
	4'-OH-QI conjugates		2.22±0.4 (98.8±2.0)	2.90±0.6	15.1±1.6	16.0±1.5	119.0±8.0	
	M1		0.13±0.04 (3.34±0.4)	0.30±0.07	0.24±0.02	1.80±0.8	3.28±0.1	
	M3		0.15±0.02 (1.56±0.1)	0.15±0.03	0.41±0.06	3.57±0.21	1.95±0.1	
	M8		n.d. (0.63±0.1)	n.d.	n.d.	n.d.	1.02±0.2	
	5-OH-QI conjugates		0.28±0.04 (5.53±0.5)	0.45±0.05	0.65±0.08	5.37±0.9	6.25±0.5	

Zileuton is restricted for the treatment of asthma due to the severe hepatotoxicity that occurs in some of the patients. The mechanisms of toxicity most likely involves a sequence of biotransformation reactions forming 2-acetylbenzothiophene (2-ABT) that is further oxidized to reactive metabolite(s) (Figure 11). The mercapturate of 2-acetylbenzothiophene was identified in urine of rats dosed with zileuton *(223)*. Joshi *et al.* investigated detoxification reaction between 2-ABT-S-oxide and GSH (*19*). All tested cytosolic GSTs, GSTA1-1, GSTM1-1, and GSTP1-1 catalysed this reaction (Table 8). Although GSTA1-1 did catalyse the reaction between 2-ABT-S-oxide and GSH, kinetic data were not much different compared to non-enzymatic reaction. The contribution by GSTM1-1 and GSTP1-1 was found to be similar.

Incubations of GSTs with RIs can not be applied to highly reactive, short-lived reactive drug metabolites, such as nitrenium ions, or metabolites that are not available commercially or poorly accessible by organic synthesis. In these cases, the involvement of human GSTs in the GSH conjugation of reactive drug metabolites is studied by using cytochrome P450s as bioactivation system.

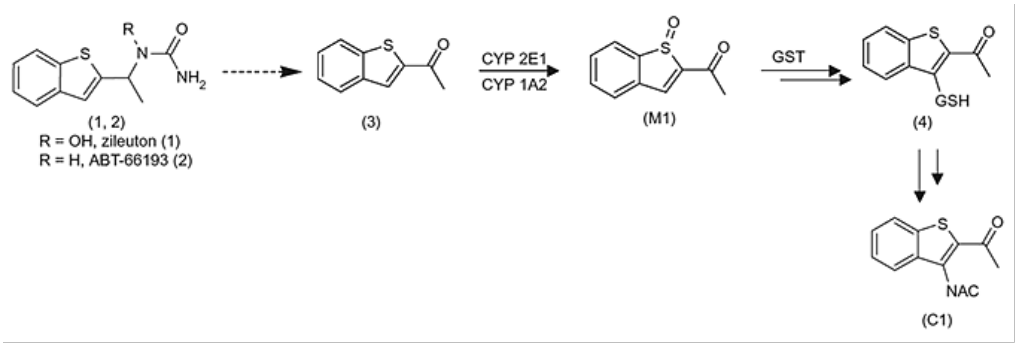

Figure 11. Zileuton bioactivation pathway. Adapted from (*19*).

As mentioned in section 4.1, GSTM1-1/GSTT1-1 double-null genotype correlate statistically significantly with abnormally high levels of alanine aminotransferase and aspartate aminotransferase in troglitazone treated diabetic patients (*164*). Subsequently, *in vitro* studies were performed to demonstrate the activity of GSTM1 and GSTT1 in inactivation of reactive troglitazone metabolites formed by HLM *(21, 224)*. The covalent binding levels (an index of reactive metabolite formation) of troglitazone and rosiglitazone were measured *in vitro*, using human liver samples with a diversity of P450 phenotypes and GST genotypes *(224)*. Addition of hGSTA1 or hGSTM1 significantly decreased the microsomal covalent binding for both troglitazone and rosiglitazone. Only for troglitazone the formation of a GSH adduct (Met2, Figure 5) of the reactive 5-glutathionyl-thiazolidine-2,4-dione was increased, which means higher exposure to the reactive metabolite in null genotypes. Contrary to expectations, covalent binding of troglitazone in GSTM1 or GSTT1 null hepatocytes was lower compared to wild-type hepatocytes. ATP-depletion was only observed in GSTM1 and GSTT1 null hepatocytes and independent of phase I enzyme activities as CYP3A and CYP2C8 activities, which affect reactive metabolite formation, were comparable among the hepatocytes. Measurement of exposure to the reactive metabolite by trapping as M2 or direct measurement of cytotoxicity in GSTM1- and GSTT1-genotyped hepatocytes could thus assist a better prediction of troglitazone-induced hepatotoxicity. Okada *et al. (21)* investigated the direct involvement of recombinant human GST-isoforms in the GSH conjugation of reactive metabolites of troglitazone (Table 8). It was reported that addition of hGSTA1, hGSTA2, hGSTM1 or hGSTP1 increased the formation of five GSH-conjugates produced from troglitazone reactive metabolites after incubation with human liver microsomes. The addition of GSTT1 did not show any catalytic effect. GST-isoforms contributed differently to the GSH-conjugation of the individual reactive metabolites of troglitazone, and GSTM1 was the most important GST-isoform in the GSH-conjugation of a specific reactive metabolite produced from the cytotoxic, quinone-metabolite of troglitazone (Met4, Figure 5). Both studies indicate that the higher formation of particular reactive metabolites, which are conjugated specifically by GSTM1 and/or GSTT1, could be more important as the risk factor for hepatotoxicity in the individuals lacking these enzymes.

In Chapter 3 of this thesis, we investigated the ability of recombinant human GSTs to catalyze the GSH-conjugation of the reactive nitrenium ion of clozapine (CLZ),

formed *in vitro* by human and rat liver microsomes and drug-metabolizing P450 BM3 mutants (*20*). Purified P450 102A1M11H, a BM3 mutant, was selected because it is able to metabolise CLZ to all relevant metabolites at a much higher rate than human and rat liver microsomes *(226)*. In the presence of three of the GSTs, i.e. hGSTP1-1, hGSTM1-1, and hGSTA1-1, total GSH-conjugation was strongly increased in all bioactivation systems tested. Results obtained in BM3-incubations are summarised in Table 8. Polymorphic hGSTT1-1 did not show any activity. Interestingly, the addition of hGSTs resulted in major changes in the regioselectivity of GSH-conjugation with all used bioactivation systems. Also, two GSH-conjugates which were previously only observed in *in vivo* studies were completely dependent on the presence of hGSTs (Figure 12). These results strongly suggest that genetic polymorphisms of hGSTP1-1 and hGSTM1-1 might contribute to the interindividual differences in susceptibility to clozapine-induced adverse drug reaction.

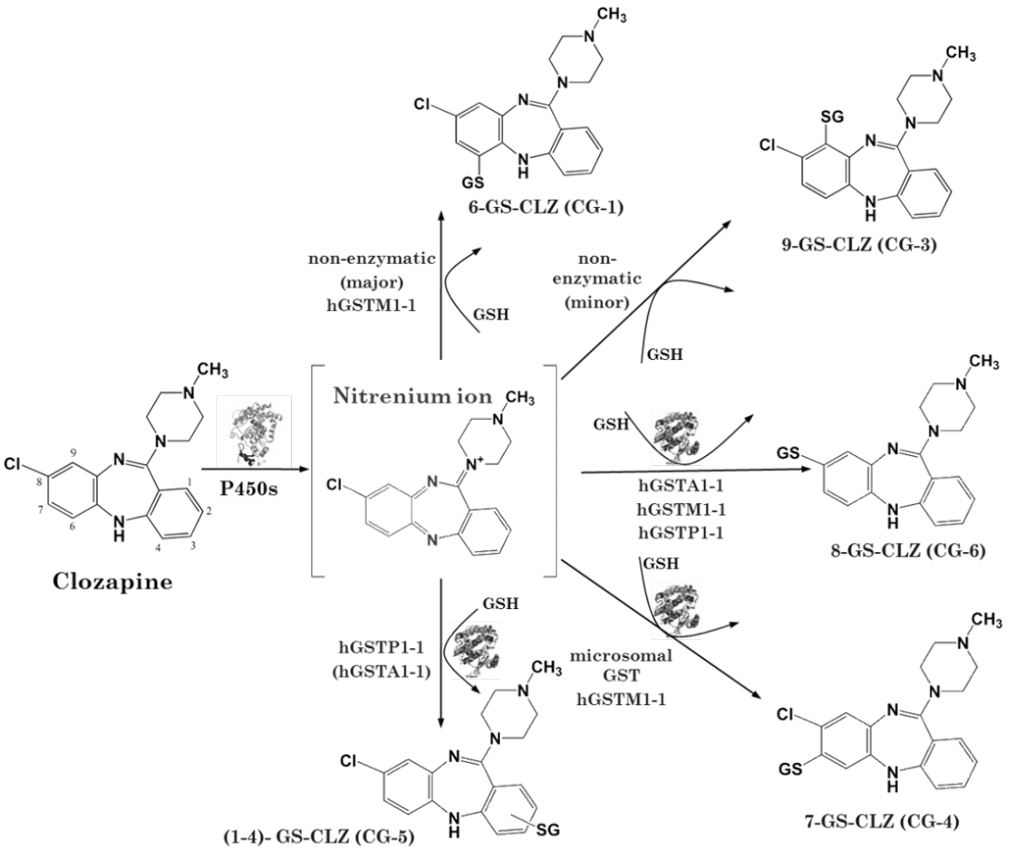

Figure 12. Role of hGSTs in the regioselective GSH-conjugation of the reactive nitrenium ion of clozapine (CLZ) formed by CYP450s. Adapted from (*20*).

A similar approach was used to test the effect of hGSTs on the inactivation of diclofenac (DF) reactive metabolites (Chapter 4) *(225)*. Diclofenac was bioactivated using human liver microsomes and P450 CYP102A1, a drug metabolizing bacterial mutant to four reactive intermediates: quinone imines from two major hydroxy-metabolites, 4´-hydroxydiclofenac (4'-OH-DF) and 5-hydroxydiclofenac (5-OH-DF), a reactive o-imine methide formed by the oxidative decarboxylation of diclofenac, and a novel minor reactive metabolite that leads to chlorine substitution by GSH. The role of GSTs in conjugation reactions between these reactive intermediates and GSH was investigated (Table 8). Three of the tested hGSTs, i.e. hGSTA1-1, hGSTM1-1 and hGSTP1-, showed activity in detoxification of the diclofenac reactive metabolites, with hGSTP1-1 showing the highest and hGSTA1-1 the lowest activity, Figure 13. hGSTT1-1 did not show significant activity. hGSTP1-1 showed the highest activity towards reactive metabolites of 5-OH-DF, while hGSTM1-1 catalysed mainly the formation GSH-conjugates of 4´OH-DF. hGSTs also catalyzed GSH-conjugation of the o-imine methide formed by oxidative decarboxylation of diclofenac as well as the substitution of one its chlorine atoms by GSH. hGSTP1-1 showed the highest activity for the formation of these conjugates. In summary, hGSTP1-1 was highly active in inactivating all four reactive intermediates that can be formed by P450s, and while not higly abundant in liver, it might play an important role in the protection of the gastrointestinal tract against DF-induced toxicity. On the other hand, deficiency of hGSTM1-1 might be a risk factor for DF-induced hepatotoxicity, particularly in conditions when cellular GSH becomes depleted and inactivation of reactive DF-metabolites will be more dependent on GST-catalyzed GSH-conjugation.

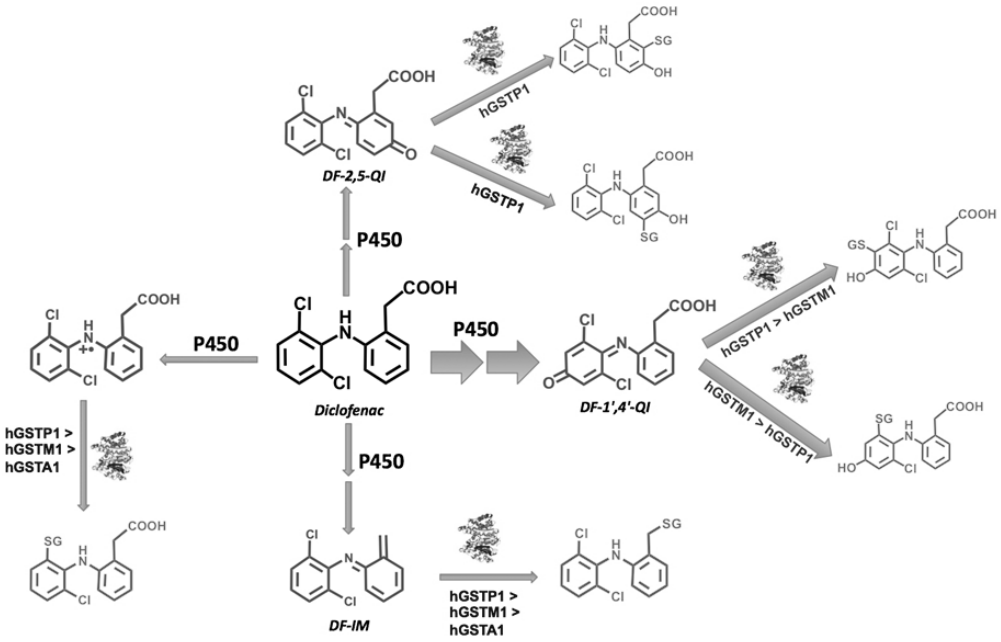

Figure 13. Role of hGSTs in the GSH-conjugation of the reactive intermediates of diclofenac. Adapted from *(225)*.

We have also investigated the ability of four allelic forms of hGSTP1-1, resulting from Ile105Val and Ala114Val substitutions, to catalyze the GSH-conjugation of reactive metabolites of acetaminophen, clozapine, and diclofenac formed by their bioactivation in incubations by human liver microsomes and drug metabolizing P450 BM3 mutants (Chapter 5). The results suggested that differences in GSH-conjugation due to hGSTP1-1 polymorphism, which was not higher than 30% of total GSH conjugates, might not result in differential susceptibility to adverse drug reactions caused by these drugs. Single mutation at residue 105 affected more the ability of the enzyme to catalyse GSH-conjugation comparing to single mutation at 114. Interestingly, hGSTP1-1 allelic forms showed altered regioselectivity towards formation of individual GSH conjugates of clozapine, implying changes in binding orientation of the substrates due to the mutations.

From all tested substrates in described *in vitro* studies, clinical studies were performed only for APAP and DF by now. Case control studies with large number of patients, correlating GST genotypes with susceptibility to side effects by these drugs are necessary to prove the true importance of these enzymes as a risk factor.

5. Conclusion

A number of clinical studies has shown associations between GST-genotypes and clinical outcomes. The link between genetic variants of GSTs with various malignancies has been examined in over 500 studies (*141*, *160*). Correlation between GST-genotypes and susceptibility for diseases was also demonstrated for multiple sclerosis *(227)*, Parkinson's disease *(228)*, rheumatoid arthritis *(229)*, asthma *(230)* and a few other diseases. Lately, several epidemiological studies in humans have also pointed to possible associations between GST-polymorphisms, such as GSTM1 and GSTT1 deletions, with increased risk for the occurrence of idiosyncratic adverse drug reactions (*10*, *12*, *163*, *164*). However, a direct relationship between an increased risk of drug-induced toxicity and GST-polymorphisms has not been established yet. Multifactorial and multigenic processes seems to be involved in complex ADRs including those involved in cellular signaling, adaptation and regeneration/repair processes. A better understanding of the factors affecting GST-expression and activity, accompanied by more incisive genetic analysis, may reveal further connections between GST genotypes and individual susceptibility to drug toxicity. At the same time, efforts are invested in evaluating detoxification mechanisms of reactive drug metabolites by GSTs in *in vitro* studies as well as in *in vivo* animal studies (*16*, *19–21*, *199*, *202*). A better understanding of mechanisms of detoxification and the role of GSTs, as well as larger case studies with enough statistical power in well-defined patient groups-cases and controls for drugs causing idiosyncratic toxicities will help to confirm these relationships.

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Chapter 3

ROLE OF HUMAN GLUTATHIONE S-TRANSFERASES IN THE INACTIVATION OF REACTIVE METABOLITES OF CLOZAPINE

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Abstract

Conjugation of reactive drug metabolites to GSH is considered an important detoxification mechanism that can be spontaneous and/or mediated by glutathione S-transferases (GSTs). In case GSTs play an important role in GSH conjugation, genetically determined deficiencies in GSTs may be a risk factor for adverse drug reactions (ADRs) resulting from reactive drug metabolites. So far, the role of GSTs in the detoxification of reactive intermediates of clozapine, a drug causing idiosyncratic drug reactions (IDRs), has not been studied. In the present study, we studied the ability of four recombinant human GSTs (hGSTA1-1, hGSTM1-1, hGSTP1-1, and hGSTT1-1) to catalyse the GSH conjugation of reactive metabolites of clozapine, formed in vitro by human and rat liver microsomes and drug-metabolising P450 BM3 mutant, P450 102A1 M11H. Consistent with previous studies, in absence of GSTs three GSH conjugates were identified derived from the nitrenium ion of clozapine. In presence of three of the GSTs, hGSTP1-1, hGSTM1-1, and hGSTA1-1, total GSH conjugation was strongly increased in all bioactivation systems tested. Highest activity was observed with hGSTP1-1, whereas hGSTM1-1 and hGSTA1-1 showed slightly lower activity. Polymorphic hGSTT1-1 did not show any activity in catalysing GSH conjugation of reactive clozapine metabolites. Interestingly, addition of hGSTs resulted in major changes in the regioselectivity of GSH conjugation of the reactive clozapine metabolite, possibly due to the different active site geometries of hGSTs. Two GSH conjugates found were completely dependent on the presence of hGSTs. Chlorine substitution of the clozapine nitrenium ion, which so far was only observed in in vivo studies, appeared to be the major pathway of hGSTP1-1-catalysed GSH conjugation, whereas hGSTA1-1 and hGSTM1-1 also showed significant activity. The second GSH conjugate, previously also only found in in vivo studies, was also formed by hGSTP1-1 and to a small extent by hGSTA1-1. These results demonstrate that human GSTs may play a significant role in the inactivation of reactive intermediates of clozapine. Therefore, further studies are required to investigate whether genetic polymorphisms of hGSTP1-1 and hGSTM1-1 contribute to the interindividual differences in susceptibility to clozapine-induced adverse drug reactions.

1. Introduction

Clozapine (CLZ) is an atypical antipsychotic drug, lacking extrapyramidal adverse effects and used in the treatment of refractory schizophrenia and with treatment-resistant patients *(1-3)*. In spite of its advantages, an important side effect of CLZ is agranulocytosis occurring in 1-2% of the patients *(2, 3)*. In addition, hepatotoxicity has been reported as a side effect of CLZ with 37% of the patients showing enhanced serum transaminases and 0.06% of the patients getting liver failure *(4)*. So far, several in vitro studies have been performed to identify the mechanism of toxicity. Although the exact mechanism is not known yet, formation of reactive metabolites such as nitrenium ions has been proposed as a possible explanation for these ADRs *(5-8)*.

The large number of *in vivo* and *in vitro* studies, performed in humans and animal models, collectively show that CLZ undergoes extensive oxidative metabolism, followed by phase 2 reactions *(7-12)*, as summarized in Figure 1. In human, N-demethylation and N-oxidation represent the major metabolic pathways. In addition, hydroxylation reactions have been demonstrated at positions 6, 7, 8, and 9 of the chlorinated ring of CLZ *(9-11)*. These hydroxy-metabolites are subject to subsequent glucuronidation and

sulfation reactions, Figure 1. In vitro studies have shown that several human cytochrome P450s are involved in oxidative metabolism of CLZ: P450 3A4 and P450 1A2 are involved in N-oxidation, whereas P450 2D6, P450 1A2, and P450 3A4 are involved in N-demethylation of CLZ *(13)*.

Next to these stable metabolites, CLZ is known to be bioactivated to reactive intermediates by myeloperoxidases and cytochrome P450s *(6-8)*. In vitro incubations of CLZ with hepatic microsomes (human and rat) in the presence of GSH or human neutrophils and myeloid cells have shown formation of several GSH conjugates, with GSH conjugated at the 6-, 7-, and 9-position *(6, 8)*, Figure 1. These conjugates might result from a reactive nitrenium intermediate which can be conjugated to GSH at different positions of the quinoid ring *(6)*. In case of peroxidase- and P450-mediated bioactivation of CLZ, GSH conjugation mainly occurs at the 6-position of the chlorinated aromatic ring and to a lower extent to the 9-position *(8)*. An additional minor GSH conjugate formed in vitro by human and rat liver microsomes was tentatively assigned to the 7-position *(8)*. Two other GSH adducts were found only in vivo in bile of rats and mice, and were considered to be GSH conjugates resulting from still unidentified reactive intermediates *(8)*. One GSH conjugate results from substitution of the chlorine by GSH. Identification of 8-methylthio-deschloroclozapine (8-SCH_3-desCLZ, Figure 1) in urine of patients *(9)*, most likely resulting from initial substitution of the 8-chlorine by GSH, suggest that this bioactivation pathway is also represented in human. Another GSH conjugate found only in vivo was proposed to result from conjugation to the non-chlorinated ring of CLZ *(8)*.

Although formation of multiple GSH conjugates of CLZ has been demonstrated in many in vitro studies, the role of GSTs in catalysing GSH conjugation of reactive CLZ metabolites has remained unexplored. GSTs are a family of enzymes whose main role is detoxifying electrophilic xenobiotics forming stable and more hydrophilic GSH conjugates for easier excretion from the body *(16-19)*. An increasing number of GST genes are being recognized as polymorphic. In humans, marked interindividual differences exist in the expression of class Alpha, Mu, Pi and Theta GSTs *(20)*. Genetic polymorphism of GSTs has been associated with increased susceptibility of several forms of cancers, alcoholic liver disease, and toxic hepatitis caused by chemicals and drugs *(21-25)*. Several clinical studies have demonstrated an increased susceptibility to idiosyncratic drug-induced liver injury by the combined GSTM1-T1 double-null genotype *(22, 23)*.

So far, only few in vitro studies have been performed to investigate the role of GSTs in inactivation of electrophilic drug metabolites *(26-29)*. Several rat and human GSTs catalyze the GSH conjugation reaction of synthetical N-acetylbenzoquinoneimine, the reactive metabolite of acetaminophen *(26)*. In case of valproic acid, it was found that non-enzymatic GSH conjugation to its reactive diene metabolite results in a single GSH conjugate. Addition of GSTs, however, resulted in a second GSH conjugate, which was also found in vivo in rats treated with valproic acid *(27)*. For felbamate *(28)* and zileuton (29), the rate of GSH conjugation to their electrophilic metabolites, 2-phenylpropenal and 2-acetylbenzothiophene, respectively, was increased in presence of all three studied human GST isoforms (hGSTA1-1, hGSTM1-1 and hGSTP1-1).

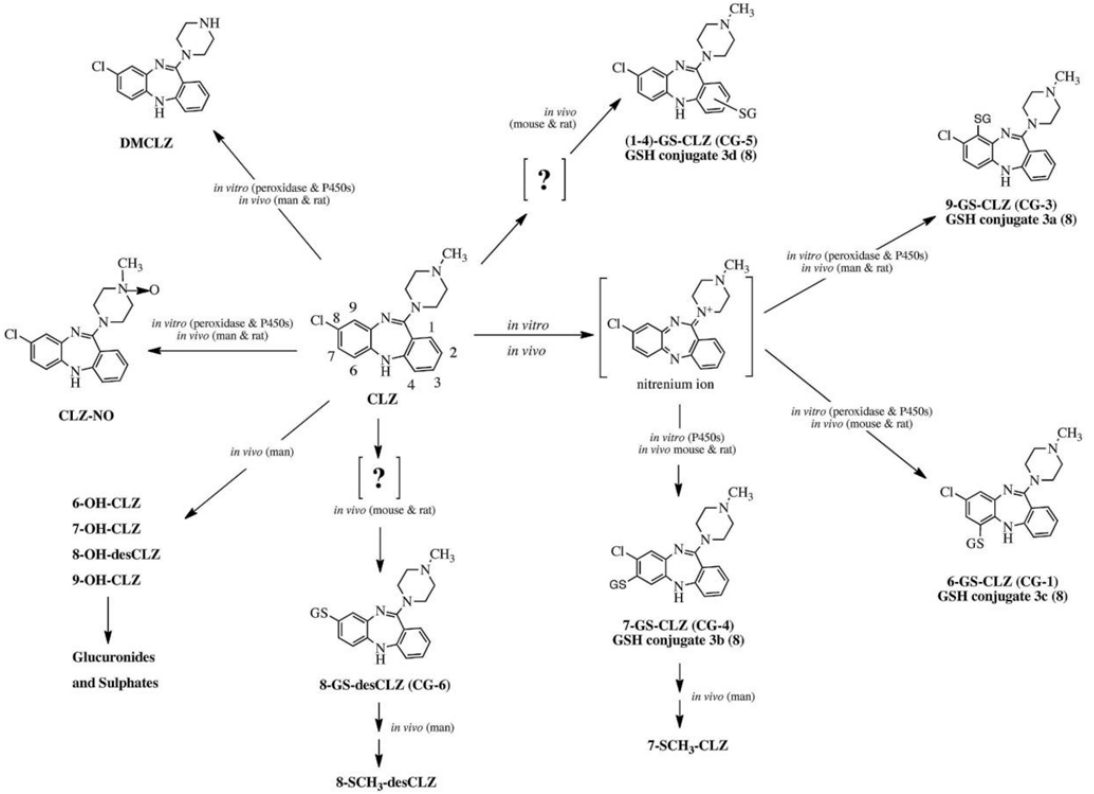

Figure 1. Scheme of the oxidative metabolism of clozapine (CLZ) to stable and reactive metabolites, and subsequent phase II metabolism, as found in in vitro and in vivo studies (adapted from refs. *(8-11)*).
Major stable metabolites and GSH conjugates are shown with structures, others are indicated by abbreviations. The proposed reactive intermediate of CLZ is depicted in brackets and the structures of GSH-adducts are presented as described in reference (8). Conjugates CG-5 and CG-6 were proposed to result from as yet unidentified reactive intermediates (8). DMCLZ, N-desmethylclozapine; CLZ-NO, clozapine N-oxide; 8-SCH_3-desCLZ, 8-thiomethyldeschloroclozapine; 7-SCH_3-CLZ, 7-thiomethylclozapine; 6-OH-CLZ, 6-hydroxyclozapine; 7-OH-CLZ, 7-hydroxyclozapine; 8-OH-desCLZ, 8-hydroxydeschloroclozapine; 9-OH-CLZ, 9-hydroxyclozapine.

In all of these studies, the GST-incubations were performed in presence of a synthetical electrophilic drug metabolite. However, this approach will not be applicable to highly reactive, short-lived reactive drug metabolites, or metabolites which are not or poorly accessible by organic synthesis. Therefore, in the present study, the role of human GSTs in the GSH conjugation of reactive CLZ metabolites was studied in incubations of CLZ with human and rat liver microsomes and a drug-metabolising bacterial P450 BM3 mutant (P450 102A1 M11H) as bioactivation systems. Purified P450 102A1 M11H was selected because it is able to bioactivate CLZ to all relevant metabolites at much higher activity than human and rat liver microsomes *(12)*. The results demonstrate that hGSTs play an important role in inactivation of reactive metabolites of CLZ and explain the formation of several GSH conjugates previously only found in in vivo studies.

2. Materials and methods

2.1. Enzymes and plasmids

The bacterial P450 BM3 mutant, P450 102A1 M11H, was prepared and purified as described previously *(12)*. Rat liver microsomes were prepared according to protocol already used in our laboratory *(30)*. Human liver microsomes (Lot No. 0710619), pooled from 50 donors, were obtained from Xenotech (Lenexa, USA) and contained 20 mg/mL protein. E. Coli XL-1 Blue cells containing the expression plasmids for human GSTA1-1, GSTM1-1 (B allele) and GSTP1-1 (A allele) were a kind gift from Prof. Mannervik (Department of Biochemistry and Organic chemistry, Uppsala University, Sweden). The plasmid pet20b-hGSTT1 *(33)*, coding for human GSTT1-1 with a C-terminal his-tag, was a kind gift from Prof. Hayes (Biomedical Research Centre, University of Dundee, Scotland, UK).

All other chemicals and reagents were of analytical grade and obtained from standard suppliers.

2.2. Expression and purification of human GSTA1-1, GSTM1-1 and GSTP1-1

Overnight precultures of E. Coli XL-1 Blue cells containing the expression plasmids for human GSTs were prepared in 5 mL LB medium supplemented with 50 µg/mL ampicillin and grown at 37°C and 175 rpm. For large scale enzyme expression, 500 ml 2YT medium fortified with 50 µg/mL ampicillin was inoculated with 5 mL of an overnight culture and cells were grown at 37°C until OD600 reached 0.3. Enzyme expression was then induced by addition of IPTG to a final concentration of 0.2 mM and cells were incubated overnight at 37°C and 150 rpm. The following procedures were all carried out at 4 °C. Cells were harvested by centrifugation at 4000 g for 15 minutes. Pellets were resuspended in 10 mM Tris-HCl pH 7.8 containing 1 mM DTT and 0.2 mg/mL lysozyme and allowed to incubate for 1 hour. Cells were subsequently disrupted by sonication (Branson sonifier 250, 4x 30 sec, at 60% full power) and cell debris removed by centrifugation in an eppendorf centrifuge (50 min at 20 000 g). Lysate was incubated with a 50% slurry of GSH sepharose 4B (GE healthcare) on a rollerbank for 90 minutes. Nonspecifically bound proteins were removed by washing of the beads with three changes of 10 mM Tris-HCl pH 7.8 containing 1 mM DTT. GSTs were eluted with 50 mM Tris-HCl pH 8 supplemented with 10 mM GSH and 1 mM DTT. The GSH was subsequently

removed by repeated washing with 10 mM Tris-HCl pH 7.8 containing 1 mM TCEP in a Vivaspin 20 filtration tube (10,000 MWCO PES, Sartorius) at 4000 g. The washing procedure was continued until the GSH concentration was below 500 nM. Enzymes were aliquoted and stored at -20°C until use.

Protein concentrations were determined according to the method of Bradford *(31)* with reagent obtained from Bio-Rad. The speciific activity of the purified GSTs was assayed according to Habig et al. *(32)*. The specific activities of the purified recombinant human GSTs using CDNB as a substrate were as follows: 20.4 μmol/min/mg protein for GSTA1-1, 55.6 μmol/min/mg protein for GSTM1-1, 26.9 μmol/min/mg protein for GSTP1-1.

2.3. Expression and purification of human GSTT1-1

E. Coli BL21 DE3 competent cells were transformed with pet20b-hGSTT1 by heatshock. For expression, 400 ml LB containing 50 μg/mL ampicillin was inoculated with 3 mL of an overnight culture and cells were grown at 37°C and 150 rpm. IPTG was added to the culture in a final concentration of 0.5 mM when OD600 reached 0.7. After an induction period of approximately 6 hours, cells were harvested (4000 g, 15 min, 4°C) and pellets frozen overnight at -20°C.

The following steps were conducted at 4°C. Cell pellets were reconstituted in NaP buffer (50 mM sodium phosphate buffer pH 8, 0.2 M NaCl) supplemented with 0.2 mg/mL lysozyme. Following an incubation period of 30 minutes, cells were disrupted using a French Press (1000 psi, 3 repeats). Cell debris was removed by ultracentrifugation (120 000 g; 70 minutes) and the supernatant passed through a 0.45 μm filter (Whatman).

The GST was purified using Ni-NTA agarose (Sigma). A 50% slurry of Ni-NTA in NaP buffer was added to the lysate and incubated on the rollerbank for 2 hours. The solution was then applied to a disposable column (Pierce, Rockford, USA). Nonspecifically bound proteins were removed by extensive washing with NaP buffer fortified with 2 mM imidazole. His-tagged hGST T1-1 was eluted with NaP buffer supplemented with 250 mM imidazole and then dialysed overnight against NaP buffer. For storage, this buffer was exchanged for 25 mM Tris-HCl pH 7.7 containing 1 mM TCEP and 20% glycerol by repeated washing in a Vivaspin 20 filtration tube (10,000 MWCO PES, Sartorius) at 4000 g and by overnight dialysis. Purified enzyme was stored at -80 °C until use. The protein concentration of purified hGST T1-1 was determined according to the method of Bradford *(31)* with reagent obtained from Bio-Rad. The activity of hGSTT1-1, using 1,2-epoxy-3-(p-nitrophenoxy)propane as a substrate, was determined essentially as described *(34)*. The specific activity of his-tagged hGSTT1-1 using 1,2-epoxy-3-(p-nitrophenoxy)propane was 1.83 μmol/min/mg protein.

2.4. Bioactivation of clozapine by P450 102A1 M11H in presence of GSH and human recombinant glutathione S-transferases

Incubations using purified P450 102A1 M11H as bioactivation system were performed at a final enzyme concentration of 250 nM, as described previously *(12)*. All incubations were performed in 100 mM potassium phosphate buffer (pH 7.4) and at a

final volume of 250 µL. The substrate CLZ was incubated at a concentration of 500 µM. The final DMSO concentration from the CLZ stock-solution was less than 1% in the incubations. Non-enzymatic GSH conjugation was investigated at different GSH concentrations (0.0125, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 mM). Reactions were initiated by the addition of 500 µM NADPH (final concentration) and performed for 30 minutes at room temperature. In this time period, product formation was linear as described previously *(12)*. Reactions were terminated by addition of 25 µL 10% $HClO_4$, and centrifuged for 15 minutes at 4000 rpm. Supernatants were analysed by HPLC and LC-MS, as described below.

Enzymatic GSH conjugation by recombinant human GSTs was investigated by adding 8 µM (final concentration) hGSTA1-1, hGSTM1-1, hGSTP1-1, or hGSTT1-1 to the incubations. Incubations with hGSTs were performed in presence of 100 µM of GSH. hGSTT1-1 was also incubated in presence of 5 mM GSH because of its lower affinity to GSH (33). Finally, hGSTs, showing activity in catalysing GSH conjugation of CLZ metabolites, were also tested at 0.25, 0.5, 1, 2, 4, and 8 µM to investigate linearity of product formation with protein concentration.

2.5. Bioactivation of clozapine by human and rat liver microsomes in presence of GSH and human recombinant glutathione S-transferases.

To confirm that the catalytic effects of hGSTs, as observed in P450 BM3-incubations, were also applicable for incubations with mammalian liver fractions, CLZ was also incubated with human liver microsomes and and rat liver microsomes, each at a final microsomal protein concentration of 1 mg/mL, 100 µM GSH, and in absence or presence of 8 µM hGST. Reactions were initiated by the addition of 500 µM NADPH (final concentration) and were incubated for 30 min at 37 °C. Reactions were terminated by addition of 25 µL 10% $HClO_4$, and centrifuged to remove precipitated proteins (4000 rpm, 15 min). Supernatants were analysed by HPLC and LC-MS, as described below.

2.6. Analytical methods

The metabolites of CLZ were analysed by reversed-phase liquid chromatography. A Luna 5 µm C18 column (150 mm x 4.6 mm i.d.) from Phenomenex was used as stationary phase, protected by a 4.0 mm × 3.0 mm i.d. security guard (5 µm) C18 guard column (Phenomenex, Torrance, CA, USA). The gradient used was constructed by mixing the following mobile phases: solvent A (1 % acetonitrile, 99 % water, and 0.2 % formic acid); solvent B (99 % acetonitrile, 1 % water, and 0.2 % formic acid). The first 5 min were isocratic at 0 % solvent B; from 5 to 30 min the concentration of solvent B linearly increases to 100 %; from 30 to 35 min linear decreased to 0 % B and maintained at 0 % for re-equilibration until 40 min. The flow rate was 0.5 mL/min. Samples were injected at an injection volume of 50 µL.

Samples were analyzed using LC-MS/MS for identification and UV/VIS detector on 254 nm for quantification. A standard curve of CLZ was used to estimate the concentrations of the formed GSH conjugates, assuming that the extinction coefficients of the GSH adducts are equal to that of CLZ. The standard curve of CLZ was linear between 1 and 100 µM; the limit of quantitative detection by UV/VIS was estimated to be 0.2 µM

(data not shown). The Shimadzu Class VP 4.3 software package was used for determination of peak areas in the UV chromatograms.

For identification of metabolites an Agilent 1200 Series Rapid resolution LC system was connected to a hybrid quadrupole-time-of-flight (Q-TOF) Agilent 6520 mass spectrometer (Agilent Technologies, Waldbronn, Germany), equipped with electrospray ionization (ESI) source and operating in the positive mode. The MS ion source parameters were set with a capillary voltage at 3500 V; nitrogen was used as the desolvation and nebulizing gas at a constant gas temperature of 350 °C, drying gas 8 L/min and nebulizer 40 psig. Nitrogen was used as a collision gas with collision energy voltage of 25V. MS spectra were acquired in full scan analysis over an m/z range of 50–1000 using a scan rate of 1.003 spectra/s. The MassHunter Workstation Software (version B.02.00) was used for system operation and data collection. Data analysis was performed using Agilent MassHunter Qualitative analysis software.

3. Results

3.1. Role of GSTs in inactivation of CLZ metabolites formed by P450 102A1 M11H

It was previously shown that purified P450 102A1 M11H metabolizes CLZ to all metabolites produced by human and rat liver microsomes but at significantly higher levels *(12)*. As shown in Table 1, when incubated in presence of 5 mM GSH, but in absence of hGSTs, approximately 30% of CLZ concentration was converted to nine metabolites of which four were GSH dependent, CG-1, CG-2, CG-3, and CG-4, consistent with the previous study *(12)*. The characteristics and identity of the different metabolites found are listed in Table 2.

In incubations with 100 μM GSH the concentration of metabolites C1 to C-5 did not change significantly indicating that the activity of P450 102A1 M11H is not influenced by GSH. As expected, at 100 μM GSH significantly lower amounts of the corresponding GSH conjugates were found; the concentration of GSH conjugate CG-4 was too low to quantify. However, the total concentration of GSH conjugates was only 4.8-fold lower than that found at 5 mM GSH, suggesting that even at 100 μM GSH already a significant fraction of the reactive intermediates is trapped by GSH. To test this hypothesis, the dependence of the non-enzymatic GSH conjugation on GSH concentration was investigated using concentrations ranging from 0 and 5 mM. As shown in Figure 2, between 0 and 100 μM of GSH, the concentration of GSH-conjugates increased almost linear with increasing concentration of GSH. At GSH concentrations higher than 1 mM, only relatively small increases in concentration of GSH conjugates was observed suggesting that at the high GSH concentrations the reactive intermediates are trapped almost quantitatively. Assuming that the observed saturation curve in Figure 2 is described by the equation $y = a*(1-e^{-b*x})$, the maximal yield of GSH-conjugates would be 38 μM at infinitive GSH concentration. From this it is estimated that at 100 μM GSH approximately 18 % of the reactive intermediates formed is trapped non-enzymatically by GSH, whereas at 5 mM GSH approximately 85% is trapped by GSH.

To investigate whether hGSTs are able to catalyse formation of GSH conjugates, incubations were performed in presence of 8 μM hGST and 100 μM GSH, as done

previously *(29)*. At this low GSH concentration, hGST activity will still be close to maximal, whereas the competing non-enzymatic reaction will be minimized.

Table 1. Effect of glutathione concentration on concentration of metabolites formed in incubations of clozapine with bacterial P450 102A1 M11H mutant in absence of human GSTs.

	Concentration of clozapine metabolites (μM) [a]	
	100 μM GSH	5 mM GSH
GSH-conjugates		
CG-1	5.9 ± 0.2	26.3 ± 0.5
CG-2	0.3 ± 0.2	1.4 ± 0.1
CG-3	0.5 ± 0.2	3.0 ± 0.2
CG-4	n.d. [b]	1.2 ± 0.1
CG-5	n.d.	n.d.
CG-6	n.d.	n.d.
CG-7	n.d.	n.d.
CG-8	n.d.	n.d.
Other metabolites		
C-1	18.8 ± 0.3	19.5 ± 0.9
C-2	84.5 ± 0.5	88.2 ± 1.6
C-3	1.6 ± 0.2	2.1 ± 0.1
C-4	2.7 ± 0.1	2.9 ± 0.1
C-5	3.3 ± 0.2	2.8 ± 0.2
Total	118 ± 1.9	147 ± 3.8

[a] Concentration of metabolites formed after 30 minutes of incubations of 200 nM P450 102A1 M11H with 500 μM clozapine in presence of 100 μM or 5 mM GSH.
Quantification was based on peak areas in the LC-UV-chromatograms using a standard curve of clozapine, assuming that the extinction coefficients of the substrate and the metabolites at 254 nm are identical.
[b] n.d., not detectable (limit of detection: 0.2 μM).

3.1.1. *Effect of human GST P1-1*

As shown in Figure 3A, addition of 8 μM hGSTP1-1 to the incubation of CLZ with P450 BM3 and 100 μM GSH resulted in major changes in the profile of GSH conjugates. Addition of hGSTP1-1 resulted in the formation of significant amounts of four additional GSH conjugates, CG-5, CG-6, CG-7, and CG-8. Amounts of CG-1, CG-2, and CG-3 did not change significantly. By assuming that extinction coefficients of all GSH conjugates at 254 nm are equivalent, the total amount of GSH conjugates was increased 3.7-fold by hGSTP1-1 when compared to corresponding incubations in absence of hGST, Table 3. CG-6 and CG-5 were the major GSH conjugates in presence of hGSTP1-1, representing 49.6 and 31.6% of the total amount of GSH conjugates, respectively, Table 3.

Table 2. Metabolites of clozapine formed by P450 102A1 M11H mutant in presence and absence of glutathione and recombinant human GSTs.

Metabolites	t_{ret} (min)	Exact mass m/z [a]	Elemental composition	Change of parent drug	Identity [b]
GSH-conjugates					
CG-1	17.6	632.230	$C_{28}H_{35}ClN_7O_6S$	CLZ + GS	C-6 glutathionyl clozapine
CG-2	17.2	618.216	$C_{27}H_{33}ClN_7O_6S$	CLZ-CH_2 + GS	
CG-3	17.4	632.230	$C_{28}H_{35}ClN_7O_6S$	CLZ + GS	C-9 glutathionyl clozapine
CG-4	16.7	632.230	$C_{28}H_{35}ClN_7O_6S$	CLZ + GS	C-7 glutathionyl clozapine
CG-5	17.9	632.230	$C_{28}H_{35}ClN_7O_6S$	CLZ + GS	C(1-4) glutathionyl clozapine
CG-6	15.0	598.269	$C_{28}H_{35}N_7O_6S$	CLZ-Cl + GS	C-8 glutathionyl deschloroclozapine
CG-7	15.4	903.348	$C_{38}H_{50}N_{10}O_{12}S_2$	CLZ-Cl + 2GS	
CG-8	14.7	584.253	$C_{27}H_{33}N_7O_6S$	CLZ-Cl-CH_2 + GS	
Other metabolites					
C-1	19.9	343.147	$C_{18}H_{20}ClN_4O$	CLZ + O	clozapine N-oxide
C-2	18.2	313.135	$C_{17}H_{18}ClN_4$	CLZ-CH_2	N-desmethylclozapine
C-3	16.4	301.134	$C_{16}H_{18}ClN_4$	CLZ-C_2H_2	
C-4	16.2	287.118	$C_{15}H_{16}ClN_4$	CLZ-C_3H_4	
C-5	19.5	329.132	$C_{17}H_{18}ClN_4O$	CLZ-CH_2 + O	

Metabolites were formed by incubating clozapine (500 µM) for 30 minutes with 250 nM P450 102A1 M11H and 500 µM NADPH.
t_r , retention time in minutes.
[a] *m/z* values correspond to the singly protonated molecular ion [(M+1H)/1]. Molecular composition and change of the parent drug are proposed.
[b] Identification was based on identity of mass spectra and order of elution as described previously by Maggs et al. *(8)*.

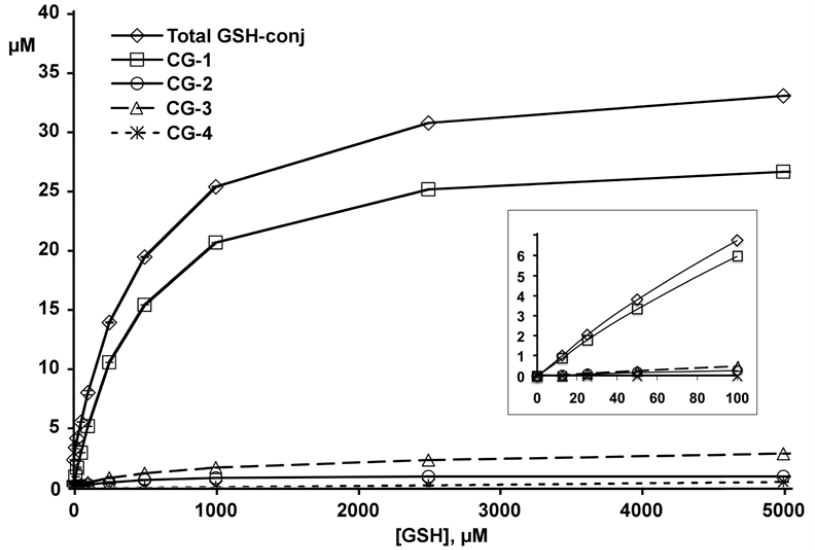

Figure 2. Dependence of non-enzymatic GSH conjugation of reactive clozapine metabolites formed by P450 102A1 M11 on GSH concentration. Insert: detail from 0 to 100 µM GSH.

LC-MS analysis of CG-6 showed a protonated molecular ion [M + H]+ of m/z 598.3 ([CLZ-SG – Cl + H]+). The MS/MS of this ion displayed product ions of m/z 580.3 ([CLZ-SG – Cl – H2O + H]+); m/z 541.2 ([CLZ-SG – Cl – C_3H_7N (piperazine ring) + H]+); m/z 469.2 ([CLZ-SG – Cl – glutamic acid + H]+); m/z 412.2 ([CLZ-SG – Cl – glutamic acid – C_3H_7N(piperazine ring) + H]+); m/z 325.2 ([CLZ-SG – glutamic acid – glycine – C_3H_7NO(GSH-moiety) + H]+) and 268.1 ([CLZ-SG – glutamic acid – glycine – C_3H_7NO(GSH-moiety) – C_3H_7(piperazine ring) + H]+). Loss of the γ-glutamyl moiety ([M+1-129] +) giving m/z 469.2 and scission of the S-CH2 linkage with hydrogen transfer to the CLZ thiyl moiety 9 [M+1-273]+ giving m/z 325.2 are typical characteristics for this GSH adduct. Based on the identical m/z value and fragmentation pattern, this product is identified as C-8 glutathionyl deschloroclozapine, which previously was only found in bile of rat and mice administered CLZ *(12)*. This product was not found in incubations in absence of P450 BM3 or NADPH, and therefore should represent a GSH conjugate of an oxidative CLZ metabolite. Formation of C-8 glutathionyl deschloroclozapine can be rationalised by enzymatic substitution of the chlorine-atom of the CLZ nitrenium ion by GSH, followed by reduction by NADPH and/or GSH to restore aromaticity.

LC-MS analysis of the second major GSH conjugate, CG-5, showed a protonated molecular ion [M + H]+ of m/z 632.2 [CLZ-SG + H]+ and therefore apparently corresponds to a regioisomer of CG-1 and CG-3, which both result from addition of GSH tot the CLZ nitrenium ion. CG-5 showed the following fragmentation pattern: m/z 614.2 ([CLZ-SG – H2O + H]+); m/z 575.2 ([CLZ-SG – C_3H_7N(piperazine ring) + H]+); m/z 503.2 ([CLZ-SG – glutamic acid +H]+); m/z 446.1 ([CLZ-SG – glutamic acid – C_3H_7N(piperazine ring) + H]+); m/z 359.1 ([CLZ-SG – glutamic acid – glycine – C_3H_7NO(GSH-moiety) + H]+) and 302.1 ([CLZ-SG – glutamic acid – glycine – C_3H_7NO(GSH-moiety) – C_3H_7N (piperazine ring) +

H]+). Because CG-1 and CG-3 were previously identified by 1H-NMR as GSH conjugates at the C-6 and C-9 positions of the nitrenium ion, CG-5 might correspond to an adduct to the C-7 position, the only remaining position on the chlorinated ring, or a GSH conjugate to the non-chlorinated ring, as suggested previously *(8)*.

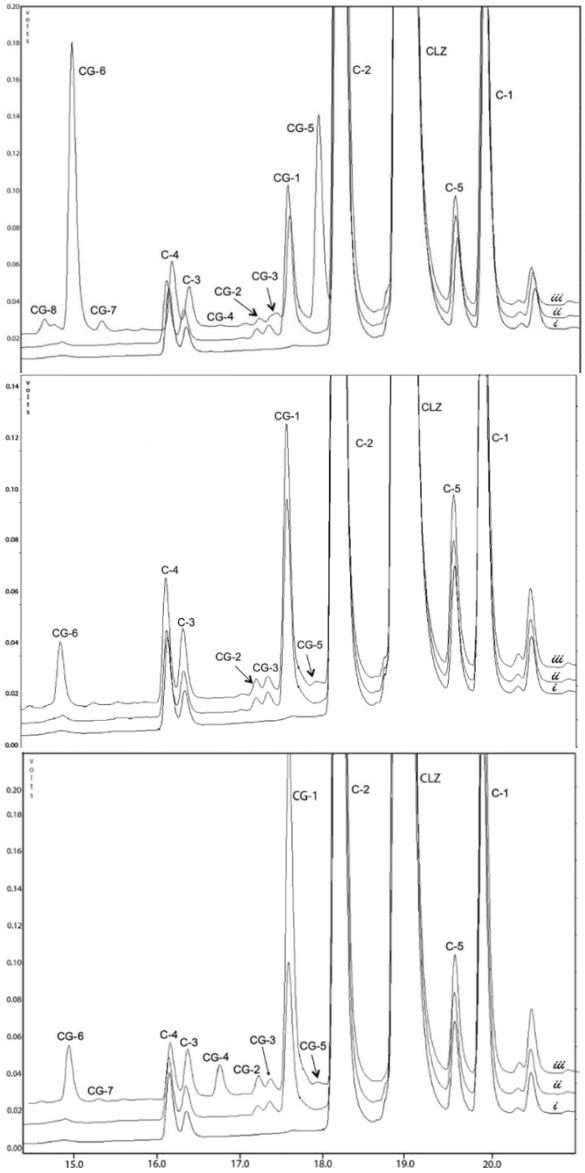

Figure 3. HPLC-UV chromatograms of incubations of clozapine using bacterial P450 102A1 M11H mutant as bioactivation system, in absence and presence of GSH and/or hGSTs. (A) Addition of 8 μM hGSTP1-1. (B) Addition of 8 μM hGSTA1-1. (C) Addition of 8 μM hGSTM1-1.
Traces: i: incubations in absence of GSH and GST; ii: incubations with 100 μM GSH; iii: incubations in the presence of 100 μM GSH and 8 μM hGSTs.

Table 3. Effect of recombinant human glutathione S-transferases (hGST) on the formation of GSH conjugates of reactive metabolites formed by oxidative bioactivation of clozapine.

Oxidative bioactivation system	Recombinant human GST	Total of GSH-conjugates (%)[a]	Relative amounts of GSH-conjugates (%)[b]				
			CG-1 (m/z 632)	CG-3 (m/z 632)	CG-4 (m/z 632)	CG-5 (m/z 632)	CG-6 (m/z 598)
BM3 M11$_{his}$ (250 nM)	None	100 ± 0.8	95.3 ± 0.7	4.7 ± 0.1	n.d.[c]	n.d.	n.d.
	hGST A1-1	152 ± 3.5	77.8 ± 2.0	2.9 ± 0.1	n.d.	0.8 ± 0.1	18.5 ± 0.1
	hGST M1-1	274 ± 2.5	79.8 ± 0.5	1.5 ± 0.1	6.5 ± 0.2	n.d.	12.2 ± 0.1
	hGST P1-1	368 ± 1.5	17.5 ± 0.1	1.1 ± 0.0	0.2 ± 0.1	31.6 ± 0.1	49.6 ± 0.1
Human liver microsomes (1 mg/mL)	None	100 ± 0.5	76.4 ± 0.1	4.0 ± 0.1	19.7 ± 0.3	n.d.	n.d.
	hGST A1-1	134 ± 1.0	66.0 ± 0.3	2.5 ± 0.1	13.3 ± 0.1	1.2 ± 0.1	17.0 ± 0.2
	hGST M1-1	145 ± 4.8	71.3 ± 2.4	2.2 ± 0.1	14.2 ± 0.2	n.d.	12.3 ± 0.6
	hGST P1-1	372 ± 4.5	14.7 ± 0.1	0.5 ± 0.1	6.0 ± 0.2	18.3 ± 0.3	60.5 ± 0.5
Rat liver microsomes (1 mg/mL)	None	100 ± 0.2	95.1 ± 0.1	4.9 ± 0.1	n.d.	n.d.	n.d.
	hGST A1-1	135 ± 2.0	76.9 ± 0.5	3.2 ± 0.2	n.d.	1.8 ± 0.2	18.1 ± 0.5
	hGST M1-1	153 ± 5.5	79.8 ± 3.1	2.8 ± 0.1	3.8 ± 0.1	n.d.	13.5 ± 0.3
	hGST P1-1	243 ± 2.4	25.9 ± 0.1	1.7 ± 0.1	n.d.	25.4 ± 0.1	47.0 ± 10.7

Clozapine (0.5 mM) was incubated for 30 minutes with different bioactivation systems in presence of 100 µM of glutathione, and in absence or presence of different recombinant human glutathione S-transferases (at 8 µM). Relative quantification was performed by integrating peaks of LC-UV-chromatograms and assuming that the extinction coefficients of the different GSH conjugates at 254 nm are identical.

[a] 100% * [Sum of peak areas of all GSH-conjugates] / [Sum of peak areas of GSH-conjugates in absence of recombinant human GST].

[b] 100% * [Peak area of individual GSH-conjugate] / [Sum of peak areas of all GSH-conjugates].

Assigned structures, based on identity of mass spectra and order of elution [8]: CG-1, C-6 glutathionyl clozapine; CG-3, C-9 glutathionyl clozapine; CG-4, C-7 glutathionyl clozapine; CG-5, C1-4-glutathionyl clozapine; CG-6, C-8 glutathionyl deschloroclozapine.

[c] n.d., not detectable because below level of detection (0.2 µM).

The two minor GSTP1-1 dependent adducts CG-7 (tret 15.4 minutes) and CG-8 (tret 15.4 minutes), Fig. 3A, showed protonated molecular ions of m/z ratio 903.3 and 584.2, respectively, and did not show a chlorine isotope pattern. CG-7 most likely represents a secondary metabolite of CG-6, resulting from addition of an additional glutathionyl group. CG-8 most likely results from chlorine substitution of the corresponding nitrenium ion of desmethylclozapine (C-2), the major metabolite of CLZ, Table 1.

3.1.2. Effect of human GST A1-1

Figure 3B shows the effect of 8 μM hGSTA1-1 on GSH conjugation of reactive metabolites of CLZ. Similar to GSTP1-1, a significant amount of CG-6 was produced although to a 5-fold lower extent. Only minor amounts of CG-5 were produced by hGSTA1-1. In contrast to hGSTP1-1, addition of 8 μM hGSTA1-1 resulted in a 25% increase of CG-1, the major GSH conjugate in absence of hGST. By assuming equivalent extinction coefficients for each GSH conjugate, the total amount of GSH conjugates was increased by 52% by the addition of hGSTA1-1.

3.1.3. Effect of human GST M1-1

As shown in Figure 3C, addition of 8 μM hGSTM1-1 to P450 102A1 M11H incubations resulted in a more than 2-fold increase in the amount of CG-1. Furthermore, hGST M1-1 catalysed the formation of CG-6 at amounts comparable to that formed by GST A1-1. Interestingly, GST M1-1 also produced significant amounts of GSH adduct CG-4, which was also found as a minor metabolite in incubations in presence of 5 mM GSH, Table 1, and in trace amounts in incubations with hGST P1-1, Table 3. LC-MS-analysis of this GSH conjugate also showed a protonated molecular ion [M + H]+ of m/z 632.2 [CLZ-SG + H]+, and a fragmentation pattern highly similar to that of the other GSH conjugates with the same molecular weight: m/z 614.2 ([CLZ-SG – H2O + H]+); m/z 575.2 ([CLZ-SG – C_3H_7N(piperazine ring) + H]+); m/z 503.2 ([CLZ-SG – glutamic acid +H]+); m/z 446.1 ([CLZ-SG – glutamic acid – C_3H_7N(piperazine ring) + H]+); m/z 359.1 ([CLZ-SG – glutamic acid – glycine – C_3H_7NO(GSH-moiety) + H]+) and 302.1 ([CLZ-SG – glutamic acid – glycine – C_3H_7NO(GSH-moiety) – C_3H_7N (piperazine ring) + H]+). CG-4 might also correspond to an adduct to the C-7 position, or a GSH conjugate to the non-chlorinated ring, as suggested previously *(8)*.

Because of its ability to catalyse formation of CG-1, CG-4 and CG-6, the total amount of GSH conjugate was increased 2.7-fold, assuming extinction coefficients of GSH conjugates are comparable.

3.1.4. Effect of human GST T1-1

Addition of 8 μM GST T1-1 did not show any effect on the formation of CLZ GSH conjugates, both in presence of 100 μM or 5 mM GSH (data not shown). The role of this enzyme was therefore not further evaluated.

3.2. The effects of hGST concentration on GSH conjugation of CLZ metabolites

Three of the GSH conjugates, CG-4, CG-5, and CG-6, were only found in incubations of CLZ with P450 102A1 M11H when hGSTs were added to the incubation, Table 3. To study the effect of protein concentration of hGST on the formation of these GSH conjugates, incubations were performed at hGST concentrations ranging from 0.25 to 8 μM. Figure 4A shows the effects of different concentrations of hGSTs on the formation of GSH conjugate CG-6. For hGSTP1-1, hGSTA1-1, and hGSTM1-1 formation of CG-6 was linear with the concentration of hGST. GSTP1-1 showed the highest activity, being approximately 5 times more active than the other two isoenzymes. GSTM1-1 and GSTA1-1 showed similar activity.

Formation of the GSH adduct CG-5 was only catalyzed by GSTP1-1 and, at 40-fold lower activity, by GSTA1-1, Table 3. When varying the concentration of hGSTP1-1, the amount of CG-5 was proportional to the hGSTP1-1 concentration, Figure 4B. In case of hGSTA1-1, CG-5 formation was proportional to GST concentration at concentrations higher than 2 μM. Because of its lower activity compared to GSTP1-1, at concentrations lower than 2 μM GSTA1-1, this GSH conjugate was below the level of detection.

Formation of GSH conjugate CG-4 which was only produced to a significant extent by GSTM1-1 also increased with GSTM1-1 concentration, although not linearly, Figure 4C. At the lowest GST concentration, the amount of CG-4 was below the level of detection.

3.3. Role of hGSTs in inactivation of CLZ metabolites formed by human and rat liver microsomes

Table 3 shows the effect of hGSTs on the GSH conjugation of reactive CLZ metabolites formed by human and rat liver microsomes. In both human and rat liver microsomal incubations, hGST P1-1 was the most active enzyme in catalysing GSH conjugation, increasing the total amount of GSH conjugates 3.7-fold and 2.4-fold, respectively, when compared to the corresponding incubations in absence of hGST. By adding hGST P1-1, CG-6 and CG-5 again appeared to be the major GSH conjugates, consistent with the observations with P450 102A1 M11H incubations. Addition of hGST A1-1 or hGST M1-1 to the microsomal incubations increased the total GSH conjugation to approximately the same extent, from 34 to 53%, with slightly higher activity for GST M1-1. In case of human liver microsomes incubations, significant amount of CG-4 was already found in absence of recombinant hGSTs, consistent with previous studies *(8)*. This GSH conjugate, which was originally assigned to C-7 of the chlorobenenoid ring, was previously proposed to be a product of microsomal glutathione S-transferase *(8)*.

4. Discussion

It is generally accepted that the ADRs caused by CLZ are the result of the bioactivation to reactive metabolites in the different target tissues. The mild hepatotoxicity observed in 37% of patients treated with CLZ might be the result from local bioactivation by hepatic P450s, whereas the idiosyncratic agranulocytosis might result from myeloperoxidases-mediated bioactivation in neutrophils and their bone marrow precursors. Based on the observation that in presence of GSH the same GSH

conjugates are produced in both P450 and peroxidase-mediated reactions, a common reactive intermediate is implicated in both types of adverse drug reactions *(8)*. As reactive intermediate, a nitrenium ion was proposed, formed by a two-electron oxidation pathway. In microsomal incubations, the nitrenium ion can be trapped chemically by GSH leading to several GSH conjugates. The principle GSH conjugate results from GSH addition to the C-6-position while a minor conjugate was assigned to GSH addition to the C-9 position. Furthermore, a third conjugate with identical mass was tentatively assigned to a conjugate to the C-7 position *(8)*. Furthermore, two GSH conjugates were only identified in in vivo in bile of treated rats and mice, and were considered to involve an unknown reactive intermediate.

Although GSH conjugation of reactive CLZ metabolites has been observed in many in vitro studies, the role of the cytosolic GSTs has not yet been investigated. Because several cytosolic GSTs are known to be polymorphic, genetically determined deficiency in GSTs might be considered as a risk factor for drug-induced idiosyncratic drug reactions if these enzymes play an important role in GSH conjugation of reactive intermediates. The results of the present investigation, as summarized in Table 3, clearly show for the first time that hGSTs have a significant activity in catalysing GSH conjugation of reactive CLZ metabolites formed by cytochrome P450. The most active human GST in all experiments appeared to be hGSTP1-1, which, at 8 μM, resulted in a 3.7-fold increase in the total amount of GSH conjugates formed in incubations with P450 BM3 and human liver microsomes. This increase is only slightly lower than the 4.8-fold increase observed by raising the GSH concentration 50-fold, from 100 μM to 5 mM, Table 1. Next to hGST P1-1, hGSTM1-1, and hGSTA1-1 were able to catalyse GSH conjugation, Table 3. Although their activity appeared to be lower than that of hGST P1-1, when measured at equal concentration, the role of GSTA1-1 and GSTM1-1 in vivo might be more important due to their 13- and 8-fold higher protein concentration in the liver, Table 4 *(36, 37)* and the fact that the enzymatic GSH-conjugation is proportional to the concentration of GST, Figure 4.

Table 4. Estimated concentration of individual GST-isoenzymes in human liver (adapted from Van Ommen et al., *(36)*

	Hepatic GST concentration			
	(μg/mg cytosolic protein)	(mg/gr liver)[a]	μM[b]	%
hGST A1-1	20.3	1810	~ 72	45
hGST A2-2	10.7	952	~ 38	24
hGST M1-1	12.1[c]	1080	~ 43	27
hGST P1-1	1.5	133	~ 5.3	3.3
hGST T1-1	0.3	26.7	~ 1.1	0.7

a Calculated using a hepatic cytosolic protein concentration of 89 μg cytosolic protein/gram liver *(37)*.
b Estimated using a hGST molecular weight of 25 kD and assuming 1 gr liver/mL.
c Sum of GST M1a-1a and GST M1b-1b which are indistinguishable in their catalytic properties *(38)*.

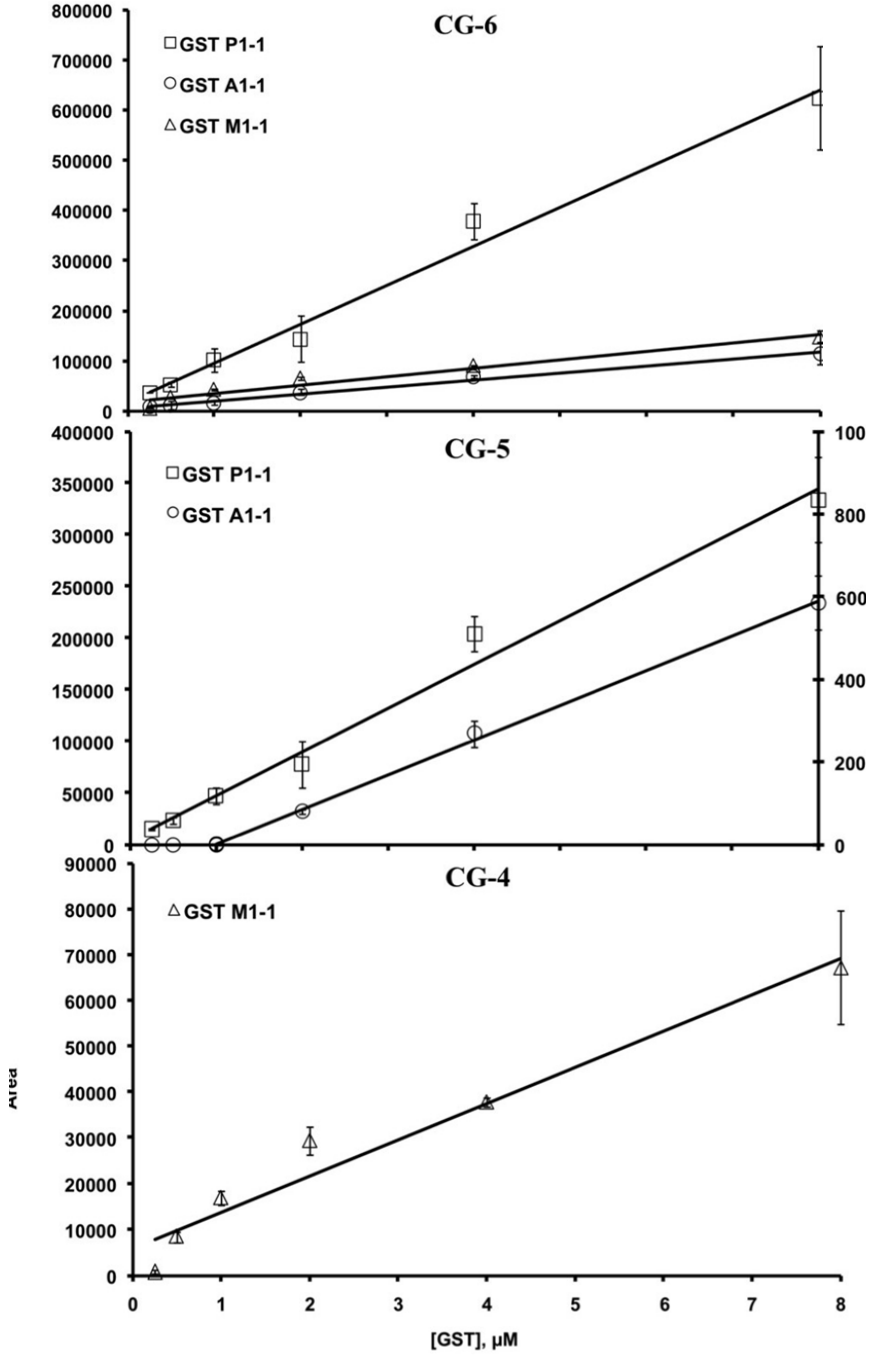

Figure 4. Effect of different GST-concentrations on formation of GSH-adducts form in incubation of clozapine with bacterial P450 M11.: (A) Influence of concentration of hGSTP1-1, A1-1 and M1-1 on formation of CG-6; (B) Influence of concentration of hGST P1-1 (scale: left axis) and A1-1 (scale: right axis) on formation of CG-5; (C) Influence of concentration hGST M1-1-concentration on formation of CG-4.

Two of the active GSTs, hGSTP1-1 and hGSTM1-1, are known to be genetically polymorphic. Individuals lacking the gene of hGSTM1-1 therefore theoretically might have an increased risk for CLZ-induced hepatotoxicity. For hGSTP1-1, four alleles have been described resulting from the polymorphic substitutions I105V and A113V *(40, 41)*. However, it remains to be established whether these polymorphic substitutions affect the ability of hGSTP1-1 to inactivate the reactive nitrenium ion of CLZ. Because hGSTT1-1 did not show any activity in catalysing GSH conjugation, deficiency of this GST is not likely to be a risk factor for CLZ. The clinical relevance of these GST polymorphisms to CLZ-induced ADRs, however, still remains to be confirmed in case-control studies.

Next to an increase in the total amount of GSH conjugation of reactive CLZ metabolites, addition of the hGSTs also resulted in striking changes in regioselectivity of GSH conjugation, Table 3. Consistent with previous studies, in absence of GSTs in all bioactivation systems used, the major GSH conjugate formed was the adduct at the C-6-position of CLZ (CG-1), whereas the adduct at the C-9 position (CG-3) was a minor adduct. In presence of hGSTP1-1, however, the major GSH conjugate observed appeared to be C-8 glutathionyl deschloroclozapine (CG-6), representing approximately 50-60% of the total of GSH conjugates. The formation of this GSH conjugate can be rationalised by a substitution reaction of the chlorine of the nitrenium ion of CLZ, followed by a reduction reaction, presumably by GSH and/or NADPH, Figure 5. In absence of NADPH or P450-enzyme fraction this GSH conjugate was not found, excluding a direct chlorine-substitution reaction on CLZ itself. Next to hGSTP1-1, also hGSTA1-1 and hGSTM1-1 were able to catalyse this substitution reaction, at apparently 5-fold lower activity, Figure 3. From the fact that CG-6 was observed only in bile of clozapine-treated animals, but not in in vitro microsomal incubations, it was previously concluded that this GSH conjugate was derived from a distinct pathway of bioactivation in vivo *(8)*. The present study, however, clearly demonstrates that CG-6 most likely originates enzymatic GSH conjugation of the reactive CLZ nitrenium ion. Although the present in vitro incubations were performed at low GSH concentration to minimize non-enzymatic GSH conjugation, the fact that CG-6 and CG-4 were found in bile of rats and mice in amounts comparable to that of CG-1 *(8)*, show that GSTs contribute significantly to GSH conjugation in vivo. Furthermore, C-8 methylthio deschloroclozapine and C-7 methylthio clozapine are the only thioethers so far identified in urine of man treated with CLZ *(9, 10)*, and are most likely resulting from catabolism of CG-6 and CG-4, respectively. The fact that formation of both CG-6 and CG-4 are completely dependent on presence of GSTs, suggest that GSTs play an important role in GSH conjugation of reactive CLZ metabolites in man *(9)*. However, the excretion of products derived from GSH conjugates in urine of CLZ-treated patients still remains to be further characterized. The highly sensitive LC/MS/MS-methodology recently developed for analysis of N-acetyl-L-cysteine conjugates of CLZ and other drugs might be useful in human studies when high analytical sensitivity and selectivity is required *(39)*. In the latter study, human urine was spiked with N-acetyl-L-cysteine conjugates of CLZ generated by incubating rat liver microsomes in presence of N-acetyl-L-cysteine conjugates. Unfortunately, no urine-samples of CLZ-treated patients were analysed, which would have shown the actual urinary profile of N-acetyl-L-cysteine conjugates of CLZ. Consistent with the present study, no N-acetyl-L-cysteine conjugate was found resulting from chloro-substitution of the CLZ nitrenium ion *(39)*, confirming the dependence of conjugating enzymes for this specific regioisomeric conjugate.

The results summarized in Table 3, show that four GSH conjugates are formed with m/z 632, consistent with GSH conjugates 3a-d described previously *(8)*. GSH conjugate CG-4 was found in incubations of CLZ with human liver microsomes in absence of cytosolic GSTs, and therefore most likely corresponds to GSH conjugate 3b which was found by Maggs et al. *(8)* in incubations of CLZ with human and rat liver microsomes *(8)*. This GSH conjugate was tentatively identified by these authors as the thioether substituted ortho to the chlorine, i.e. C-7 glutathionyl clozapine, and was suggested to be a product of enzymic GSH conjugation by the microsomal glutathione S-transferase *(8)*. As shown in Table 3 and Figure 4C, hGSTM1-1 is also contributing to formation of CG-4. As mentioned above, a methylthio-adduct at the C-7 position of clozapine has been identified in urine of man *(9,10)* supporting the relevance of this metabolic pathway for man.

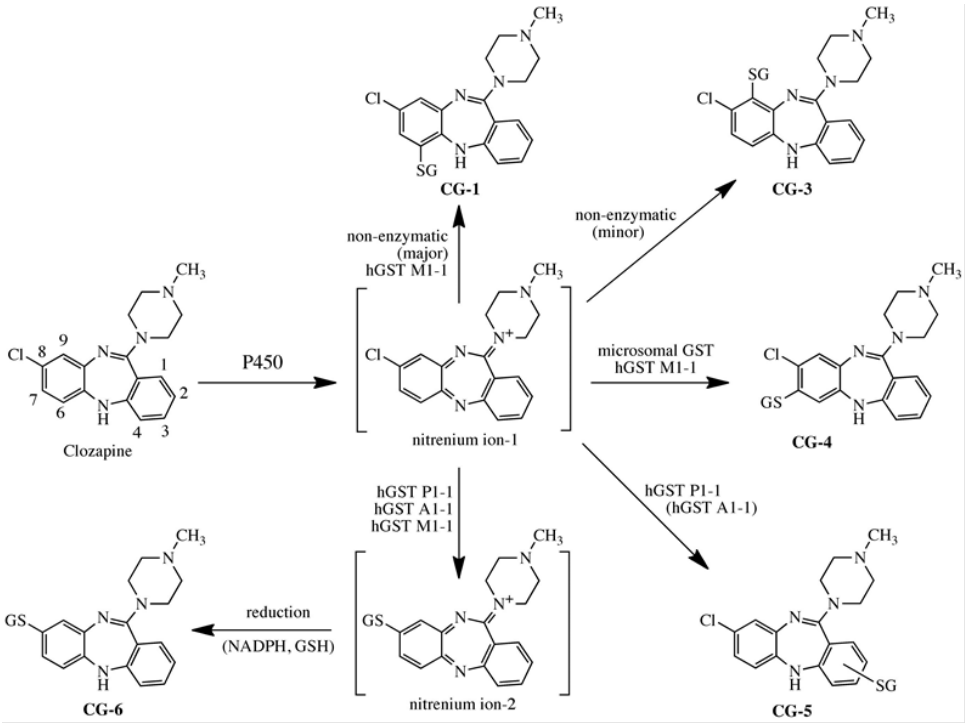

Figure 5. Role of hGSTs in the regioselective GSH conjugation of the reactive nitrenium ion formed by cytochrome P450. Structures of CG-4 and CG-5 were tentatively assigned in reference *(8)*.

The fact that CG-1, CG-3 and CG-4 all represent GSH conjugates in which GSH is bound to the chlorinated ring, implicates that the fourth GSH conjugate with m/z 632, CG-5, results from GSH conjugation to the non-chlorinated ring. This GSH conjugate therefore most likely corresponds to conjugate 3d of Maggs et al., which was only found in vivo in bile of clozapine-treated rats and mice *(8)*. In the present study, CG-5 was found at significant amounts (18 to 32% of total of GSH conjugates) when CLZ incubations were

performed in presence of hGSTP1-1 and at much lower amounts in presence of hGSTA1-1, Table 3. GSH conjugation to the non-chlorinated ring of CLZ might be explained by reaction to the CLZ nitrenium ion, involving delocalization of the positive charge to the non-chlorinated ring, or by a distinct P450-dependent reactive intermediate such as an arene oxide. The fact that aromatic hydroxylation to the non-chlorinated ring was not observed in the present in vitro incubations and has never been found in previous in vitro and in vivo studies *(10, 11)* seems to rule out involvement of arene oxides and suggests that all GSH-conjugates originate from a common reactive nitrenium ion, as illustrated in Figure 5. Therefore, the different regioselectivity in GSH conjugation observed is most likely a reflection of the different binding orientation of the nitrenium ion in the active-sites of these GSTs.

In summary, the results of the present study indicate that at least three hGSTs are able to catalyse the GSH conjugation of the reactive CLZ nitrenium ion resulting in different regioisomeric GSH conjugates, Figure 5. Genetically determined deficiency of hGST or drug-drug interaction at the level of hGST, therefore, might be risk factors for adverse side effects associated with CLZ-treatment. Case-control studies correlating GST-genotypes with susceptibility to CLZ side-effects however remain to be performed. The fact that several GSH conjugates are formed only in presence of hGST implicates that analysis of corresponding thioethers (N-acetyl-L-cysteine- and/or thiomethyl-conjugates) in urine of CLZ-treated patients might support these case-control studies.

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Chapter 4

EFFECT OF HUMAN GLUTATHIONE S-TRANSFERASES ON GLUTATHIONE-DEPENDENT INACTIVATION OF CYTOCHROME P450-DEPENDENT REACTIVE INTERMEDIATES OF DICLOFENAC

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Abstract

Idiosyncratic adverse drug reactions due to the anti-inflammatory drug diclofenac have been proposed to be caused by the generation of reactive acyl glucuronides and oxidative metabolites. For the oxidative metabolism of diclofenac by cytochromes P450 at least five different reactive intermediates have been proposed previously based on structural identification of their corresponding GSH-conjugates. In the present study, the ability of four human glutathione S-transferases (hGSTs) to catalyse the GSH-conjugation of the different reactive intermediates formed by P450s was investigated. Addition of pooled human liver cytosol and recombinant hGSTA1-1, hGSTM1-1, and hGSTP1-1 to incubations of diclofenac with human liver microsomes or purified CYP102A1 M11 L437N as model system significantly increased total GSH conjugation. Strongest increase of total GSH-conjugation was observed by adding hGSTP1-1, whereas hGSTM1-1 and hGSTA1-1 showed lower activity. Addition of hGSTT1-1 did only show a minor effect. When considering the effects of hGSTs on GSH-conjugation of the different quinoneimines of diclofenac, it was found that hGSTP1-1 showed the highest activity in GSH-conjugation of the quinoneimine derived from 5-hydroxydiclofenac (5-OH-DF). hGSTM1-1 showed highest activity in inactivation of the quinoneimine derived from 4′-hydroxydiclofenac (4′-OH-DF). Separate incubations with 5-OH-DF and 4′-OH-DF as substrates confirmed these results. hGSTs also catalyzed GSH conjugation of the o-imine methide formed by oxidative decarboxylation of DF as well as the substitution of one of the chlorine atoms of DF by GSH. hGSTP1-1 showed the highest activity for the formation of these minor GSH-conjugates. These results suggest that hGSTs may play an important role in the inactivation of DF quinone imines and its minor reactive intermediates especially in stress conditions when tissue levels of GSH are decreased.

1. Introduction

Diclofenac (DF) is a nonsteroidal anti-inflammatory drug (NSAID) which is widely prescribed to treat inflammation and pain in diseases like rheumatoid arthritis and acute muscle pain. The major toxic side-effect of DF is gastrointestinal injury which is clinically presented as ulceration and bleeding of the stomach and small intestines *(1, 2)*. Furthermore, 15% of patients regularly taking DF develop elevated levels of liver enzymes in plasma *(3, 4)*. About 6.3 per 100 000 users develop severe liver injury with an 8% case fatality rate *(5)*. Although the exact mechanism underlying the idiosyncratic hepatotoxicity of DF remains to be established, bioactivation to protein-reactive metabolites and subsequent immune-mediated reactions are hypothesized to play an important role *(6, 7)*.

The combined results of the large number of studies addressing the bioactivation of DF show that DF shows a very complex pattern of bioactivation, involving multiple reactive intermediates and multiple bioactivating enzymes *(8, 9)*. Quantitatively the most important pathway of bioactivation of DF is glucuronidation by UGT2B7 to a protein-reactive acyl glucuronide *(10)*. However, the fact that inhibitors of UGT increased rather than decreased cytotoxicity of DF in hepatocytes, whereas inhibitors of P450 were protective, suggest that protein modification by oxidative metabolites of DF might be more critical for acute cytotoxicity than modification by its acyl glucuronide *(11)*. In an

association study it was shown that the UGT2B7*2 allele was associated with an eight-fold increased risk of DF hepatotoxicity *(12)*. Although the authors considered this as an high activity allele, based on in vitro studies with 4-hydroxyestradiol *(13)*, a more recent study showed that UGT2B7*2 was actually more than ten times less active than wild-type UGT2B7*1 in the acyl glucuronidation of the NSAID flurbiprofen *(14)*. In case UGT2B7*2 has also lower activity for DF, the higher susceptibility of this genotype might be explained by the higher contribution of P450-bioactivation.

Although inhibition of formation of protective prostaglandins is generally regarded is the underlying mechanism of the more frequently occurring gastrointestinal injury caused by NSAIDs, it was shown recently that tissue-specific knock-out of P450 reductase in intestines strongly protected mice against intestinal toxicity of DF *(15)*. Therefore, bioactivation by P450s might also play an important role in gastrointestinal side effects of DF.

Based on the structures of the various GSH-conjugates identified in incubations of DF with human liver microsomes or recombinant P450s at least five different oxidative bioactivation mechanisms for DF have been proposed, as summarized in Figure 1. 4'-Hydroxydiclofenac (4'-OH-DF), which is the major oxidative metabolite of DF in microsomal incubations and which is formed specifically by CYP2C9, is further oxidized to diclofenac 1',4'-quinone imine (DF-1',4'-QI) that can react to GSH by both addition and chlorine-substitution reactions *(16, 17)*. Similarly, 5-hydroxydiclofenac (5-OH-DF), which is a minor metabolite of DF formed mainly by human CYP2C8 and CYP3A4 *(18, 19)*, is further oxidized to diclofenac 2,5-quinone imine (DF-2,5-QI) that reacts to GSH by addition reactions to two regioisomeric GSH-conjugates, **M1** and **M3** *(16)*. CYP2C9 was also proposed to also produce a reactive arene oxide, diclofenac-2',3'-oxide (DF-2',3'-oxide), as an explanation for the formation of 2'-hydroxy-3'-(glutathione-S-yl)-monoclofenac *(20)*. A fourth oxidative bioactivation pathway of DF, which appears to be mainly catalysed by CYP3A4, involves oxidative decarboxylation to a reactive o-imine methide (DF-IM, Figure 1), which reacts to GSH to form 2-(2,6-dichlorophenylamino)-benzyl-S-thioether glutathione (DPAB-SG) *(21, 22)*. Finally, a GSH conjugate was identified in which one chlorine of DF was substituted by GSH *(23, 24)*. Three reactive metabolites were recently proposed in which the chlorine of DF is activated by the electron-withdrawing *ortho*-nitrogen-atom formed by dehydrogenation (forming electrophilic o-imine methide), N-oxygenation or one-electron oxidation, Figure 1 *(24)*. A reactive radical intermediate of DF was previously proposed as mechanism for horseradish peroxidase-dependent inactivation of acetylcholinesterase *(25).* Because P450s do also contain peroxidase activities, a similar mechanism might be applicable for DF-bioactivation.

Although the studies described above all have shown that the oxidative reactive intermediates of DF react to GSH non-enzymatically, it has not yet been reported whether these reactions can be catalyzed by human glutathione *S*-transferases (hGSTs). The activity of several hGSTs is strongly genetically determined due to gene deletions (null alleles) and single nucleotide polymorphisms *(26)*. Several *in vitro* studies have shown that purified and recombinant hGSTs can significantly increase GSH-conjugation of reactive metabolites of acetaminophen *(27)*, valproic acid *(28)*, felbamate *(29)*, zileuton *(30)*, clozapine *(31)* and troglitazone *(32)* which supporting the hypothesis that genetically determined deficiency of GSTs might be risk factors for drug toxicity. An association study by Lucena et al. reported that patients carrying a double GSTT1-M1 null

genotype were at 8.8-fold increased risk to develop idiosyncratic drug-induced liver injury when using NSAIDs *(33)*. However, in this study only four cases of DF-induced hepatotoxicity were included. Therefore, additional studies are required to further support the protective role of hGSTs against DF-induced hepatotoxicity.

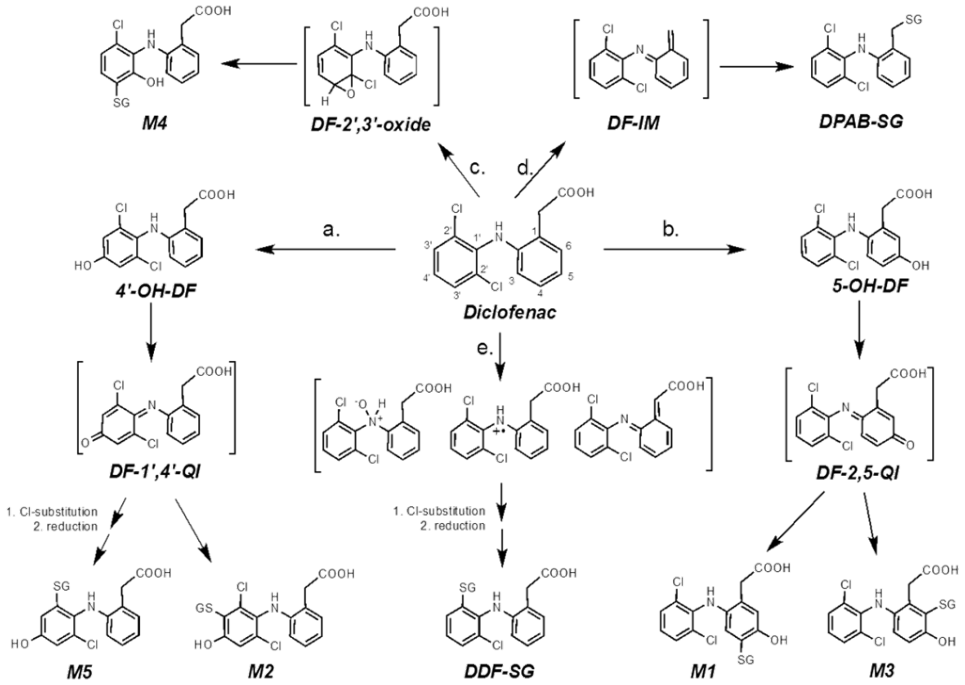

Figure 1. Cytochrome P450-dependent bioactivation of diclofenac (DF) to reactive intermediates, and structures of identified GSH-conjugates. **M1**, 4-glutathion-S-yl-5-hydroxy-diclofenac; **M2**, 3'-glutathion-S-yl-4-hydroxy-diclofenac; **M3**, 6-glutathion-S-yl-5-hydroxy-diclofenac; **M4**, 2'-hydroxy-3'-glutathione-S-yl)monoclofec; **M5**, 2'-glutathion-S-yl-4'-hydroxy-deschlorodiclofenac; **DPAB-SG**, 2-(2,6-dichloro-phenylamino)benzyl-S-thioether glutathione; **DDF-SG**, 2'-glutathion-S-yl-deschlorodiclofenac.

The aim of the present study was to investigate whether human GSTs, hGSTA1-1, hGSTM1-1, hGSTP1-1 and hGSTT1-1 might be involved in catalysing GSH-conjugation of the different reactive intermediates formed by oxidative metabolism of DF by human liver microsomes (HLM). In addition, purified drug-metabolizing CYP102A1 mutants were used as alternative bioactivating system for DF. The advantage of using purified soluble bacterial CYP102A1-mutants as biactivating system is that reactive intermediates formed cannot not be scavenged by other proteins as will be the case when using microsomal enzymes. Also, the study of the activity of individual hGSTs on GSH conjugation will not be confounded by microsomal GST present in liver microsomes *(31)*.

Furthermore, because of its relatively high specific activity only a low concentration of this bioactivating enzyme is required, minimizing trapping of reactive intermediates by the CYP102A1-mutant itself. The results of this study indicate that the different hGSTs studied showed differential effects, depending on the nature of reactive intermediate involved.

2. Materials and methods

2.1. Materials

DF sodium salt was obtained from Sigma (Steinheim, Germany), whereas 4'-OH-DF and 5-OH-DF were from Toronto Research Chemicals (North York, Canada). All other reagents and chemicals were of analytical grade and obtained from standard suppliers. Human liver microsomes (HLM; 20 mg/ml protein) pooled from 50 donors were purchased from Xenotech (lot no. 0710619). Escherichia coli XL-1 Blue cells containing the expression plasmids for human GST A1-1, M1-1 (B allele), and P1-1 (A allele) were a kind gift from Prof. Bengt Mannervik (Department of Biochemistry and Organic chemistry, Uppsala University, Sweden). The plasmid pet20b-hGSTT1 coding for human GST T1-1 with a C- terminal his-tag, was a kind gift from Prof. John D. Hayes (Biomedical Research Centre, University of Dundee, Scotland, United Kingdom). All other chemicals and reagents were of analytical grade and obtained from standard suppliers.

Pooled human liver cytosol was prepared from pieces of human liver tissue kindly provided by Prof. Geny M.M. Groothuis (Department of Pharmacy, University of Groningen, The Netherlands). The characteristics of the human liver donors are described elsewhere *(34).* Liver samples were homogenized in two volumes (w/v) of icecold 100 mM potassium phosphate buffer, (KPi buffer, pH 7.4). Liver cytosol was obtained by differential centrifugation according to standard procedures. Pooled human liver cytosol was prepared by combining cytosol of ten individuals. The specific activity of cytosolic GSTs was assayed according to Habig et al. *(35)* and was 447 nmol/min/mg cytosolic protein using 1 mM CDNB as substrate. Protein content of the pooled human liver cytosol was 21.5 mg/mL using bovine serum albumin as protein standard.

2.2. *Expression and purification of human GSTs and drug metabolizing CYP102A1-mutants.*

Human GSTA1-1, GSTM1-1, GSTP1-1, and GSTT1-1 were expressed and purified as described previously *(31)*. The specific activities of the purified recombinant hGSTs using 1mM CDNB as a substrate were: 20.4 µmol/min/mg protein for GSTA1-1, 55.6 µmol/min/mg protein for GSTM1-1, and 27.9 µmol/min/mg protein for GSTP1-1. The specific activity of hGSTT1-1 was determined using 1,2-epoxy-3-(p-nitrophenoxy)propane as a substrate as described *(36)* and was 1.83 µmol/min/mg protein.

Drug-metabolising mutants of CYP102A1 were used as model system to generate reactive metabolites of DF in presence of GSH and hGST. Although CYP102A1M11H was previous shown to be able to bioactivate DF to all human-relevant reactive intermediates, the levels of the GSH-conjugates were relatively low *(37)*. Therefore, first a library of drug-metabolising CYP102A1-mutants was screened to identify a mutant with higher

product formation. The details of the library of CYP102A1-mutants used can be found elsewhere *(38, 39)*. Mutant CYP102A1 M11 L437N, which was identified as the most active mutant, was purified by HIS-select nickel affinity chromatography, as described previously *(37)*. The P450 concentrations were determined according to the method of Omura and Sato *(40)*.

2.3. *Oxidative bioactivation of Diclofenac in the Presence of GSH and Human Recombinant GSTs.*

DF (500 μM) was incubated with HLM at final protein concentration of 2 mg/mL and with CYP102A1-mutants at concentration of 500 nM. Incubations were performed in 100 mM potassium phosphate buffer (KPi buffer, pH 7.4) and in an incubation volume of 250 μL. The non-enzymatic GSH conjugation was first investigated in incubations of DF with CYP102A1-mutant in presence of different concentrations of GSH (0.0125, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 20 mM). All reactions were initiated by the addition of an NADPH regenerating system constisting of 0.5 mM NADPH, 20 mM glucose-6-phosphate and 2 Units/mL glucose-6-phosphate dehydrogenase (final concentrations). Incubations were performed for 1 hour at 24°C for CYP102A1-mutants and at 37°C for HLM. The reactions were terminated by adding an equal volume of ice cold methanol containing 2% (v/v) of 50 mM ascorbic acid in water, and centrifuged for 15 min at 14000 rpm. The supernatants were analyzed by HPLC and LC-MS/MS, as described below. Enzymatic GSH conjugation catalysed by recombinant human GSTs was investigated by adding 8 μM (final concentration) hGSTs A1-1, M1-1, P1-1, or T1-1 to the incubations. Incubations with hGSTs were performed in the presence of 100 μM GSH, except for hGSTT1-1 which was incubated in the presence of 5 mM GSH because of its lower affinity to GSH *(36)*. Incubations of 500 μM DF with 2 mg/mL HLM were also performed in presence of 4 mg/mL pooled human liver cytosol (PHLC) since the hepatic cytosolic protein content is two times that of the hepatic microsomal content (40 mg microsomal protein/gr liver vs 81 mg microsomal protein/gr liver) *(41)*. Based on previous quantification of GSTs in human liver cytosol, which showed concentration of 45 to 50 μg of GST/gr liver *(42, 43),* the total concentration of GSTs at 4 mg cytosolic protein/mL is estimated to be approximately 6.7 to 8.1 μM, which is close to the 8 μM used in incubations with recombinant human GSTs.

The bioactivation of 4'-OH-DF and 5-OH-DF was also investigated individually by bioactivating these hydroxylated DF metabolites at 100 μM with 500 nM of CYP102A1 M11 L437N in presence of 100 μM GSH, NADPH regeneration system and in absence and presence of 8 μM hGSTs. Incubations of 4'-OH-DF and 5-OH-DF were terminated by adding an equal volume of ice cold methanol containing 2% (v/v) of 50 mM ascorbic acid in water, centrifuged for 15 min at 14000 rpm and analyzed by HPLC and LC-MS/MS, as described below.

2.4. *Analytical methods*

Samples of incubations were analyzed by reversed phase HPLC using a Symmetry Shield RP18 column (C18; 4.6 x 100 mm; 3.5 μm; Waters) as the stationary phase. A SecurityGuard Cartridge system (C18; 4.0 x 3.0 mm; Phenomenex) was used to protect the column. Analytes were eluted by a binary gradient, composed of solvent A

(1% acetonitrile, 0.2% formic acid, 98.8% water) and solvent B (99% acetonitrile, 0.2% formic acid, 0.8%water). Total flow rate was 0.5 mL/min (in case of UV detection) or 0.4 mL/min (in case of mass spectrometric detection). The gradient was programmed as follows: 0–5 min: isocratic at 0% solvent B; 5–30 min: linear increase from 0% to 100% solvent B; 30–35 min: linear decrease from 100% to 0% solvent B; 35–40 min: isocratic at 0% solvent B. A UV/Vis detector set to 254 nm was used to detect and quantify DF and its formed metabolites. Peak areas were determined for quantification using Shimadzu Class VP 4.3 software. A standard curve of DF was used to estimate the concentrations of metabolites assuming that the extinction coefficients of DF and its metabolites were similar. The standard curve of DF when detected by UV/VIS was linear between 0 and 500 μM; the limit of quantitative detection of DF by UV/VIS was estimated to be 1 μM (data not shown). Stock solutions of GSH-conjugates of DF were prepared by concentration of a large scale incubation of DF, GSH and CYP102A1 M11 L437N (data not shown). By assuming the same extinction coefficient for DF and DF-metabolites, standard curves were constructed by serial dilution of calibrated stock solution of each GSH-conjugate.

LC-MS/MS operating in the positive mode was used for the identification of the formed metabolites of DF using the same separation conditions as for HPLC analysis. An Agilent 1200 Series Rapid Resolution LC system connected to a hybrid quadrupole time-of-flight (Q-TOF) Agilent 6520 mass spectrometer (Agilent Technologies) with an electrospray ionization (ESI) source was used to acquire spectra at a rate of 1.003/second over an m/z range of 50 to 1000. The MS ion source parameters were as described previously *(31)*. LC-MS/MS system was operated using MassHunter Workstation Software version B.02.00, while data analysis was performed using Agilent MassHunter Qualitative Analysis software. The standard curves of DF and calibrated solutions of GSH-conjugates of DF was linear between 0 and 100 μM when detected by LC-MS/MS. The limit of quantitative detection of DF and DF metabolites by LC-MS/MS was estimated to be 20 nM based on peak areas of extracted ion chromatograms of each metabolites (data not shown).

3. Results

3.1. *Selection of drug metabolising mutant of CYP102A1 as a model for oxidative bioactivation of DF*

Although it was shown previously that CYP102A1 M11H was able to bioactivate DF to all human relevant oxidative metabolites, the yields of GSH-conjugates were relatively low *(37)*. Therefore, it was investigated whether other mutants of CYP102A1 were more efficient in bioactivation of DF. When screening a library of drug-metabolizing mutants of CYP102A1, which previously showed a wide diversity in activity and substrate selectivity *(38, 39)*, the most active mutant appeared to be CYP102A1 M11 L437N being almost 2-fold more active than CYP102A1 M11H, Figure 2A. CYP102A1 M11 L437N contains one extra mutation, L437N, compared to CYP102A1M11H which was used in previous studies *(24, 37)*. For all mutants 4-OH-DF was the major metabolite, whereas 5-OH-DF was formed at 4-fold lower activities, Figure 2B. CYP102A1 M11 L437N was selected as biocatalyst to bioactivate DF to study the ability of human GSTs to catalyze GSH-conjugation of P450-dependent reactive intermediates. In absence of NADPH-regenerating system, NADPH consumption measurements in incubations of DF with

CYP102A1-mutants showed complete NADPH consumption within two minutes, explaining the relatively low conversion of DF and the low yields of GSH conjugates reported previously *(37)*. By adding an NADPH regenerating system, a more than 8-fold increase in GSH conjugates formation was observed in the presence of 5 mM GSH. Also the percentage of DF that was metabolized was increased from 8 to 75% (data not shown). Therefore, all subsequent incubations were performed in presence of NADPH-regenerating system.

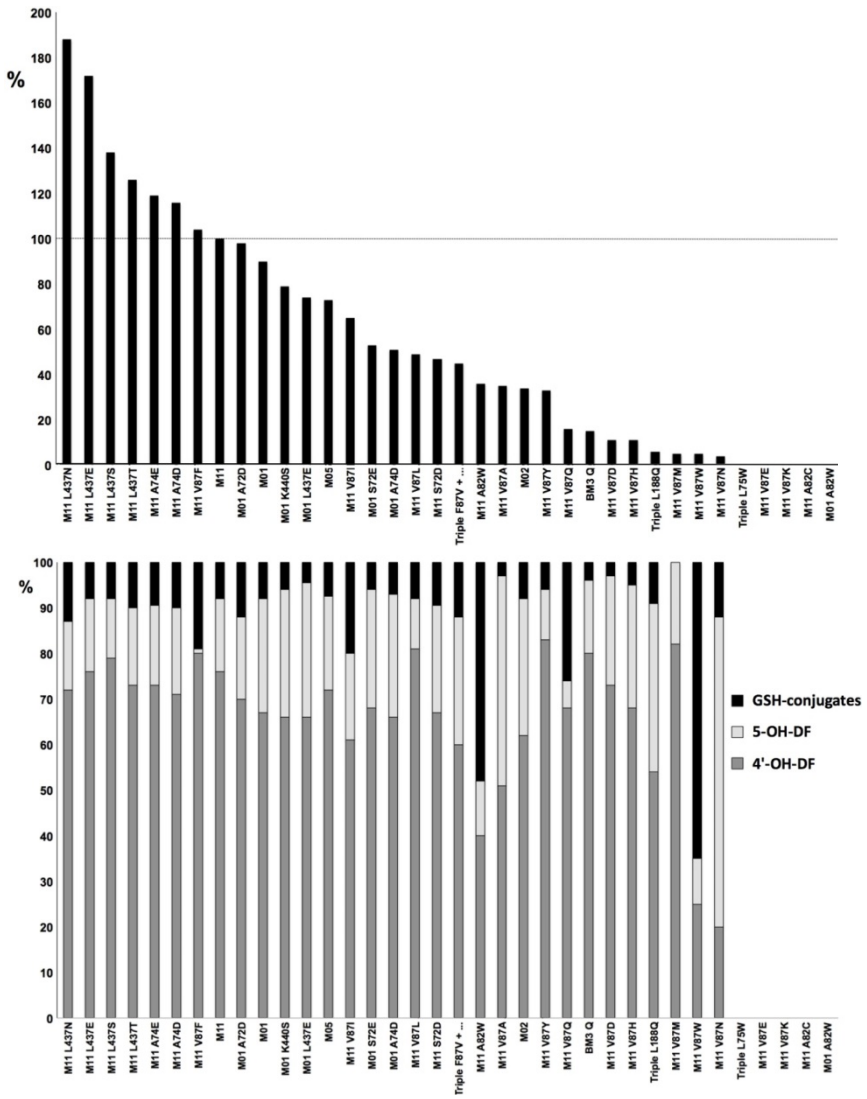

Figure 2. A) Activity of a library of drug metabolizing mutants of CYP102A1 with 500 µM of diclofenac as substrate; **B)** Profile of metabolites produced by a library of drug metabolizing mutants of CYP102A1 with 500 µM of diclofenac as substrate.

As shown in Table 1, eight different GSH conjugates were formed in incubations of DF with purified CYP102A1 M11 L437N when incubated in presence of 5 mM of GSH. By comparison with GSH conjugates formed in incubations with 5-OH-DF as substrate, three of the conjugates could be attributed to GSH-conjugation to DF-2,5-QI, the quinone imine formed from 5-OH-DF, Figure 1. Two conjugates with m/z of 617,08 correspond to **M1** and **M3,** as characterized previously *(13)*. The third GSH-conjugate, in this paper assigned **M8**, was identified as a double conjugated GSH-conjugate which can be rationalized by secondary oxidation of **M1** and/or **M3** to their corresponding quinone imines and subsequent GSH-addition, see Figure 3. By comparison with GSH conjugates formed in incubations 4'-OH-DF as substrate, four of the conjugates found in incubations of DF with CYP102A1 M11 L437N could be attributed to GSH-conjugation to DF-1',4'-QI, the quinone imine formed from 4'-OH-DF, Figure 1. The GSH-conjugate with m/z 617,08 corresponds to **M2**, which was characterized previously *(16)*. The GSH-conjugate with m/z 583,13, here as assigned **M5**, can be rationalized by chlorine-substutution of DF-1',4'-QI followed by reduction by NADPH and/or GSH *(17)*. Two double conjugated GSH-conjugates, assigned **M6** and **M7**, can be rationalized by addition and chlorine-substitution to the quinoneimine preceding **M5**, see Figure 3.

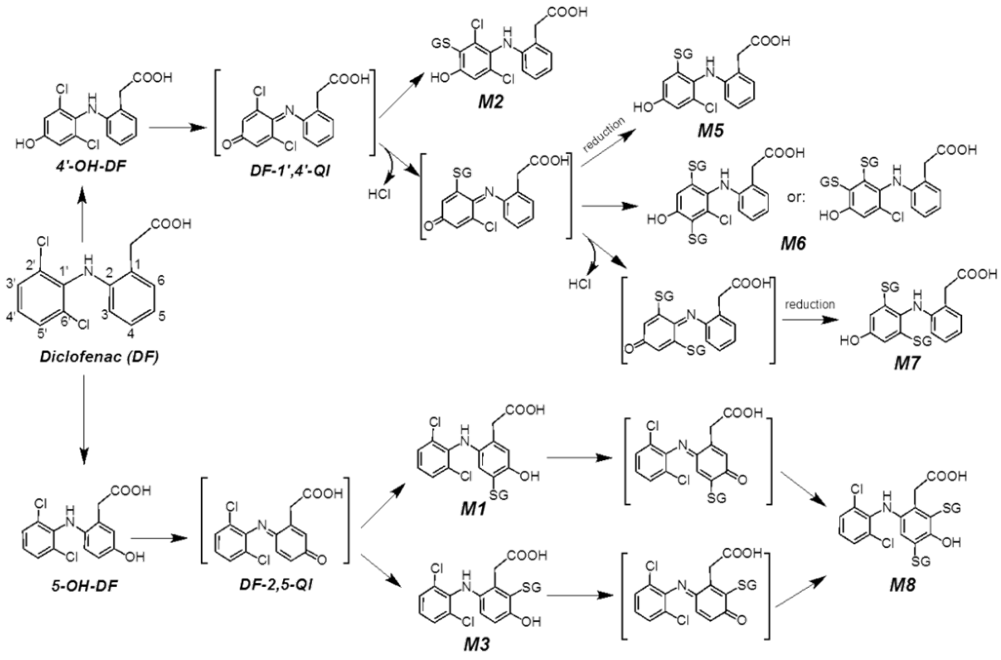

Figure 3. Metabolic scheme rationalizing the formation of primary and secondary GSH-conjugates in incubations of diclofenac with drug metabolizing mutant CYP102A1 M11 L437N.

Next to the GSH-conjugates derived from the quinone imines of 5-OH-DF and 4'-OH-DF, a GSH-conjugate with m/z of 557,132 was found in incubations of DF with CYP102A1 M11 L437N. The mass of this conjugate corresponds to that of DPAB-SG, which results from the reactive imine methide DF-IM formed by oxidative decarboxylation of DF *(21, 22)*.

Figure 4 shows the concentration dependence of the non-enzymatic GSH conjugation of reactive DF metabolites when performing incubations with GSH concentrations ranging from 0 to 20 mM. At all GSH-concentrations, approximately 90% of the total conjugates is formed through the 4'-OH-DF bioactivation pathway. Assuming that the observed saturation curve in Figure 4 is described by the equation $y = a \times (1 - e^{-bx})$, 165 µM would be the maximal yield of GSH conjugates at infinitive GSH concentration indicating that approximately 30% of the initial DF concentration was converted to reactive intermediates under the present incubation conditions. At 100 µM GSH approximately 2.1 % of the reactive metabolites are trapped whereas at 5 mM GSH 66.0 % is trapped. To study the effect of hGSTs on GSH-conjugation subsequent incubations were performed at 100 µM GSH, except for hGST T1-1 which was incubated at 5 mM GSH because of its lower affinity to GSH *(36)*.

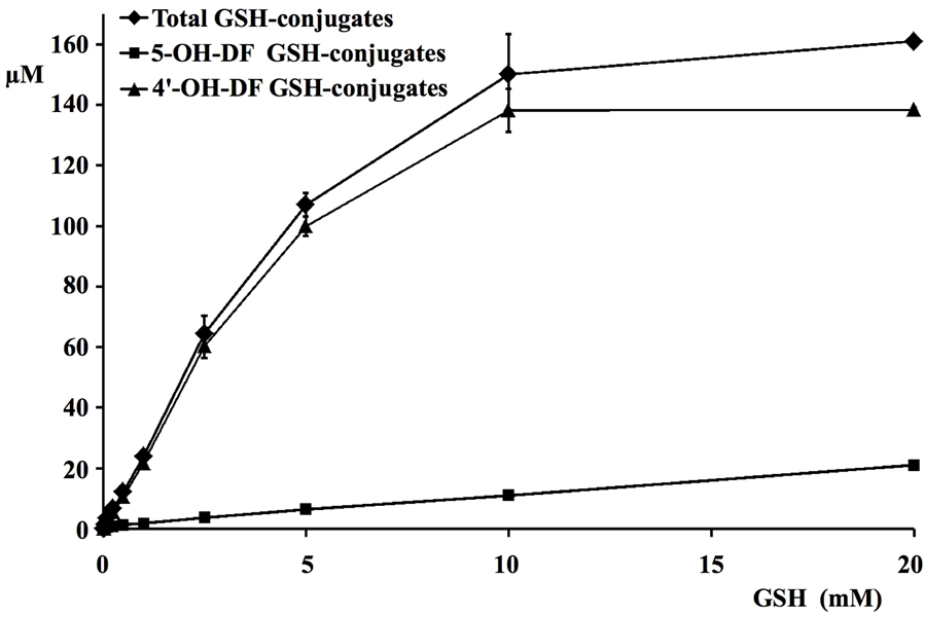

Figure 4. GSH-concentration dependence on the non-enzymatic GSH conjugation of reactive DF-metabolites formed by drug metabolizing mutant CYP102A1 M11 L437N. The increase in the peak area was investigated for 4'-OH-DF, 5-OH-DF and total GSH conjugates when the concentration of GSH was changed from 0.0125 to 20 mM. Each point represents the mean ± S.D. (n = 2).

Table 1. Characteristics of GSH conjugates of diclofenac formed after bioactivation by CYP102A1 M11 L437N in presence of 5 mM GSH.

GSH-conjugates	*Reactive intermediate*	t_{ret} *(min)*	*Concentration of GSH conjugate (μM)*	*m/z*	*Change of parent drug*
M1	DF-2,5-QI	19.7	3.34 ± 0.07	617.087 $[M+H]^+$	DF+O+SG
M3	DF-2,5-QI	20.4	1.56 ± 0.32	617.087 $[M+H]^+$	DF+O+SG
M8	DF-2,5-QI	18.0	0.63 ± 0.15	461.583 $[M+H]^{2+}$	DF+O+2SG
	Total GSH-conjugates of DF-2,5-QI		5.53 ± 1.93		
M2	DF-1',4-QI	20.0	11.3 ± 0.42	617.087 $[M+H]^+$	DF+O+SG
M5	DF-1',4-QI	19.7	63.0 ± 1.15	583.127 $[M+H]^+$	DF+O+SG-HCl
M6	DF-1',4-QI	17.3	7.4 ± 0.53	444.603 $[M+H]^{2+}$	DF+O+2SG-HCl
M7	DF-1',4-QI	16.3	17.0 ± 0.46	427.622 $[M+H]^{2+}$	DF+O+2SG-2HCl
	Total GSH-conjugates of DF-1',4-QI		98.8 ± 2.8		
DPAB-SG	DF-IM	21.7	0.66 ± 0.25	557.132	DF-COOH+SG
DDF-SG	Unknown	19.5	n.d.[a]	567.138	DF-Cl+SG

Metabolites were formed after 1 hour incubation of 500 μM DF with 500 nM CYP102A1 M11 L437N in the presence of 5 mM GSH. Quantification was based on peak areas in the LC-UV chromatograms using the DF standard curve, assuming that the extinction coefficients of the substrate and the metabolites at 254 nm are the same.
t_{ret}, retention time in minutes.

[a] Only detectable in incubations supplemented with human GST.

3.2. Effect of human GSTs on GSH-conjugation of reactive metabolites of DF formed by CYP102A1 M11 L437N.

To study whether addition of hGST enzymes increased the GSH conjugation of reactive DF metabolites formed by purified CYP102A1 M11 L437N, incubations were performed in the presence and absence of 8 μM hGSTs. As shown in Table 2, addition of hGSTs A1-1, M1-1, and P1-1 to incubations of DF with CYP102A1 M11 L437N resulted in a significant increase in GSH conjugation when compared to incubations without added hGSTs. hGST P1-1 and hGST M1-1 were found to be the most active in GSH conjugation, resulting in 8.7-fold and 6.4-fold increase in total GSH-conjugation, respectively. Addition of 8 μM hGSTA1-1 only showed 1.4-fold increase in GSH-conjugation. hGST T1-1, which was incubated in presence of 5 mM GSH, showed an approximately 20% increase in the formation of GSH conjugates.

As shown in Table 2, addition of the different hGSTs to the incubations resulted in significant changes in the profile of the individual GSH-conjugates. When considering the GSH-conjugates derived for DF-1',4'-QI, it was observed that addition of 8 μM of hGSTP1-1 and hGSTM1-1 both increased GSH-conjugation approximately 7-fold. In incubations with hGSTM1-1 conjugate **M5** represented 80% of the DF-1',4'-QI-dependent GSH-conjugates, indicating a very strong preference for the chlorine-substitution pathway, Figure 1. The less active hGSTA1-1 also showed an increase of formation of **M5**. In contrast, in presence of 8 μM GSTP1-1 conjugate **M2,** which is formed by GSH-addition, represented 83% of DF-1',4'-QI-dependent GSH-conjugates indicative for a strong difference in regioselectivity, consistent with previous observations with the reactive intermediate of clozapine *(31)*.

hGSTP1-1 was found to have the highest activity in inactivation of DF-2,5-QI showing an almost 20-fold increase in GSH-conjugation when compared to the non-enzymatical GSH-conjugation. The sum of **M1** and **M3** formed in presence of 8 μM GSTP-1 was almost equal to that formed in presence of 5 mM GSH, implicating that addition of this enzyme increased trapping efficiency of 100 μM GSH almost 50-fold. For hGSTM1-1 and hGSTA1-1 a 2.3-fold and 1.6-fold increase was found respectively. For both hGSTP1-1 and hGSTM1-1 conjugate **M3** was the major GSH-conjugate formed. For hGSTA1-1 conjugate **M1** appeared to be the major DF-2,5-QI-derived conjugate.

DPAB-SG, the conjugate formed via the decarboxylation bioactivation pathway *(18, 19)*, was only found at very low concentration in incubations with 100 μM GSH. Table 3 shows the peak areas of the extracted ion chromatograms (EIC) of m/z 557,13. Again hGSTP1-1 appeared to be the most active enzyme, resulting in an 27-fold increase of formation DPAB-SG. hGSTM1-1 and hGSTA1-1 were less active, showing 6.6-fold and 2.90-fold increase of this GSH-conjugate. hGSTT1-1 showed only a 25% increase of DSAB-SG formation.

Recently, deschlorodiclofenac glutathione (DDF-SG) was identified as a minor P450-dependent GSH-conjugate of DF *(24)*. In the present study, this conjugate with m/z 567.13 was found only in bioactivation reactions of DF in the presence of hGSTs, Figure 5. Due to the low abundance of this metabolite, peak areas of the EIC of m/z 567.13 were used to quantify the relative amounts formed by the different GSTs. hGSTP1-1 was the most active enzyme in catalysing formation of this conjugate, being approximately 18.6- and 2.5-fold more active than hGSTA1-1 and hGSTM1-1, respectively.

Table 2. Influence of human glutathione S-transferases on formation of GSH-conjugates after P450-dependent bioactivation of diclofenac.

		GSH-Conjugates of DF-1',4'-QI				*GSH-Conjugates of DF-2,5-QI*				
	Total GSH-Conjugate (μM)	**M2** (μM)	**M5** (μM)	**M6** (μM)	**M7** (μM)	**Total** (μM)	**M1** (μM)	**M3** (μM)	**M8** (μM)	**Total** (μM)
Bioactivation by CYP102A1 M11 L437N										
100 μM GSH	2.5 ± 0.2	0.45±0.2	1.12±0.2	0.64±0.1	n.d.	22±0.4	0.13±0.04	0.15±0.02	n.d.	28±0.04
+ hGSTA1-1	3.4 ± 1.3	0.34±0.4	1.91±0.3	0.64±0.2	n.d.	2.90±0.6	0.30±0.07	0.15±0.03	n.d.	0.45±0.05
+ hGSTM1-1	15.9 ± 2.3	2.39±0.2	11.96±1.2	0.74±0.2	n.d.	15.1±1.6	0.24±0.02	0.41±0.06	n.d.	0.65±0.08
+ hGSTP1-1	21.7 ± 2.6	13.32±2.1	1.39±0.6	1.28±0.1	n.d.	16.0±1.5	1.80±0.8	3.57±0.21	n.d.	5.37±0.9
5 mM GSH	104.3 ± 0.4	11.27±1.0	63.01±5.8	7.41±0.2	17.0±0.8	98.8±2.0	3.34±0.4	1.56±0.1	0.63±0.1	5.53±0.5
+ hGSTT1-1	125.8 ± 4.0	13.63± 3.0	74.48±2.0	8.78±0.2	22.1±1.1	119.0±8.0	3.28±0.1	1.95±0.1	1.02±0.2	6.25±0.5
Bioactivation by human liver microsomes (HLM)										
100 μM GSH	0.48±0.1	0.02±0.01	0.42±0.12	n.d.	n.d.	0.44±0.08	0.02±0.01	0.02±0.01	n.d.	0.04±0.02
+ hGSTA1-1	0.54±0.1	0.05±0.02	0.42±0.20	n.d.	n.d.	0.47±0.09	0.04±0.02	0.03±0.02	n.d.	0.07±0.02
+ hGSTM1-1	1.11±0.1	0.08±0.03	0.97±0.10	n.d.	n.d.	1.05±0.09	0.02±0.01	0.03±0.01	n.d	0.05±0.01
+ hGSTP1-1	1.95±0.2	0.98±0.10	0.41±0.10	n.d.	n.d.	1.40±0.20	0.09±0.01	0.46±0.04	n.d.	0.55±0.03
5 mM GSH	1.14±0.2	0.17±0.02	0.69±0.20	n.d.	n.d.	0.87±0.24	0.12±0.1	0.16±0.02	n.d.	0.27±0.0
+ hGSTT1-1	1.30±0.1	0.15±0.04	0.92±0.10	n.d.	n.d.	1.07± 0.15	0.10±0.2	0.12±0.02	n.d.	0.22±0.02

3.3. *Effect of human GSTs on GSH-conjugation of reactive metabolites of 4'-OH-DF and 5-OH-DF formed by CYP102A1 M11 L437N.*

Because multiple reactive intermediates are formed from DF, which may compete with active site of hGSTs, incubations were also performed with 100 µM of 4'-OH-DF and 5-OH-DF as substrates. As shown in Figure 6, with both substrates the strongest increase in amounts of GSH-conjugates were found in incubations in which hGSTP1-1 was added, consistent with the incubations with DF as substrate. With 4'-OH-DF as substrate, hGSTP1-1 increased total GSH-conjugation almost 7-fold, Figure 6A, whereas in case of 5-OH-DF a more than 9-fold increase was found, Figure 6B. Interestingly, the total amount of 5-OH-DF-related GSH-conjugates formed in presence of 100 µM GSH and 8 µM hGSTP1-1 even surpassed the amount formed in incubations containing 5 mM GSH, Figure 6B. This implicates that addition of hGSTP1-1 increased the trapping efficiency of DF-2,5-QI by GSH more than 50-fold. As was found in incubations with DF as substrate, the GSH-addition reaction, forming conjugate **M2**, appeared to be the major pathway of inactivation of DF-1',4'-QI by hGSTP1-1, Figure 6A.

Table 3. Influence of human glutathione S-transferases on formation of GSH-conjugates DPAB-SG (m/z 557.13) and DDF-SG (m/z 567.13) after P450-dependent bioactivation of diclofenac.

	DPAB-SG[a] *(m/z 557,13)*	*DDF-SG*[b] *(m/z 567,13)*
Bioactivation of DF by CYP102A1 M11 L437N		
100 µM GSH	100	n.d.
+ hGSTA1-1	288	100
+ hGSTM1-1	663	748
+ hGSTP1-1	2712	1864
5 mM GSH	6084	n.d.
+ hGSTT1-1	7929	n.d.
Bioactivation of DF by human liver microsomes (HLM)		
100 µM GSH	n.d.	n.d.
+ hGSTA1-1	n.d.	n.d.
+ hGSTM1-1	5	24
+ hGSTP1-1	9	149
5 mM GSH	108	n.d.
+ hGSTT1-1	99	n.d.

a. Values represent percentages of peak areas of EIC of m/z 557.13 with the peak area of the incubation of CYP102A1 M11 L437N with 100 µM GSH set at 100%.
b. Values represent percentages of peak areas of EIC of m/z 567.13 with the peak area of the incubation of CYP102A1 M11 L437N with hGSTA1-1 and 100 µM GSH set at 100%.
n.d., below limit of detection.

For hGSTM1-1 the strongest effect on GSH-conjugation was observed in incubations with 4'-OH-DF as substrate, Figure 6A. A 4.5-fold increase of total of GSH-conjugates from DF-1',4'-QI was found, which was mainly due to the chlorine-substitution

pathway leading to GSH-conjugate **M5**. In incubations with 5-OH-DF as substrate, hGSTM1-1 did not show a significant effect on GSH-conjugation. Addition of hGSTA1-1 and hGSTT1-1 to incubations of purified CYP102A1 M11 L437N with 4'-OH-DF and 5-OH-DF did not show significant effects.

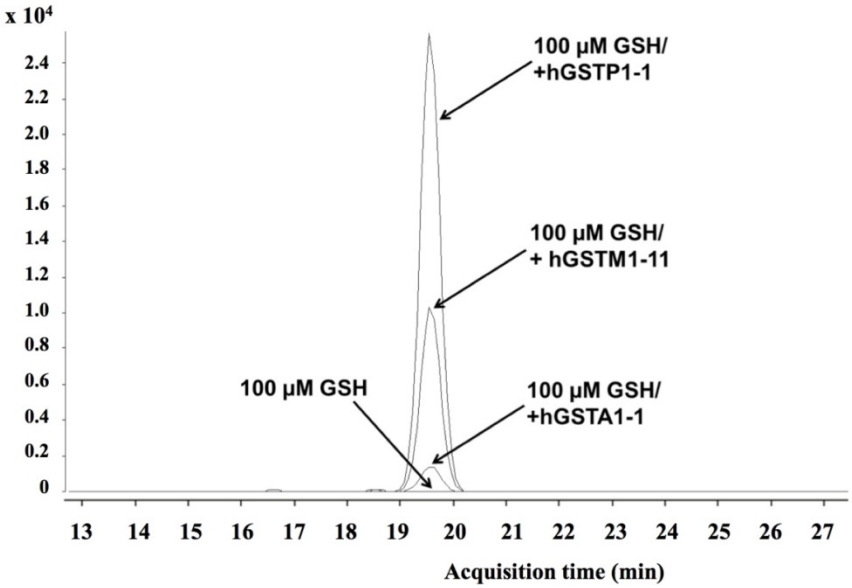

Figure 5. Effect of hGSTs on the formation of DDF-SG in incubations of DF with CYP102A1 M11 L437N and GSH. Traces represent extracted ion chromatograms (EIC) of the exact mass of DDF-SG (m/z 567.12, [M+H]+). Incubations were performed in the presence of 100 µM GSH and absence or presence of 8 µM: hGSTA1-1, hGSTM1-1 and hGSTP1-1.

3.4 *Effect of hGSTs and pooled human liver cytosol on the formation of GSH conjugates in incubations of DF with HLM.*

In Table 2, the effect of addition of hGSTs on amounts of GSH conjugates in incubations of DF with HLM is shown. In presence of 100 µM of GSH and in absence of hGSTs relatively low concentrations of GSH-conjugates were found. The econdary GSH-conjugates **M6**, **M7** and **M8** were not found, which may explained by the significantly lower activity of the microsomal P450s. GSH-conjugate **M5** was the only conjugate found with m/z of 583. The fact that this conjugate was identical to that formed from 4'-OH-DF seems to rule out the involvement of DF-2',3'-oxide as a reactive intermediate. Compared with the incubations of DF with CYP102A1 M11 L437N and 100 µM GSH a significantly higher ratio of conjugate **M5** to **M2** was found. Also, increasing GSH-concentration from 100 µM to 5 mM only increased the total of GSH-conjugates only 4-fold. These observations might be explained by the involvement of microsomal GST in inactivation of the reactive DF-metabolites. When adding 8 µM hGSTs to the HLM-incubations, hGSTP1-1 was found to be the most active enzyme in catalyzing GSH conjugation consistent with the

results obtained with CYP102A1 M11 L437N as biocatalyst. The total amount of GSH-conjugates was increased 4-fold when compared to incubations in absence of hGSTP1-1. Similar to the incubations with purified, hGSTP1-1-catalyzed GSH-conjugation of DF-1',4'-QI formed **M2** as major metabolite, whereas DF-2,5-QI was mainly conjugated to **M3**. The addition of 8 µM hGSTM1-1 to incubations with HLM increased the total GSH conjugation about 2.3-fold. Influence of hGSTA1-1 was not significant and hGSTT1-1 again did show only a minor effect significant activity.

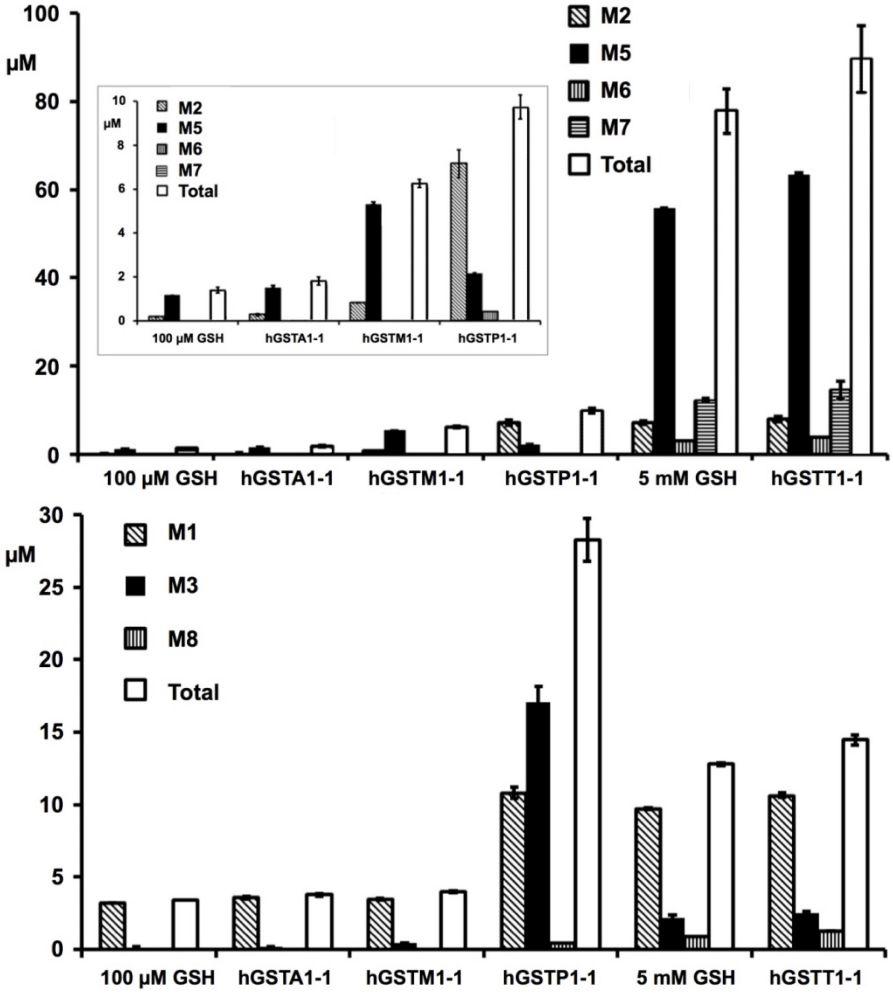

Figure 6. GSH conjugation of reactive metabolites formed in incubations of CYP102A1 M11 L437N with 100 µM 4'-OH-DF (A) and 5-OH-DF (B) in the absence and presence of hGSTs. The substrates were incubated for 60 min with 500 nM CYP102A1 M11 L437N. Relative quantification was performed by integrating peaks of LC-UV chromatograms and assuming that the extinction coefficients of the different GSH conjugates at 254 nm are identical. The insert in (A) shows a magnification of the incubation with low yields of GSH-conjugates.

Addition of 4 mg/mL pooled human liver cytosol (PHLC) to the incubations of DF with HLM increased total amount of GSH-conjugates 1.7-fold, which was determined by an increase of GSH-conjugates derived from DF-1',4'-QI. No significant increase in GSH-conjugates derived from DF-2,5-QI was observed.

4. Discussion

An association study of Lucena et al. suggested that the double null genotypes of hGSTM1 and hGSTT1 might lead to an almost 9-fold increased risk for NSAID hepatotoxicity *(33)*. However, only four of the 19 cases of NSAID-induced hepatotoxicity in this study were related to use of DF. The role of genetic polymorphism of hGSTs in DF-induced liver toxicity therefore requires confirmation by further expanding the association studies or by supporting mechanistic studies. In the present study, for the first time the ability of hGSTs to catalyse the inactivation of P450-dependent reactive intermediates of DF is shown, by using a purified drug metabolizing mutant of bacterial CYP102A1 and HLM as bioactivation systems. Using these enzyme fractions all metabolites of DF that have been reported in previous in vitro studies could be identified, including the GSH-conjugates of four different reactive intermediates of DF. The only GSH-conjugate that could not be found in incubations of DF with both HLM and the highly active CYP102A1 M11 L437N was conjugate M4 which was previously proposed to result from GSH-conjugation to DF-2',3'-oxide *(20)*. However, the assignment of the structure of M4 was only based on LC-MS/MS fragmentation and not supported by NMR-analysis. A GSH-conjugate of DF with exactly the same mass, m/z 583, was also found at the same time by another group *(17)*. This GSH-conjugate, in the present study assigned **M5**, was shown to results from chlorine-substitution of DF-1',4'-QI by GSH, followed by reduction, Figure 1. Therefore it is likely that the structure of **M4** was incorrectly assigned and that DF-2',3'-oxide is not a relevant reactive intermediate of DF.

When using mutant CYP102A1 M11 L437N as biocatalyst to produce the human-relevant reactive intermediates of DF, it was found that three of the four hGSTs studied were able to significantly catalyse the formation of GSH conjugates. When tested at concentrations of 8 µM, the most active enzyme appeared to be hGSTP1-1 which was able to strongly increase the amounts of GSH-conjugate of all four different reactive intermediates of DF. In particular, GSH-conjugation of the reactive quinoneimine formed from 5-OH-DF, DF-2,5-QI, was increased to levels equal to those formed at 50-fold higher GSH-concentration. When bioactivation studies were performed with 5-OH-DF instead of DF, a similar increase in GSH-conjugation by hGSTP1-1 was observed. Whether hGSTP1-1 plays an important protective role in human liver against reactive DF-metabolites is not very likely, however. Firstly, compared to the Alpha- and Mu-class GSTs, hGSTP1-1 has a relatively low expression level in adult human liver, which is estimated to be between 2 and 6 µM *(42, 43)*. This hepatic amount of hGSTP1-1 appears to be restricted to bile duct cells, Kupffer cells, macrophages and endothelial cells while hepatocytes appear to be devoid of hGSTP1-1 *(44).* Much more abundant GSTs in the adult human liver are hGSTA1-1, hGSTA2-2 and hGSTM1-1, which have hepatic concentrations of approximately 70-110, 20-35 and 30-45 µM, respectively *(42, 43)*. Therefore, although their specific activity is lower than that of hGSTP1-1, their much higher hepatic concentration and localization in hepatocytes implicate that they may contribute to inactivation of reactive DF-metabolites to a much higher extent. As shown in Table 2, the

addition of pooled human liver cytosol (PHLC) to the microsomal incubations, also resulted in 1.7-fold increase in GSH-conjugation. Based on the previous quantification of human GSTs in liver cytosol *(42, 43)*, the total concentration of GSTs in the incubations fortified with 4 mg/mL PHLC is expected to range from 6.7 to 8.2 μM. The fact that no significant increase in GSH-conjugates of DF-2,5-QI was found supports the very low concentration of GSTP1-1 in human liver cytosol.

Although expression of hGSTP1-1 is low in hepatocytes, it is abundantly expressed in the bile duct and in extrahepatic tissues *(44)*. It is highly expressed in the epithelia of the gastrointestinal tract. Interestingly, it was shown recently that P450-dependent bioactivation might play an important role in the gastrointestinal toxicity of DF in mice *(15)*. The number intestinal ulcers caused by DF was significantly reduced in intestinal epithelium-specific P450 reductase knockout mice, and in mice pretreated with grapefruit juice, which is known to inhibit intestinal P450s. In human, CYP3A4 is the major intestinal enzyme, accounting for 80% of total immunoquantified P450s *(45).* CYP3A4 is considered to play a major role in the bioactivation of DF to 5-OH-DF, which is further activated to DF-2,5-QI both enzymatically and non-enzymatically *(19)*. DF-2,5-QI appears to be responsible for the majority of covalent binding to proteins as shown by immunochemical and mass spectrometric methods *(19, 24)*. Therefore, hGSTP1-1, which is highly active in inactivating DF-2,5-QI and is highly expressed in intestinal epithelia might play a crucial role in protection against gastrointestinal toxicity of DF. Therefore it remains to be established whether genetic polymorphisms of hGSTP1-1 might determine susceptibility to the gastrointestinal side-effects of DF.

The association study of Lucena et al. in 2008 suggested that combined null genotype of hGSTM1-1 and hGSTT1-1 strongly increased risk for idiosyncratic liver injury by NSAIDs, including DF *(33)*. In the present study is was found that hGSTM1-1 was specifically active in catalysing GSH-conjugation of DF-1',4'-QI, the quinoneimine formed from the major metabolite 4'-OH-DF, Table 2 and Figure 6A. Also, similar increases in GSH-conjugation were observed for the minor bioactivation pathways leading to DPAB-SG and DDF-SG, Table 3. The lower effect of hGSTM1-1 and other GSTs in the incubation with HLM, when compared to those with CYP102A1 as bioactivating enzyme, may be explained by the fact that significantly lower concentrations of reactive intermediates are formed. In addition, microsomal GST might contribute to catalysis of GSH-conjugation, as was observed in similar bioactivation studies with clozapine *(31)*. The fact that hGSTM1-1 significant increased of GSH-conjugation of several reactive DF-metabolites seems to support the hypothesis of Lucena et al. that the null genotype of hGSTM1-1 is associated with increased risk for NSAID hepatotoxicity *(33)*. Addition of 8 μM hGSTT1-1, however, showed only a relatively small increase in GSH-conjugation of the quinone imines of DF, Table 2 and Figure 4. Considering the fact that hepatic concentration of hGSTT1-1 is 5.4 μg/mg cytosolic protein *(46)*, corresponding to a hepatic concentration of approximately 15 μM, it seems unlikely that the null genotype of hGSTT1-1 results in strongly reduced inactivation of reactive DF-metabolites. However, it cannot be excluded that GST T1-1 expression may modulate susceptibility to hepatotoxicity in ways unrelated to detoxification of electrophiles. Other GSTs have been shown to interfere with signal transduction pathways by protein-protein interaction and by S-glutathionylation of proteins *(47)*. For example, both hGSTP1-1 and hGSTA1-1 can suppress JNK signalling, whereas hGSTM1-1 can bind to and inhibit apoptosis signal-regulating kinase 1. These non-catalytic functions may explain why GSTP knock-out mice appeared to be resistant to

hepatotoxicity of acetaminophen *(48)*, which was unexpected because GSTP was shown to be highly active in the detoxification of synthetical N-acetylbenzoquinoneimine, the reactive intermediate of acetaminophen *(26)*.

In conclusion, the results of the present study show that several hGSTs have the ability to catalyse the GSH-conjugation of several P450-dependent reactive intermediates of DF. The fact that hGSTP1-1 was highly active in inactivating all four reactive intermediates that can be formed by P450s, this enzyme might play an important role in the protection of the gastrointestinal tract against DF-induced toxicity. Deficiency of hGSTM1-1 might be a risk factor for DF-induced hepatotoxicity, particularly in conditions when cellular GSH becomes depleted and inactivation of reactive DF-metabolites will be more dependent on GST-catalyzed GSH-conjugation. However, because not all homodimers and heterodimers of GSTs which are present in human liver were included in the present study, additional studies are required to quantify the protective role of polymorphic GSTs against reactive metabolites of DF. Furthermore, cellular cytotoxicity models in which levels of specific human GSTs are manipulated by transfection, specific knock-out or pharmacological inhibitors might give further insight in the protective role of GSTs in a cellular context.

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Chapter 5

EFFECT OF HUMAN GLUTATHIONE S-TRANSFERASE HGSTP1-1 POLYMORPHISMS ON THE DETOXIFICATION OF REACTIVE DRUG METABOLITES

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Abstract

Recent association studies suggest that genetically determined deficiencies in GSTs might be a risk factor for idiosyncratic adverse drug reactions resulting from the formation of reactive drug metabolites. hGSTP1-1 is polymorphic in the human population with a number of single nucleotide polymorphisms that yield an amino acid change in the encoded protein. Three allelic variants of hGSTP1-1 containing an Ile105Val or Ala114Val substitution, or a combination of both, have been the most widely studied and showed different activity when compared to wild-type hGSTP1-1*A (Ile105/Ala114). In the present study, we studied the ability of these allelic variants to catalyze the GSH conjugation of reactive metabolites of acetaminophen, clozapine, and diclofenac formed by bioactivation in in vitro incubations by human liver microsomes and drug metabolizing P450 BM3 mutants. The results show that effects of change of amino acid at residue 105 and 114 on conjugation reactions was substrate dependent. A single substitution at residue 105 affects the ability to catalyze GSH conjugation, while when both residue 105 and 114 were substituted the effect was additionally enhanced. Single mutation at position 114 did not show a significant effect. The different hGSTP1-1 mutants showed slightly altered regioselectivities in formation of individual GSH conjugates of clozapine which suggests that the binding orientation of the reactive nitrenium ion of clozapine is affected by the mutations. For diclofenac, a significant decrease in activity in GSH-conjugation of diclofenac 1',4'-quinone imine was observed for variants hGSTP1-1*B (Val105/Ala114) and hGSTP1-1*C (Val105/Val114). However, since the differences in total GSH conjugation activity catalysed by these allelic variants were not higher than 30%, interindividual differences in inactivation of reactive intermediates by hGSTP1-1 are not likely to be a major factor in determining interindividual difference in susceptibility to adverse drug reactions induced by the drugs studied.

1. Introduction

Glutathione S-transferases (GSTs) are a superfamily of dimeric phase II enzymes, which play an important role in the cellular defense system (*1, 2*). The human GSTs exhibit broad catalytic diversity due to the existence of eight cytosolic classes: α (*alpha*), μ (*mu*), π (*pi*), σ (*sigma*), θ (*theta*), κ (*kappa*), ο (*omega*) and z (*zeta*) and microsomal GSTs (*3*). The major role of these GSTs is to catalyze the conjugation of reduced glutathione (GSH) to electrophilic compounds and the reduction of organic hydroperoxides, thereby preventing cytotoxicity or mutagenicity. Furthermore, upregulation of GSTs in tumor tissues can contribute to resistence against alkylating antitumor agents *(2).* It is well established that many drugs are bioactivated by cytochrome P450s to short-lived reactive metabolites that may be responsible for adverse drug reactions (ADRs) (*4*). The balance between the rate of formation of these highly reactive metabolites and their inactivation by GSH conjugation, which may be catalyzed by GSTs, will determine the level of protein adduct formation and may determine the risk for ADRs (*4, 5*).

In humans, several GSTs are known to be genetically polymorphic due to point mutations, gene deletion and gene duplication *(1, 6).* Therefore, interindividual variations in the GSTs due to genetic polymorphism have been proposed as potentially important factors determining susceptibility to cancer and ADRs (*7-10)*. One of the polymorphic

GSTs, human GSTP1-1 (hGSTP1-1). is highly expressed in most extrahepetic tissues *(11,12)* and overexpressed in many tumor forms *(1,2,9).* Because hGSTP1-1 has high activity in the inactivation of many environmental carcinogens and antitumor agents, its genetic polymorphisms have been subject of many studies to test whether they might affect susceptibility of individuals to different types of cancer and the efficacy of chemotherapy (*6, 9*). For hGSTP1-1, a number of single nucleotide polymorphisms (SNPs) have been identified that yield an amino acid change in the encoded protein, i.e. Ile105Val (*13*), Ala114Val (*14*), Asp147Tyr (*15*), Phe151Leu (*16*), and Gly169Asp (*17*). Four allelic forms of hGSTP1-1, resulting from combinations of the first two SNPs, have been subject to most studies so far. The variants that result from polymorphisms at position 105 and 114 are hGSTP1*A (Ile105/Ala114), hGSTP1*B (Val105/Ala114), hGSTP1*C (Val105/Val114) and hGSTP1*D (Ile105/Val114). The allele frequencies of the genotypes in Caucasian poulations are: *hGSTP1*A*, 0.685; *hGSTP1*B*, 0.262, and *hGSTP1*C*, 0.0687 (*18*). Although frequently tested in in vitro studies the occurrence of allele hGSTP1*D remains to be established.

The effect of the mutations at position 105 and 114 of hGSTP1 has been studied *in vitro* using approximately thirty different electrophilic drugs and model substrates, including 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid, chlorambucil, thiopeta, acrolein and diol epoxides of several polycyclic aromatic hydrocarbons (PAHs), see supplementary Table S1. However, the activity of hGSTP1-1 variants in inactivation of very short-lived reactive drug metabolites, which can only be tested in presence of a bioactivating system, has not yet been evaluated. In the present study, the effect of GSTP1-1 allelic variants on the GSH-dependent inactivation of the highly reactive metabolites of acetaminophen (APAP), clozapine (CLZ) and diclofenac (DF) was studied because *in vitro* studies have shown that hGSTP1 showed high activity in inactivation of the reactive metabolites of these drugs formed by cytochrome P450s (*19-21*).

APAP is bioactivated by cytochrome P450 to N-acetyl-p-benzoquinone imine (NAPQI), a highly reactive intermediate which covalently binds to proteins, thereby causing hepatotoxicity (*4, 22*). Among the GSTs investigated, GSTP1-1 was the most active catalyst in GSH-conjugation of synthetical NAPQI in both rats and humans (*19*). Although GSTP1-1 is expressed in only low amounts in human liver (*12*), a recent clinical study by Buchard *et al.* suggests that homozygous carriers of the hGSTP1*C variant have a decreased risk for APAP hepatotoxicity (*23*). However, it has not been tested whether GSTP1-variants differ in the activity in inactivation of NAPQI.

The use of the antipsychotic drug CLZ is restricted because of the risk of life-threatening agranulocytosis and hepatotoxicity, both of which are considered to the result of bioactivation to a reactive nitrenium ion by myeloperoxidase and cytochrome P450s, respectively *(24-26)*. The nitrenium ion of CLZ is efficiently inactivated by GSH, forming multiple regioisomeric GSH conjugates as summarized in Table 1. Dragovic et al. showed that hGSTP1-1 has high activity in the inactivation of reactive nitrenium ion by catalyzing both chlorine-substitution and GSH-addition reactions, Figure 1 *(20)*. GSTP1-1 is highly expressed in white blood cells *(27)* and hence polymorphism of GSTP1-1 might be a risk factor for occurrence of agranulocytosis in case mutations strongly affect the inactivation of the nitrenium ion.

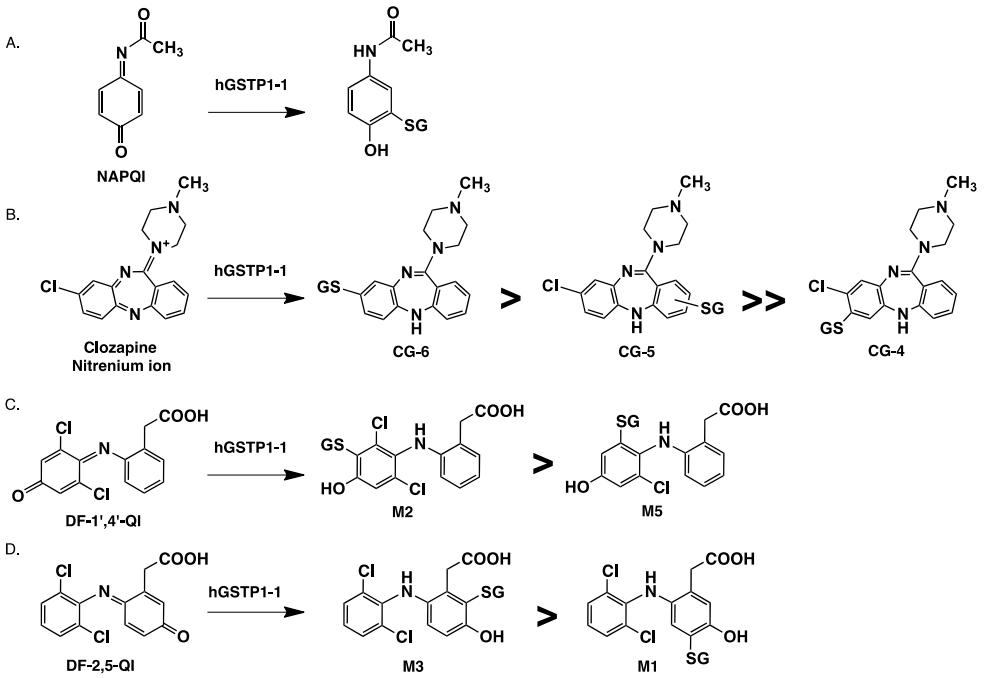

Figure 1. Reactive intermediates of acetaminophen (a), clozapine (b) and diclofenac (c,d) and identified structures of GSH-conjugates formed by wild-type hGSTP1-1. NAPQI, N-acetyl-p-benzoquinoneimine; DF-1',4'-QI, diclofenac 1',4'-quinoneimine; DF-2,5-QI, diclofenac 2,5-quinoneimine.

Treatment with the nonsteroidal anti-inflammatory drug DF is associated with serious gastrointestinal injury and rare cases of idiosyncratic hepatotoxicity *(28,29)*. Although the exact mechanisms of these ADRs remain to be established, formation of reactive metabolites by cytochrome P450s is considered to play an important role in the onset of these toxic events *(29,30)*. Based on the structure of GSH-conjugates identified in microsomal incubations at least four different reactive intermediates have been proposed for DF. Quantitatively the major ones are quinoneimines resulting from dehydrogenation of 4'-hydroxy diclofenac and 5-hydroxy diclofenac, Figure 1 *(31)*. Minor GSH-conjugates result from a quinonemethide formed via oxidative decarboxylation *(32)*, and an as yet unidentied reactive intermediate resulting in chlorine substitution of DF *(33)*. We recently showed that hGSTP1-1 has high activity in catalysing GSH-conjugation of all reactive intermediates. Because hGSTP1-1 is abundantly expressed in the gastrointestinal tract, polymorphisms of hGSTP1-1 might be risk factor for the occurrence of gastrointestinal injury in case the amino acid substitutions influence GSH-conjugation of the different reactive intermediates formed by P450s.

The aim of the present study was to investigate whether the allelic variants of hGSTP1-1 have different activity in the GSH conjugation of reactive metabolites generated by P450s in incubations of APAP, CLZ, and DF. Because the reactive intermediates of CLZ

and DF can be conjugated to GSH at different positions, Figure 1, also the effect of the mutations on regioselectivity of GSH-conjugation will be studied, which might be indicative for different binding orientations. Reactive intermediates were generated using human liver microsomes (HLM), and purified drug metabolizing mutants of bacterial CYP102A1 as bioactivation systems. The CYP102A1 mutants were selected because of their ability to produce human relevant GSH conjugates at high activity and to circumvent interference of microsomal GSTs *(5, 20)*.

2. Materials and methods

2.1. Enzymes, plasmids and chemicals

The bacterial CYP102A1 mutants CYP102A1 M11H, CYP102A1 M11H Phe87, and CYP102A1 M11 L437N were prepared and purified as described previously *(20, 35, 36)*. Human liver microsomes (HLM), pooled from 50 donors, were obtained from Xenotech (Lenexa, United States) and contained 20 mg/mL protein. Escherichia coli XL-1 Blue cells containing the expression plasmid for human GSTP1-1, was kind gift from Prof. Mannervik (Department of Biochemistry and Organic chemistry, Uppsala University, Sweden). All other chemicals and reagents were of analytical grade and obtained from standard suppliers.

2.2. Site directed mutagenesis of hGSTP1-1 isoforms

Three variants of wild type hGSTP1-1 (hGSTP1*A), namely hGSTP1*B (Val105, Ala114), hGSTP1*C (Val105, Val114) and hGSTP1*D (Ile105, Val114), were constructed by site directed mutagenesis. The single mutants hGSTP1*B and hGSTP1*D were constructed using the expression vector pKKD-hGST P1-1 as the template. The double mutant hGSTP1*C was constructed by introducing Ile104Val mutation in pKKD-hGSTP1*D (Ile105, Val114). The mutations were introduced by the Quikchange mutagenesis protocol using the following forward primers:

Ile104Val : CGC TGC AAA TAC GTC TCC CTC ATC TAC

Ala113Val : C ACC AAC TAT GAG GTG GGC AAG GAT GAC

The mutated amino acid position is indicated in bold. The reverse primers were exactly complementary to the forward primers. After mutagenesis, the presence of right mutations was verified by DNA sequencing (Service XS, Leiden, The Netherlands).

2.3. Expression and purification of hGSTP1-1 enzymes

Human GSTP1*A and its variants hGSTP1*B, hGSTP1*C and hGSTP1*D were expressed in E. coli XL-1 Blue cells. pKKD expression plasmids containing the different hGSTP1-1 genes, were transformed into E.coli XL-1 Blue cells. Expression and purification were essentially conducted as previously described *(20)*. Purity of the enzymes was checked by SDS-polyacrylamide gel electrophoresis on a 12% gel with Coomassie staining as well as by reversed-phase liquid chromatography using a method adapted from Jenkins et al. *(37)*.

Protein concentrations were determined according to the method of Bradford *(38)* with reagent obtained from Bio-Rad. The specific activity of the purified GSTs was assayed according to Habig et al. *(39)*. The specific activities of the purified recombinant human GSTP1*A and its polymorphic variants using CDNB as a substrate were as follows: 20.1 μmol/min/mg protein for hGSTP1*A, 10.6 μmol/min/mg protein for hGSTP1*B, and 9.2 μmol/min/mg protein for hGSTP1*C and 17.3 μmol/min/mg protein for hGSTP1*D.

2.4. Bioactivation of the drugs and GSH conjugates formation in the presence of hGSTP1-1 variants

Incubations were performed using purified CYP102A1 M11H as bioactivation system for APAP *(34)*. Mutant CYP102A1 M11H Phe87 was used for CLZ *(35)* and CYP102A1 M11 L437N for DF *(21)* as we have shown that these enzymes have high activity and selectivity for bioactivation to reactive intermediates. CLZ and APAP incubations were performed at a final enzyme concentration of 250 nM while for DF incubations were performed at a final BM3 concentration of 500 nM. All incubations were performed in 100 mM potassium phosphate buffer (pH 7.4) at a final volume of 250 μL. The substrates concentration was 500 μM. The final DMSO concentration from the stock solutions was not more than 2% in the incubations. The allelic variants of hGSTP1-1 were initially tested at 0.25, 0.5, 1, 2, 4, and 8 μM with CLZ as substrate to investigate the linearity of product formation with protein concentration and to compare their activities. A final concentration of 8 μM of the allelic variants of hGSTP1-1 was used in the incubations to investigate enzymatic GSH conjugation of the other substrates. Incubations with hGSTP1-1 were performed in the presence of 100 μM GSH. Reactions with CLZ were started by adding 0.5 mM NADPH (final concentration) and performed for 30 min at 24°C, as described previously (20). For the other substrates, an NAPDH regenerating system (final concentrations: 0.5 mM NADPH, 20 mM glucose-6-phosphate and 2 units/mL of glucose-6-phosphate dehydrogenase) was used to start the reaction and incubations were performed for 60 min at 24 °C. Reactions containing APAP and CLZ were terminated by the addition of 25 μL of 10 % ice-cold $HClO_4$. In case of DF 250 μL ice-cold methanol with 2% 50 mM ascorbic acid in water was added to stop incubations. All samples were centrifuged for 15 min at 14000 rpm. The supernatants were analyzed by HPLC and LC-MS, as described below.

Incubations were also performed using HLM as bioactivation system. 500 μM CLZ was incubated in 100 mM Kpi buffer, pH 7.4, for 30 minutes at 37°C with 1 mg/mL microsomal proteins, 100 μM GSH and in the absence and presence of 8 μM hGSTP1-1. Reactions were started with 0.5 mM NADPH (final concentration). For DF and APAP, incubations were performed in 100 mM Kpi buffer, pH 7.4, at a final volume of 250 μL with final concentrations of 500 μM substrate, 2 mg/mL microsomal proteins, 100 μM GSH and in the absence and presence of 8 μM hGSTP1-1. Reactions were initiated by the addition of NADPH regenerating system (final concentrations: 0.5 mM NADPH, 20 mM glucose-6-phosphate and 2 units/mL of glucose-6-phosphate dehydrogenase) and were incubated for 60 min at 37°C. Reactions were terminated depending on the substrate as described above and centrifuged at 14000 rpm for 15 min to remove precipitated proteins.

2.7. Analytical methods

The analyses of the GSH-conjugates were performed by reversed-phase liquid chromatography using a Luna 5 µm C18 column (4.6 x 150 mm) as stationary phase, except for DF where a Symmetry Shield™ RP18 3.5 µm column (4.6 x 100 mm) was used. For incubations of CLZ and DF, a gradient method was used where two mobile phases were mixed: eluent A (1% acetonitrile, 99% water, 0.2% formic acid) and eluent B (1% water, 99% acetonitrile, 0.2% formic acid). The gradient used was: 0 to 5 min isocratic at 0% eluent B; 5 to 30 min a linear increase to 100% eluent B; 30 to 30.5 min linear decrease to 0% eluent B; 30.5 to 40 min isocratic re-equilibration at 0% solvent B. The flow rate was 0.5 mL/min. For analysis of metabolites of APAP, an isocratic method was used with a mobile phase consisting of 18% methanol, 82% water, and 0.1 % trifluoroacetic acid (TFA) and a flow rate of 1 mL/min. Samples were injected at an injection volume of 50 µL and detected with a UV/Vis detector at a wavelength of 243 nm (APAP) or 254 nm (CLZ, DF). Quantification is based on the substrate standard curves, assuming that the extinction coefficients of the GSH adducts are equal to that of the substrate.

All metabolites were identified using an Agilent 1200 Series Rapid resolution LC system was connected to a hybrid quadrupole-time-of-flight (Q-TOF) Agilent 6520 mass spectrometer, equipped with electrospray ionization (ESI) source and operating in the positive mode as described in Dragovic et al. *(20)*. The characteristics of the GSH-conjugates analyzed in the incubations are tabulated in Table 1.

Table 1. Characteristics of the GSH conjugates formed by bioactivation of the drugs used in this study.

Abbreviation	GSH conjugates	t_{ret} (min)	m/z ($[M+H]^+$)
Clozapine			
CG-1	6-(glutathione-S-yl)-clozapine	14.3	632.23
CG-3	9-(glutathione-S-yl)-clozapine	14.0	632.23
CG-4	7-(glutathione-S-yl)-clozapine NI	13.5	632.23
CG-5	(1-4)-(glutathione-S-yl)-clozapine	14.6	632.23
CG-6	8-(glutathione-S-yl)-deschloroclozapine	11.8	598.27
Diclofenac			
M1	5-hydroxy-4/6-(glutathione-S-yl)-diclofenac	19.7	617.09
M2	4'-hydroxy-3'-(glutathione-S-yl)-diclofenac	20.0	617.09
M3	5-hydroxy-4/6-(glutathione-S-yl)-diclofenac	20.4	617.09
M5	4'-hydroxy-2'-(glutathione-S-yl)-diclofenac	19.7	583.13
DPAB-SG	2-(2,6-dichlorophenylamino)-benzyl-S-thioether glutathione	21.7	557.13
DF-SG	2'-(glutathione-S-yl)-deschlorodiclofenac	19.5	567.14
Acetaminophen			
APAP-SG	3-(glutathion-S-yl)-acetaminophen		

3. Results

3.1. Activity of hGSTP1-1 variants in inactivation of clozapine nitrenium ion

Consistent with previous results, addition of hGSTP1-1 to incubations of CLZ with CYP102A1 M11H Phe87 resulted in major changes of the profile and concentrations of GSH conjugates *(20)*. For each of the allelic variants of hGSTP1-1, the increase in formation of GSH conjugates CG-4, CG-5 and CG-6 was proportional with the hGSTP1-1 concentrations when varied from 0.5 to 8 μM (data not shown). Table 2 shows the effects observed at 8 μM of hGSTP1-1 variants. The amount of the total GSH conjugates of CLZ was increased 2.4 to 2.7-fold, when compared to the non-enzymatic conjugation. However, none of the three variants showed a statistically significant difference compared to wild-type hGSTP1*A. As shown in Figure 2A, the variants only showed slightly different profiles of GST-dependent GSH conjugates. For hGSTP1-1*B and hGSTP1-1*C CG-6 was produced at 2-fold higher amount than CG-5. For mutants hGSTP1-1*A and hGSTP1-1*D conjugate CG-6 was produced at 2.7- and 2.5-fold higher amounts, respectively, than CG-5. The minor GSH conjugate, CG-4, was increased with the highest activity by hGSTP1*B and hGSTP1*C being 2 times higher comparing to hGSTP1*A, after subtraction of non-enzymatic conjugation.

As can be seen from Table 2, the variants of hGSTP1-1 enzyme also did not show significant differences in total GSH conjugate formation when bioactivation reactions were performed with HLM which might be due to the almost 4-fold lower amounts of GSH-conjugates formed. However, similar differences in the ratio of GST-dependent conjugates CG-6 and CG-5 were observed. Again, hGSTP1-1*A and hGSTP1-1*D produced conjugate CG-6 at relatively higher amounts than CG-5. The effect on relative amount of CG-4 could not be confirmed because this conjugate is mainly produced by the microsomal GST present in HLM *(19)*.

3.2. Activity of hGSTP1-1 variants in inactivation of reactive metabolites of diclofenac

As shown in Table 2, all hGSTP1-1 variants showed very high activity in catalysing GSH-conjugation of reactive DF metabolites when using CYP102A1 as bioactivating system. The total amount of GSH-conjugates was increased 7- to almost 9-fold. hGSTP1*A and hGSTP1*D showed the highest activity for the formation of total GSH conjugates. When corrected for the non-enzymatic conjugation hGSTP1*B and hGSTP1*C appeared to have 20% and 30% lower activity, respectively, compared to the other variants.

DF is bioactivated by CYP102A1 to different reactive intermediates, of which diclofenac 1',4'-quinoneimine (DF-1'4'-QI), formed via 4'-hydroxylation, and diclofenac 2,5-quinoneimine (DF-2,5-QI), formed via 5-hydroxylation, are quantitatively the major ones. As shown in Table 2, the GSH-conjugation of DF-1'4'-QI was more affected by the polymorphic mutations with hGSTP1*C exhibiting the lowest activity in formation of the corresponding GSH-conjugates M2 and M5. When correcting for the non-enzymatic reaction, hGSTP1*C showed almost 40% lower activity when compared to the wild type hGSTP1*A. Activities of hGSTP1*B and hGSTP1*D were 22% and 17% lower, respectively. No differences in the ratio of M2 and M5 were observed, Figure 3A.

Table 2. Effect of recombinant hGSTP1-1 variants on the total of GSH conjugates formed in incubations of drugs with CYP102A1-mutants and human liver microsomes.

Bioactivation system	Drug	GSH-conjugates	Recombinant human GSTP1-1				
			No hGST	hGSTP1-1*A (Ile105/Ala114)	hGSTP1-1*B (Val105/Ala114)	hGSTP1-1*C (Val105/Val114)	hGSTP1-1*D (Ile105/Val114)
None							
	CDNB	DNB-SG	N.D.	20.1± 1,0	10.6 ± 0.5	9.2 ± 0.4	17.3 ± 0.9
CYP102A1-mutants							
	CLZ	Total[a]	13.1 ± 0.6	32.1 ± 1.3	31.0 ± 2.8	31.3 ± 2.9	35.3 ± 1.1
	DF	Total[b]	2.2 ± 0.1	19.1 ± 0.8	15.9 ± 0.9	14.2 ± 0.5	18.2 ± 0.7
		DF-1',4'-QI **(M2** + **M5)**	1.7 ± 0.2	13.8 ± 0.9	11.1 ± 0.6	9.2 ± 0.4	11.7 ± 0.6
		DF-2,5-QI **(M1** + **M3)**	0.4 ± 0.03	5.2 ± 0.6	4.9 ± 0.4	4.9 ± 0.3	6.5 ± 0.6
	APAP	APAP-SG	8.2 ± 0.7	84.8 ± 4.3	90.9 ± 5.1	73.5 ± 3.4	82.3 ± 2.5
HLM							
	CLZ	Total[a]	3.7 ± 0.1	7.9 ± 0.6	8.2 ± 0.5	8.1±0.3	8.1 ± 0.2
	DF	Total[b]	0.48 ± 0.08	2.02 ± 0.13	1.90 ± 0.17	1.94±0.20	1.97 ± 0.14
		DF-1',4'-QI **(M2** + **M5)**	0.46 ± 0.04	1.49 ± 0.10	1.40 ± 0.08	1.47±0.09	1.46 ± 0.10
		DF-2,5-QI **(M1** + **M3)**	0.02 ± 0.01	0.53 ± 0.07	0.49 ± 0.05	0.47±0.04	0.51 ± 0.03
	APAP	APAP-SG	1.9 ± 0.1	10.5 ± 1.6	11.4 ± 1.7	10.0±1.8	10.2 ± 2.4

Metabolites were formed after 1 hour incubation of 500 µM DF with 500 nM CYP102A1 M11 L437N in the presence of 5 mM GSH.
Quantification was based on peak areas in the LC-UV chromatograms using the DF standard curve, assuming that the extinction coefficients of the substrate and the metabolites at 254 nm are the same.
t_{ret}, retention time in minutes.
[a] Only detectable in incubations supplemented with human GST.

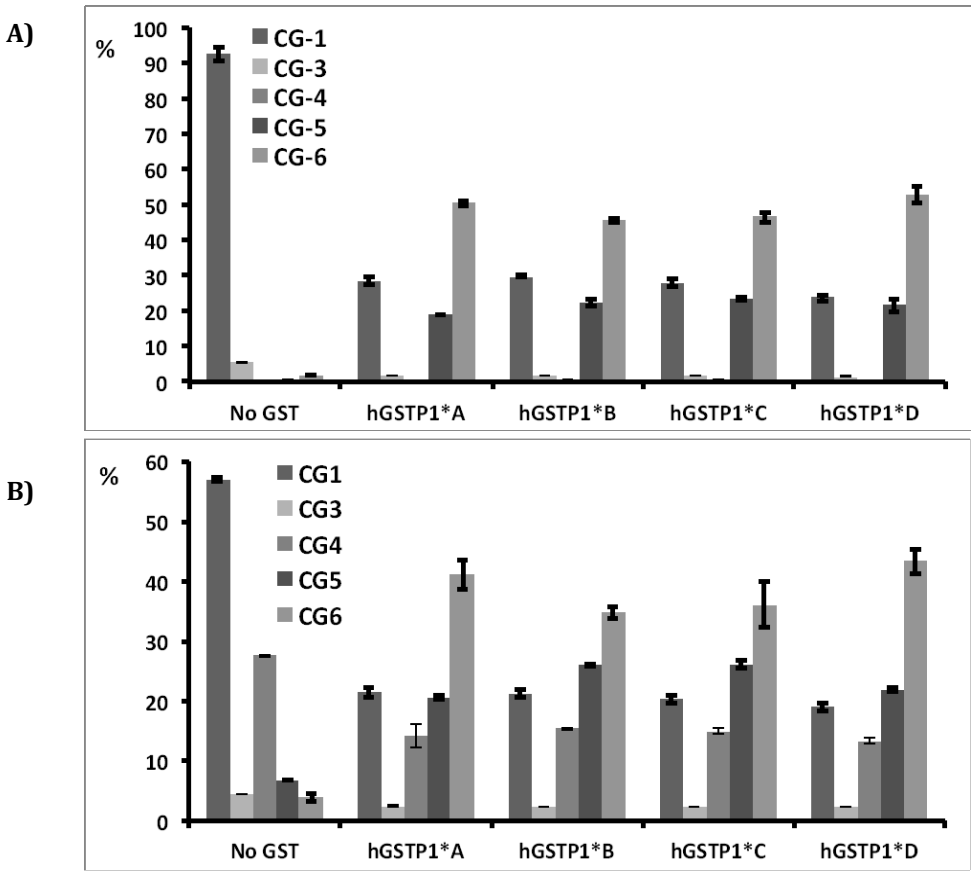

Figure 2. Effect of 8 μM of hGSTP1*A, hGSTP1*B, hGSTP1*C, and hGSTP1*D on the regioselectivity of GSH-conjugation of the nitrenium ion of CLZ. CLZ was bioactivated to reactive metabolites using CYP102A1 M11H Phe87 mutant (**A**) or HLM (**B**).

What concerns the GSH-conjugation of DF-2,5-QI, only hGSTP1*D appeared to have 25% higher activity than wild-type hGSTP1*A, whereas hGSTP1*C and hGSTP1*B showed similar activity. As shown in Figure 3B, small changes were observed in the regioselectivity of GSH-conjugation of DF-2,5-QI since the relative amount of GSH-conjugate M1 was slightly higher in variants hGSTP1*A and hGSTP1*D. No significant differences were observed between the hGSTP1-1 polymorphic variants for the formation of the minor GSH conjugates DF-SG (m/z 567) and DPAB-SG (m/z 557) which are formed by the two minor bioactivation pathways (data not shown) *(32,33)*.

In case of HLM as bioactivation system, addition of 8 μM of hGST-variants increased the total GSH-conjugation of reactive metabolites of DF about 4-fold, Table 1. No significant differences were observed between the activities and regioselectivities of the hGSTP1-1 variants, which may be explained by the lower levels of reactive intermediates produced and the analytical error, Figure 4. The fact that GSH-conjugation of DF-1'4'-QI is increased to a smaller extent by hGSTP1-1, whereas a significantly

different ratio of conjugates M2 and M5 was found, point to contribution of microsomal GST in the HLM incubations, Figure 4B. Again no significant differences were observed between the polymorphic variants of hGSTP1-1 for the formation of DF-SG and DPAB-SG (data not shown).

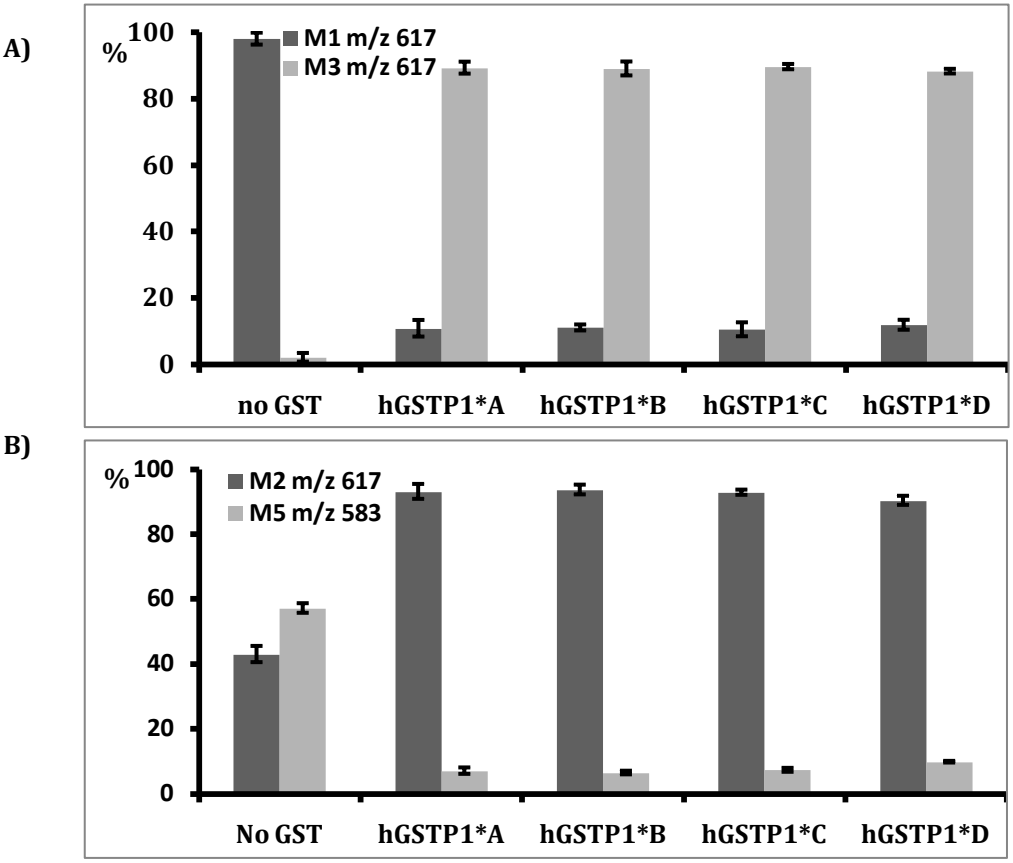

Figure 3. Effect of 8 μM hGSTP1*A, hGSTP1*B, hGSTP1*C, and hGSTP1*D on the regioselectivity of GSH conjugation of DF-1',4'-QI (A) and DF-2,5-QI (B), using CYP102A1 M11 L437N mutant as bioactivation system.

3.4 Activity of hGSTP1-1 variants in inactivation of acetaminophen reactive metabolites

The addition of 8 μM of hGSTP1-1 mutants to the incubation of APAP with CYP102A1 M11H, in the presence of 100 μM GSH, increased the concentrations of NAPQI-SG about 10-fold, Table 2. Variants hGSTP1*A, hGSTP1*B and hGSTP1*D did not show statistically significant differences in conjugation of NAPQI. Only hGSTP1*C showed a 15% decreased activity compared to hGSTP1*A. When using HLM as bioactivation system, however, no significant differences were found in the activity of the hGSTP1-1 variants.

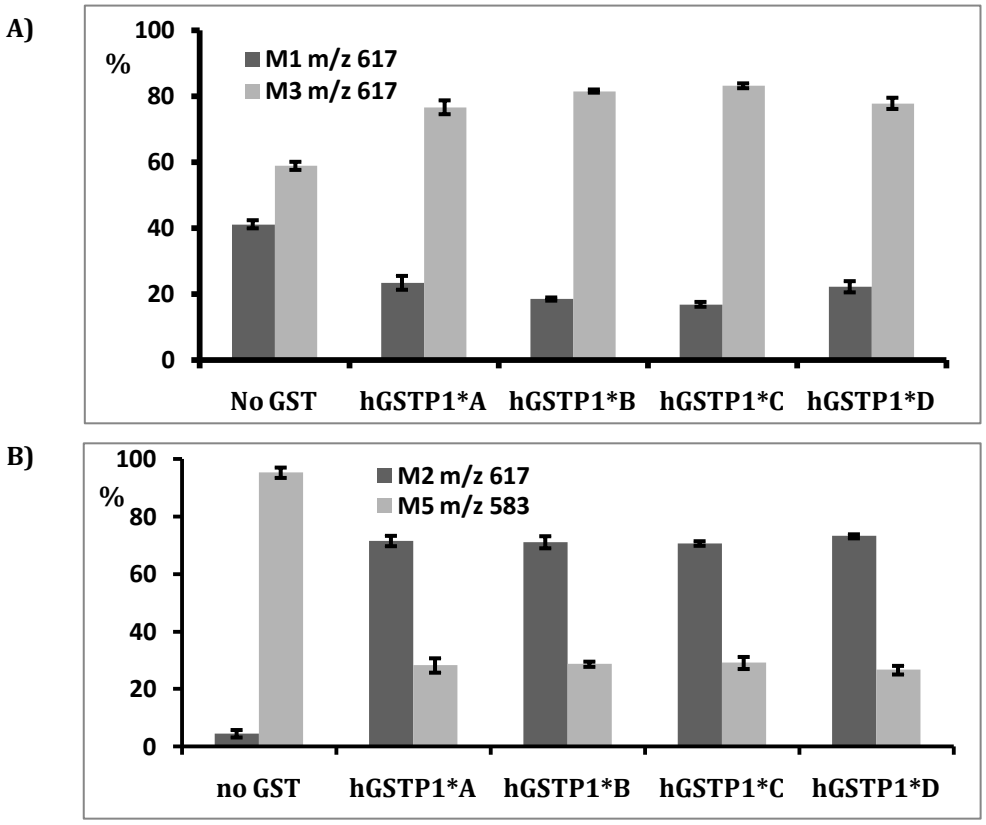

Figure 4. Effect of 8 μM hGSTP1*A, hGSTP1*B, hGSTP1*C, and hGSTP1*D on the regioselectivity of GSH conjugation of DF-1',4'-QI (A) and DF-2,5-QI (B), using human liver microsomes as bioactivation system.

4. Discussion

Because hGSTP1-1 is highly expressed in normal extrahepatic tissues and overexpressed in many tumors types, in some cases in response to antitumor drugs. Therefore, a large number of studies have been performed to investigate whether genetic polymorphism of hGSTP1-1 has consequences for the inactivation of carcinogenic diolepoxides of PAHs or alkylating antitumor drugs, such as thiotepa and chlorambucil, since these might be revelant for susceptibility to carcinogenesis and efficacy of antitumor treatment. Table 3 summarizes the relative activities observed for the hGSTP1-1-variants with amino acid substitutions Ile105Val and/or Ala114Val when compared to wild-type hGSTP1-1*A, which is set at 100% for all compounds. The data shown in Table 3 are based on the kcat/Km-values reported because this enzyme kinetical parameter will most likely the most relevant parameter to describe differences in enzyme activity at the low, physiologically relevant, concentrations of reactive carcinogens and toxicants. Only data is shown of studies in which at least three of the hGSTP1-1 variants were compared; for a more complete list see Table 4.

Table 3. Relative activity of polymorphic variants of hGSTP1-1 in GSH-conjugation of electrophilic drugs and reactive drug metabolites.

Electrophilic drug / Reactive intermediate	hGSTP1*A (Ile105/Ala114)	hGSTP1*B (Val105/Ala114)	hGSTP1*C (Val105/Val114)	hGSTP1*D (Ile105/Val114)	Literature
Clozapine nitrenium ion / CLZ NI	100 ± 10	94 ± 22	96 ± 30	117 ± 21	This study [a]
Diclofenac-1',4'-quinoneimine / DF-1',4'-QI	100 ± 9	78 ± 7‡	62 ± 5‡	83 ± 7	This study [a]
Diclofenac-2,5-quinoneimine / DF-2,5-QI	100 ± 13	94 ± 9	94 ± 7	127 ± 13	This study [a]
N-Acetyl-p-benzoquinoneimine / NAPQI	100 ± 7	108 ± 8	85 ± 5‡	97 ± 4	This study [a]
Ethacrynic acid	100 ± 13	150 ± 20‡	253 ± 13‡	93 ± 13	(*43*)
Thiotepa	100 ± 8	53 ± 9‡	38 ± 16‡		(*48*)
Chlorambucil	100 ± 7	13 ± 4‡	7 ± 2‡	40 ± 10‡	(*49*)
Atrazine	100 ± 4	103 ± 14	100 ± 11	97 ± 14	(*50*)
1-chloro-2,4—dinitrobenzene / CDNB	100 ± 5	53 ± 3‡	46 ± 2‡	86 ± 4	This study [a]
	100 ± 14	37 ± 7‡	44 ± 9‡	56 ± 10‡	(*51*)
	100 ± 5	27 ± 3‡	32 ± 5‡	85 ± 3	(*43*)
	100 ± 8	30 ± 3‡	24 ± 3‡		(*14*)
	100 ± 35	21 ± 4‡	64 ± 19‡	157 ± 26	(*52*)
7-chloro-4-nitrobenz-2-oxa-1,3-diazole / NBD-Cl	100 ± 5	15 ± 2‡	104 ± 4	47 ± 3‡	(*43*)
Acrolein	100 ± 2	99 ± 3	71 ± 2‡	90 ± 2	(*53*)
Crotonaldehyde	100 ± 13	106 ± 13	75 ± 13‡	75 ± 6‡	(*53*)
Diol epoxides of polycyclic aromatic hydrocarbons					
(+)-anti-benzo[a]pyrene diolepoxide / (+)-anti-BPDE	100 ± 12	130 ± 20‡	500 ± 100‡	150 ± 70	(*52*)
(+/-)-chrysene diolepoxide / (+/-)-anti-CDE	100 ± 25	767 ± 219‡	438 ± 110‡	151 ± 27	(*52*)
(+)-anti-5-methylchrysene diolepoxide / (+)-anti-5-MeCDE	100 ± 11	109 ± 4	82 ± 16		(*54*)
(-)-anti-5-methylchrysene diolepoxide / (-)-anti-5-MeCDE	100 ± 80	200 ± 40‡	180 ± 100‡		(*54*)
(±)-anti-benzo[c]chrysene diolepoxide-1 / (+/-)-anti-B[c]CDE-1	100 ± 17	233 ± 33‡	167 ± 17‡		(*55*)
(±)-anti-benzo[c]chrysene diolepoxide-2 / (+/-)-anti-B[c]CDE-2	100 ± 10	61 ± 5‡	23 ± 4‡		(*55*)

Numbers of published data are based on reported k_{cat}/K_m-values. [a.] Data of incubations with CYP102A1 M11H-mutants as bioactivation system. ‡Signifinantly increased or decreased.

Table 4. Catalytic efficiency (k_{cat}/K_m) of polymorphic variants of human glutathione S-transferases P1-1 (hGSTP1-1)

		hGSTP1-1 variant					
Substrate	Abbreviation	*A Ile105/Ala114	*B Val105/Ala114	*C Val105/Val114	*D Ile105/Val114	Unit	Reference
1-chloro-2,4--dinitrobenzene	CDNB	55,1 ± 4,6	16,7 ± 1,4	13,2 ± 1,7		($mM^{-1}.s^{-1}$)	(a)
1-chloro-2,4--dinitrobenzene	CDNB	70 ± 20	13 ± 2	110 ± 15	40 ± 12	($mM^{-1}.s^{-1}$)	(b)
1-chloro-2,4--dinitrobenzene	CDNB	98,2 ± 7,5	42,5 ± 4,2			($mM^{-1}.s^{-1}$)	(c)
1-chloro-2,4--dinitrobenzene	CDNB	66,0 ± 3,0	18,0 ± 2,0	21,0 ± 3,0	56,0 ± 2,0	($mM^{-1}.s^{-1}$)	(d)
1-chloro-2,4--dinitrobenzene	CDNB	98,2 ± 14,0	35,9 ± 6,8	43,4 ± 9,3	55,0 ± 9,8	($mM^{-1}.s^{-1}$)	(e)
Ethacrynic acid		15,0 ± 2,0	22,5 ± 3,0	38,0 ± 2,0	14,0 ± 2,0	($mM^{-1}.s^{-1}$)	(d)
Ethacrynic acid		45,5 ± 9.6	54,6 ± 9,0			($mM^{-1}.s^{-1}$)	(c)
Thiotepa		11,8 ± 1,0	6,3 ± 1,1	4,5 ± 0,7		($M^{-1}.s^{-1}$)	(f)
Chlorambucil		15,0 ± 1,0	2,0 ± 0,6	1,0 ± 0,3	6,0 ± 1,6	($mM^{-1}.s^{-1}$)	(g)
Atrazine		18,0 ± 0,8	18,5 ± 2,5	18,0 ± 2,0	17,5 ± 2,5	(nmol/min/mg)	(h)
7-chloro-4-nitrobenz-2-oxa-1,3-diazole	NBD-Cl	250 ± 12	37 ± 5	260 ± 10	118 ± 7	($mM^{-1}.s^{-1}$)	(d)
7-chloro-4-nitrobenz-2-oxa-1,3-diazole	NBD-Cl	362 ± 37	177 ± 19			($mM^{-1}.s^{-1}$)	(c)
Acrolein		129,0 ± 3,0	128,0 ± 4,0	92,0 ± 3,0	116,0 ± 3,0	($mM^{-1}.s^{-1}$)	(i)
Crotonaldehyde		16,0 ± 2,0	17,0 ± 2,0	12,0 ± 2,0	12,0 ± 1,0	($mM^{-1}.s^{-1}$)	(i)
4-nitrocinnamaldehyde	4-NCA	19,5 ± 3,0	16,6 ± 2,0			($mM^{-1}.s^{-1}$)	(c)
4-vinylpyridine		202,0 ± 31	234,7 ± 33,8			($mM^{-1}.s^{-1}$)	(c)
Phenethylisocyanate		463 ± 39	503 ± 51			($mM^{-1}.s^{-1}$)	(c)
(+)-anti-benzo[a]pyrene diolepoxide	(+)-anti-BPDE	7,5 ± 1,9	20,0 ± 2,2			($mM^{-1}.s^{-1}$)	(j)
(+)-anti-benzo[a]pyrene diolepoxide	(+)-anti-BPDE	10 ± 1	13 ± 2	50 ± 10	15 ± 8	($mM^{-1}.s^{-1}$)	(b)
(+)-anti-benzo[a]pyrene diolepoxide	(+)-anti-BPDE	11 ± 1	14 ± 2			($mM^{-1}.s^{-1}$)	(k)
(-)-anti-benzo[a]pyrene diolepoxide	(-)-anti-BPDE	2,7 ± 0,3	3,0 ± 0,3			($mM^{-1}.s^{-1}$)	(k)
(+)-syn-benzo[a]pyrene diolepoxide	(+)-syn-BPDE	64,0 ± 0,3	100,0 ± 11,0			($mM^{-1}.s^{-1}$)	(j)
(+)-anti-Chrysene diolepoxide	(+)-anti-CDE	3,6 ± 1,0	9,9 ± 1,8			($mM^{-1}.s^{-1}$)	(j)
(+/-)-Chrysene diolepoxide	(+/-)-anti-CDE	4,0 ± 1.0	28 ± 4			($mM^{-1}.s^{-1}$)	(l)
(+/-)-Chrysene diolepoxide	(+/-)-anti-CDE	3 ± 1	28 ± 8	16 ± 3	6 ± 2	($mM^{-1}.s^{-1}$)	(b)
(+)-syn-Chrysene diolepoxide	(+)-syn-CDE	11,0 ± 3,2	11,0 ± 1,0			($mM^{-1}.s^{-1}$)	(j)
(+)-anti-5-methylchrysene diolepoxide	(+)-anti-5-MeCDE	31 ± 3	18 ± 3	26 ± 3		($mM^{-1}.s^{-1}$)	(m)
(-)-anti-5-methylchrysene diolepoxide	(-)-anti-5-MeCDE	17 ± 2	25 ± 3	11 ± 1		($mM^{-1}.s^{-1}$)	(m)
(-)-anti-Benzo[c]chrysene diolepoxide	(-)-anti-B[c]CDE	1,9 ± 0,3	1,8 ± 0,3			($mM^{-1}.s^{-1}$)	(n)
(+)-syn-Benzo[c]chrysene diolepoxide	(+)-syn-B[c]CDE	0,53 ± 0,09	0,87 ± 0,13			($mM^{-1}.s^{-1}$)	(n)

Table 4 (continued). Catalytic efficiency (k_{cat}/K_m) of polymorphic variants of human glutathione S-transferases P1-1 (hGSTP1-1)

		hGSTP1-1 variant					
Substrate	Abbreviation	*A Ile105/Ala114	*B Val105/Ala114	*C Val105/Val114	*D Ile105/Val114	Unit	Reference
(±)-anti-benzo[c]chrysene diolepoxide-1	(+/-)-anti-B[c]CDE-1	0,12 ± 0,02	0,28 ± 0,04	0,20 ± 0,02		($mM^{-1}.s^{-1}$)	(o)
(±)-anti-benzo[c]chrysene diolepoxide-2	(+/-)-anti-B[c]CDE-2	0,092 ± 0,010	0,055 ± 0,008	0,022 ± 0,008		($mM^{-1}.s^{-1}$)	(o)
(-)-anti-Dibenzo[a,l]pyrene diolepoxide	(-)-anti-DB[a.l]PDE	1,2 ± 0,2	1,1 ± 0,3			($mM^{-1}.s^{-1}$)	(n)
(+)-syn-Dibenzo[a,l]pyrene diolepoxide	(+)-syn-DB[a.l]PDE	1,1 ± 0,3	2,1 ± 0,5			($mM^{-1}.s^{-1}$)	(n)
(-)-anti-benzo[g]chrysene diolepoxide	(-)-anti-B[g]CDE	1,3 ± 0,5	1,2 ± 0,5			($mM^{-1}.s^{-1}$)	(n)
(+)-syn-benzo[g]chrysene diolepoxide	(+)-anti-B[g]CDE	0,5 ± 0,2	1,5 ± 0,7			($mM^{-1}.s^{-1}$)	(n)
(-)-anti-benzo[g]chrysene diolepoxide	(-)-anti-B[g]CDE	0,36 ± 0,04	0,31 ± 0,04	0,21 ± 0,03	0,28 ± 0,04	($mM^{-1}.s^{-1}$)	(p)
(+)-anti-Benzo[c]phenanthrene diolepoxide	(+)-anti-B[c]PhDE	0,55 ± 0,10	0,54 ± 0,03			($mM^{-1}.s^{-1}$)	(n)
(+/-)-anti-Benzo[c]phenanthrene diolepoxide	(+/-)-anti-B[c]PhDE	0,24 ± 0,01	0,11 ± 0,01	0,15 ± 0,03	0,12 ± 0,02	($mM^{-1}.s^{-1}$)	(p)
(-)-syn-Benzo[c]phenanthrene diolepoxide	(-)-syn-B[c]PhDE	0,24 ± 0,03	0,49 ± 0,04			($mM^{-1}.s^{-1}$)	(n)
(+)-anti-Dibenz[a,h]anthracene diolepoxide	(+)-anti-DBADE	1,4 ± 0,40	1,9 ± 0,7			($mM^{-1}.s^{-1}$)	(j)
(+)-syn-Dibenz[a,h]anthracene diolepoxide	(+)-syn-DBADE	2,0 ± 0,6	5,4 ± 0,7			($mM^{-1}.s^{-1}$)	(j)

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The results of the present study show that the amino acid substitutions Ile105Val and Ala114Val have differential effects on the activity of hGSTP1-1, dependent on the reactive intermediate involved, consistent with the previous studies which have been performed with chemically stable electrophilic compounds, see Table 3. For CLZ no significant differences were observed in the total rate by which the CLZ nitrenium ion is conjugated to GSH by hGSTP1-1. The only significant effect which was observed in both CYP102A1 and HLM incubations was a slight change in regioselectivity in GSH-conjugation to the CLZ nitrenium ion. In both hGSTP1*B and hGSTP1*C, which share the Ile105Val substitution, an increase in the relative amount of GSH-conjugates CG-4 and CG-5 was found while substitution of chlorine by GSH and formation of CG-6 was decreased, Figure 2. Although no association studies have been carried out yet to study the role of hGSTP1-1-polymorphism as risk factor for CLZ-induced agranulocytosis, the present data see to rule out that genetically determined differences in hGSTP1-1 activity in white blood cells might determine susceptibility.

As shown in Tables 2 and 3, variant hGSTP1-1*B and hGSTP1-1*C both showed a significant decreased total activity in GSH-conjugation of reactive metabolites of DF, which could be mainly attribited to a decreased GSH-conjugation of DF-1',4'-QI, which is quantitatively the major reactive intermediate formed. Both hGSTP1-1*B and hGSTP1-1*C contain amino acid substitution Ile105Val which was also shown to be detrimental for the GSH-conjugation of the antitumor agents chlorambucil and thiotepa, and for the general GST-substrate CDNB but benificial for the catalysis of GSH-conjugation of ethacrynic acid and several carcinogenic diolepoxides of PAHs, Table 3. According to the available crystal structures of hGSTP1*A, hGSTP1*B and hGSTP1*C *(42, 43)*, amino acid residue 105 is localized in the H-site of hGSTP1-1 and can affect enzyme activity in several ways, dependent on the substrate. First, the Ile105Val substitution can affect the shape of the active-site, because the Val105 has more conformational freedom than Ile105. For large substrates, Val105 can point away from the active site, which allows the accommodating of larger substrates, such as BPDE, for catalysis *(42)*. Also, amino acid residue 105 is close to residue Tyr109, which for some substrates plays an important role in the catalytic mechanism of hGSTP1-1 by stabilizing the intermediate complex *(44)*. Furthermore, substitution Ile105Val has shown to affect the hydrophobicity/hydrophilicity of the H-site of hGSTP1-1 by influencing the number of active-site water molecules which can affect substrate binding and/or product release *(45)*. Which of these mechanisms contribute to the decreased GSH-conjugation of DF-'1,'4-QI and the slight change in regioselectivity of GSH-conjugation of CLZ-nitrenium ion is not known.

A recent association study has shown that the frequency of hGSTP1*C-allele was significantly lower in APAP-poisoned patients than in insensitive patients, which suggests that this genotype may reduce the risk of APAP-hepatotoxicity *(23)*. The results of the present study shows that the hGSTP1*C variant had the lowest activity in catalysing the GSH-conjugation of NAPQI, Table 1. However, this activity was only 15% lower than that of hGSTP1-1*A, which is not likely to be of toxicological relevance, also considering the fact that the current study was performed at reduced GSH-concentration, to minimize non-enzymatic GSH-conjugation, and that hGSTP1-1 is expressed in the human liver only to a significant extent in the biliary tree *(11)*.

In conclusion, the results of the present study show that the amino acid substitutions Ile105Val and Ala115Val in hGSTP1-1 have only relatively minor effect on

the activity of GSH-conjugation of the reactive metabolites of APAP, CLZ and DF. Differences in the rate of inactivation of their reactive intermediates are, therefore, unlikely to play a role in the susceptibility to their toxic side-effects. However, in recent years it has been shown that GSTP1-1 also can directly influence signalling pathways underlying stress response, apoptosis, inflammation, and cell proliferation by protein-protein interaction and protein glutathionylation *(46)*. Interestingly, it was recently shown that only variants hGSTP1-1*A and hGSTP1-1*C were able to reactivate the antooxidant enzyme peroxiredoxin VI, whereas variants hGSTP1-1*B and hGSTP1-1*D were without effect, suggesting that these polymorphisms may influence human population susceptibility to oxidant stress *(47)*. Therefore, these factors rather than interindividual differences in the rate of GSH-conjugation of reactive drug metabolites by hGSTP1-1 can play a role in susceptibility to adverse drug reactions induced by the drugs studied.

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Chapter 6

ROLE OF RESIDUE 87 IN THE ACTIVITY AND REGIOSELECTIVITY OF CLOZAPINE METABOLISM BY DRUG METABOLIZING CYP102A1 M11H: APPLICATION FOR STRUCTURAL CHARACTERIZATION OF CLOZAPINE GSH CONJUGATES

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Abstract

In the present study, a site-saturation mutagenesis library of drug metabolizing BM3 M11 with all 20 amino acids at position 87 was applied as biocatalyst for the production of stable and reactive metabolites of clozapine. Clozapine is an atypical antipsychotic drug where formation of reactive metabolites is considered to be responsible for several adverse drug reactions. Reactive intermediates of clozapine can be inactivated by GSH to multiple GSH conjugates, by non-enzymatic and glutathione S-transferase (GST) mediated conjugation reactions. The structures of several GST dependent metabolites have not yet been elucidated unequivocally. The present study shows that the nature of amino acid at position 87 of BM3 M11 strongly determines both activity and regioselectivity of clozapine metabolism. Some mutants showed preference for N-demethylation and N-oxidation, whereas others showed high selectivity for bioactivation to reactive intermediates. The mutant containing Phe87 showed both high activity and high selectivity for the bioactivation pathway and was used for the large scale production of GST dependent GSH conjugates by incubation in presence of recombinant human glutathione S-transferase P1-1. Five human relevant GSH adducts were produced at high levels enabling structural characterization by 1H-NMR. This work shows that drug metabolizing BM3-mutants, in combination with GSTs, are very useful tools for the generation of GSH conjugates of reactive metabolites of drugs in order to enable their isolation and structural elucidation.

1. Introduction

Cytochrome P450 (P450s) are involved in the metabolism of approximately 80% of the drugs currently on the market (*1, 2*). In some cases drugs can be oxidized by P450s to electrophilic reactive intermediates, which subsequently can react with nucleophilic functional groups in biomolecules such as proteins and DNA. Also, stable metabolites might possess pharmacological activities that might be responsible for undesired adverse drug reactions. It is for these reasons that also the characterization of the biological properties of major metabolites is considered to be important for drug safety assessment (*3*). Therefore, methods are required to obtain the relevant drug metabolites in sufficient yield to allow structural elucidation and to study their pharmacological and toxicological properties. Metabolite production can be achieved by organic synthesis, electrochemical oxidation of parent drug and by biosynthesis using specific P450s. In particular, mutants of the bacterial cytochrome P450 BM3 (P450 BM3) are considered to have good perspective for the large scale production of human relevant drug metabolites, as this very stable enzyme possesses the highest activity ever recorded for a P450 (*4*). By combinations of site-directed and random mutagenesis many BM3 mutants have been obtained which are able to convert drugs and drug-like molecules to human relevant metabolites (*5-8*). In our previous work, four mutants of BM3 have been evaluated as biocatalysts for the bioactivation of several drugs to reactive intermediates (*9*). Drugs tested were acetaminophen, diclofenac and clozapine (CLZ), and formation of reactive intermediates was analyzed by measurement of GSH conjugates. For all drugs tested, most stable metabolites and reactive intermediates were produced at much higher activity by the BM3 mutants than by human and rat liver microsomes, supporting their potential for use in characterization of toxicologically relevant metabolites (*9*).

Recently, the highly active drug-metabolizing mutant BM3 M11 was used to investigate the role of human glutathione S-transferases in the inactivation of CLZ (*10*). CLZ is an atypical antipsychotic drug showing a low incidence of extrapyramidal side effects combined with excellent antipsychotic efficacy in schizophrenic and manic treatment-resistant patients (*11-13*). Approximately 1-2% of patients develop agranulocytosis. Enhanced serum transaminases were monitored with 37% of the patients while 0.06% of the patients had liver failure (*14*). It is still unknown which factors predispose part of the patient population to these forms of CLZ toxicity. Based on the identification of several GSH conjugates, formation of a reactive nitrenium ion by peroxidases, hypochlorite and P450s has been proposed as a possible explanation for these ADRs (*15-18*). *In vitro* and *in vivo* studies of CLZ have shown the formation of four GSH-conjugates with identical mass with MH^+ ion at m/z 632.2 and one deschlorinated GSH conjugate with MH^+ ion at m/z 598.3. All conjugates can be explained by direct conjugation of GSH at different positions of a reactive nitrenium ion and by chloro-substitution of the nitrenium ion followed by reduction, see Figure 1 (*10, 17*). Unequivocal structure determination by ^{1}H-NMR has been published for only two of the glutathione conjugates of CLZ (*15, 16, 19*). The major GSH conjugate (CG-1, Figure 1) was found to be conjugated at position 6 of the chlorinated aromatic ring, whereas a minor GSH conjugate (CG-3, Figure 1) was found to be conjugated at position 9 (*15, 16*). A third GSH conjugate with a MH^+ ion at m/z 632.2 was only identified in *in vitro* incubations with human and rat liver microsomes and was tentatively assigned to position 7 (CG-4, Figure 1). Two GSH-conjugates, with MH^+ ion at m/z 632.2 and 598.3, were first discovered in bile of treated mice and rats and were originally proposed to originate from unidentified reactive intermediates formed *in vivo* (*17*). However, we recently demonstrated that these GSH conjugates, CG-5 and CG-6 in Figure 1, are formed at high levels when CLZ incubations with purified BM3 M11 and human liver microsomes were supplemented with human glutathione S-transferases (*10*). Three of the four tested human glutathione S-transferases (hGSTs) showed strongly increased total GSH conjugation and also resulted in formation of different regioisomeric GSH conjugates of CLZ, Figure 1 (*10*).

For two of the GSH conjugates that have been found previously, the structure has not been elucidated by NMR. Conjugate CG-4, which was found in incubations of CLZ with human liver microsomes, was tentatively assigned as conjugate at position 7 (*17*). GSH conjugate CG-5, which was identified in bile of rats and mice, was proposed to result from GSH conjugation to the non-chlorinated ring (*17*). The aim of the present study was to identify the structures of these GSH conjugates by ^{1}H-NMR, by performing large scale incubations of CLZ with selective BM3 M11-mutants in presence of glutathione S-transferase. Because GST P1-1 appeared to be the most active hGST in the formation of enzyme-dependent GSH conjugates (*10*), this enzyme was selected for large scale production of GSH conjugates.

Although BM3 M11 was previously shown to produce high levels of CLZ metabolites, the most abundant metabolites appeared to be N-demethylclozapine and CLZ N-oxide (*9, 10*). As a consequence, also GSH conjugates derived from these metabolites were produced, which strongly complicates isolation of CG-4 and CG-5. Recently, we showed that by changing the active site residue at position 87 of BM3 M11 the regioselectivity of testosterone hydroxylation was strongly modified *(20)*.

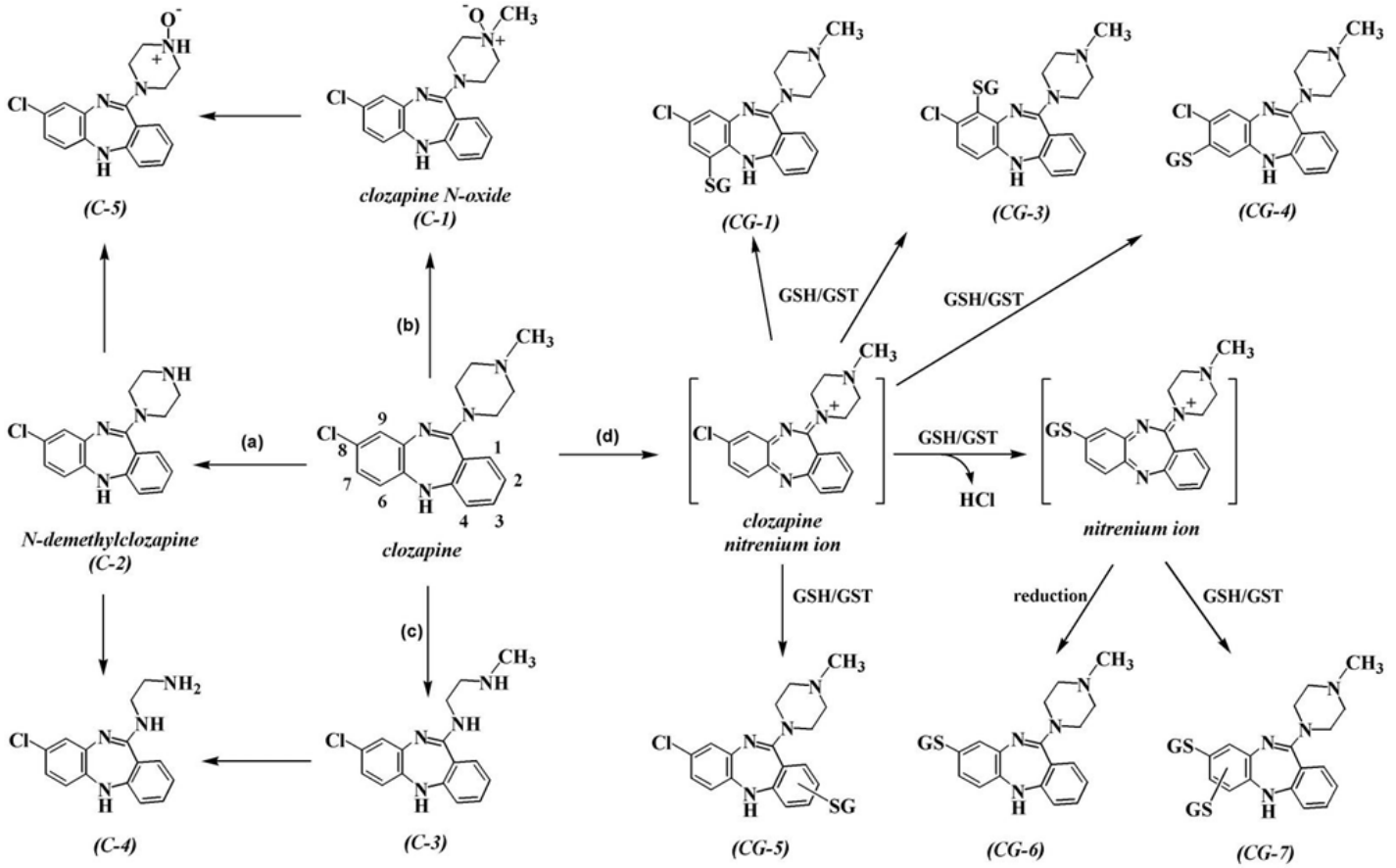

Figure 1. Oxidative pathways of metabolism of CLZ by cytochrome P450 and non-enzymatic and enzymatic conjugation reactions of reactive CLZ nitrenium ion by glutathione (GSH) and glutathione S-transferase (GST): a) N-demethylation; b) N-oxidation; c) oxidative opening of piperazine-ring; d) dehydrogenation to nitrenium ion.

Therefore, in the present study, we first evaluated the effect of mutation at position 87 on the regioselectivity of CLZ metabolism in order to identify the most suitable biocatalyst for the bioactivation of CLZ and subsequent structural characterization of the formed GSH conjugates. The results show that the nature of the residue at position 87 strongly influences regioselectivity of CLZ metabolism and that by using a more selective P450 BM3 M11-mutant all five human relevant GSH conjugates of CLZ could be produced in high levels, enabling structural elucidation by ^{1}H-NMR.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade and obtained from standard suppliers.

2.2. Library construction

Site-directed mutants of BM3 M11 at position 87 were constructed by mutagenic PCR using the Stratagene QuikChange XL site–directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using 20 complementary pairs of mutagenesis primers (*20*). The mutagenic PCR was applied to a pBluescript vector containing the gene of the drug metabolizing BM3 M11, flanked by EcoR1 and BamH1 restriction sites. BM3 M11 contains mutations R47L, E64G, F81I, F87V, E143G, L188Q, Y198C, E267V, H285Y and G415S when compared to wild-type BM3 (*9*). The sequence of the forward primer was as follows: 5'-GCA GGA GAC GGG TTA **XXX** ACT AGT TGG ACG CAT-3'. The **XXX** represents the codon that was used to introduce the specific mutation at position 87. The reverse primer for the mutagenic PCR was a 34-mer 5'-CAT GCG TCC AAC TAG T**YY Y**TA ACC CGT CTC CTG C-3' in which the **YYY** is the reverse complement of codon **XXX**. The underlined bases indicate a new SpeI digestion site. The following codons (**XXX**) were used: Ala, GCC; Arg, CGG; Asn, AAC; Asp, GAC: Cys, UGC; Gln, CAG; Glu, GAG; Gly, GGG; His, CAC; Ile, AUC; Leu, CUG, Lys, AAG; Met, AUG; Phe, UUC; Pro, CCC, Ser, UCC; Thr, ACC; Trp, UGG; Tyr, UAC, and Val, GUG. After mutagenic PCR, the plasmids were digested with EcoR1 and BamH1 restriction enzymes and the genes of mutated BM3 M11 were cloned into a pET28a+ vector, which encodes for a N-terminal His-tag. The desired mutations in the P450 domain were confirmed by DNA sequencing (Baseclear, Leiden, The Netherlands).

2.3. Expression, isolation and purification of enzymes

Expression of the P450 BM3 M11 mutants was performed by transforming competent *E.coli* BL21 cells with the pET28+-vectors, as described previously (*20*). Proteins were purified using Ni-NTA agarose, after which P450 concentrations were determined using carbon monoxide (CO) difference spectrum assay. Purity of the enzymes was checked by SDS-PAGE electrophoresis on 12% gel and Coomassie-staining. Protein purity was higher than 98% in all samples obtained.

Human GST P1-1 was prepared and purified as described previously (*10*). Protein concentration was determined according to the method of Bradford (*21*) with reagent obtained from Bio-Rad. The specific activity of the purified GST was assayed

according to Habig et al. (*22*). The specific activity of the purified recombinant human GST P1-1 using CDNB as a substrate was 27.9 μmol/min/mg protein.

2.4. Metabolism of CLZ by BM3 M11-mutants in presence of human GST P1-1

Incubations using BM3 M11 mutants as bioactivation system were performed at a final enzyme concentration of 250 nM, as described previously (*9*). All incubations were performed in 100 mM potassium phosphate buffer (pH 7.4) and at a final volume of 250 μL. The substrate CLZ was incubated at a concentration of 500 μM. GST P1-1 (8 μM) and GSH (100 μM) were added to the incubations in order to trap reactive CLZ nitrenium ion. Reactions were initiated by the addition of a NADPH regenerating system (0.2 mM NADPH, 1 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, final concentrations) and performed for 30 min at room temperature. In this time period, product formation was linear, as described previously (*9*). Reactions were terminated by addition of 25 μL of 10% $HClO_4$, and centrifuged for 15 min at 14000 rpm. The supernatants were analyzed by reversed-phase liquid chromatography using a Luna 5 μm C18 column (150 mm x 4.6 mm i.d.; Phenomenex, Torrance, CA, USA) as stationary phase, protected by a 4.0 mm × 3.0 mm i.d. security guard (5 μm) C18 guard column (Phenomenex). The gradient used was constructed by mixing the following mobile phases: eluent A (0.8 % acetonitrile, 99 % water, and 0.2 % formic acid) and eluent B (99 % acetonitrile, 0.8 % water, and 0.2 % formic acid). The first 5 min were isocratic at 0 % eluent B; from 5 to 30 min the percentage of eluent B linearly increases to 100 %; from 30 to 35 min linear decrease to 0 % B and maintained at 0 % for re-equilibration until 40 min. The flow rate was 0.5 mL/min.

Samples were analyzed using LC-MS/MS for identification and using UV/vis detection at 254 nm for quantification. The Shimadzu Class VP 4.3 software package (Shimadzu, Kyoto, Japan) was used for determination of peak areas in the UV chromatograms. A standard curve of CLZ was used to estimate the concentrations of the metabolites, assuming that the extinction coefficients of the metabolites at 254 nm are equal to that of CLZ. UV/vis spectra of clozapine and its metabolites, as determined online by diode array detection (180-400 nm), all showed similar spectra with maxima at 240, 260 and 295 nm [data not shown]. The standard curve of CLZ was linear between 1 and 100 μM; the limit of quantitative detection by UV/vis was estimated to be 0.1 μM [data not shown].

For identification of the metabolites, an Agilent 1200 series rapid resolution LC system was connected to a hybrid quadrupole-time-of-flight (Q-TOF) Agilent 6520 mass spectrometer (Agilent Technologies, Waldbronn, Germany), equipped with an electrospray ionization (ESI) source and operating in the positive mode. The MS ion source parameters were set with a capillary voltage at 3500 V; nitrogen was used as the desolvation and nebulizing gas at a constant gas temperature of 350°C, drying gas 8 L/min and nebulizer 40 psig. Nitrogen was used as a collision gas with collision energy of 25V. MS spectra were acquired in full scan analysis over an m/z range of 50 to 1000 using a scan rate of 1.003 spectra/s. The MassHunter Workstation Software (version B.02.00, Agilent Technologies) was used for system operation and data collection. Data analysis was performed using Agilent MassHunter Qualitative analysis software.

2.5. Preparative scale biotransformation

The CLZ GSH conjugates produced on a preparative scale by large scale incubation with the most selective BM3 M11 mutant as biocatalyst. A 5 mL reaction volume containing purified enzyme (1 μM), CLZ (500 μM), GSH (100 μM), GST P1-1 (8 μM), and an NADPH regenerating system (0.2 mM NADPH, 1 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase) was prepared in potassium phosphate buffer (100 mM, pH 7.4). The reaction was allowed to continue for 6 h at 25°C. To achieve maximal conversion of CLZ, the incubation was supplemented every hour with 40 μL of 120 μM M11 Phe87, 20 μL of 25 mM GSH, 500 μL of the NADPH regenerating system and 100 μL of 200 μM GST P1-1. The final incubation volume was 8.4 mL. The reaction was terminated by adding 0.84 mL of 10 % $HClO_4$ and centrifuged for 15 min at 14000 rpm. The supernatant was applied to a Strata X C-18 solid-phase extraction column (200mg/3mL, Phenomenex). The column was washed with 5 ml of H_2O to remove salts and proteins. CLZ and its metabolites were subsequently eluted using 2 mL of methanol. The sample was evaporated to dryness and reconstituted in 2 mL of eluent A (0.8% acetonitrile, 99% water, and 0.2% formic acid). The sample was applied by manual injection on a preparative chromatography column Luna 5 μm C18(2) column (250 mm x 100 mm i.d.) from Phenomenex, which was previously equilibrated with 100% eluent A. A flow rate of 2 mL/min and a gradient using eluent A (0.8% acetonitrile, 99% water, and 0.2% formic acid) and B (99% acetonitrile, 1% water, and 0.2% formic acid) was applied for separation of formed CLZ metabolites. The first 10 min were isocratic at 0% eluent B; from 10 to 65 min, the percentage of eluent B increased linearly to 65%; from 65 to 70 min further increase of eluent B to 100%; from 70 to 80 min, there was a linear decrease to 0% B, and re-equilibration was maintained until 120 min. Metabolites were detected using UV detection (254 nm) and collected manually. Collected fractions were first analyzed for purity and identity by the analytical HPLC and LC-MS/MS methods as described above. The samples were evaporated to dryness under nitrogen stream and dissolved in 1 mL deuterium oxide to exchange acidic hydrogen atoms by deuterium atoms. Samples were evaporated to dryness in the vacuum concentrator, the residues were redissolved in 500 μL of methyl alcohol-d_4 and ^{1}H-NMR spectra were recorded at room temperature. ^{1}H-NMR-analysis was performed on Bruker Avance 500 (Milan, Italy), equipped with cryoprobe. ^{1}H-NMR measurements were carried out at 500.23 MHz.

3. Results

3.1. Expression of BM3 M11 mutants

A saturation mutagenesis library with a different residue at position 87 of BM3 M11 was recently created in our laboratory *(20)*. All 20 mutants were expressed in E.coli BL21 with pET28+-vectors; the P450 quantification was done by CO difference spectrum. For the mutants Pro87, Asp87 and Ser87, the reduced CO difference spectra only showed a peak at 420 nm, suggesting that these amino acids negatively affect the folding and/or stability of BM3 M11. The mutant containing Asn87 showed a significant peak at 420 nm with intensity of almost equal to that at 450 nm. Mutants containing Met87, His87 and Gly87 showed a small shoulder at 420 nm next to the peak at 450 nm. All other mutants only produced peaks with maxima ranging from 448 nm to 450 nm *(20)*.

3.2. Activity and regioselectivity of metabolism of CLZ by BM3 M11-mutants

When CLZ was incubated at analytical scale with the 20 different BM3 M11 mutants in presence of GSH and hGST P1-1, 13 different metabolites were found in total (Table 1), which is consistent with our previous studies (*9, 10*). Five of the metabolites result from N-oxidation (C-1), N-demethylation (C-2) and piperazine ring cleavage (C-3), and combinations of these (C-4 and C-5), Figure 1. In total, eight different GSH conjugates were found resulting from bioactivation of CLZ to reactive intermediates. Five of these GSH conjugates result from addition reactions of the CLZ nitrenium ion with GSH (CG-1, CG-3, CG-4, CG-5) and chlorine-substitution (CG-6). The LC-MS/MS spectra of these five GSH-conjugates are tabulated in Table 2; the assignment of the fragments have been described elsewhere (*9, 10, 17*). GSH conjugates designated CG-2 and CG-8 were found to be secondary GSH conjugates, resulting from bioactivation of N-demethylclozapine to its corresponding nitrenium ion and subsequent addition (CG-2) and chlorine-substitution (CG-8). CG-7 corresponds to a di-GSH conjugate that most likely results from GSH conjugation to the GSH containing nitrenium ion formed after chlorine substitution of the CLZ nitrenium ion, Figure 1.

As shown in Table 1, the nature of amino acid residue at position 87 has strong influence on both the activity and regioselectivity of formation of CLZ metabolites. As indicated in the last column, the highest activity was generally observed with mutants containing apolar amino acids at position 87. The mutants containing Ala87, Val87 and Ile87 showed the highest activity, followed by Phe87 and Trp87. Mutants containing Leu87, Met87, Gly87 and Pro87 showed only very low activity (<6% conversion). Among the mutants containing polar uncharged residues, Tyr87 and Gln87 were the most active, showing 25.7% and 9.4% conversion, respectively. The mutants containing negative charged residue Asp87 and Glu87 had low activity with 7.4% and 2.4% conversion, respectively. For the mutant Asp87, no total activity could be calculated, as the P450 concentration could not be measured. The mutants containing positive charged residue His87, Arg87 were poorly active in the metabolism of CLZ (<2% conversion). The mutants having Lys87, Ser87, and Thr87 at position 87 did not show any activity.

Figure 2 shows the effect of amino acid residue 87 on the relative amounts of the stable metabolites formed via pathways a, b and c (Figure 1) and the relative amounts of GSH conjugates resulting from bioactivation of CLZ (pathway d, Figure 1).

For all the mutants the major stable metabolite was N-demethylclozapine (C-2) followed by CLZ N-oxide (C-1), Figure 2A. Significant differences were observed in the ratios of C-2 to C-1. In case of mutants containing Ile87, Leu87, Met87 and Glu87, N-demethylation was up to 10-fold more abundant than N-oxidation. In contrast, with mutants containing Phe87, His87, Asp87, Gly87 and Gln87 the ratio of N-demethylation to N-oxidation ranged from 1.2 to 2. The other stable metabolites (C-3, C-4 and C-5) represented only minor metabolites for all the mutants.

As illustrated in Figure 2B, the metabolic profile of the formed GSH adducts appears to be relatively constant for all the active mutants, supporting the hypothesis that all GSH conjugates originate from the CLZ nitrenium ion (*10*). In all cases, GSH conjugate CG-6 is the major metabolite and accounts for on average 41 ± 5 % of the total GSH conjugates. Considering the fact that conjugate CG-7 most likely also originates from the same intermediate nitrenium ion (Figure 1), the chlorine substitution pathway represents 47 ± 4% of the total GSH conjugation in presence of hGST P1-1. GSH conjugate

CG-5, which was tentatively assigned to the conjugate in which the GSH moiety is attached to the non-chlorinated aromatic ring, represents on average 25 ± 3% of the GSH conjugates. Conjugate CG-1, which is the major conjugate formed in non-enzymatic GSH-conjugation, represents on average to 22 ± 2% of the GSH conjugates. GSH conjugates CG-3 and CG-4, as shown in Figure 1, represented less than 2% of the total of GSH conjugates. GSH conjugate CG-8 that most likely results from the chlorine substitution of the nitrenium ion of N-demethylclozapine represents 3.5 ± 2.3% of the total GSH conjugates.

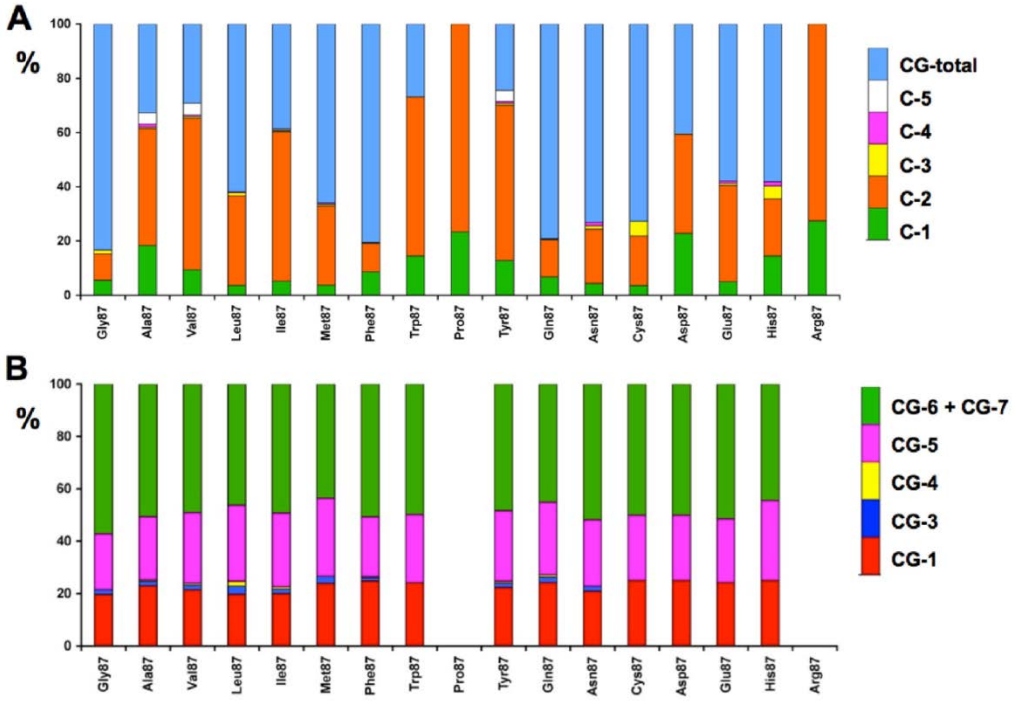

Figure 2. Relative amounts of CLZ metabolites formed by CYP102A1 M11H mutants with different amino acid residues at position 87. Incubations were carried out for 30 minutes in presence of 100 µM GSH and 8 µM recombinant human GST P1-1. (A) relative amounts of stable CLZ-metabolites (C-1 to C-5) and total of GSH-conjugates (CG-total); (B) relative amounts of individual GSH-conjugates.

The secondary GSH conjugates, CG-2 (MH^+ ion at m/z 618.23) and CG-8 (MH^+ ion at m/z 584.25) derived from N-demethylclozapine, and CG-7 (MH^+ ion at m/z 903.35) derived from CG-6 (MH^+ ion at m/z 598.27), have not been found in human studies and therefore were not further characterized.

To select the most appropriate BM3 M11 mutant for large scale production of GSH conjugates, it was investigated which mutant showed a combination of high overall activity and high selectivity towards the bioactivation pathway (route d in Figure 1). Figure 3 shows the ratio of total of GSH conjugates to stable metabolites for each active mutant, ranked from low to high ratio.

Table 1. Effect of aminoacid at position 87 in CYP102A1 M11H on the GSH-conjugation to reactive metabolites formed by oxidative bioactivation of clozapine.

m/z (MH^+)	C-1 343	C-2 313	C-3 301	C-4 287	C-5 329	CG-1 632	CG-2 618	CG-3 632	CG-4 632	CG-5 632	CG-6 598	CG-7 903	CG-8 584	Specific activity*)	% conv ersion
Apolar side chain															
Gly87	0.4	0.7	0.1	0.0	0.0	1.1	0.0	0.1	0.0	1.2	2.7	0.5	0.4	1.2	1.4
Ala87	35.1	82.5	1.1	2.0	7.8	13.7	0.7	1.0	0.3	14.4	26.1	4.0	2.5	31.9	38.2
Val87	15.1	90.1	0.9	1.0	7.1	9.3	0.7	0.8	0.3	11.7	15.8	5.5	2.9	26.9	32.3
Leu87	1.0	9.0	0.3	0.1	0.0	3.2	0.1	0.5	0.3	4.7	6.3	1.2	0.6	4.6	5.5
Ile87	6.8	71.4	0.5	0.3	0.6	9.6	0.5	0.8	0.4	0.0	20.7	2.9	1.6	21.6	25.9
Phe87	8.7	10.5	0.2	0.2	0.0	18.2	0.2	0.9	0.3	16.7	37.2	5.3	2.2	16.8	20.1
Trp87	11.6	46.9	0.0	0.0	0.0	5.0	0.0	0.0	0.0	5.4	7.6	2.7	0.8	13.3	16.0
Met87	0.7	5.5	0.1	0.1	0.0	2.8	0.0	0.3	0.0	3.5	5.1	0.0	0.7	3.2	3.8
Pro87	1.4	4.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	n.d. **)	1.2
Polar uncharged side chain															
Tyr87	16.5	73.4	0.9	0.9	5.2	6.7	0.3	0.5	0.2	8.1	11.5	3.0	1.1	21.4	25.7
Gln87	3.2	6.4	0.1	0.1	0.0	8.5	0.1	0.7	0.3	9.7	13.9	1.9	2.1	7.9	9.4
Asn87	0.7	3.2	0.2	0.2	0.0	2.3	0.0	0.2	0.0	2.8	4.7	1.0	0.7	2.6	3.2
Cys87	0.2	1.0	0.3	0.0	0.0	1.0	0.0	0.0	0.0	1.0	2.0	0.0	0.0	0.9	1.1
Ser87	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Thr87	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Polar charged side chain															
Asp87	8.5	13.6	0.0	0.0	0.0	3.7	0.0	0.0	0.0	3.7	7.1	0.3	0.3	n.d. **)	7.4
Glu87	0.6	4.3	0.1	0.1	0.0	1.7	0.0	0.0	0.0	1.7	3.1	0.5	0.0	2.0	2.4
His87	0.9	1.3	0.3	0.1	0.0	0.9	0.0	0.0	0.0	1.1	1.6	0.0	0.0	1.0	1.2
Arg87	1.7	4.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.2

Concentration of metabolites (μM) formed after 30 minutes incubations of 200 nM CYP102A1 with 500 μM clozapine, 8 μM GST P1-1 in presence of 100 μM GSH.
Values represent averages of three individual experiments; standard errors were always lower than 10%.
*(nmol product/nmol CYP102A1/30min),;
** P450 concentration could not be quantified (CO difference spectrum only showed a peak at 420 nm).

As shown in Figure 3, for the four most active mutants, having active site amino acids Ala87, Val87, Tyr87 and Ile87, less than 35% of the total of metabolites represented GSH conjugates. For mutants containing Gln87, Phe87 and Gly87, however, approximately 80% of the metabolites found were GSH conjugates, indicating that for these mutants bioactivation to CLZ nitrenium ion (route d, Figure 1) is the major pathway of metabolism. Because the mutant containing Phe87 has the highest activity, this mutant was selected for large scale production of GSH conjugates for structural elucidation by ^{1}H-NMR.

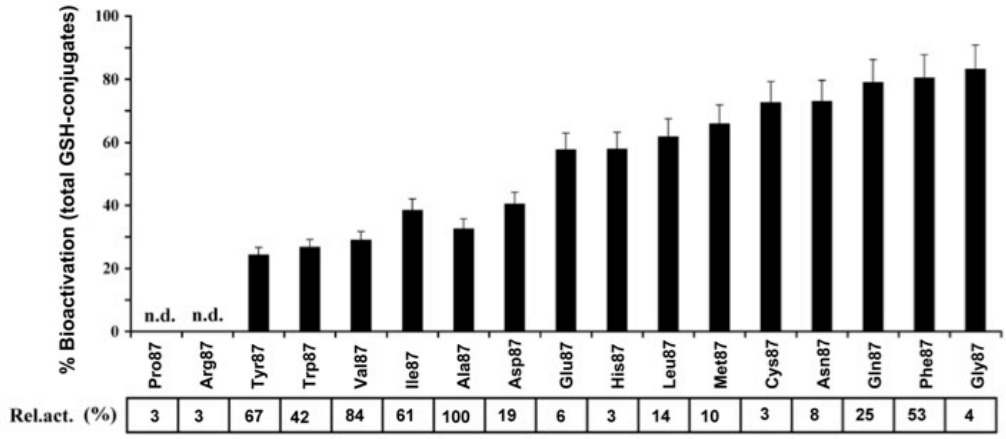

Figure 3. Percentage of total of GSH-conjugates formed in incubations of CLZ with BM3 M11 mutants with different amino acid residues at position 87, ranked in order of activity. *Numbers below each bar* represent the activity of the BM3 M11 mutants relative to the most active mutant BM3 M11 Ala87.

3.3. Effect of human GST A1-1

Figure 4 shows the preparative HPLC chromatogram, with UV-detection at 254 nm, obtained after large scale incubation of CLZ with mutant Phe87.

After isolation of the individual metabolites by preparative HPLC, their purity and identity was first analyzed by analytical HPLC and LC/MS/MS method, resulting in the assignment of metabolites and parent compound as presented in Figure 5. By hourly additions of enzymes and cofactors, over 90% of CLZ was converted, according to the strong decrease in parent compound. Based on the peak areas approximately 98% of the metabolites found were GSH conjugates. This higher percentage of GSH conjugation, compared to the analytical scale incubations, can be explained by further bioactivation of the stable metabolite C-2, producing CG-8 and CG-2. The low yield of CLZ N-oxide (C-1) might be explained by non-enzymatic reduction of the N-oxide by NADPH and GSH that was added hourly to the incubation (*18*).

For the five primary GSH conjugates of CLZ, having MH^+ ion at m/z 632.23 (CG-1, CG-3, CG-4 and CG-5) and MH^+ ion at m/z 598.25 (CG-6), ^{1}H NMR spectra were recorded

to identify the position of GSH conjugation. Figure 5 shows the signals of the aromatic hydrogen atoms of the CLZ-moiety of these GSH conjugates. The correlation spectroscopy (COSY) spectra of these signals, which were recorded to facilitate the assignment of the aromatic hydrogen atoms as shown in Table 2, are shown in Figure 6-8. The signals of the aliphatic hydrogen atoms were consistent with the glutathionyl moiety (data not shown).

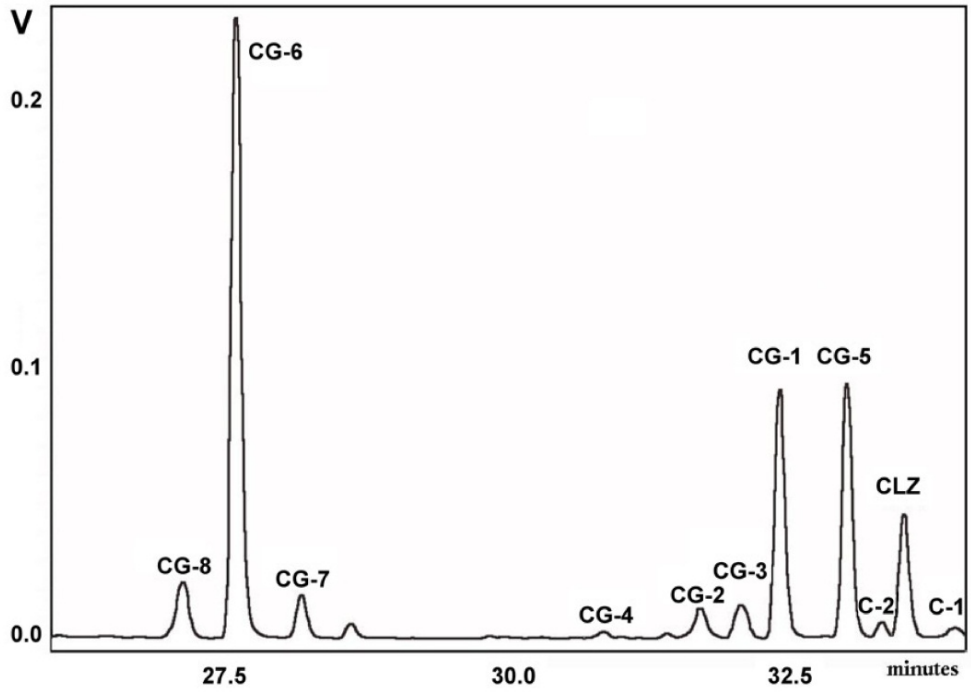

Figure 4. Preparative HPLC-UV chromatogram of metabolites obtained by large scale incubation of CLZ with BM3 M11 Phe87 in presence of hGST P1-1 and GSH.

Figure 5A shows the ^{1}H-NMR spectrum of conjugate CG-1, which is the major GSH conjugate formed in absence of glutathione S-transferases (*9, 10*). This conjugate, which eluted after 32.5 min in the preparative HPLC (Figure 4), was previously identified as C-6 glutathionyl clozapine. The spectrum shown in Figure 5A is in full agreement with the ^{1}H-NMR spectra of C-6 glutathionyl clozapine which was identified previously as the major GSH conjugate formed by peroxidases and electrochemical oxidation of CLZ (*15, 16, 19*). Two doublets at 6.96 and 7.23 ppm correspond to the protons at positions 9 and 7, with a small coupling constant of 2.5 Hz due to proton in the *meta*-position. Fischer et al. previously assigned doublet at 6.96 ppm to H_7 and the signal at 7.23 ppm to H_9 (*15*). Madsen et al., however, assigned doublet at 6.96 ppm to H_9 and the signal at 7.23 ppm to H_7 (*19*). Which signal corresponds to which proton could not be determined unequivocally, only based on chemical shift and coupling pattern (*16*). However, this does not affect the identification of the position of GSH conjugation because each theoretically possible GSH conjugate is expected to have its own unique combination of multiplicity

and coupling pattern. Therefore, for signals that could not be assigned unequivocally to specific aromatic protons, two possibilities are shown in Table 2. The assignments before the slashes correspond to the first possibility, whereas the assignments after the slashes correspond to the second possibility, according to the COSY spectra (Figure 6).

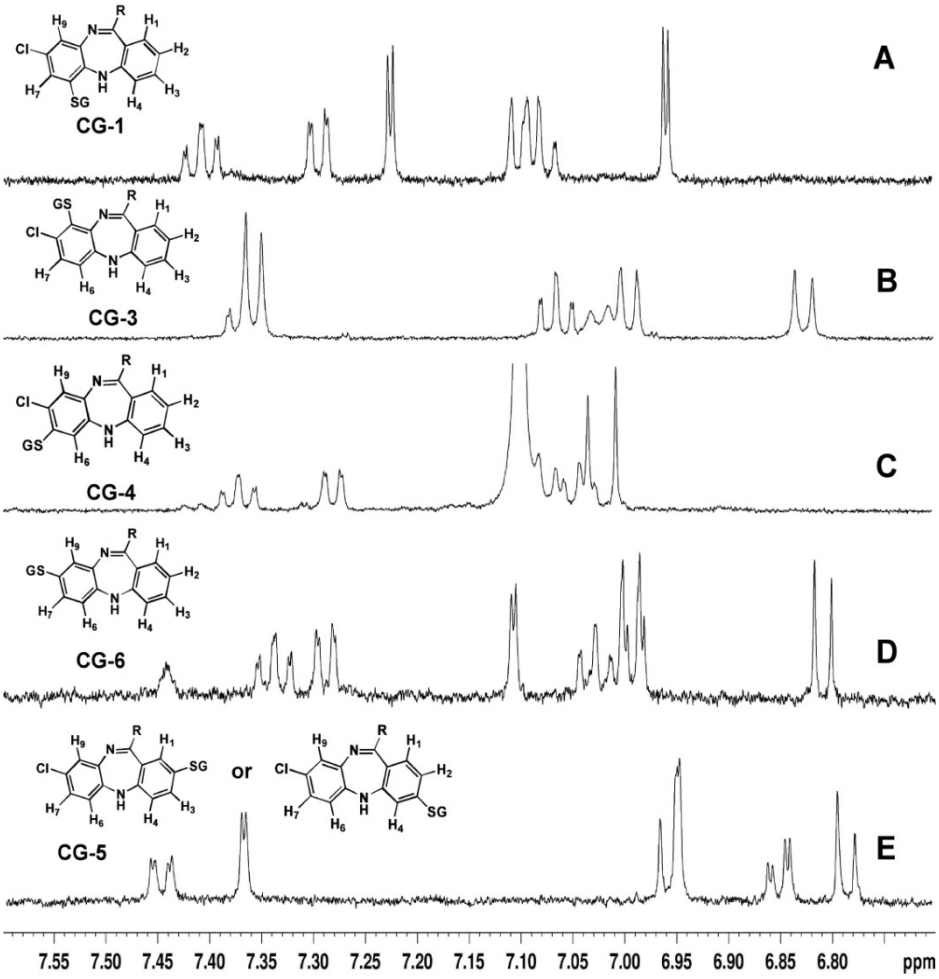

Figure 5. Aromatic regions of the ^{1}H-NMR spectra of clozapine GSH-conjugates. The conjugates were obtained by purification by preparative HPLC of metabolites formed by BM3 M11 Phe87, GSH and hGSTP1-1. (A) CG-1, C-6 glutathionyl CLZ; (B) CG-3, C-9 glutathionyl CLZ; (C) CG-4, C-7 glutathionyl CLZ; (D) CG-6, C-8 glutathionyl deschloroclozapine; (E) CG-5, C-2 glutathionyl CLZ or C-3 glutathionyl CLZ.

Table 2. LC-MS/MS characteristics and ^{1}H NMR spectra of the aromatic hydrogen atoms of the GSH conjugates of clozapine

		1H-NMR-spectra		LC-MS/MS-mass spectra
Conjugate	Assignment [a]	δ ppm (intensity)	multiplicity: coupling constants	m/z (intensity) [b]
CG-1	H_1/H_4	7.30 (1H)	d of d: $^{2}J_{HH}$, 7 Hz; $^{3}J_{HH}$, 1.5 Hz	623.23 (MH^+; 100%);
	H_2/H_3	7.08 (1H)	d of t: $^{2}J_{HH}$ 7.5 Hz, $^{3}J_{HH}$ 1.5 Hz	614.22 (8%); 575.17 (6%);
	H_3/H_2	7.41 (1H)	d of t: $^{2}J_{HH}$ 7.5 Hz, $^{3}J_{HH}$ 1.5 Hz	503.18 (37%); 446.12 (1%);
	H_4/H_1	7.10 (1H)	d: $^{2}J_{HH}$ 7.5 Hz	359.12 (29 %); 302.06 (12%)
	H_7/H_9	7.23 (1H)	d: $^{2}J_{HH}$ 2.5 Hz	
	H_9/H_7	6.96 (1H)	d: $^{2}J_{HH}$ 2.5 Hz	
CG-3	H_1/H_4	7.36 (1H)	d: $^{2}J_{HH}$ 8 Hz	623.23 (MH^+; 100%);
	H_2/H_3	7.07 (1H)	d of t: $^{2}J_{HH}$ 7 Hz, $^{3}J_{HH}$ 1.5 Hz	614.22 (1%); 503.18 (23%);
	H_3/H_2	7.37 (1H)	t: $^{2}J_{HH}$ 7 Hz	446.12 (1%); 359.12 (41%);
	H_4/H_1	6.99 (1H)	d: $^{2}J_{HH}$ 8 Hz	302.06 (6%)
	H_6	7.02 (1H)	d: $^{2}J_{HH}$ 8 Hz	
	H_7	6.83 (1H)	d: $^{2}J_{HH}$ 8 Hz	
CG-4	H_1/H_4	7.28 (1H)	d of d: $^{2}J_{HH}$ 8 Hz, $^{3}J_{HH}$ 1.5 Hz	623.23 (MH^+; 100%);
	H_2/H_3	7.04 (1H)	t: $^{2}J_{HH}$ 7 Hz	614.22 (8%); 575.17 (3%)
	H_3/H_2	7.37 (1H)	d of t: $^{2}J_{HH}$ 7 Hz, $^{3}J_{HH}$ 1.5 Hz	503.18 (44%); 446.12 (4%);
	H_4/H_1	7.07 (1H)	d: $^{2}J_{HH}$ 8 Hz	359.12 (17 %); 302.06 (4%);
	H_6/H_9	7.01 (1H)	s	243.06 (5%)
	H_9/H_6	7.03 (1H)	s	
CG-5	H_1/H_4	6.95 (1H)	d: $^{3}J_{HH}$ 1 Hz	623.23 (MH^+; 100%);
	H_3 or H_2	6.85 (1H)	d of d: $^{2}J_{HH}$ 8.5 Hz, $^{3}J_{HH}$ 2 Hz	614.22 (6%); 575.17 (10%);
	H_4/H_1	6.78 (1H)	d: $^{2}J_{HH}$ 9 Hz	503.18 (27%); 446.12 (25%);
	H_6	6.96 (1H)	d: $^{2}J_{HH}$ 9 Hz	359.12 (15 %); 302.06 (13%);
	H_7	7.45 (1H)	d of d: $^{2}J_{HH}$ 9 Hz, $^{3}J_{HH}$ 2 Hz	243.06 (34%)
	H_9	7.37 (1H)	d: $^{2}J_{HH}$ 2 Hz	
CG-6	H_1/H_4	7.00 (1H)	d of d: $^{2}J_{HH}$ 8 Hz, $^{3}J_{HH}$ 2 Hz	598.25 (MH^+; 100%);
	H_2/H_3	7.35 (1H)	d of t: $^{2}J_{HH}$ 7.5 Hz, $^{3}J_{HH}$ 1.5 Hz	580.24 (8%); 469.20 (77%);
	H_3/H_2	7.04 (1H)	d of t: $^{2}J_{HH}$ 7.5 Hz, $^{2}J_{HH}$ 1 Hz	412.20 (4%); 325.15 (24%);
	H_4/H_1	7.30 (1H)	d of d: $^{2}J_{HH}$ 7.5 Hz, $^{3}J_{HH}$ 1 Hz	268.09 (6%)
	H_6	6.82 (1H)	d: $^{2}J_{HH}$ 8 Hz	
	H_7	7.01 (1H)	d: $^{2}J_{HH}$ 8 Hz	
	H_9	7.12 (1H)	d: $^{3}J_{HH}$ 2 Hz	

a) Absolute assignment of protons was not possible, therefore each signal is indicated by two assignments corresponding to two possible solutions. Assignments before the slash
correspond to solution 1; assignments after the slash correspond to solution 2.
b) Electrospray spectra (LC-MS/MS) were acquired using nitrogen as collision gas with collision energy of 25V.

Figure 5B shows the ^{1}H-NMR spectrum of purified CG-3, which eluted at 32.2 min with preparative HPLC. This conjugate was previously found to be a minor GSH conjugate, with MH^+ ion at m/z of 632.2, in incubations of CLZ with liver microsomes and BM3 M11 when incubated in absence of glutathione S-transferases (*9, 10*). The spectrum shown in the Figure 5B can only be explained by conjugation of GSH at the C-9 position of clozapine. Two signals at 6.83 and 7.02 ppm showed only a coupling of 8 Hz and are therefore assigned to the neighboring H_6 and H_7 protons. The COSY spectrum of conjugate CG-3 (Figure 7A) was identical to that of C-9 glutathionyl clozapine as published previously *(16)*. The corresponding chemical shifts and coupling constants for the observed signals are given in Table 2.

Two more conjugates having a MH^+ ion at m/z 632.2 eluted at retention times 30.9 min and 33.1 min (Figure 4) and appeared to correspond to conjugates CG-4 and CG-5 (*10*). On the basis of the order of elution and small differences in fragmentation patterns in LC-MS/MS (*17*), the structure of these GSH conjugates were previously tentatively assigned to C-7 glutathionyl clozapine (CG-4) and a conjugate with GSH bound to the non-chlorinated ring (CG-5). However, so far no ^{1}H NMR spectra have been reported confirming the exact positions. Spectra for this two conjugates are shown in the Figure 5C and 5E, respectively.

Figure 5E shows the ^{1}H-NMR-spectrum of the aromatic region of CG-5, which is found at high levels when GSH conjugation is catalyzed by GST P1-1 (Figure 4). This spectrum can only be explained by conjugation of GSH at position 2 or 3 of the non-chlorinated aromatic ring. In each conjugate in which the non-chlorinated aromatic ring is not substituted, two triplets are found corresponding the protons H_2 and H_3 that are both strongly coupled by two ortho-protons, Figure 5A-D. These typical triplets could not be found in the spectrum of CG-5 (Figure 5E), indicating that one of these protons is substituted. Furthermore, the signals at 6.96, 7.37 and 7.45 ppm could be attributed to protons H_6, H_9 and H_7 of the chlorinated ring, according to the COSY-spectrum (Figure 7B). This confirms that addition of the GSH is at the non-chlorinated aromatic ring. Although the loss of characteristic triplets show that GSH is conjugated to position 2 or 3, the ^{1}H-NMR and COSY-spectra could not differentiate between positions 2 or 3 for GSH binding. When GSH is bound at the 2 position, the signal at 6.85 ppm will correspond to proton H_3, because this signal has a *ortho*-coupling of 8.5 Hz due to H_4 and a weak coupling of 1.5 Hz due to the *meta* proton in position 1. The doublet with ortho-coupling of 8.5 Hz at 6.78 ppm corresponds to H_4, coupled by H_3, whereas the doublet with weak coupling of 1.5 Hz at 6.95 ppm would correspond to H_1 by meta-coupling by proton H_3. When GSH is bound at the 3-position, the signal at 6.85 ppm will correspond to proton H_2, because with *ortho*-coupling of 8.5 Hz due to H_1 and a weak coupling of 1.5 Hz due to the *meta* proton in position 4. The doublet with an ortho-coupling of 8.5 Hz at 6.78 ppm corresponds to H_1, coupled by H_2, whereas the doublet with a weak coupling of 1.5 Hz at 6.95 ppm corresponds to H_4 by meta-coupling by proton H_2.

Conjugate CG-4 is the fourth GSH conjugate with MH^+ ion at m/z 632.2, and was previously tentatively assigned to C-7 glutathionyl clozapine (*10*). However, theoretically this can also represent a conjugate with GSH bound to one of the other positions of the non-chlorinated aromatic ring. This GSH conjugate eluted at 30.9 min with preparative HPLC, Figure 4. Because this conjugate is produced at very low yield, several large scale incubations were performed to obtain enough material to record a ^{1}H-NMR-spectrum with sufficient signal-to-noise ratio. Although this small amount of the conjugate

appeared to be contaminated by an unknown compound, the COSY-spectrum allowed us to solve the spectrum, despite the strong contaminant signal at 7.10 ppm, (Figure 5C, Figure 8A). Based on these spectra this GSH conjugate is identified as C-7 glutathionyl clozapine, consistent with the previous proposals (*10, 17*). Firstly, two triplets at 7.04 and 7.37 could be assigned to the protons H_2 and H_3. Two sharp singlets at 7.01 and 7.03 ppm are attributed to isolated protons that do not have ortho- or meta-coupling. If GSH was bound to position 7, protons at position 6 and 9 would lose their ortho and meta couplings by H_7. Two signals centered at 7.28 and 7.07 ppm represent proton H_1 and H_4, which showed both *ortho*- and *meta*-coupling by H_2 and H_3, Table 2. Although the signal at 7.07 partially overlaps with the signal of the impurity, the COSY-spectrum confirmed that signal centered at 7.07 ppm is a doublet with *ortho*-coupling.

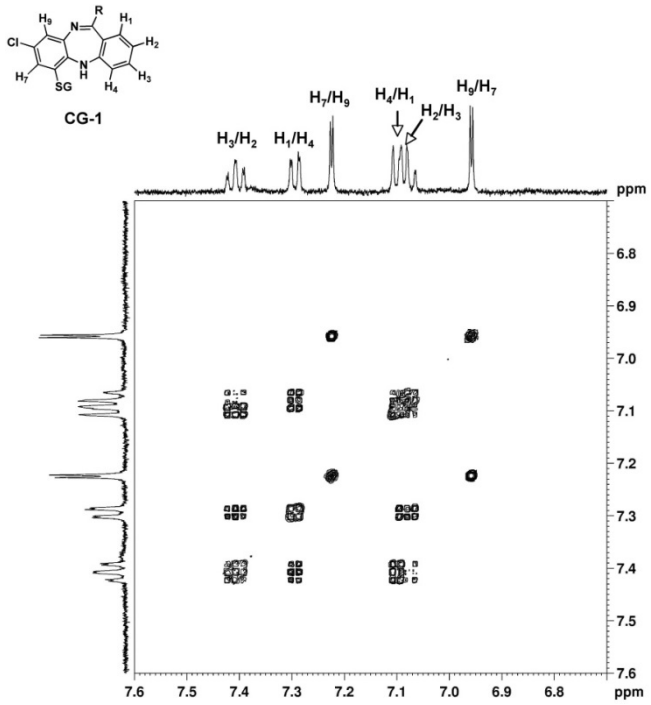

Figure 6. COSY NMR-Spectrum of the aromatic region of C-6 glutathionyl clozapine (CG-1). Absolute assignment of protons was not possible, therefore each signal is indicated by two assignments corresponding to the two possible solutions. Assignments before the slash correspond to solution 1; assignments after the slash correspond to solution 2.

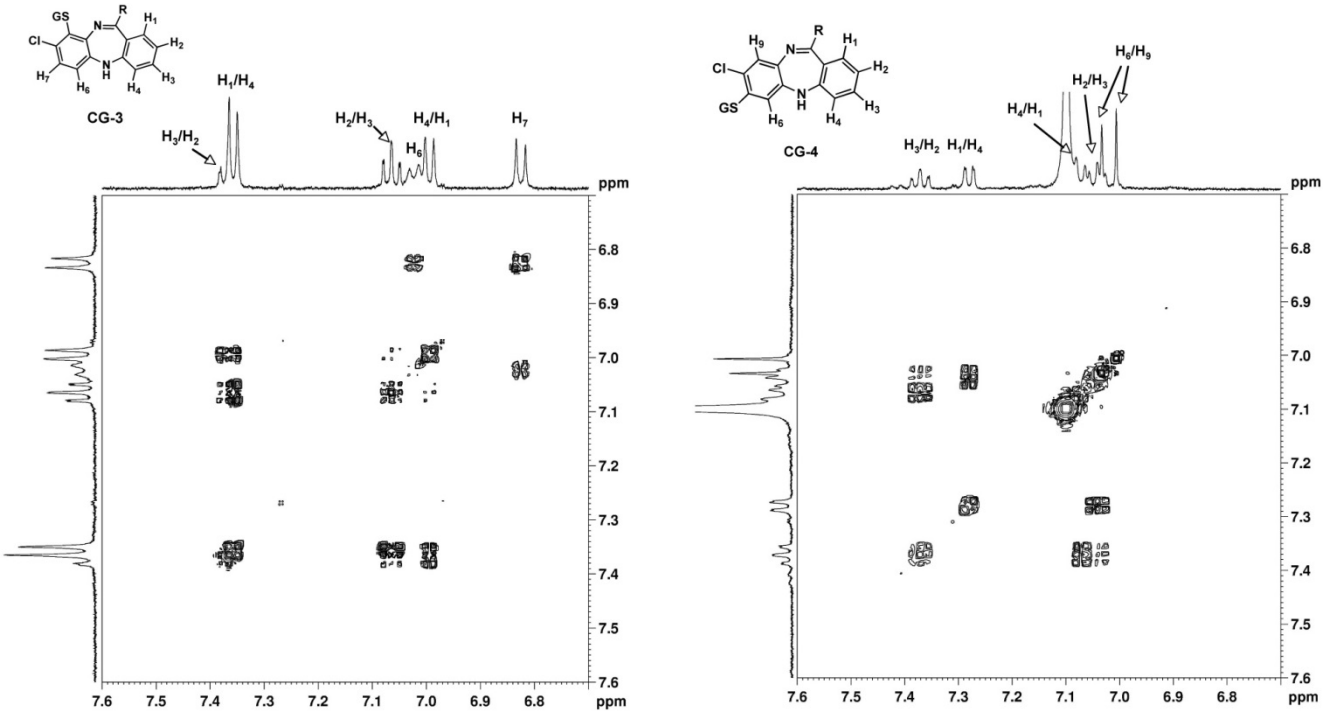

Figure 7. COSY NMR-spectrum of the aromatic region of: A) C-9 glutathionyl clozapine (CG-3). The COSY-spectrum is identical to that previously published by Liu and Utrecht (16); and B) C-7 glutathionyl clozapine (CG-4). The out-of-scale signal at 7.12 ppm results from an impurity. Absolute assignment of protons was not possible; therefore each signal is indicated by two assignments corresponding to the two possible solutions. Assignments before the slash correspond to solution 1; assignments after the slash correspond to solution 2.

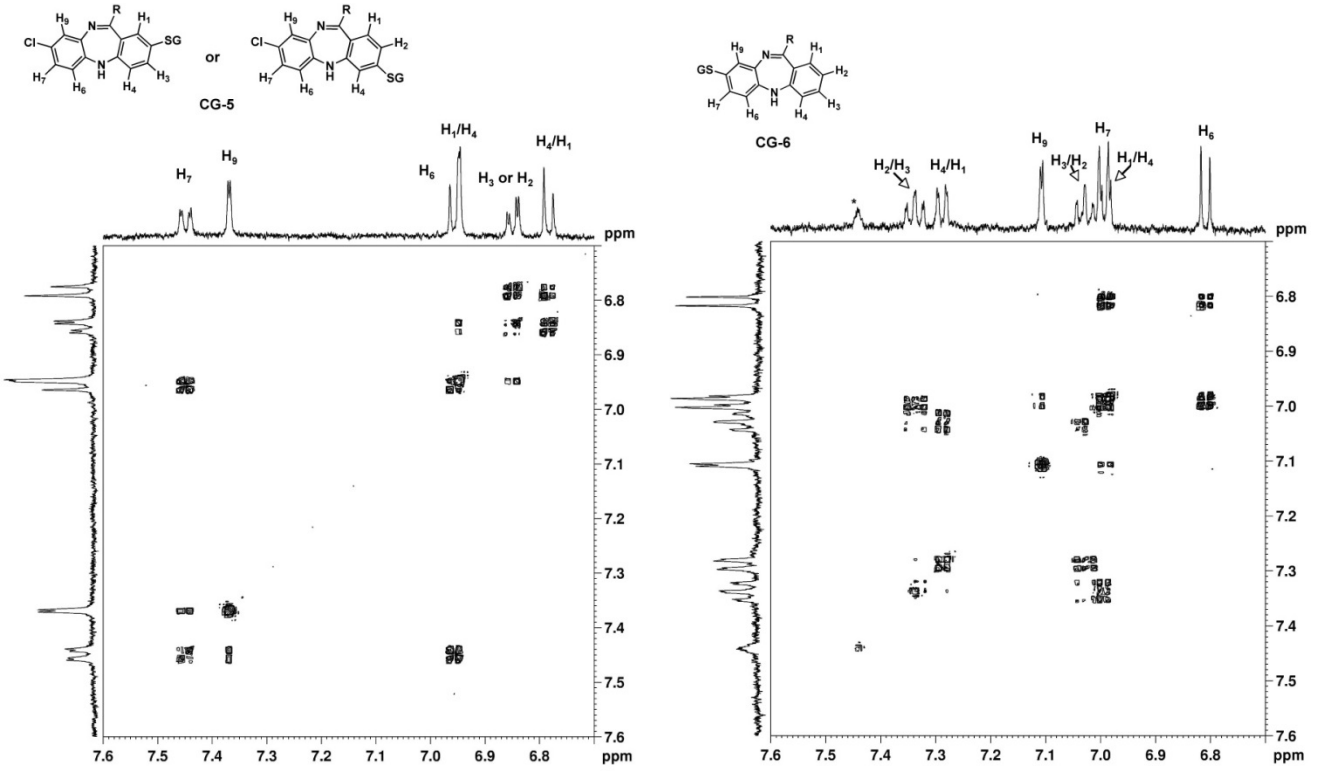

Figure 8. COSY NMR-Spectrum of the aromatic region of: A) C-2 or C-3 glutathionyl deschloroclozapine (CG-5); and B) C-8 glutathionyl deschloroclozapine (CG-6).
Absolute assignment of protons A) H1, H2, H3 and H4 and B) H1 and H4 was not possible, therefore each signal is indicated by two assignments corresponding to the two possible solutions. Assignments before the slash correspond to solution 1; assignments after the slash correspond to solution 2.

The fifth GSH-conjugate for which a ^{1}H-NMR spectrum is recorded is CG-6, which was the major GSH conjugate found in the incubations in presence of hGST P1-1, and which showed a MH^+ ion at m/z 598.25 by LC-MS/MS-analysis. In the preparative HPLC system used, this GSH conjugate eluted at 27.6 min, Figure 4. Figure 5D and Figure 8B show the ^{1}H-NMR and COSY spectra obtained. Two triplets centered at 7.04 and 7.35 ppm with small *meta*-couplings correspond to the protons H_3 and H_2 at the non-chlorinated aromatic ring. The signals centered at 7.00 and 7.30 ppm correspond to protons H_1 and H_4, as demonstrated by the combination of both *ortho* and *meta* coupling by protons H_2 and H_3. The signals at 6.82, 7.01 and 7.12 ppm correspond to protons H_6, H_7 and H_9 respectively, based on the coupling patterns and COSY-spectrum (Figure 8B). On the basis of this spectrum and mass spectrum it was confirmed that this conjugate correspond to C-8 glutathionyl deschloroclozapine.

4. Discussion

Currently, there is an increasing interest in developing novel methodologies to produce human relevant drug metabolites on a large scale in order to enable structural characterization of metabolites and test their pharmacological and toxicological properties. One of the approaches is to make use of genetically engineered cytochromes P450s that are developed for the catalysis of regio- and stereoselective hydroxylation of chemicals at high activity. In particular the bacterial cytochrome P450 BM3 from *Bacillus megaterium* has high potential as a biocatalyst for these purposes because this enzyme is the most active P450 discovered so far and because the substrate selectivity and metabolic profile can be manipulated by site-directed and/or random mutagenesis (*6, 7*). One of the BM3 mutants that show high activity in drug metabolism is BM3 M11, which contains 10 different amino acid substitutions compared to wild-type BM3. This BM3 mutant was shown to be highly active in metabolizing a variety of drugs to human relevant metabolites, including reactive intermediates (*5, 9, 10*). Recently, we have performed a saturation mutagenesis study in which the active-site residue at position 87 was mutated to all 20 possible amino acids (*20*). In BM3 M11 the residue at this position is Val87, which was introduced at an early stage of the mutagenesis process, to expand the substrate selectivity to drug metabolism (*23*). In the saturation mutagenesis study in which all amino acids were evaluated at position 87, we recently demonstrated that the type of amino acid at position 87 has strong effect on substrate selectivity when comparing a series of alkoxyresorufins (*20*). In this study it was also demonstrated that the nature of the amino acid at position 87, strongly influences the regioselectivity of testosterone hydroxylation of BM3 M11.

In the present study, the library of BM3 M11 mutants with different amino acids at position 87 was evaluated with CLZ as substrate. CLZ is a drug that can be metabolized by peroxidases and P450s to multiple metabolites, including reactive nitrenium ions that might be involved in adverse drug reactions associated with CLZ therapy. BM3 M11 has been shown to produce high levels of most human relevant metabolites of CLZ, which are represented in Figure 1. All metabolites can be explained by four different initial oxidative pathways: N-demethylation (a), N-oxidation (b), piperazine-ring opening (c) and dehydrogenation to a reactive nitrenium ion (d). Although BM3 M11 with residue Val87 produced significant amounts of reactive nitrenium ion (as identified as GSH conjugates), the major pathways of metabolism are N-demethylation and N-oxidation,

which explain approximately 70% of the total of metabolites. The aim of this study was to investigate whether residue 87 also controls the regioselectivity of CLZ metabolism, and to investigate whether a mutant could be identified with higher selectivity toward the bioactivation to the toxicologically relevant CLZ nitrenium ion. A more selective P450 BM3 mutant would be more useful for the generation of high levels of CLZ GSH-conjugates that still require structural confirmation by ^{1}H-NMR. So far, the structure of only two of the GSH-conjugates shown in Figure 1 has been elucidated by ^{1}H-NMR and mass spectrometry. However, the structures of the GSH-conjugates found in bile of rats and mice, and which also have MH^+ ions at m/z 632.2 (*17*) have not yet been characterized by ^{1}H-NMR.

As shown in Table 1, changing the amino acid residue at position 87 of BM3 M11 has strong effects on the total activity and regioselectivity of CLZ oxidation. The mutants Ala87, Val87, and Ile87 were found to be the most active, as was found previously with alkoxyresorufins and testosterone (*20*). These mutants have a small and apolar residue in position 87. This seems consistent with the previous hypothesis that replacement of the bulky Phe87 in wild type BM3 by smaller amino acids creates space for the bulky substrates that allows better positioning with respect to the activated oxygen species, resulting in higher activities and coupling efficiencies (*24-26*). However, in the present study relatively high activities were also found in the BM3 M11 mutants containing the relatively bulky amino acids Phe87, Tyr87 and Trp87. Previously, replacing Phe87 by Tyr87 in wild-type BM3 was found to be detrimental for activity towards long-chain fatty acids, probably by disruption of the hydrophobic interaction by the phenol-group. In case of BM3 M11 the presence of these bulky amino acids apparently is less restrictive for bulky substrates because in presence of Phe87 and Tyr87 both testosterone (*20*) and CLZ are metabolized at high activity. Apparently, by the combination of ten mutations present in BM3 M11 the topology of the active site and/or substrate access channel has changed significantly, explaining the much wider substrate selectivity compared to wild-type BM3.

As shown in Figure 2A, the nature of amino acid 87 has strong effect on regioselectivity of CLZ metabolism. When considering the stable metabolites that are formed via pathways a, b and c (Figure 1), the major metabolite with all mutants was N-demethylclozapine (C-2), although the N-oxide (C-1) was also produced at significant levels. However, the ratio of N-demethylation to N-oxidation appeared to be quite dependent on the nature of amino acid residue at position 87. For example, for the mutants containing Leu87 and Ile87, N-demethylation was almost 10-fold higher than N-oxidation. In the mutant containing Phe87, N-demethylclozapine and CLZ N-oxide were formed in almost the same amount. However, the relative contribution of N-oxidation in all incubations might be somewhat underestimated, because all incubations were performed in presence of GSH which is known to reduce CLZ N-oxide back to CLZ (*27*). Therefore, in case of Phe87 N-oxidation of CLZ might even be higher in absence of reductive agents. In case of the human P450s, it has been shown that CYP1A2 preferentially metabolizes CLZ by N-demethylation, whereas CYP3A4 is mainly responsible for production of CLZ N-oxide (*27*). However, the factors that determine the ratio of N-demethylation and N-oxidation are still unclear. Different presentation of the piperazine N-methyl group to the oxidative species at the active site might explain why some human P450s preferentially catalyze N-demethylation, whereas others predominantly catalyze N-oxidation.

One of the aims of the present study was to identify mutants with high activity and selectivity for bioactivation of CLZ to the reactive nitrenium ion. As shown in Figure 2A several mutants produced high levels of GSH conjugates (CG-total), indicative for relative high selectivity in the formation of the reactive nitrenium ion. Other mutants showed strong preference in catalyzing formation of N-demethylclozapine and CLZ N-oxide. However, from the results it is unclear what features of the amino acid side-chain determines selectivity for bioactivation. For example, the BM3 M11-mutant containing the bulky Phe87 showed high selectivity and activity in formation of GSH conjugates, whereas the mutants containing the bulky Tyr87 and Trp87 preferentially catalyzed formation of stable metabolites. Future detailed protein modeling studies including those evaluating protein dynamics and substrate mobility might help to rationalize the different regioselectivities observed. Figure 2B shows the relative amounts of the different GSH conjugates that are formed in incubations of CLZ with BM3 mutants in presence of recombinant hGST P1-1. Consistent with our previous study, the major pathway of GST P1-1 catalyzed GSH conjugation is substitution of the chlorine-atom of the CLZ nitrenium ion (*10*). The resulting GSH bound nitrenium ion is subsequently reduced by NADPH or GSH, to form CG-6, or further conjugated to GSH, to form CG-7 (Figure 1). The fact that with all mutants the same ratio of GSH conjugates is formed strongly suggests that all form from the same reactive intermediate.

Mutant Phe87 was selected for large scale biosynthesis of GSH conjugates because this mutant combined high activity with high preference for the bioactivation pathway, Figure 3. Previous studies, aiming at the characterization of GSH conjugates of CLZ, showed that non-enzymatic GSH conjugation to the CLZ nitrenium ion, formed by peroxidases or electrochemically, mainly produced a GSH conjugate bound at the C-6 position of CLZ and minor amounts of conjugate bound at the C-9 position. The structures of these two GSH conjugates have been elucidated by ^{1}H-NMR. However, *in vivo* studies with rats and mice have shown that in bile two major GSH conjugates are excreted that do not correspond to these two conjugates (*17*). Also, incubations with rat liver microsomes showed small amounts of a fifth GSH conjugate with MH^+ ion at m/z 632.2 (*17*). It was initially concluded that these GSH conjugates might originate from as yet unidentified reactive intermediate produced *in vivo*. However, we recently demonstrated that these alternative GSH conjugates probably are resulting from GST catalyzed inactivation of the CLZ nitrenium ion (*10*). By using mutant Phe87, we were able to produce significant amounts of all GSH conjugates, for which the structures were not yet elucidated unequivocally by ^{1}H-NMR. Because four GSH conjugates were found with MH^+ ion at m/z 632.2, it was previously concluded that for at least one of the conjugates, GSH is bound to the non-chlorinated aromatic ring of CLZ. The present study shows that conjugate designed CG-5, which is a major product in presence of hGST P1-1 has the GSH moiety bound to the non-chlorinated ring at the position 2 or 3 (Figure 5). For the minor conjugates CG-4, we were able to confirm binding at the 7 position, as it was tentatively assigned based on fragmentation pattern in LC-MS/MS (*17*).

In conclusion, the present study shows that mutation of residue 87 in drug metabolizing mutant BM3 M11 has strong influence on activity and regioselectivity of CLZ metabolism. Using a mutant that combined high activity and high selectivity for CLZ bioactivation, we were able to produce sufficient amounts of as yet tentatively assigned GSH conjugates to characterize their structures by ^{1}H-NMR. This study confirms the high potential of BM3 mutants as tool to characterize human-relevant metabolites.

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Chapter 7

CHARACTERISATION OF HUMAN CYTOCHROME P450S INVOLVED IN THE BIOACTIVATION OF CLOZAPINE

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Abstract

Clozapine is known to cause hepatotoxicity in a small percentage of patients. Oxidative bioactivation to reactive intermediates has been proposed as possible mechanism. However, in contrast to their role in formation of N-desmethylclozapine and clozapine N-oxide, the involvement of individual P450s in the bioactivation to reactive intermediates is much less well characterized. The results of the present study show that six out of fourteen recombinant human P450s were able to bioactivate clozapine. CYP3A4 and CYP2D6 showed the highest specific activity. Enzyme kinetical characterization of these P450s showed comparable intrinsic clearance implicating that CYP3A4 would be more important because of its higher hepatic expression compared to CYP2D6. Inhibition experiments using pooled human liver microsomes confirmed the major role of CYP3A4 in hepatic bioactivation of clozapine. By studying bioactivation of clozapine in human liver microsomes from 100 different individuals, an 8-fold variability in bioactivation activity was observed. In two individuals bioactivation activity exceeded N-demethylation and N-oxidation activity. Quinidine did not show significant inhibition of bioactivation in any of these liver fractions, suggesting that CYP2D6 polymorphism is not an important factor in determining susceptibility to hepatotoxicity of clozapine. Therefore, interindividual differences and drug-drug interactions at the level of CYP3A4 might be factors determining exposure of hepatic tissue to reactive clozapine metabolites. Previously, we have shown that hGSTs, specifically polymorphic hGST M1-1, have a significant role in catalyzing GSH conjugation of reactive metabolites of clozapine formed by cytochrome P450s. Combination of interindividual variability in the activity of these enzymes due to genetic polymorphism and/or drug-drug interactions might explain high risk of the small group of patients to severe clozapine toxicity.

1. Introduction

Clozapine (CLZ) is an atypical antipsychotic drug, which causes fewer extrapyramidal side effects than other neuroleptics *(1-3)*. However, because of its risk for severe agranulocytosis it is recommended only as a second line drug for patients with schizophrenia who do not respond to typical neuroleptic drugs *(2, 3)*. Next to agranulocytosis, mild hepatotoxicity has been reported as a side effect of CLZ in 37 % of patients. However, in 0.06 % of patients it may progress to liver failure *(4)*. Although the exact mechanism is not known yet, formation of reactive metabolites has been proposed as a possible explanation for these adverse drug reactions *(5-8)*.

Analysis of urinary metabolites of clozapine-treated patients has shown that clozapine is extensively metabolised by P450s *(9, 10)*. Several studies have been performed in order to identify the role of individual P450s in the oxidative metabolism of CLZ to its major metabolites, N-desmethylclozapine (DMCLZ; C-2) and clozapine N-oxide (CLZ-NO; C-1), see Figure 1 *(7, 11-16)*. The combined results of these *in vitro* studies showed that both CYP1A2 and CYP3A4 are playing major roles in the biotransformation of CLZ to these two metabolites. *In vivo* studies point to a major role of CYP1A2 in the pharmacokinetics of CLZ *(17)*. Furthermore, the involvement of polymorphic CYP2D6 and its role in the formation of unidentified metabolites other than N-demethylclozapine and N-oxide using cells that specifically express CYP2D6 was also described *(18)*. No

association has been found for metabolizer status with regard to debrisoquine (CYP2D6) or (S)-mephenytoin (CYP2C19) *(19, 20)*.

Several studies have shown that CLZ is bioactivated by human liver microsomes (HLM) to a reactive nitrenium ion *(7, 8, 21, 22)*. Furthermore, formation of an iminium ion resulting from dehydrogenation of the piperazine-ring of CLZ was demonstrated using cyanide as trapping agent, which is able to trap hard electrophiles *(23-25)*. So far no bioactivation studies have been performed using recombinant human P450 isoenzymes because in none of previous studies GSH or cyanide was included to trap reactive intermediates. Therefore, the aim of the present study was to identify the isoenzymes of human P450s which are involved in the hepatic bioactivation of CLZ. The formation of reactive intermediates of CLZ was determined by quantifying GSH conjugation in incubations with individual recombinant human CYPs and by performing inhibition studies in incubations with pooled HLM using P450-isoform selective inhibitors. Formation of reactive intermediates was measured by using GSH and cyanide as trapping agents to measure the formation of nitrenium ions and iminium ions, respectively. Finally, to study the interindividual variability in hepatic biotransformation and bioactivation of CLZ, incubations were performed with HLM of 100 individuals was quantified.

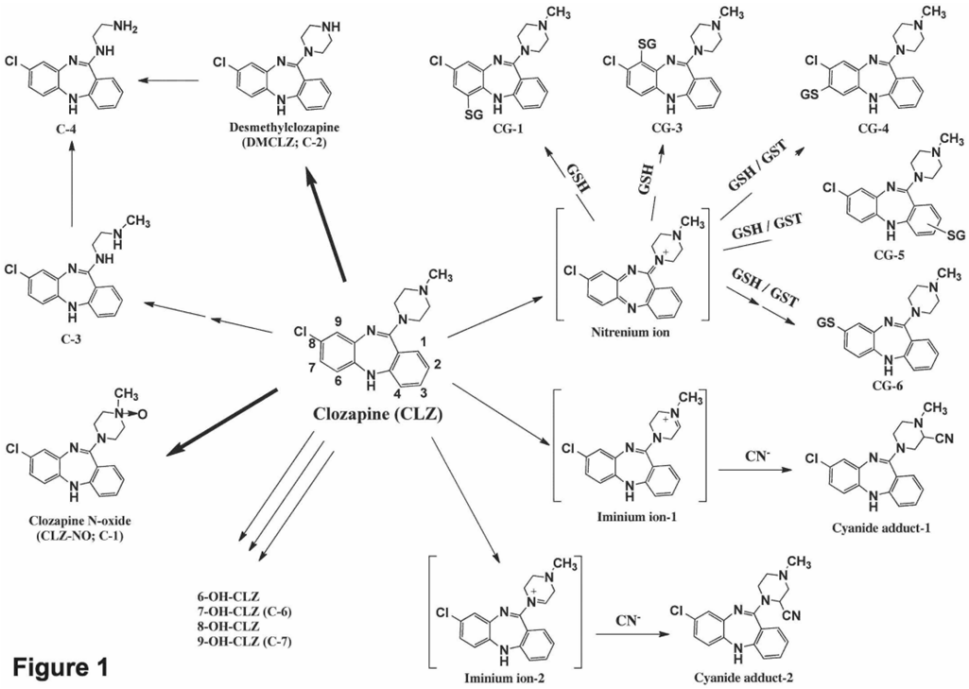

Figure 1. Metabolic scheme of identified oxidative metabolites of clozapine formed by cytochrome P450s. Structures of proposed reactive nitrenium and iminium ions are shown between brackets, and are based on identified structures of adducts to GSH and cyanide.

2. Materials and methods

2.1. Materials

Supersomes containing cDNA-expressed human cytochrome P450 (CYP) enzymes were purchased from BD Biosciences (Breda, Netherlands). These enzymes were CYP1A1 (Lot No. 35400), CYP1A2 (Lot No. 21667), CYP2A6 (Lot No. 33769), CYP3A4 (Lot No. 38275), CYP3A5 (Lot No. 44743), CYP1B1 (Lot No. 26314), CYP2B6 (Lot No. 62543), CYP2C8 (Lot No. 62556), CYP2C9*1(Arg_{144}) (Lot No. 41274), CYP2C18 (Lot No. 11301), CYP2C19 (Lot No. 62542), CYP2D6*1 (Lot No. 38273), CYP2E1 (Lot No. 44748) and CYP2J2 (Lot.No. 456264). Human liver microsomes (HLM; Lot No. 0710619), pooled from 50 donors, were obtained from Xenotech (Lenexa, USA) and contained 20 mg protein/mL. DMCLZ and CLZ-NO were purchased from Sigma Aldrich (Netherlands). 7-Hydroxyclozapine (7-OH-CLZ; C-6) and 9-hydroxyclozapine (9-OH-CLZ; C-7) were prepared by the Udenfriend reaction as described previously *(26)*. All other chemicals and reagents were of analytical grade and obtained from standard suppliers. Expression and purification of human GSTP1-1 was done as described previously *(22)*. Protein concentrations were determined according to the method of Bradford *(27)* with reagent obtained from Bio-Rad (München, Germany). The specific activity of the purified recombinant human GST P1-1, which was assayed according to Habig et al. *(28)*, was 27.9 μmol/min/mg protein, using CDNB as a substrate.

2.2. Incubations of CLZ with recombinant human P450s

Incubations with recombinant human P450 were performed at CLZ concentrations of 10 and 100 μM (22). Duplicate incubations were performed in 100 mM potassium phosphate buffer (pH 7.4) at a final volume of 200 μL. The individual cDNA-expressed CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP1B1, CYP2B6, CYP2C8, CYP2C9*1, CYP2C18, CYP2C19, CYP2D6*1, and CYP2E1 were incubated for 30 minutes at 37 ^{o}C with CLZ at a final P450 concentration of 50 nM. Formation of reactive nitrenium ion was determined by including 100 μM GSH and 8 μM GSTP1-1 in the incubations. GSTP1-1 was previously shown to be highly active in conjugation of the CLZ nitrenium ion *(22)*. Besides GSH, potassium cyanide (1 mM) was also used as trapping reagent to detect formation of the reactive iminium ion resulting from oxidative bioactivation of the piperazine ring *(24)*. All incubations were initiated by the addition of 500 μM NADPH (final concentration). After 30 minutes, the reactions were terminated by the addition of 20 μL 10% ice-cold $HClO_4$. To precipitate denaturated proteins, the samples were centrifuged for 15 min at 14000 rpm. The supernatants were analysed by HPLC, as described below.

2.3. Determination of enzyme kinetic parameters of oxidative metabolism of CLZ.

For the most active recombinant P450s and HLM, the enzyme kinetic parameters of product formation were determined. First the ranges were determined where the enzyme activity is still linear with enzyme concentration and incubation time. Based on these experiments, enzym concentrations used were were 50 nM recombinant P450 and 1 mg microsomal protein of HLM. Incubation time was 15 minutes. The incubations were

performed using CLZ concentrations ranging from 1 to 1000 μM. Specific activities were calculated and plotted against substrate concentrations. Enzyme kinetic parameters K_m and V_{max} were determined by nonlinear regression according to the Michaelis-Menten equation, using GraphPad Prism software (San Diego, CA).

2.4. Inhibition of metabolite formation in incubations of CLZ with pooled human liver microsomes by isoenzyme-specific inhibitors of P450s

The contribution of individual P450s in metabolite formation were also studied by incubating CLZ with pooled HLM in presence or absence of specific inhibitors of individual P450 enzymes. The final concentration of HLM was 1 mg protein/mL. Incubations were performed in 100 mM potassium phosphate buffer (pH 7.4) and at a final volume of 250 μL. The concentration of CLZ was 100 μM and the final concentration of DMSO in incubations (used for stock solution of CLZ) was less than 1%. GSH conjugation was performed in addition of 100 μM GSH and 8 μM GSTP1-1. P450 selective inhibitors, furafylline (FURA, 10 μM), ketoconazole (KTZ, 2 μM and 20 μM), sulfaphenazole (SPZ, 10 μM), tranylcypromine (TCP, 25 μM), quinidine (2 μM), and diethyldithiocarbonate (DDC, 20 μM), were used to investigate the involvement of CYP1A2, CYP3A and CYP3A4, CYP2C9, CYP2C19, CYP2D6, and CYP2E1, respectively. These inhibitors and inhibitor concentrations have been previously shown to offer isoenzyme-selective inhibition *(29-32)*. All inhibitors except DDC were dissolved in methanol and the final concentration of the solvent in the incubations was not exceeding 1 %. DDC was dissolved in water. Reactions were initiated by the addition of 500 μM NADPH (final concentration) and incubated for 30 min at 37 °C. Incubations containing the mechanism-based inhibitors furafylline, tranylcypromine, and DDC were preincubated for 15 min in the presence of NADPH before addition of CLZ. The reactions were terminated by the addition of 25 μL of 10 % $HClO_4$ and centrifuged for 15 min at 14000 rpm. The supernatants were analyzed by HPLC and LC-MS, as described below. Control incubations without CLZ were performed under the same conditions to ensure that the presence of inhibitors did not interfere with the quantification of formed metabolites. Incubations without inhibitor were performed as a control as well. All incubations were performed in duplo.

2.5. Incubations of CLZ with individual human liver microsomes

Liver microsomes were prepared of liver pieces from 100 individuals from a liver bank (approved by the Ethical Review Board) established at the Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden as described previously *(33)*. Protein contents were determined according to the method of Bradford *(27)* with reagent obtained from Bio-Rad using bovine serum albumin as standard. The microsomes were stored in potassium phosphate buffer (100 mM, pH 7.4) at -80°C until use.

Incubations with HLM obtained from 100 individuals were performed for 30 min at 37°C with a final concentration of 0.1 mg microsomal protein/mL, in the presence of 5 mM GSH. The concentration of CLZ was 100 μM. The reaction was initiated with 0.5 mM NADPH and terminated by perchloric acid, as described above. Incubations were also

performed in presence of 2 μM quinidine to investigate the involvement of CYP2D6 in the bioactivation of clozapine.

2.6. Analytical methods

All samples were analysed by reversed-phase HPLC as described previously, using LC-MS/MS for identification and UV/Vis detection at 254 nm for quantification of formed metabolites *(22)*. Standard curves of commercially available DMCLZ and CLZ-NO references were used for quantification of these two metabolites. A standard curve of CLZ was used to estimate the concentrations of the formed GSH and cyanide conjugates, assuming that their extinction coefficients are equal to that of CLZ.

3. Results

3.1. Oxidative metabolism of clozapine by pooled human liver microsomes

Previously, CLZ was shown to be metabolised by pooled HLM to both CLZ-NO, DMCLZ and GSH conjugates *(21, 22)*. Consistent with these studies, CLZ-NO (C-1) and DMCLZ (C-2) were the major stable metabolites formed by pooled HLM, Figure 2. In total six GSH conjugates of CLZ were found in incubations of HLM in presence of GST P1-1. The structure of conjugates CG-1, CG-3, CG-4, CG-5 and CG-6 are shown in Figure 1. According to its *m/z* value of 452.18 (2+), conjugate CG-7 represents a secondary metabolite of CG-6, resulting from the presence of an additional glutathionyl group. In addition, two minor stable metabolites were formed resulting from partial degradation of the piperazine ring, CLZ-C_2H_2 (C-3) and CLZ-C_3H_4 (C-4). Analysis by the highly sensitive LCMS-Q-TOF instrument revealed the formation of two minor peaks with the *m/z* value of 343.14 (C-6, and C-7), eluting between 15.5 and 18.5 minutes which correspond to hydroxylated CLZ metabolites (Figure 3). Only C-6 could also be detected by UV-detection, Figure 2. Using the references obtained by the Udenfriend reaction *(26)*, the metabolites correspond to 7-hydroxyclozapine (C-6) and 9-hydroxyclozapine (C-7).

3.2. Effect of isoenzyme-selective inhibitors on metabolism of clozapine by pooled human liver microsomes

Figure 4 shows the effect of the isoenzyme-selective inhibitors on the formation of DMCLZ, CLZ-NO and total of GSH conjugates by pooled HLM. The results obtained are expressed as % of control HLM incubation in which no inhibitor was added.

The only inhibitor showing very significant inhibition of CLZ bioactivation was KTZ, which reduced the formation of total GSH conjugates by 58.8 % at 2 μM. At a concentration of 20 μM, KTZ inhibited GSH conjugation by 67.0 %. QND and DDC inhibited formation of GSH conjugates only to a low extent, 18.0 and 19.3 %, respectively. Results for inhibition of individual conjugates formation were matching these for total GSH conjugates, Figure 4B. These results indicate that CYP3A4 is the major isoenzyme involved in bioactivation of CLZ by pooled HLM.

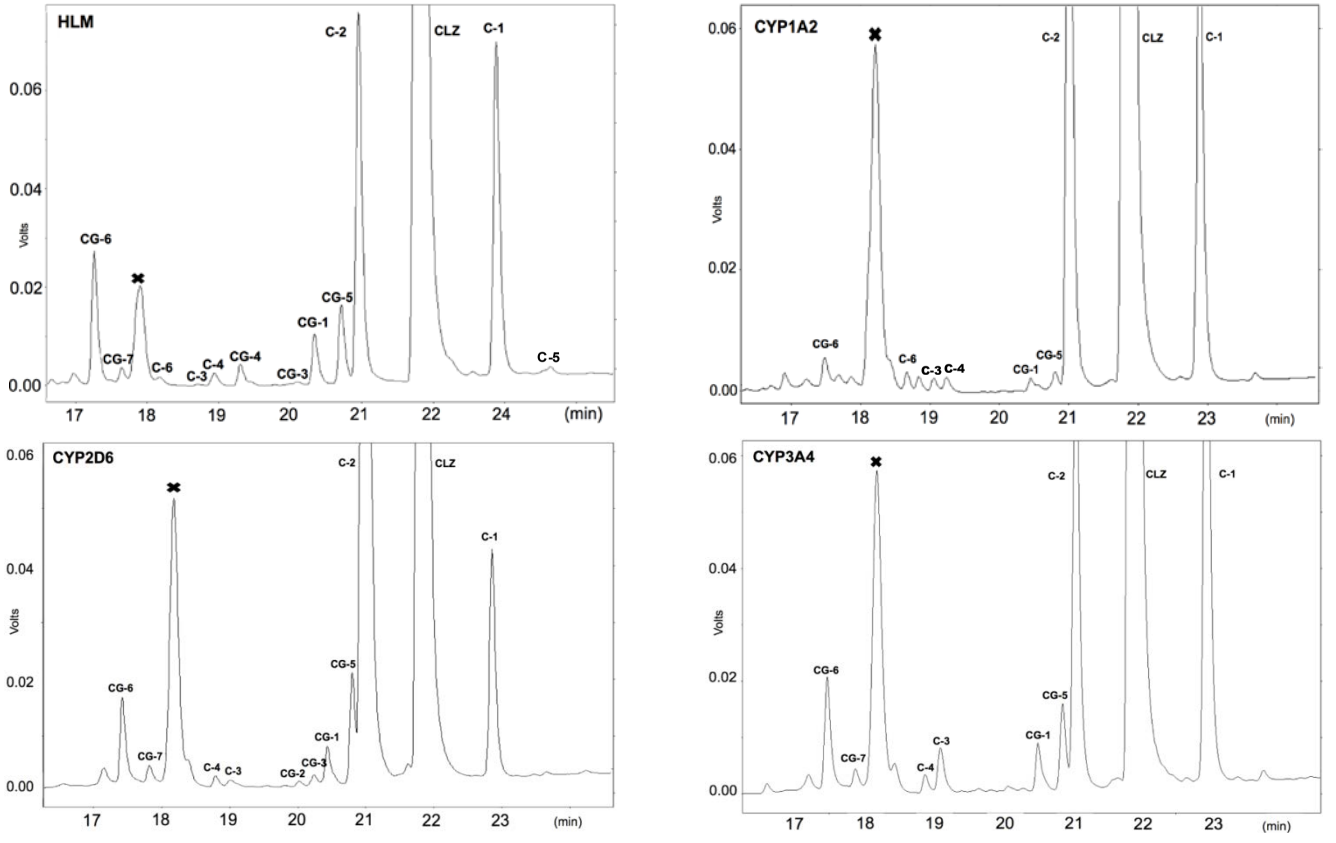

Figure 2. HPLC Chromatogram showing clozapine metabolites formed by HLM (A), CYP1A2 (B), CYP2D6 (C), and CYP3A4 (D). 100 μM CLZ was incubated for 30 minutes in the presence of 100 μM GSH and 8 μM hGSTs. Identification and codes of all metabolites correspond to previously described (refs. *21, 22*). Background peak 'X' was also present in the control without the substrate.

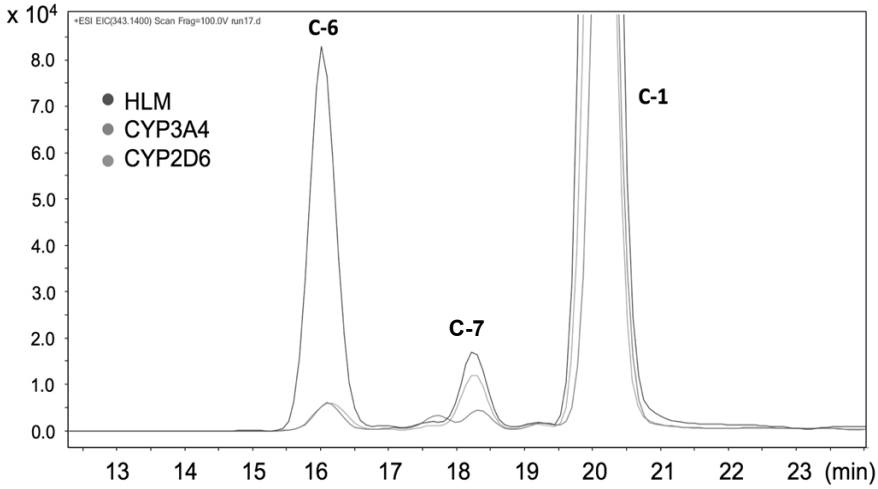

Figure 3. Extracted ion chromatograms of *m/z* 343,14 of incubations of CLZ with HLM, recombinant human CYP3A4 and CYP2D6. C-1, clozapine N-oxide; C-6, 7-hydroxyclozapine; C-7, unidentified; C-8, 9-hydroxyclozapine.

Inhibition studies on formation of the two major stable metabolites of CLZ, revealed involvement of CYP1A2 and CYP3A4 in the N-demethylation and mainly CYP3A4 in the N-oxidation of CLZ. FURA inhibited the demethylation by 36.2 % while KTZ reduced the formation of DMCLZ by 23.6 % and 44.2 % at 2 and 20 μM, respectively. QND and SPZ only slightly changed formation of DMCLZ, 9.3 and 6.8 %, respectively while TCP and DDC did not inhibit formation of this metabolite. Only KTZ showed significant inhibition on formation of CLZ-NO, 61.9 % at concentration of 2 μM and 69.7 % at 20 μM. All other inhibitors did not show any effect on N-oxidation of CLZ.

3.3. Oxidative metabolism of clozapine by recombinant human P450 enzymes

Oxidative metabolism of CLZ by recombinant human P450s were determined at substrate concentrations of 10 and 100 μM CLZ. The 10 μM concentration reflects the therapeutically relevant hepatic concentrations level of CLZ *(34, 35)* whereas 100 μM was commonly used in previous in vitro studies *(11-16)*. Serum concentrations of CLZ extend up to about 3 μM with the common therapeutic doses *(34, 35)*. Taking into account that hepatic tissue concentrations are ten times higher, the clinically relevant hepatic tissue concentrations were estimated to range up to 30 μM *(36)*. The specific activities by which each metabolite was formed by the individual recombinant P450s are shown in Table 1. All metabolites, which are formed by HLM, were also represented in incubations with recombinant P450 enzymes as shown in Figure 1.

3.3.1. Bioactivation of clozapine

Figure 5 shows the relative activities of individual recombinant human P450s in bioactivation of CLZ using GSH and potassium cyanide as trapping agents. Six of the recombinant P450s were able to bioactivate CLZ to variable extent. At both 10 and 100 μM CLZ, CYP3A4 appeared to be the most active enzyme, followed by CYP2D6.

CYP1A1, CYP1A2, CYP3A5, and CYP1B1 all showed less than 20% of the activity of CYP3A4 activity at these two substrate concentrations. CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, and CYP1E2 did not show any measurable formation of GSH conjugates. Specific activities (nmol/min/nmol P450) for formation of total GSH conjugates are given in Table 1 while details of the individual GSH conjugates are presented in Table 2. The ratios of the three formed GSH conjugates did not significantly change between the different enzymes, suggesting that all GSH conjugates are formed from the same reactive intermediate, as proposed previously *(37)*.

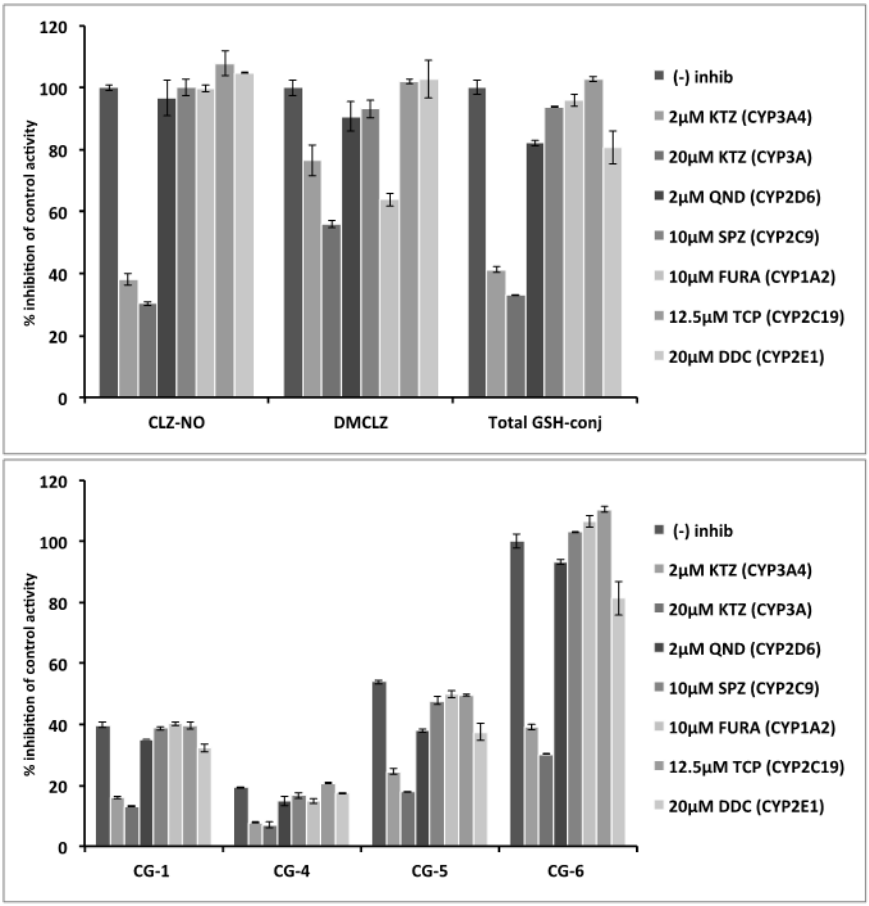

Figure 4. Effect of CYP450 inhibitors on the metabolism of clozapine by HLM to: A) clozapine N-oxide (CLZ-NO), N-desmethylclozapine (DMCLZ) and total of GSH conjugates (CG-1, CG-4, CG-5 and CG-6); and B) individual GSH conjugates. Data are expressed as % of control activity and represent mean of duplicate determinations.

Table 1 Comparison of specific activities for major stable and reactive metabolites formation by individual CYPs[a]

	DMCLZ		**CLZ-NO**		**Total GSH conjugate**[b]		**Cyanide conjugate**[b]
CLZ conc.	10μM	100μM	10μM	100μM	10μM	100μM	100μM
CYP 1A1	0.64±0.02	0.56±0.03	0.24±0.01	0.20±0.02	0.30±0.008	0.32±0.01	0.024±0.003
CYP 1A2	1.12±0.004	8.71±0.1	0.55±0.001	3.70±0.1	0.060±0.003	0.25±0.004	0.20±0.005
CYP 1B1	1.16±0.009	0.94±0.05	0.84±0.007	0.64±0.07	0.15±0.01	0.15±0.004	0.010±0.0007
CYP 2A6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.17±0.004
CYP 2B6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CYP 2C8	0.92±0.01	0.89±0.005	0.10±0.005	0.075±0.006	n.d.	n.d.	0.058±0.002
CYP 2C9	0.33±0.02	0.22±0.003	0.065±0.005	0.057±0.01	n.d.	n.d.	0.010±0.001
CYP 2C18	4.08±0.05	5.71±0.02	0.025±0.005	0.065±0.01	n.d.	n.d.	0.41±0.02
CYP 2C19	6.62±0.03	5.11±0.02	0.71±0.03	0.58±0.005	n.d.	n.d.	0.030±0.003
CYP 2D6	8.43±0.2	32.71±1.1	0.51±0.009	1.62±0.1	0.47±0.01	1.60±0.05	0.64±0.07
CYP 2E1	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.
CYP 2J2	0.70±0.07	1.40±0.1	0.05±0.007	0.10±0.01	0.009±0.001	0.03±03	0.05±0.002
CYP 3A4	0.98±0.01	5.35±0.007	2.37±0.02	5.62±0.01	0.87±0.01	1.92±0.008	0.18±0.003
CYP 3A5	0.24±0.04	1.29±0.02	0.32±0.04	0.88±0.03	0.15±0.009	0.30±0.005	0.08±0.002

a) Specific activities (nmol/min/nmol P450) were calculated by peak areas at 254 nm assuming that extinction coefficients of GSH-conjugates and cyanide adducts are the same as that of CLZ.

b) Involvement of individual CYPs in CLZ bioactivation is expressed as formation of total of GSH conjugates and cyanide adduct after 30 minutes incubations of 10 μM or 100 μM CLZ, 50 nM recombinant human CYPs, in the presence of 100 μM GSH and 8 μM hGST P1-1 or 1 mM KCN, respectively. The values represent averages of two measurements; standard deviations were less than 10%; n.d. not detectable.

Table 2 Involvement of individual CYPs in CLZ bioactivation and formation of individual GSH conjugates (pmol/min/pmol CYP) after 30 minutes incubations of 10 μM or 100 μM CLZ, 50 nm with CYPs, in the presence of 100 μM GSH and 8 μM GST P1-1.

	Total GSH conjugates		**CG-1**		**CG-5**		**CG-6**	
CLZ conc.	10μM	100μM	10μM	100μM	10μM	100μM	10μM	100μM
CYP 1A1	0.30±0.008	0.32±0.01	0.080±0.008	0.097±0.008	0.049±0.0004	0.049±0.004	0.17±0.001	0.18±0.01
CYP 1A2	0.060±0.003	0.25±0.004	0.029±0.002	0.074±0.004	0.021±0.001	0.093±0.001	0.031±0.003	0.18±0.004
CYP 2A6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CYP 3A4	0.87±0.01	1.92±0.008	0.19±0.009	0.40±0.001	0.20±0.006	0.64±0.006	0.48±0.01	0.89±0.008
CYP 3A5	0.15±0.009	0.30±0.005	0.057±0.001	0.052±0.002	0.023±0.005	0.082±0.005	0.074±0.009	0.17±0.0002
CYP 1B1	0.15±0.01	0.15±0.004	0.013±0.0003	0.014±0.004	0.051±0.001	0.036±0.0001	0.083±0.01	0.10±0.003
CYP 2B6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CYP 2C8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CYP 2C9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CYP 2C18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CYP 2C19	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CYP 2D6	0.47±0.01	1.60±0.05	0.14±0.002	0.27±0.03	0.18±0.01	0.70±0.04	0.15±0.002	0.64±0.05
CYP 2E1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CYP 2J2	0.009±0.001	0.03±0.03	0.003±0.001	0.007±0.002	0.004±0.001	0.02±0.01	0.003±0.0003	0.008±0.005

When incubations of CLZ with individual P450s were performed in the presence of potassium cyanide, a cyano adduct of the clozapine iminium ion with m/z 352.13 was observed. As shown in Figure 5B, the highest activity was found CYP2D6 and CYP2C18. CYP1A2, CYP2A6, and CYP3A4 showed approximately % of CYP2D6 activity, respectively. All other CYPs had very low or no activity. Specific activities (pmol/min/pmol P450) of all P450s for formation of cyanide conjugate are given in Table 1.

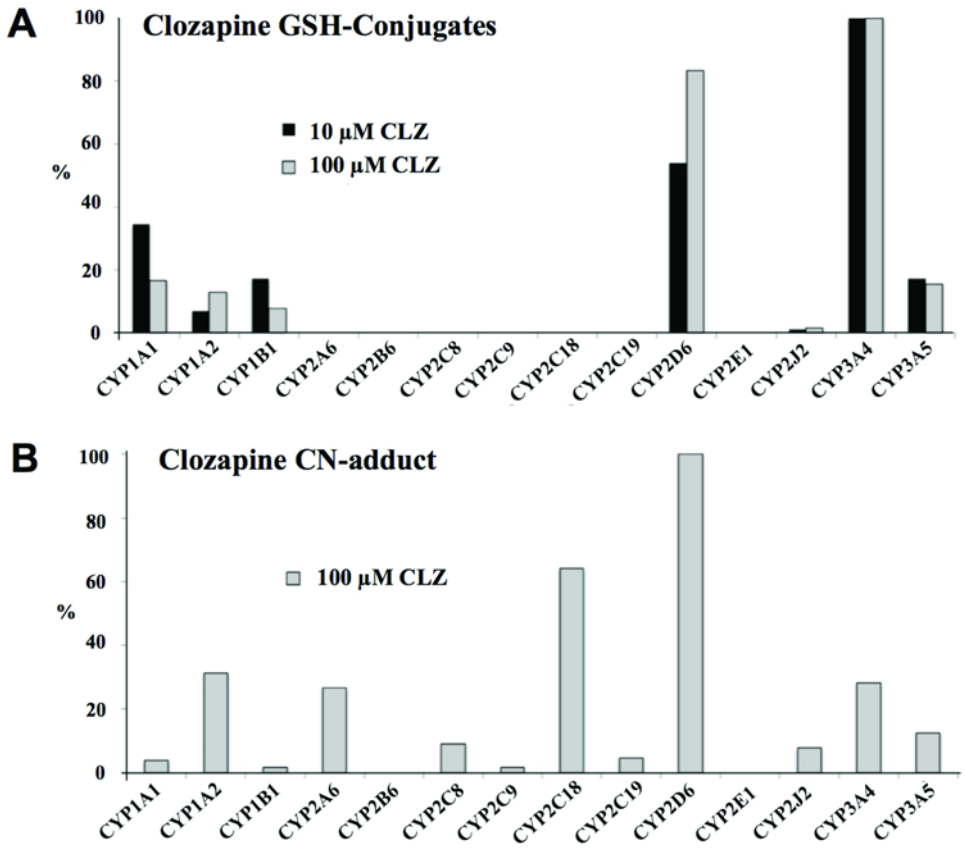

Figure 5. Formation of GSH- and cyanide-reactive metabolites of clozapine by recombinant human P450s. Rates of formation were measured as total of GSH conjugates (CG-1, CG-5 and CG-6) (A) and cyanide adduct (B) when incubated with 10 µM and 100 µM CLZ. Values represent the mean of duplicate measurements and are given as %, where 100 % correspond to the activity of the most active enzyme.

3.3.2. Formation of stable metabolites of clozapine by recombinant human P450s

The results from incubations with CLZ concentration of 100 µM (Figure 6A) show that specific activity of DMCLZ formation was highest with CYP2D6 and lower with

CYP1A2, CYP3A4, CYP2C18, and CYP2C19, being 27, 16, 17, and 16 % of CYP2D6 activity, respectively. CYP1A1, CYP3A5, CYP1B1, CYP2C8, and CYP2C9 showed very low activity, while the other P450s were inactive. At 10 μM CLZ incubations, highest activities of N-demethylation were observed with CYP2D6, CYP2C18 and CYP2C19, respectively.

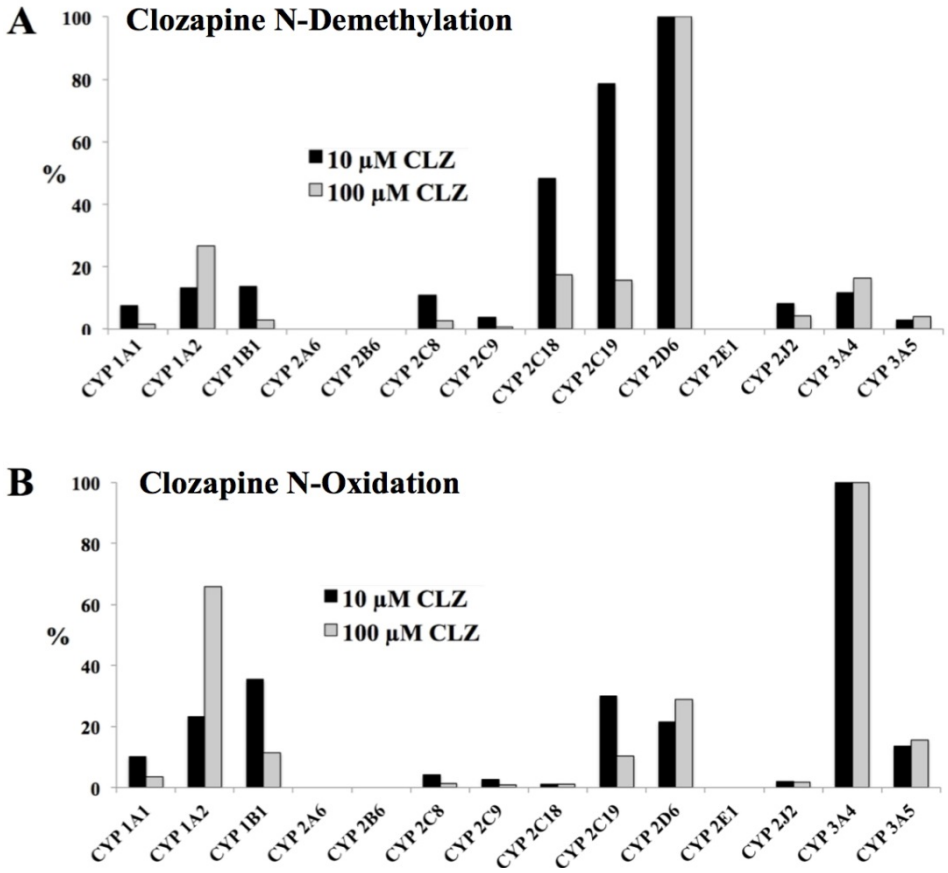

Figure 6. Formation of clozapine N-oxide (CLZ-NO) and N-desmethylclozapine (DMCLZ) by recombinant human P450s. Rates of formation of DMCLZ (A) and CLZ-NO (B) when incubated with 10 μM (dark grey) and 100 μM CLZ (light grey). Values represent the mean of duplicate measurements and are given as %, where 100 % correspond to the specific activities (pmol/min/pmol CYP) of the most active enzyme (Table 1).

CYP3A4 exhibited the highest catalytic activity with respect to CLZ N-oxidation, followed by CYP1A2 and CYP2D6 which showed 66 and 29 % of CYP3A4 activity at 100 μM CLZ, respectively, Figure 6B. CYP3A5, CYP1B1, and CYP2C19 were less efficient having only 15, 11, and 10 % of CYP3A4 activity, respectively. CYP1A1, CYP2C8, CYP2C9, and CYP2C18 showed. CYP2A6, CYP2B6, and CYP1E2 did not mediate any measurable

conversion of CLZ. In 10 μM incubations, CYP1B1 and CYP2C19 activity increased to 35 and 30 % of CYP3A4 activity, respectively while CYP1A2 activity decreased to 24 % of CYP3A4 activity. Specific activities (pmol/min/pmol P450) of all CYPs for both stable metabolites are given in Table 1.

Using the highly sensitive LCMS-Q-TOF instrument two minor hydroxy metabolites with m/z of 343,14 were also produced by the recombinant human P450s (Figure 3). Due to low yields, evaluation of the enzymes involved in their formation was determined based on LC/MS peak areas. The metabolite with the retention time at 18.2 min, which was identified as 7-hydroxyclozapine (C-6) was produced mainly by CYP1A1 in 10 μM CLZ incubations while in 100 μM CLZ incubations CYP1A2 was the most important for its production. The second hydroxy metabolite which was identified as 9-hydroxyclozapine (C-7), and having retention time at 20.7 min, was produced mainly by CYP3A4. It was also found in small quantities in incubations with CYP1A1, CYP1A2, CYP2B6, CYP2C18, CYP2D6, and CYP2E1.

3.4. Enzyme kinetic characterization of P450-dependent metabolism of clozapine by pooled human liver microsomes and recombinant human CYP2D6 and CYP3A4

The enzyme kinetic parameters of pooled HLM and two most active CYP enzymes for the bioactivation of CLZ (CYP3A4 and CYP2D6) were determined by varying CLZ substrate concentrations from 1 to 1000 μM. For both recombinant CYPs substrate inhibition was observed at concentrations above 250 μM (data not shown). The enzyme kinetic parameters for these isoenzymes were therefore estimated from the initial part of the substrate-velocity plots; the last two points at 750 and 1000 μM were excluded. The K_M and V_{max} values and the intrinsic clearance, V_{max}/K_M, of the P450 enzymes are shown in Table 3.

In the pooled HLM incubations, the enzyme kinetic parameters for total GSH conjugate formation were 125.7 μM and 1266 nmol/min/mg protein for K_M and V_{max} values, respectively. For CLZ N-demethylation a K_M of 268.5 μM and V_{max} of 3215 nmol/min/mg protein was found. For CLZ N-oxidation these values were 250.1 μM and 2130 nmol/min/mg protein, respectively, Table 3. These results led to the 1.1 and 1.2-fold higher intrinsic clearance (V_{max}/K_M) for total GSH conjugates than for DMCLZ and CLZ-NO, respectively. The K_M and V_{max} values for DMCLZ and CLZ-NO are somewhat higher than previously determined in literature *(11, 15, 16)*, although higher values (>300 μM) have also been reported for N-oxide formation *(15, 16)*. Also, our results are in agreement with Zhang et al. *(11)* who showed that K_M values for DMCLZ and CLZ-NO are similar. When comparing enzyme kinetic parameters of recombinant CYP3A4 and CYP2D6, both K_M and V_{max} values for the formation of total GSH conjugates were somewhat higher for CYP3A4 (30.3 μM and 3.1 nmol/min/nmol CYP) than those determined for CYP2D6 (21.9 μM and 2.8 nmol/min/nmol CYP). Because of its lower K_M value, a 26 % higher intrinsic clearance was found for CYP2D6.

Concerning the stable metabolites, the K_M for N-demethylation was about 2-fold higher for CYP3A4 (47.6 μM) than for CYP2D6 (25.3 μM) while for the V_{max} values it was opposite, 19.9 nmol/min/nmol CYP for CYP3A4 and 32.8 nmol/min/nmol CYP for CYP2D6. This resulted in a 3.1-fold lower intrinsic clearance for CYP3A4. For CLZ-NO

formation, CYP3A4 displayed somewhat higher K_M and V_{max} values (92.7 μM and 9.5 nmol/min/nmol CYP) than CYP2D6 (84.1 μM and 6.1 nmol/min/nmol CYP), resulting in a 29 % increase of intrinsic clearance.

3.5. *Interindividual variability in clozapine bioactivation by human liver microsomes*

To investigate the interindividual variability in microsomal biotransformation of CLZ, hundred individual HLM fractions were incubated at a CLZ concentration of 100 μM. The amounts of the formed metabolites (DMCLZ, CLZ-NO, and total GSH conjugates) are displayed in Figure 7, with the samples ranked from highest to lowest GSH-conjugate formation. The total of GSH conjugates formation varied over an 8.3-fold range (0.26-2.16 μM; median 0.62 μM), whereas DMCLZ formation varied over a 5.1-fold range (0.43-2.19 μM; median 0.95 μM) and CLZ-NO over a 9.4-fold range (0.17-1.59 μM; median 0.50 μM). The larger variation for CLZ-NO formation than for DMCLZ is in agreement with previous results obtain with liver fractions obtained from fourteen individuals by Zhang et al. *(11)*. Interestingly, two HLM-fractions, indicated by stars in Figure 7, showed very high GSH conjugate formation when compared to formation of DMCLZ and CLZ-NO. When correlating formation of GSH-conjugates to DMCLZ and CLZ-NO relatively low correlations were found: DMCLZ vs. total CLZ-SG, r2 = 0,384; CLZ-NO vs. total CLZ-SG, r2 = 0,624. The low correlations might be explained by the fact that multiple P450s are involved in these metabolites, as shown in Figures 4 and 5. Because inhibition of CYP2D6 had only minor effect in pooled HLM, also inhibition studies with quinidine were performed to investigate if in any of the liver fractions CYP2D6 plays a significant role in bioactivation of CLZ. The treatment of the panel of human liver microsomal samples (n = 100 subjects) with quinidine did not show significant inhibition of the formation of total GSH conjugates (data not shown).

4. Discussion

Bioactivation of CLZ to reactive metabolites in the different target tissues is generally accepted as a cause for its ADRs. Occasional cases of liver injury in patients treated with CLZ might be the result from local bioactivation to a reactive nitrenium ion by hepatic P450s *(8)*. In addition, bioactivation of CLZ to a reactive iminium ion, which can be trapped by cyanide, in microsomal incubations, as has been described *(23-25)*, might contribute to hepatotoxicity. Although these reactive CLZ metabolites has been demonstrated in many *in vitro* studies using HLM, only very limited information is available on the role of individual P450s in bioactivation of CLZ *(7)*.

Table 3 Enzyme kinetic parameters for formation of clozapine metabolites by HLM and recombinant human CYP3A4 and CYP2D6.

Metabolite	Kinetic Parameters	Enzyme fractions		
		CYP3A4	CYP2D6	Pooled HLM
Total GSH conjugates	K_M (μM)	30.3 ± 5.1	21.9 ± 2.7	126 ± 22
	V_{max} (nmol/min/nmol CYP)	3.1 ± 0.1	2.8 ± 0.1	1266 ± 89 [a]
	V_{max}/K_M (ml/min/nmol CYP; x10^3)	101.9	128.8	10071 [b]
Clozapine N-Oxide	K_M (μM)	92.7 ± 5.5	84.1 ± 3.9	250 ± 39
	V_{max} (nmol/min/nmol CYP)	9.5 ± 0.2	6.1 ± 0.1	2130 ± 164 [a]
	V_{max}/K_M (ml/min/nmol CYP; x10^3)	102.5	72.8	8516 [b]
N-Demethyl Clozapine	K_M (μM)	47.6 ± 4.4	25.3 ± 0.4	268 ± 37
	V_{max} (nmol/min/nmol CYP)	19.9 ± 0.6	32.8 ± 0.2	3215 ± 226 [a]
	V_{max}/K_M (ml/min/nmol CYP; x10^3)	419	1298	11974 [b]

a. V_{max} values for HLM are expressed as nmol/min/mg protein.
b. V_{max}/K_M values for HLM are expressed in ml/min/mg protein

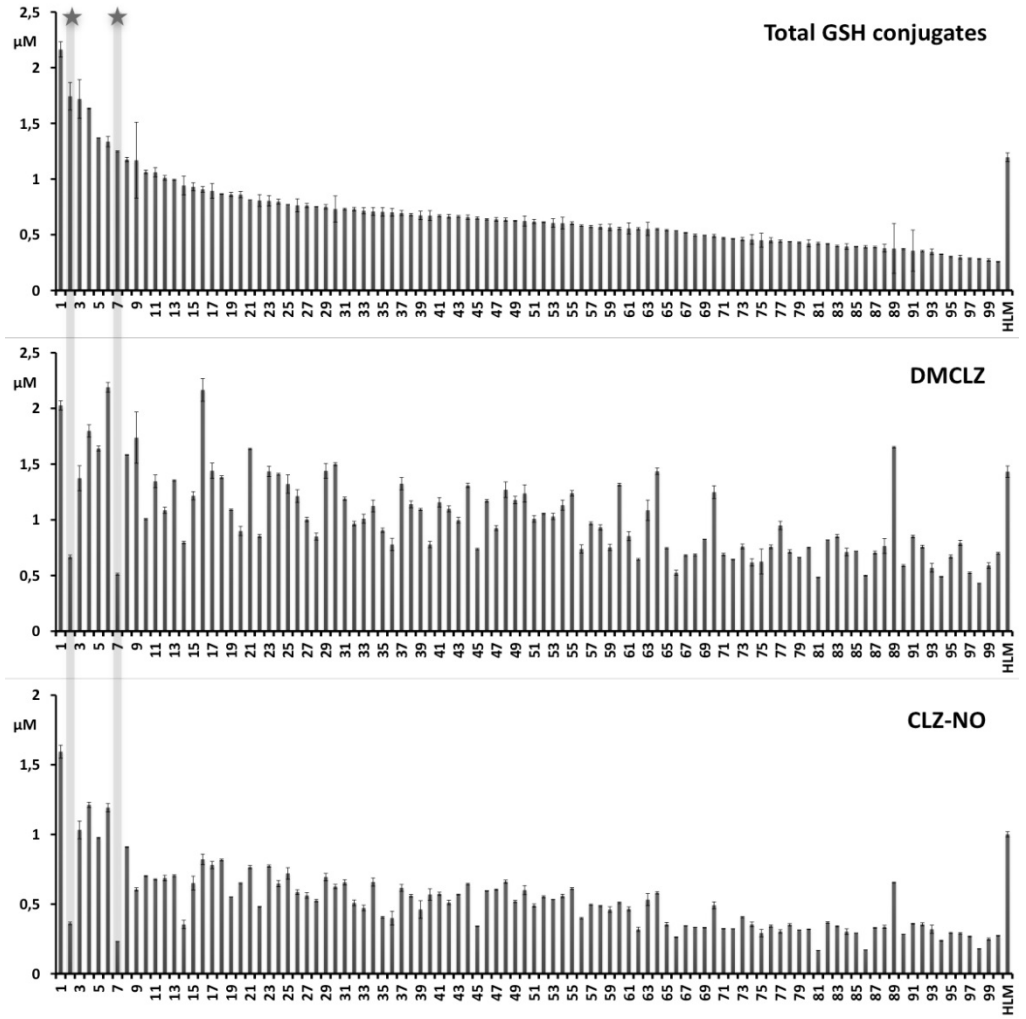

Figure 7. Metabolism of CLZ by different individual human liver microsomes at substrate concentration of 100 µM. Concentrations of total of GSH conjugates (A), DMCLZ (B) and CLZ-NO (C) were measured after 30 minutes of incubation of 0.1 mg/mL HLM with 100 µM CLZ in presence of 5 mM GSH. Two individuals with high bioactivation activity compared to N-demethylation and N-oxidation are marked by grey column and asterix.

Several previous studies already described involvement of individual human CYPs in formation of the stable metabolites DMCLZ and CLZ-NO *(11-16)*. The first studies performed evaluated only a limited number of recombinant human CYPs. The most recent study of Zhang et al. *(11)* was the most complete in which 14 commercially available CYPs and three flavin-containing monooxygenases were evaluated. The results of the present study are consistent with those of Zhang et al: N-oxidation of CLZ was catalyzed mainly by CYP3A4 and by CYP1A2 to a lesser extent, whereas N-demethylation

was catalysed by several enzymes of which CYP2D6 displayed the greatest catalytic activity. As CYP1A2 and CYP3A4 are implicated as the major enzymes involved in CLZ biotransformation, it was concluded that the variability in activity of these two enzymes in individuals is responsible to large extent for the observed differences in the profile of pharmacokinetic drug interactions *(11)*. However, because in none of these previous *in vitro* studies GSH was included to study formation of GSH-conjugates, the bioactivation pathway has been overlooked so far.

As shown in Figure 7, bioactivation of CLZ, when quantified by the total of GSH conjugates, appears to be a relatively important pathway when compared to N-demethylation and N-oxidation pathways. DMCLZ formation which is the major pathway of CLZ metabolism was on average only 1.6-fold higher than bioactivation. In the set of 100 HLM fractions, two individuals even showed higher activity of bioactivation when compared to N-demethylation and N-oxidation pathways. To determine which P450s contribute to the bioactivation pathways, studies with recombinant P450s and enzyme-specific inhibitors were performed. As shown in Figure 5A, recombinant human CYP3A4 showed the highest specific activity in the formation of GSH conjugates, whereas also relatively high activity was observed with recombinant CYP2D6. Lower bioactivation activity was observed with CYP1A1, CYP1A2, CYP3A5, and CYP1B1. When using cyanide as trapping agent, CYP2D6 also seems to be the most active enzyme involved in the bioactivation of CLZ to reactive iminium ion, with CYP2C18 also showing high activity, Figure 5B. When determining enzyme kinetic parameters for the three pathways of CLZ metabolism, it appeared that CYP2D6 displayed a 1.3- and 3.1-fold higher intrinsic clearance for GSH conjugates and DMCLZ, respectively, when compared to CYP3A4. For CLZ-NO the difference in intrinsic clearances was 1.4-fold higher for CYP3A4 comparing to CYP2D6.

Although CYP2D6 showed relatively high specific activity in all three oxidative pathways of CLZ-metabolism, Table 1, the CYP2D6-specific inhibitor quinidine did not show significant inhibition of these pathways in incubations of CLZ with pooled HLM and any of the 100 individual HLM fractions (data not shown). These results support previous observations of Pirmohamed et al. *(7)* in which no significant difference was observed in covalent protein binding between incubations of microsomes of a limited number of individuals which were genotyped as poor en extensive metabolisers of CYP2D6. Also, no significant differences in the pharmacokinetic parameters of CLZ were observed between poor and extensive metabolizers of debrisoquine *(20)*, suggesting that the genetic polymorphism of CYP2D6 has little clinical relevance for CLZ pharmacokinetics and CLZ bioactivation.

Considering the fact that CYP3A4 is on average almost 20-fold more abundant in HLM than CYP2D6 *(38)*, whereas its intrinsic clearance in CLZ-bioactivation is only slightly lower than CYP2D6, we conclude that CYP3A4 is most likely the major enzyme involved in hepatic CLZ bioactivation. This is supported by the fact that only the CYP3A4-specific inhibitor ketoconazole was able to cause significant inhibition of bioactivation of CLZ by pooled HLM, Figure 4. These results are consistent with the observation of Pirmohamed et al. that ketoconazole significantly inhibited the formation of GSH-conjugates and protein-adducts *(7)*. Our studies also show involvement of CYP3A4 in N-demethylation and N-oxidation of CLZ as reported previously *(11, 15)*. The important role of CYP3A4 for CLZ pharmacokinetics is supported by the observations that erythromycin, a substrate and inhibitor of CYP3A4, leads to higher serum concentrations

of CLZ *(39)*, whereas induction of CYP3A4 by carbamazepine treatment increased metabolism of CLZ *(40)*.

Genetic polymorphisms of CYP3A4, but also the interactions with other xenobiotics that influence its activity might cause interindividual differences that could lead to the susceptibility for CLZ adverse drug reactions. Although it appears that CYP3A4 is without common functional polymorphisms *(41)*, it has been demonstrated that nonsynonymous alleles for CYP3A4 encode enzymes with altered catalytic properties *(11, 42, 43)*. Inducers that increase the activity of CYP3A4 *(39, 40)* could be more important than genetic polymorphism of this enzyme for the individual variability in CLZ bioactivation.

In conclusion, the results of the present study show that CYP3A4 is the main enzyme involved in the bioactivation of CLZ in human liver microsomes. Although two recent studies showed that clozapine was not cytoxic in human cell lines transfected with CYP3A4 *(44, 45)*, down regulation of Nrf-2 by siRNA resulted in cytotoxicity of clozapine in CYP3A4-transfected HepG2-cells *(44)*. This may be rationalized by the reduced activity of protective phase II-enzymes such as hGSTs. We have shown previously that several hGSTs, including polymorphic GST M1-1 and GST P1-1, have a significant activity in catalyzing GSH conjugation of reactive CLZ metabolites formed by cytochrome P450s *(22)*. Therefore a high activity of bioactivation by CYP3A4 in combination with reduced activity of protective hGSTs might explain high susceptibility of part of the patients to hepatotoxic effects of CLZ.

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Chapter 8

INTERINDIVIDUAL VARIABILITY IN CLOZAPINE METABOLITES IN PATIENT URINE AND PRECISION-CUT HUMAN LIVER SLICES

-IDENTIFICATION OF GSH-RELATED METABOLITES AND ROLE OF GENETIC POLYMORPHISM OF HUMAN GLUTATHIONE S-TRANSFERASE-

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To be submitted.

Abstract

Clozapine (CLZ) is an atypical antipsychotic drug associated with idiosyncratic agranulocythosis and hepatotoxicity which are believed to result from bioactivation to a reactive nitrenium ion by myeloperoxidase and P450s, respectively. The risk factors determining the susceptibility to these toxic side effects still remain to be elucidated. Although it has been shown that clozapine undergoes extensive metabolism in patients, little data is available on the variability in bioactivation and bioinactivation, as reflected by GSH-related metabolites. In the present study, the interindividual variability in biotransformation was investigated in incubations of CLZ with precision-cut liver slices from 14 liver donors, and by analyzing urine samples of 34 patients treated with CLZ. Also the role of genetic polymorphism of four human glutathione S-transferases (hGST) in the variability of GSH-related metabolites was studied.

The results confirm the extensive biotransformation of CLZ observed previously. More than 40 phase I and phase II metabolites of CLZ were identified in both urine and slice incubations and showed significant differences in profiles between individuals. Eleven of the urinary metabolites were derived from GSH-conjugates and reflect internal bioactivation of CLZ. Only methylthio- and cysteine-conjugates of CLZ and its major metabolites N-desmethylclozapine and clozapine N-oxide were detected by LC-MS/MS analysis; the anticipated N-acetylcysteine conjugates, however, could not be detected. Three of the urinary GSH-related products identified are dependent on catalysis by human hGSTs. However, due to the extremely large variability in amounts and profiles of GSH-related metabolites, no correlation was found with polymorphic alleles of hGSTM1, GSTT1, GSTP1 and GSTA1. Urinary GSH-related metabolites of CLZ, therefore, do not seem useful biomarkers for quantitative biomonitoring of internal exposure to reactive CLZ-metabolites.

By genotyping these four GSTs in groups of CLZ-treated patients with and without history of agranulocytosis, it was observed that in the group of the susceptible patients the GSTM1/GSTT1 double null genotype was overrepresented. A larger scale association study is required to validate whether this combination of genotypes is an important risk factor for CLZ-induced agranulocytosis.

1. Introduction

Clozapine (CLZ) is an atypical antipsychotic medicine, effective in the treatment of refractory schizophrenia (*1, 2*). Limiting side-effects associated with CLZ-use are development of agranulocytosis, with an incidence of 0.4-0.8 % (*3*). Furthermore, increased serum transaminase were observed in 37 % of the patients and liver failure occurred in about 0.06 % of the patients (*4*). Mechanistic *in vitro* studies suggest that formation of reactive metabolites such as nitrenium ions play an important role in the pathogenesis of agranulocytosis and adverse hepatic effects (*5–7*). Therefore variability in activity of the enzymes involved in bioactivation and inactivation might be important factors determining interindividual susceptibility for these adverse drug reactions.

The collective *in vitro* and *in vivo* studies performed show that CLZ undergoes extensive oxidative biotransformation, followed by the phase 2 conjugation reactions, as summarized in Figure 1. In *in vitro* studies with human liver microsomes (HLM) and recombinant human cytochrome P450s (CYPs) the major metabolites of CLZ are

desmethylclozapine (DMCLZ) and clozapine N-oxide (CLZ-NO) *(8-10)*. Both CYP1A2 and CYP3A4 were shown to play major roles in the formation of these metabolites. Bioactivation of CLZ to nitrenium ion in HLM or human neutrophils and myeloid cells has been demonstrated by measuring formation of GSH conjugates (*5-7*). In the neutrophils and myeloid cells, bioactivation of CLZ is catalyzed by the highly expressed myeloperoxidase (MPO) (*5,7*). In human liver microsomes CYP3A4 was recently identified as the major isoenzyme involved in the hepatic bioactivation of CLZ (*10*).

Several studies have investigated the metabolic profile of CLZ in healthy volunteers and/or patients (*11–16*). In serum, next to CLZ typically only DMCLZ and CLZ-NO were found as major metabolites (*12*). However, it was shown that DMCLZ and CLZ-NO together accounted for only 14% of the dose in patient urine (*14*). The first attempt to elucidate the complete metabolic pattern of CLZ in human urine and feces was performed with six volunteers which were given a single dose of 50 mg of radiolabeled [14C]clozapine (*13*). In total seven metabolites were identified in urine and feces. The four major urinary metabolites, accounting for 60% of the dose, were identified as 8-deschloro-8-hydroxy-clozapine (8-OH-DMCLZ; 11% of dose), its glucuronide (8-OH-DMCLZ-O-Gluc; 8% of dose), 7-hydroxy-desmethylclozapine sulfate (7-OH-DMCLZ-O-Sulf; 6.8% of dose) and CLZ-NO (5% of dose). In addition, a quaternary ammonium glucuronide of CLZ (CLZ-N^+-Gluc) was identified as metabolite in feces (*14*) and urine (*15*). However, because of the limited resolution of the chromatographic system used, still a significant amount of the excreted radioactivity could not be accounted for. Schaber *et al.* identified an additional ten metabolites in urine of three CLZ-treated patients by using a combination of chromatographic techniques (*16*). These minor metabolites resulted from combinations of N-oxidation, N-demethylation, hydroxylation at positions 6, 7, 8 and 9 of the benzodiazepine structure, and sulfation and glucuronidation of the oxidative metabolites, Figure 1. In addition, a metabolite was detected in which the piperazine ring of CLZ was partially degraded to an ethylenediamine derivative (EDA-BZD, Figure 1).

Using a panel of liver microsomes of 100 individuals, it was recently demonstrated that bioactivation of CLZ to nitrenium ion was quantitatively a relatively important metabolite *in vitro* when compared to N-oxidation and N-demethylation and was varying 8-fold based on the level of GSH-conjugates formed (*10*). However, metabolites derived from the GSH-conjugates of CLZ were not detected in urine and feces in the studies of Dain *et al.* (*13*) and Schaber *et al.* (*16*). In contrast, four different thioethers of CLZ were previously detected by GC-MS in basic extracts of a large volume of urine of CLZ-treated patients (*11*). Two of the identified thioether metabolites, 8-methylthio-deschloroclozapine (8-CH_3S-CLZ) and 8-methylthio-deschloro-demethylclozapine (8-CH_3S-DMCLZ) which can be rationalized as degradation products of 8-deschloro-8-glutathionyl-clozapine (8-GS-CLZ) and 8-deschloro-8-glutathionyl-desmethylclozapine (8-GS-DMCLZ), respectively, which are formed via the chlorine-substitution of the nitrenium ions of CLZ and DMCLZ (*17*). Two minor thioethers corresponded to methylthio- and methylsulfone-conjugates resulting from an addition reaction of GSH to the nitrenium ion of CLZ, most likely at the 6-position since this is the major GSH-conjugate found in *in vitro* incubations (*6*). Previously, we showed that the chlorine-substitution reaction of the CLZ nitrenium ion is fully dependent on the presence of glutathione S-transferases (hGSTs) (*17*). Therefore, genetically determined deficiency of hGSTs or drug-drug interactions at the level of hGST should be considered as possible risk factors for CLZ-induced toxicity. In analogy, clinical association studies suggest that

the combined GSTM1-T1 double-null genotype leads to an increased susceptibility to idiosyncratic drug-induced liver injury (*18-20*).

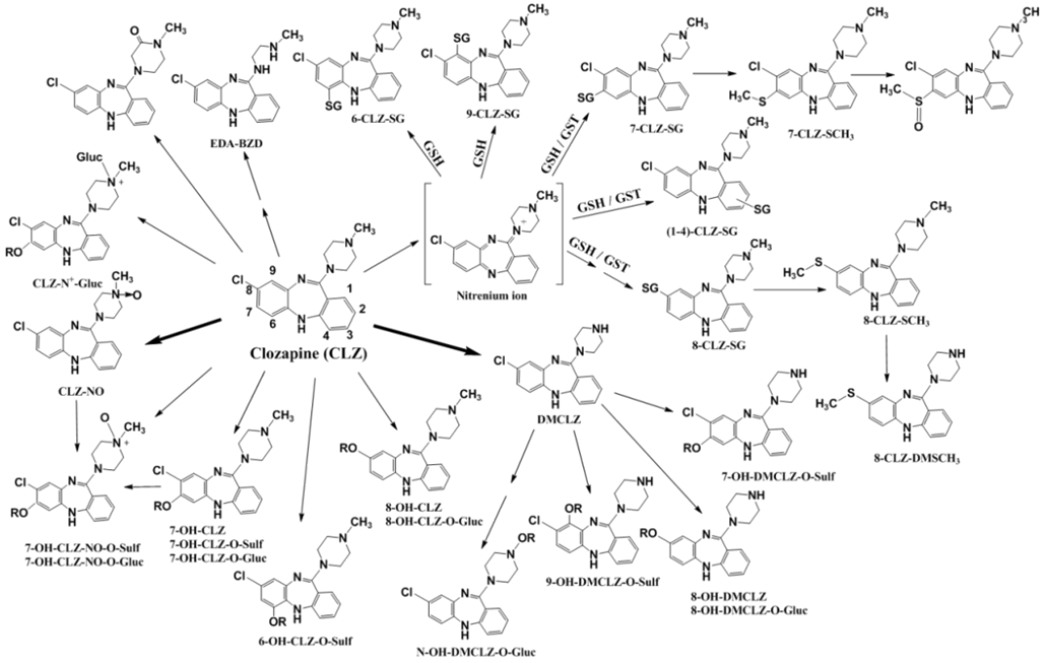

Figure 1. Metabolic pathways and clozapine metabolites identified and characterized in humans (adapted from ref. (*13–15*)). Bioactivation of CLZ to reactive nitrenium ion is depicted in brackets, the structures of GSH adducts identified *in vitro*, and role of hGSTs are presented as described in ref. (*19*). Conjugates formed by further metabolism of GSH conjugates that were described by Stock et al. (*13*) are also depicted in this scheme.

The aim of the present study was to investigate the variability in excretion of both stable and GSH-related metabolites in urine of 34 patients treated daily with CLZ. Twenty eight of these patients were genotyped with respect to their status of hGSTM1, hGSTT1, hGSTP1 and hGSTA1. Because GSH-conjugates undergo extensive catabolism by biliary, renal and intestinal enzymes (*21*), anticipated urinary metabolites of GSH-conjugates were the N-acetylcysteine conjugates (mercapturic acids), cysteine conjugates, methylthio-conjugates and the corresponding sulfoxides and sulfones of CLZ and its major phase I-metabolites DMCLZ and CLZ-NO. To enable identification of the urinary thioethers, each of the five GSH-conjugates that can be formed from the CLZ-nitrenium ion, both non-enzymatically and enzymatically (*10*), were converted to the corresponding degradation products which might be expected in urine. In addition, we studied the variability in CLZ metabolism and bioactivation in precision-cut liver slices (PCLS) of a panel of fourteen liver donors, which were also genotyped with respect to their hGST-status. PCLS represent a complex *in vitro* model which contain all cell types in their

natural environment and their metabolic profiles correlates well with *in vivo* data (*22–24*). Finally, a preliminary association study was performed to investigate the role of genetic polymorphism of four hGST as risk factor for CLZ-induced agranulocytosis.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade and obtained from standard suppliers. 7-OH and 9-OH CLZ were prepared by the modified Udenfriend reaction as described previously (*25*).

2.2. Synthesis of anticipated GSH-related metabolites of CLZ

GSH conjugates of CLZ were produced on a preparative scale by large-scale incubation using CYP102A1 M11H Phe87 mutant as a biocatalyst, and isolated by preparative HPLC as described previously (*26*). To obtain the corresponding cysteine conjugates, each GSH-conjugate was incubated overnight at 37 °C in presence of 4.4 U γ-glutamyltransferase (GGT) in potassium phosphate buffer (100 mM, pH 7.4) and in a final volume was 250 µL. The reactions were terminated by adding 25 µL of 10% ice cold $HClO_4$ and centrifuged for 15 minutes at 14.000 rpm. The supernatant was analyzed by the LC/MS/MS method described below. Using this condition the GSH-conjugates were quantitatively converted to the corresponding cysteine S-conjugates. The intermediate cysteinylglycine conjugates were only observed with shorter incubation times [data not shown]. To prepare the possible N-acetylcysteine conjugates of CLZ, 50 µL of each cysteine S-conjugate was dissolved in 2 mL icecold 2 N ice cold sodium hydroxide solution (*27*). With intervals of 10 minutes, 20 µL of acetic anhydride was added to the solution, stirred for 30 seconds and put back on ice. When the pH reached <7, it was allowed to stand on room temperature to complete the acetylation reaction. The samples were dried overnight under a nitrogen stream and redissolved in water for analysis by LC-MS/MS.

To prepare the thiolmethylconjugates, the cysteine S-conjugates were first converted to thiols using a non-enzymatic model for cysteine conjugate beta-lyase (*28*). The cysteine S-cysteine conjugates were incubated overnight in 50 mM borate buffer (pH=8.6) in presence of 167 µM pyridoxal hydrochloride and 67 µM $CuSO_4$. For synthesis of the thiolmethyl conjugates 33 mM methyl iodide in acetonitrile was added to methylate the thiol-group. The reaction was stopped with 10% ice cold $HClO_4$ (1/10 of the volume) and LC-MS/MS samples were taken to confirm the formation of the conjugates. The characteristics (retention times, exact mass and major fragments) of the prepared references of the thioethers of CLZ were used for the identification of conjugates in hPCLS and urine samples.

2.3. Collection of urine samples from clozapine-treated patients

Single-void (spot) urine samples were obtained from 34 schizophrenic patients on CLZ treatment under the care of the psychiatrist of the High Care Clinics of Rivierduinen. Average daily dose of CLZ was 530 ± 250 mg/day. All patients (31 male; 3

female) had reached steady state plasma levels of CLZ and DMCLZ-levels of 0.41 ± 0.17 and 0.26 ± 0.11 mg/L, respectively. Urine samples were collected at unspecified times with the patients' agreement. Solid phase extraction (SPE) was used for clean-up and concentration of the urine samples. 3 mL samples of urine were loaded to Strata X C18 solid-phase extraction column (200 mg/3mL; Phenomenex). The columns were washed with 3 mL of water to remove salts and proteins. CLZ and its metabolites were subsequently eluted using 2 ml of methanol. Samples were evaporated to dryness under nitrogen gas and reconstituted with 300 µL of 50:50 (v:v) methanol and water. The reconstituted solutions were analysed by LC/MS-MS system, as described below. The flow through and washing steps were checked to confirm that all CLZ-related metabolites were retained on the SPE-column.

2.4. Incubations of clozapine with human precision-cut liver slices (hPCLS)

Pieces of human liver tissue were obtained from fourteen patients (age range 17-76 years; 4 male, 10 female) undergoing partial hepatectomy for the removal of carcinoma or from liver tissue remaining as surgical waste after split liver transplantation, as described previously (*29*). The experimental protocols were approved by the Medical Ethical Committee of the University Medical Center Groningen.

The hPCLS were prepared using a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) as described previously (*30*). In ice-cold Krebs-Henseleit buffer saturated with carbogen (95% O_2 and 5% CO_2). hPCLS (5 mm diameter, 200-300 µm thick and ca. 4.5-5.5 mg wet weight) were stored in ice-cold UW solution until incubation.

CLZ was incubated with hPCLS in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria) as described previously (*30*). The hPCLS were pre-incubated at 37°C for 1h individually in a well containing 1.3 mL Williams' medium E with glutamax-1 (Gibco, Paisley, UK), supplemented with 25 mM D-glucose and 50 µg/ml gentamicin (Gibco, Paisley, UK) (WEGG medium) in a 12-well plate with shaking (90 times/min) under saturated carbogen atmosphere. After pre-incubation, hPCLS were transferred to fresh WEGG medium in the presence of 60 µM CLZ and incubated further for 24h. This CLZ concentration does not cause serious toxicity, viability higher than 80%.

Control incubations were performed in absence of CLZ. hPCLS and their corresponding media were collected and sonicated together to disrupt the tissues or cells. 10 % of the sample volume of ice-cold 10 % $HClO_4$ was added to precipitate the proteins. Samples were then centrifuged for 15 min on 14,000 rpm. Supernatants were filtered through membrane filters before HPLC-UV and/or LC-MS/MS analysis.

2.5. Genotyping of hGSTs

From 38 patients, including the 34 patients who provided urine samples, whole blood samples were obtained for GSTs genotyping during the regular checking of the patients. Seven of the patients had a history of agranulocytosis. The presence of at least one GSTM1 and/or GSTT1 allele was determined as described by Arand et al. (*31*), with minor modification. Briefly, 10 ng of DNA was taken to amplify representative sequences of the genes of GSTT1, GSTM1 and albumine (as household gene). Hotstart PCR mastermix was used from Qiagen (Venlo, The Netherlands) and PCR program was as

follows: 15 minutes at 95°C, 35 cycles of 95°-55°-72° each step for 30 seconds, followed by a final extension of 10 minutes at 72°C. The PCR products of the GSTT1 and GSTM1 alleles were detected separately by agarose gel electrophoresis. At least 5% of patient samples were duplicated in the analysis and no inconsistencies were observed.

The rs1695 genotype of GSTP1-1 (mutation A315G; Ile105Val) was determined by allele-specific PCR using a predesigned Taqman assay from LifeTechnologies (Nieuwerker a/d IJssel, the Netherlands) and analysed on 7500 real time PCR system. The presence of GSTA1 C-69T was determined by pyrosequencing on a Pyrosequencer 96MA (Oiagen, Venlo, The Netherlands) with PCR primers; 5'-AGTAGGTGGCCCCTTGGC-3' (forward) and 5'-TGTCACCGTCCTGGCTCGAC-3.' (reverse; biotinylated) Sequence primer was: forward 5'- GGCTTTTCCCTAACTTGAC-3.' Sequence to analyse was C/TCTTCTTTCA and dispensation order was GTCGTCTCA.

2.6. Analytical methods and metabolites quantification

The prepared samples from urine and hPCLS incubations were analyzed by LC-MS/MS using a Luna 5 μm C18 column (150 mm × 4.6 mm i.d.; Phenomenex), protected by a 4.0 mm × 3.0 mm i.d. security guard (5 μm) C18 guard column (Phenomenex, Torrance, CA). The gradient used was constructed by mixing the following mobile phases: solvent A (1% acetonitrile, 99% water, and 0.2% formic acid) and solvent B (99% acetonitrile, 1% water, and 0.2% formic acid). The first 5 min were isocratic at 0% solvent B; from 5 to 30 min, the concentration of solvent B linearly increased to 100%; from 30 to 35 min, there was a linear decrease to 0% B, and it was maintained at 0% for re-equilibration until 40 min. The flow rate was 0.5 mL/min. Samples were injected at an injection volume of 50 μL.

For the identification of metabolites, a hybrid quadrupole-time-of-flight (Q-TOF) Agilent 6520 mass spectrometer was used, equipped with an electrospray ionization (ESI) source and operating in the positive mode (Agilent Technologies, Waldbronn, Germany). The MS ion source parameters were set with a capillary voltage at 3500 V; nitrogen was used as the desolvation and nebulizing gas at a constant gas temperature of 350 °C; drying gas, 8 L/min; and nebulizer, 40 psig. Nitrogen was used as a collision gas with a collision energy of 25 V. MS spectra were acquired in full scan analysis over an m/z range of 50-1000 using a scan rate of 1.003 spectra/s. The MassHunter Workstation Software (version B.02.00) was used for system operation and data collection. Data analysis was performed using Agilent MassHunter Qualitative analysis software.

For quantification of the GSH-related conjugates in urine, a standard curve of CLZ based on UV peak areas was used to estimate the concentrations of the synthesized reference metabolites, assuming that the extinction coefficients are equal to that of CLZ. The obtained concentrations of the synthesized conjugate references, based on UV-peak areas, were then correlated with the corresponding EIC peaks. This was used to determine the concentrations of conjugates measured in urine based on measured EIC peak areas.

The levels of measured conjugates in urine were normalized by the corresponding levels of creatinine in the urine samples. Urinary creatinine was measured using a Creatinine (urinary) Assay Kit (Cayman Chemical, USA) as described in the protocol.

3. Results

3.1. Genotyping of human liver donors and schizophrenic patients treated with clozapine

Table 1 shows the results of the genotyping of the 12 out of 14 liver samples used for the slice experiments and the blood samples of 38 CLZ-treated patients with and without history of CLZ-induced agranulocytosis. Overall, the frequencies of the different genotypes corresponded well with those previously reported for the Caucasian population (*32-34*).

Table 1. Genotypes of alleles of polymorphic glutathione S-transferases of human liver donors and schizophrenic patients treated with and without history of clozapine-induced agranulocytosis.

Genotype		All samples number (%)	Liver donors number (%)	All patients number (%)	Patients without agranulocytosis number (%)	Patients with agranulocytosis number (%)
Total		50 (100)	12 (100)	38 (100)	31 (100)	7 (100)
GSTM1						
	Positive	22 (44)	6 (50.0)	16 (42.1)	13 (41.9)	3 (42.9)
	Null	28 (56)	6 (50.0)	22 (57.9)	18 (58.1)	4 (57.1)
GSTT1						
	Positive	41 (82)	8 (66.7)	33 (86.8)	29 (90.6)	4 (66.7)
	Null	9 (18)	4 (33.3)	5 (13.2)	3 (9.4)	2 (33.3)
GSTM1/T1						
	Double null	7 (14)	3 (25.0)	4 (10.5)	1 (3.2)	3 (42.9)
GSTA1						
	C/C	13 (26)	5 (41.7)	8 (21.0)	5 (16.1)	3 (42.9)
	C/T	26 (52)	5 (41.7)	21 (55.3)	17 (54.8)	4 (57.1)
	T/T	11 (22)	2 (16.7)	9 (23.7)	9 (29.1)	0 (0)
GSTP1						
	A/A (Ile/Ile)	33 (66)	9 (75.0)	24 (63.2)	20 (64.5)	4 (57.1)
	A/G (Ile/Val)	15 (30)	3 (25.0)	12 (31.6)	10 (32.2)	2 (28.6)
	G/G (Val/Val)	2 (4)	0 (0)	2 (5.2)	1 (3.2)	1 (14.3)

Although only seven patients had a history of CLZ-induced agranulocytosis, some remarkable differences in the distribution of genotypes were observed. Three of the seven patients with agranulocytosis showed a double null genotype for GSTM1 and GSTT1, whereas this combination of null alleles was observed in only one of the 31 patients without agranulocytosis, Table 1. Also, the T/T-genotype of GSTA1 was absent in the susceptible patients, whereas this genotype had a frequency of 29% in the group of the nonsusceptible patients. Although this study suggests that certain genotypes of hGSTs might be at increased risk for CLZ-induced agranulocytosis, this preliminary association study requires validation in a much larger case/controlled association study.

3.2. Metabolism of CLZ by precision-cut human liver slices

In vitro metabolism of CLZ was determined after 24h of incubation CLZ with precision-cat liver slices (PCLS) from 14 different liver donors. By using mass spectrometry in total 46 metabolites of CLZ could be identified tentatively, see Table 2, based on the extracted ion chromatograms (EIC) of their exact masses, the presence of a mono-chlorine isotope cluster, and the fragmentations patterns described previously (*11,16*). Thirty one metabolites could be found in all incubations, whereas fifteen of the metabolites were absent in one or more of the incubations, see Table 2. Consistent with *in vitro* incubations of CLZ with HLM (*10*), DMCLZ and CLZ-NO were the major metabolites in all slice incubations; a representative UV-chromatogram with detection at 254 nm is shown in Figure 2. The only other metabolites which were clearly detectable by UV-detection all appeared to correspond to GSH-related metabolites resulting from bioactivation of CLZ. The minor metabolites could only be detected by LC-MS/MS by analyzing EICs of known and anticipated metabolites.

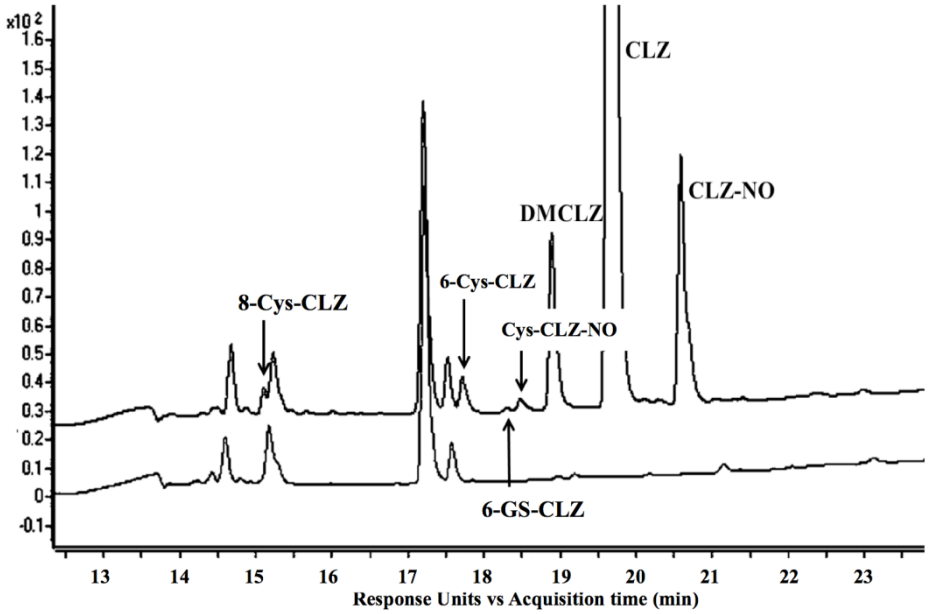

Figure 2. HPLC-UV chromatogram showing CLZ metabolites formed in 24h incubations of CLZ with human precision-cut liver slices. Lower line presents control: medium incubated with liver slice under the incubation conditions without addition of CLZ.

Figure 3 shows the relative amounts of the major metabolites DMCLZ and CLZ-NO and the total of GSH-related metabolites identified in the incubations with slices of 14 individuals. Based on the peak areas of the UV-chromatograms, the bioactivation pathway of CLZ was estimated to range from 4.7 to 13.4% of the total of CLZ-metabolism

in PCLS. Since the aim of the study was to characterize the variability of the bioactivation pathway of CLZ specifically, only the identification of GSH-related metabolites will be discussed in more detail.

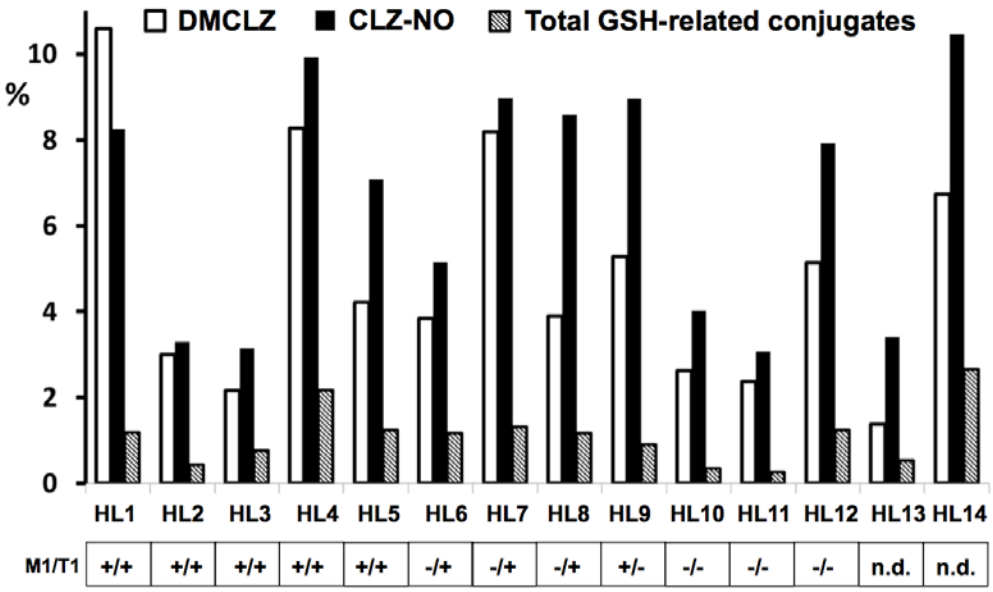

Figure 3. Metabolism of CLZ by different individual hPCLS. Amounts of DMCLZ, CLZ-NO, and total of GSH related conjugates, measured as UV peak areas, are presented as percentage of substrate conversion (60 μM) during 24h incubation.

As shown in Table 2, in total fifteen metabolites in the slice incubations are derived from GSH-conjugates formed from bioactivation of CLZ and its major metabolites DMCLZ and CLZ-NO to their corresponding nitrenium ions. Based on their identity with the synthesized references listed in Table S1, nine of the conjugates appeared to be derived from the GSH-conjugates of the CLZ-nitrenium ion. Although all five cysteine S-conjugates of CLZ could be found in varying amounts, the only intact GSH-conjugate found at small amounts was 6-GS-CLZ, indicative for a very extensive catabolism of the GSH-conjugates formed in the slice incubations.

In addition to the cysteine S-conjugates, three of the synthetical methylthio-conjugates of CLZ, 8-CH_3S-CLZ, 6-CH_3S-CLZ-SCH_3 and 7-CH_3S-CLZ could be positively identified in all slice incubations. No N-acetylcysteine-conjugates of CLZ could be identified in any of the incubations, although the mercapturic acid pathway has been shown as an intrahepatic process (*35*).

Table 2. Metabolites of CLZ identified in incubations with human precision-cut liver slices and in excreta of schizophrenic patients treated daily with CLZ.

m/z $[M+H]^+$	Elemental composition	Assigned metabolites	This study Liver slices [a]	This study Urine [b]		Previous studies [c]
GSH-Related metabolites						
325,15	$C_{18}H_{20}N_4S$	8-CH_3S-DMCLZ		0.57 ± 0.41	(33 of 34)	*(i)*
339,16	$C_{19}H_{22}N_4S$	8- CH_3S-CLZ	++	0.20 ± 0.20	(30 of 34)	*(i)*
359,11	$C_{18}H_{19}ClN_4S$	CH_3S-DMCLZ-1 [d]	++ (13 of 14)	0.18 ± 0,11	(32 of 34)	
359,11	$C_{18}H_{19}ClN_4S$	CH_3S-DMCLZ-2 [d]	++	0,56 ± 0,34	(33 of 34)	
359,11	$C_{18}H_{19}ClN_4S$	CH_3S-DMCLZ-3 [d]	+	0.28 ± 0.15	(33 of 34)	
373,13	$C_{19}H_{21}ClN_4S$	6-CH_3S-CLZ	+	0.18 ± 0.54	(4 of 34)	*(i)*
373,13	$C_{19}H_{21}ClN_4S$	7-CH_3S-CLZ	+			
405,17	$C_{19}H_{21}ClN_4O_2S$	(6,7)-CH_3SO_2-CLZ [d]		0.28 ± 0.25	(33 of 34)	*(i)*
412,18	$C_{21}H_{25}N_5O_2S$	8-Cys-CLZ	++	0.71 ± 0.49		
432,12	$C_{20}H_{23}N_5O_2S$	Cys-DMCLZ [d]	+	0.32 ± 1.29	(30 of 34)	
446,14	$C_{21}H_{24}ClN_5O_2S$	(1-4)-Cys-CLZ [d]	+			
446,14	$C_{21}H_{24}ClN_5O_2S$	6-Cys-CLZ	++	0.12 ± 0.09	(32 of 34)	
446,14	$C_{21}H_{24}ClN_5O_2S$	7-Cys-CLZ	+			
446,14	$C_{21}H_{24}ClN_5O_2S$	9-Cys-CLZ	+			
462,14	$C_{21}H_{24}ClN_5O_3S$	Cys-CLZ-NO [d]	++	0.29 ± 0.19	(31 of 34)	
618,22	$C_{27}H_{33}ClN_7O_6S$	GS-DMCLZ [d]	+ (13 of 14)			
632,20	$C_{28}H_{34}ClN_7O_6S$	6-GS-CLZ	++			
Phase I Metabolites						
287,11	$C_{15}H_{15}ClN_4$	CLZ-C_3H_4	++	0.69 ± 0.33	(32 of 34)	
295,16	$C_{17}H_{18}N_4O$	8-OH-DMCLZ-1	++ (13 of 14)	26 ± 5		*(i,ii,iii)*
295,16	$C_{17}H_{18}N_4O$	8-OH-DMCLZ-2		4.2 ± 0.9	(33 of 34)	
301,12	$C_{16}H_{17}ClN_4$	EDA-BZD (CLZ-C_2H_2)	++	0.34 ± 0.21	(33 of 34)	*(ii: 1 of 3)*
309,17	$C_{17}H_{18}N_4O$	8-OH-CLZ-1	++	3.2 ± 1.1		*(i, iii)*
309,17	$C_{17}H_{18}N_4O$	8-OH-CLZ-2		0.45 ± 0.23		
313,12	$C_{17}H_{17}ClN_4$	DMCLZ	+++	8.6 ± 4.0		*(i,ii,iii)*
329,12	$C_{17}H_{17}ClN_4O$	OH-DMCLZ-1 [d]	+ (5 of 14)	0.58 ± 0.25	(33 of 34)	
329,12	$C_{17}H_{17}ClN_4O$	OH-DMCLZ-2 [d]	+ (12 of 14)	1.2 ± 0.5	(33 of 34)	
329,12	$C_{17}H_{17}ClN_4O$	OH-DMCLZ-3 [d]	+ (12 of 14)	1.5 ± 0.8		
329,12	$C_{17}H_{17}ClN_4O$	DMCLZ-NO-1 [d]	++ (10 of 14)	0.06 ± 0.09	(22 of 34)	
329,12	$C_{17}H_{17}ClN_4O$	DMCLZ-NO-2 [d]	++	0.62 ± 0,59	(33 of 34)	
341,12	$C_{18}H_{17}ClN_4O$	2-keto-CLZ	++			*(i)*
343,13	$C_{18}H_{19}ClN_4O$	6-OH-CLZ	+	0.72 ± 0.58	(32 of 34)	
343,13	$C_{18}H_{19}ClN_4O$	7-OH-CLZ	+ (13 of 14)	0.52 ± 0.22	(31 of 34)	*(ii)*
343,13	$C_{18}H_{19}ClN_4O$	9-OH-CLZ	+	0.23 ± 0.13	(32 of 34)	
343,13	$C_{18}H_{19}ClN_4O$	CLZ-NO	+++	9.2 ± 3.1		*(i,ii,iii)*
345,14	$C_{17}H_{17}ClN_4O_2$	OH-DMCLZ-NO [d]	++	0.03 ± 0.04	(22 of 34)	
Phase II Metabolites						
409,07	$C_{17}H_{17}ClN_4O_4S$	7-OH-DMCLZ-O-Sulf [e]	+ (13 of 14)	4.6 ± 1.6		*(ii,iii)*
409,07	$C_{17}H_{17}ClN_4O_4S$	9-OH-DMCLZ-O-Sulf				*(ii,1 of 3)*
423,09	$C_{18}H_{19}ClN_4O_4S$	6-OH-CLZ-O-Sulf				*(ii,1 of 3)*
423,09	$C_{18}H_{19}ClN_4O_4S$	7-OH-CLZ-O-Sulf [e]	+	1.7 ± 0.9	(32 of 34)	*(ii)*
439,08	$C_{18}H_{19}ClN_4O_5S$	7-OH-CLZ-NO-O-Sulf [d]	+	0.56 ± 0.27	(33 of 34)	*(ii, 2 of 3)*
471,19	$C_{23}H_{26}N_4O_7$	8-OH-DMCLZ-O-Gluc	+ (10 of 14)	9.9 ± 3.1		*(ii,1 of 3)*
485,20	$C_{24}H_{28}N_4O_7$	8-OH-CLZ-O-Gluc-1	+ (6 of 14)	2.0 ± 1.2		*(ii,1 of 3)*
485,20	$C_{24}H_{28}N_4O_7$	8-OH-CLZ-O-Gluc-2		2.7 ± 0.9		
503,17	$C_{24}H_{28}ClN_4O_6$	CLZ-N+-Gluc-1	++	1.2 ± 0.7		*(ii, iii, iv)*
503,17	$C_{24}H_{28}ClN_4O_6$	CLZ-N+-Gluc-2	+	0.78 ± 1.43		*(ii, iv)*
505,15	$C_{23}H_{25}ClN_4O_7$	OH-DMCLZ-O-Gluc-1 [d]	+ (8 of 14)	3.3 ± 1.2		
505,15	$C_{23}H_{25}ClN_4O_7$	OH-DMCLZ-O-Gluc-2 [d]	+ (8 of 14)	0.33 ± 0.15		
505,15	$C_{23}H_{25}ClN_4O_7$	N-OH-DMCLZ-O-Gluc-1	++	2.2 ± 1.0		*(ii: 2 of 3)*
505,15	$C_{23}H_{25}ClN_4O_7$	N-OH-DMCLZ-O-Gluc-2	++	2.2 ± 1.2		
519,16	$C_{24}H_{27}ClN_4O_7$	7-OH-CLZ-O-Gluc [e]	++ (13 of 14)	1.8 ± 0.7	(32 of 34)	*(ii:1 of 3)*
519,16	$C_{24}H_{27}ClN_4O_7$	OH-CLZ-O-Gluc [d]	+	0.64 ± 0.27	(32 of 34)	
521,14	$C_{23}H_{25}ClN_4O_8$	OH-DMCLZ-NO-Gluc [d]	++	1.6 ± 1.0		
535,16	$C_{24}H_{27}ClN_4O_8$	7-OH-CLZ-NO-O-Gluc [e]	+ (11 of 14)	1.8 ± 0.8	(33 of 34)	*(ii: 1 of 3)*

a) +/++/+++ represent relative amount in slice incubation.
b) Values represent average peak area of extracted ion chromatogram relative to total of peak areas of all metabolites;
c) References: *(i)* Stock et al., 1977; *(ii)* Schaber et al. 2001; *(iii)* Dain et al., 1997; (*iv*) Breyer-Pfaff, 2001.
d) Position of substituent was not characterized.
e) Position of substituent was assigned based on previously identified metabolites by Dain et al. and Schaber et al.

The remaining GSH-related metabolites identified in the slice incubations result from the nitrenium ions of DMCLZ and CLZ-NO. In 13 of the 14 slices, a GSH adduct with a m/z value of 618,22 ($[M+H]^+$) was observed which corresponds to a GSH adduct of DMCLZ, as described previously (*10*, *36*). Also, a cysteine conjugate of DMCLZ, with a m/z value 432,12 ($[M+H]^+$), and four methylthio-conjugates of DMCLZ were found. One S-methyl conjugate with m/z 339,16 ($[M+H]^+$) results from the substitution of chlorine of the nitrenium ion of DMCLZ, and could be assigned as 8-CH_3S-DMCLZ. The other three thiomethyl-conjugates, all having a mono-chlorine isotope pattern and m/z value of 359,11 ($[M+H]^+$), can be rationalized from initial addition of GSH to the DMCLZ-nitrenium ion. The exact position of the substituents, however, could not be assigned because of the lack of synthetical references. A cysteine S-conjugate with m/z 462,14 appears to be the only GSH-related conjugate resulting from bioactivation of CLZ-NO and could even be identified by UV-detection, Figure 2. The position of the cysteine-moiety, however, could not be established based on the fragmentation pattern.

3.3. Interindividual Variability in CLZ bioactivation by Human Liver Slices

Table 3 shows the relative amounts of the individual GSH-related conjugates identified in the incubations of CLZ with PCLS of the fourteen liver donors. The relative amounts are based on the peak areas of the EICs of their exact masses. Although these peak areas not necessarily reflect the absolute abundance of different metabolites, comparison of the peak areas of the same metabolite between the different slice incubations reflects the interindividual variability by which each of them are produced. In all incubations, 8-Cys-CLZ, 6-Cys-CLZ and one of the thiomethyl-conjugates of DMLZ, CH_3S-DMCLZ-2, appeared to be formed in highest abundance, with an approximately 3-fold variability for each of them.

For twelve of the fourteen liver slices, the genotypes of the hGSTM1-1 and hGSTT1-1 were determined in order to investigate whether deficiency of these hGSTs leads to altered levels of GSH-related metabolites. However, according to the results presented in Table 3, no significant differences were observed between the different groups of genotypes, when comparing the average amounts of each GSH-related metabolite.

To analyze the similarity of the profiles of the GSH-related metabolites of the different liver slices, the correlations between the profiles were obtained by pairwise plotting the amounts of individual metabolites of two individuals along the x- and y-axis. As shown in Table 4, only slices from donor **HL5**, **HL8**, **HL9** and **HL12** showed very similar profiles of metabolites, as indicated by r^2-values higher than 0,9. However, the fact that each of these individuals had a different combination of genotype of hGSTs, indicate that the genotype of hGST has no predictive value for the profile of GSH-related metabolites. This can most likely be explained by the fact that also differences in activity of the bioactivation enzymes, and enzymes involved in the catabolism of the GSH-conjugates contribute to the differences in metabolic profiles between different individuals. However, no information is available on the activity of CYP3A4 in the slices of the different liver donor.

Table 3. Relative amounts of GSH related conjugates of clozapine in incubations of precision-cut slices from 14 individual human livers.[a]

	HL1	HL2	HL3	HL4	HL5	HL6	HL7	HL8	HL9	HL10	HL11	HL12	HL13	HL14	Average ± SD
M1/T1-genotype	+/+	+/+	+/+	+/+	+/+	-/+	-/+	-/+	+/-	-/-	-/-	-/-	n.d.	n.d.	
6-GS-CLZ	1.1	2.1	2.0	1.9	2.7	4.2	2.5	1.5	1.7	1.3	5.4	2.9	2.0	5.4	2.6 ± 1.4
6-Cys-CLZ	19.4	30.6	22.7	20.0	16.7	17.7	11.6	14.5	16.0	23.8	18.4	17.2	17.0	26.0	19.4 ± 5.0
7-Cys-CLZ	2.5	4.5	9.6	7.6	2.8	3.7	0.6	2.0	9.2	1.8	1.9	2.0	1.2	6.7	4.0 ± 3.0
8-Cys-CLZ	26.1	10.3	20.6	24.2	20.5	30.2	43.0	29.0	22.6	11.7	15.4	25.6	28.4	27.0	23.9 ± 8.3
9-Cys-CLZ	1.5	1.7	1.1	1.8	0.9	2.6	1.2	1.4	1.6	2.2	1.4	1.2	0.9	1.3	1.5 ± 0.5
2/3-Cys-CLZ	1.9	0.7	0.9	0.9	0.8	0.8	0.9	1.5	0.9	0.4	1.1	1.1	0.2	1.8	1.0 ± 0.5
6-CH_3S-CLZ	2.3	2.6	0.5	1.1	1.5	0.5	0.4	1.3	0.5	1.0	1.8	1.2	0.7	0.7	1.2 ± 0.7
7-CH_3S-CLZ	0.5	0.4	0.8	0.9	1.7	1.3	0.4	1.6	0.6	1.5	0.7	0.3	0.6	1.0	0.9 ± 0.5
8-CH_3S-CLZ	7.2	7.2	9.0	2.1	7.2	4.3	2.3	6.2	2.8	17.1	12.3	4.0	11.2	1.9	6.8 ± 4.4
GS-DMCLZ	0.2	0.1	0.1	1.0	0.2	0.7	0.7	0.5	0.2	0.1	n.d.	0.2	0.2	0.4	0.3 ± 0.3
Cys-DMCLZ	2.3	1.0	0.9	10.7	2.7	4.7	9.0	1.9	3.0	1.1	0.5	4.2	0.7	6.6	3.5 ± 3.2
CH_3S-DMCLZ-1	5.8	3.0	1.7	3.8	4.4	2.0	4.3	3.1	5.7	2.8	5.0	5.8	n.d.	3.2	3.6 ± 1.7
CH_3S-DMCLZ-2	10.7	25.5	23.3	15.4	25.3	20.5	16.4	23.9	28.9	26.0	31.3	25.7	34.3	11.9	22.8 ± 7.0
CH_3S-DMCLZ-3	1.1	3.3	5.1	2.0	4.1	2.8	1.7	3.5	4.1	2.3	4.0	1.9	1.1	1.8	2.8 ± 1.3
Cys-CLZ-NO	17.4	7.0	1.8	6.6	8.6	4.0	5.0	8.2	2.1	6.9	1.0	6.8	1.5	4.4	5.8 ± 4.2

a. Numbers represent percentages peak areas of EICs of specific metabolites relative to the sum of peak areas of total GSH-related metabolites.

Table 4. Analysis of the similarity of profiles of GSH-related metabolites of CLZ in incubations of slices obtained from 12 genotyped liver donors.[a]

Liver donor (GSTM1/T1)	**HL1** (+/+)	**HL2** (+/+)	**HL3** (+/+)	**HL4** (+/+)	**HL5** (+/+)	**HL6** (-/+)	**HL7** (-/+)	**HL8** (-/+)	**HL9** (+/-)	**HL10** (-/-)	**HL11** (-/-)	**HL12** (-/-)
HL1 (+/+)	1	0,437	0,526	0,711	0,649	0,690	0,650	0,723	0,450	0,434	0,333	0,674
HL2 (+/+)	0,437	1	0,799	0,548	0,736	0,522	0,201	0,528	0,64	0,866	0,762	0,641
HL3 (+/+)	0,526	0,799	1	0,739	0,854	0,808	0,488	0,778	0,873	0,775	0,819	0,821
HL4 (+/+)	0,711	0,548	0,739	1	0,726	0,877	0,756	0,766	0,734	0,448	0,469	0,816
HL5 (+/+)	0,649	0,736	0,854	0,726	1	0,848	0,598	**0,928**	0,889	0,775	0,856	**0,959**
HL6 (-/+)	0,690	0,522	0,808	0,877	0,848	1	0,867	**0,940**	0,83	0,517	0,637	0,927
HL7 (-/+)	0,650	0,201	0,488	0,756	0,598	0,867	1	0,81	0,585	0,224	0,335	0,742
HL8 (-/+)	0,723	0,528	0,778	0,766	**0,928**	**0,940**	0,81	1	0,844	0,584	0,697	**0,957**
HL9 (+/-)	0,450	0,64	0,873	0,734	0,889	0,83	0,585	0,844	1	0,603	0,803	**0,906**
HL10 (-/-)	0,434	0,866	0,775	0,448	0,775	0,517	0,224	0,584	0,603	1	0,862	0,639
HL11 (-/-)	0,333	0,762	0,819	0,469	0,856	0,637	0,335	0,697	0,803	0,862	1	0,774
HL12 (-/-)	0,674	0,641	0,821	0,816	**0,959**	0,927	0,742	**0,957**	**0,906**	0,639	0,774	1

a. Numbers represent r^2-values obtained when plotting relative amounts of GSH-related metabolites of two individuals against x- and y-axis.

3.4. Identification of metabolites of clozapine in urine of patients

As shown in Table 2 in total 44 different CLZ-metabolites could be measured by LC-MS/MS analysis of urine samples obtained from the 34 patients undergoing chronic CLZ therapy. Based on their identity with metabolites observed in the slice incubations, and based on exact masses and fragmentation pattern of CLZ-metabolites described previously *(11, 13, 16),* most could be attributed to primary and secondary phase I and phase II-metabolites of CLZ. In total 39 of the metabolites which were found in incubations of CLZ with liver slices were also found in urine; five metabolites were exclusively found in urine, as described in Table 2. Fifteen of the seventeen phase I and phase II metabolites which were previously identified by Dain et al. and Schaber et al. could be found in urine of all patients (*13, 16).* However, two sulfate conjugates which could previously be found only in one of the three patients (*16*), could not be identified in any of the urine samples.

Consistent with the previous studies (*13, 16),* 8-OH-DMCLZ appeared to be the major urinary metabolite of CLZ in urines of all patients. Interestingly, both 8-OH-CLZ, 8-OH-DMCLZ and their corresponding glucuronides appeared as two chromatographically separated peaks (Table 2). Because no regioisomers are possible for these metabolites, since the hydroxy-group has substituted the chlorine-atom of the benzodiazepine-structure at the 8-position, these peaks might resulted from boat and chair-conformations of the piperazine-ring, as was observed previously in case of ketotifen-metabolites (*38*). Relative to the sum of peak areas of all metabolites combines, the 8-OH-DMCLZ peaks represented 30 ± 5% of the total peak area. Other metabolites showing high peak areas were the glucuronide of 8-OH-DMCLZ (10 ± 3%), CLZ-NO (9 ± 3%), DMCLZ (8 ± 3%) and the sulfate conjugate of 7-OH-DMCLZ, which corresponds to the high urinary levels found previously (*13, 16*).

Interestingly, the levels of several phase I-metabolites are strongly correlated with the levels of phase II metabolites. When plotting the peak areas of the phase I metabolites versus the phase II metabolites of each urine sample, Figure 4, it was observed that the peak areas of 8-OH-CLZ and 8-OH-DMCLZ strongly correlate with the levels of their corresponding glucuronides. Also, the sulfate conjugate of 7-OH-CLZ strongly correlates with the amount of 7-OH-CLZ. The peak area of OH-DMCLZ-3 correlates with the sulfate conjugate of 7-OH-DMCLZ, suggesting that OH-DMCLZ-3 contains the hydroxy-group at the 7-position.

In contrast to the studies of Dain et al. and Schaber et al., in the present study eleven GSH-related metabolites could be identified as minor metabolites resulting from the bioactivation pathway of CLZ and its major metabolites DMCLZ and CLZ-NO. By analyzing the EICs of m/z 446,12, 6-cysteinyl-clozapine (6-Cys-CLZ) could be positively identified in 32 of the 34 urine samples because of co-elution and identical fragmentation with the synthetical reference compound. Only traces of its regioisomers 7-Cys-CLZ and 9-Cys-CLZ could be found but these were too low to integrate [data not shown]. In addition, two thiomethyl-conjugates with m/z of 373,13 and 339,16 could be assigned as 6-CH_3S-CLZ and 8-CH_3S-CLZ, respectively, by comparison with the reference compounds. These two metabolites correspond to two of the four metabolites previously identified by Stock et al. by GC-MS-analysis of basic extracts of urine (*11*). The other two metabolites observed by Stock et al., 8-CH_3S-DMCLZ, and the thiomethylsulfone CH_3SO_2-CLZ, could be identified in 33 of the 34 urine fractions by EICs of their exact masses 325,15 and 405,17.

Four of the five other GSH-related metabolites were also derived from GSH-conjugates of DMCLZ: three metabolites with exact mass of 359,11 most likely correspond to different regioisomers of thiomethyl-conjugates of DMCLZ. A minor metabolite with m/z 432,12 and chlorine-isotope pattern could by assigned as a cysteine S-conjugate of DMCLZ (Cys-DMCLZ).

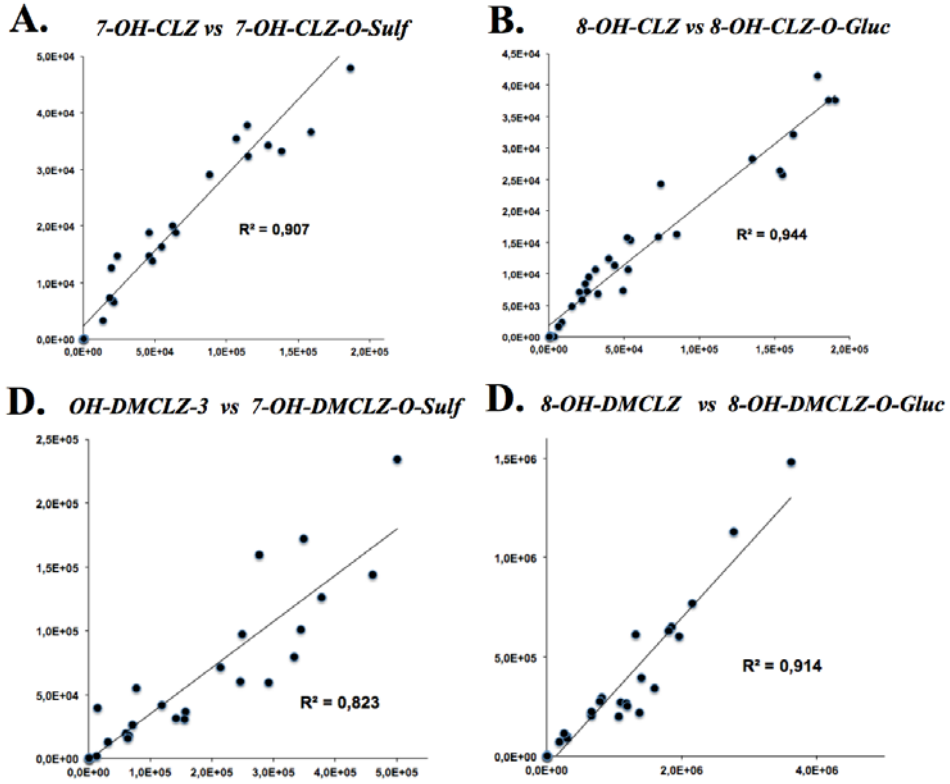

Figure 4. Correlation of urinary excretion of phase I and phase II metabolites of CLZ.
Each dot represent the peak areas of the two metabolites in a single urine sample; the x-axis shows the peak area of the phase II-metabolite; the y-axis the peak area of the phase I-metabolite.

The eleventh GSH-related metabolite had an exact mass of m/z 462,14 and chlorine-isotope pattern, which correspond to an oxygenated cysteine S-conjugate of CLZ. Based on the similar fragmentation of the piperazine-ring when compared to that of CLZ-NO, the structure of this metabolite is most likely of cysteine S-conjugate of the nitrenium-ion of CLZ-NO.

Although N-acetylcysteine S-conjugates were anticipated as end products of the GSH-conjugates of CLZ, we were not able to identify any urinary metabolite corresponding with the five references of N-acetylcysteine S-conjugates of CLZ.

3.4.1. Interindividual Variability in CLZ metabolite profile in human urine

As shown by the relative high standard deviations of the amounts of each metabolites in Table 2 significant variability was observed in the population of CLZ-treated patients, especially of the metabolites that are excreted at relatively low amounts. In Table 5, the relative amounts of the GSH-related metabolites for each patient is tabulated; only the 28 patients are included which were genotyped with respect to their genotypes of four hGSTs.

The profile of GSH-related metabolites, which collectively represent 4% of the sum of peak areas, shows a very large interindividual variability. For the only four patients which excreted 6-CH_3-CLZ, this represented a major metabolite since its peak area was 25% of the total of GSH-related material. Other individuals with remarkable profiles are patients **P8** and **P15**, who excreted a 125 and 30-times higher level of Cys-DMCLZ, respectively, than the other individuals. For **P8**, who had relatively the highest percentage of GSH-related metabolites in urine, this cysteine S-conjugate represented 75% of the total. In urine of patient **P26** only four of the GSH-related metabolites were found.

To further analyze the variability of the metabolic profiles amongst the CLZ-treated patients, for each individual the relative amount of metabolites was plotted against the corresponding averages calculated from 34 individuals.

As demonstrated by two representative examples in Figure 5, generally very good correlations were found when relative peak areas of all metabolites were included in the correlation analysis; correlation coefficients for 22 of the 34 urine samples were higher than 0.9. When excluding the major metabolite 8-OH-DMCLZ, still r^2-values above 0.85 were found [data not shown]. However, when only the GSH-related metabolites were included in the correlation analysis extreme poor correlations were observed, see Figure 5.

Because even patients with the same genotypes of hGST-alleles showed the same level of variability as the total group of patients, it was not possible to recognize a relationship between any genotype, or combination of genotypes, and the total amount of GSH-related metabolites or the profile of GSH-related profiles.

Patients **P27** and **P28** were the only genotyped patients with a history of agranulocytosis and were negative for both the GSTM1- and GSTT1-alleles. Their profile and total levels of both major metabolites, however, did not significantly differ from that of the other patients, due to the large standard deviations.

Table 5. Relative amounts of GSH-related metabolites identified in urine samples of CLZ-treated patients.[a]

Patient	GST-M1	GST-T1	GSTP1	GST-A1	6-Cys-CLZ	8-Cys-CLZ	8-CH_3S-CLZ	6-CH_3S -CLZ	CH_3SO_2-CLZ	Cys-DMCLZ	8-CH_3S -DMCLZ	CH_3S -DMCLZ-1	CH_3S-DMCLZ-2	CH_3S-DMCLZ-3	Cys-CLZ-NO
P1	Pos.	Pos.	A/A	C/T	0.16	0.98	0.12	1.27	0.22	0.08	0.71	0.16	0.61	0.29	0.22
P2	Pos.	Pos.	A/A	C/T	-	0.50	0.19	-	0.24	-	0.72	0.15	0.68	0.19	0.09
P3	Pos.	Pos.	A/A	C/T	0.13	0.61	0.06	-	0.14	0.05	0.35	0.10	0.52	0.25	0.16
P4	Pos.	Pos.	A/A	C/T	0.07	0.44	0.12	-	0.19	0.02	0.20	0.29	0.60	0.28	0.12
P5	Pos.	Pos.	A/A	C/T	0.09	0.34	0.11	1.29	**1.35**	-	0.66	0.15	1.27	0.07	0.25
P6	Pos.	Pos.	A/A	C/T	0.09	0.39	0.10	-	0.13	0.04	0.50	0.24	0.64	0.33	0.22
P7	Pos.	Pos.	A/A	T/T	0.23	1.56	0.45	-	0.08	0.06	0.44	0.25	0.38	0.26	0.12
P8	Pos.	Pos.	A/A	C/C	-	0.94	-	-	0.32	**7.41**	0.40	0.03	0.42	0.18	0.29
P9	Pos.	Pos.	A/G	T/T	**0.47**	**2.10**	-	**2.51**	0.32	0.17	0.13	0.16	0.59	0.11	**0.80**
P10	Null	Pos.	A/A	C/T	0.17	0.84	0.08	-	0.13	0.06	0.40	0.25	0.66	0.58	0.39
P11	Null	Pos.	A/A	C/T	0.08	0.55	0.06	-	0.28	0.03	0.20	0.22	0.57	0.21	0.39
P12	Null	Pos.	A/A	T/T	0.10	0.48	0.60	-	0.73	0.12	**1.79**	0.31	**1.70**	0.55	0.13
P13	Null	Pos.	A/A	T/T	0.07	0.84	0.27	-	0.13	0.02	0.86	0.22	0.28	0.07	0.22
P14	Null	Pos.	A/G	C/T	0,20	0.89	0.15	-	0.19	0.06	0.10	0.20	0.48	0.52	0.55
P15	Null	Pos.	A/G	T/T	0.15	1.13	-	-	0.05	1.76	0.05	**0.43**	0.21	0.47	0.43
P16	Null	Pos.	A/G	C/C	0.05	0.32	0.09	-	0.30	0.03	0.48	0.18	0.60	0.29	0.24
P17	Null	Pos.	A/A	C/T	0.06	0.52	0.14	-	0.12	0.06	1.13	0.11	0.23	0.23	0.32
P18	Null	Pos.	A/A	C/T	0.13	0.34	0.55	-	0.51	0.07	1.09	-	0.52	0.45	-
P19	Null	Pos.	A/A	C/T	0.14	0.58	0.12	-	0.43	0.05	0.94	0.24	0.91	0.17	0.28
P20	Null	Pos.	A/A	T/T	0.20	0.95	0.40	-	0.47	0.14	0.86	0.04	0.67	0.37	0.56
P21	Null	Pos.	A/A	C/C	0.12	0.39	0.13	-	0.06	0.03	0.40	0.23	0.80	0.25	0.23
P22	Null	Pos.	A/G	C/C	0.08	0.30	0.53	-	0.51	0.03	0.67	0.11	0.44	0.20	0.23
P23	Null	Pos.	A/G	C/C	0.16	0.47	**0.73**	-	0.20	0.04	0.75	0.41	0.43	0.39	0.29
P24	Null	Pos.	G/G	C/T	0.09	0.23	0.36	-	0.29	0.03	0.82	0.21	0.55	0.48	0.16
P25	Null	Pos.	A/A	C/T	0.16	0.40	0.14	-	0.46	0.08	0.58	0.23	0.42	0.19	0.29
P26	Pos.	Null	A/G	T/T	-	0.21	0.52	-	0.26	-	1.55	-	-	-	-
P27	Null	Null	A/G	C/T	0.05	0.39	0.17	1.13	0.28	-	0.53	0.19	1.29	0.15	0.11
P28	Null	Null	G/G	C/C	0.07	0.34	0.02	-	0.14	0.03	0.18	0.47	0.63	0.16	0.33
Average (± st.dev.)					0.12 (± 0.09)	0.65 (± 0.43)	0.22 (± 0.21)	0.22 (± 0.59)	0.30 (± 0.26)	0.37 (± 1.42)	0.62 (± 0.42)	0.19 (± 0.11)	0.61 (± 0.35)	0.29 (± 0.15)	0.27 (± 0.17)

a. Numbers represent percentage of peak areas of EIC of each GSH-related metabolite relative to the sum of peak areas of all phase I and phase II metabolites.

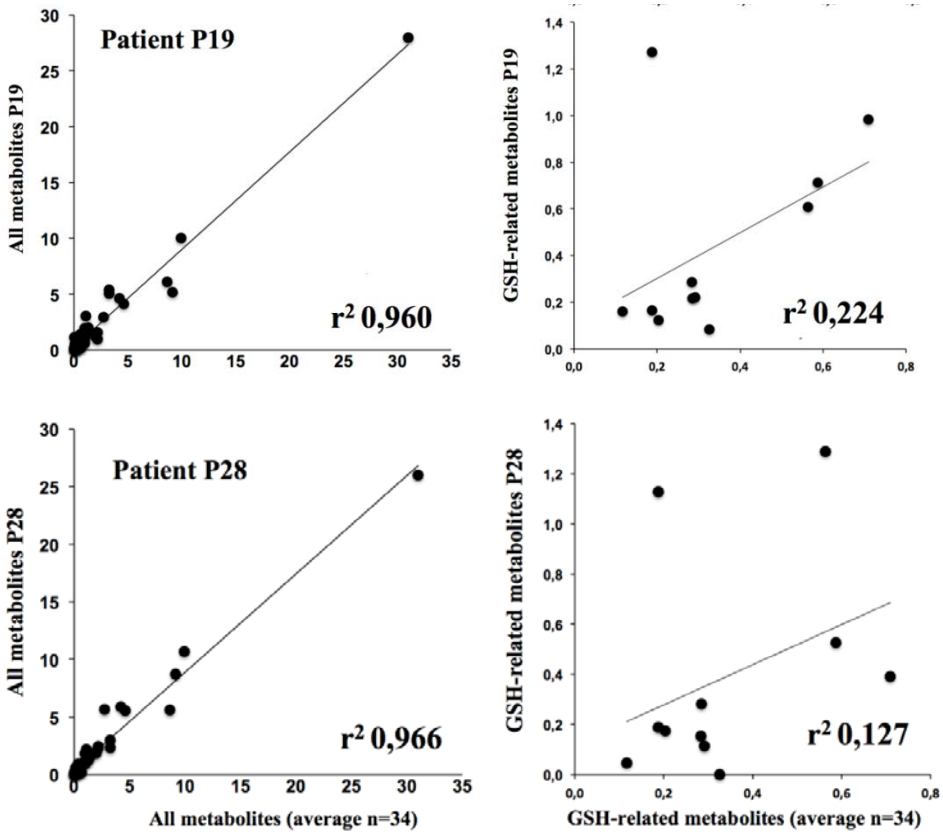

Figure 5. Correlation of profiles of patients P19 (patient without developed agranulocytosis) and P28 (patient developed agranulocytosis) with the average profile calculated from 34 individuals. Each dot represents a specific metabolite; the x-axis shows the average abundance, the y-axis the abundance of patients P19 and P28. The left plots show the correlation obtain when all 44 metabolites were included; the right plots if only percentage of GSH-related metabolites are correlated.

4. Discussion

Bioactivation of CLZ to reactive nitrenium ion by local myeloperoxidases and hepatic P450s is generally accepted as a cause for the agranulocytosis and hepatotoxicity observed in a small percentage of CLZ-treated patients (*5-7)*. It can therefore be hypothesized that patients who are susceptible to these adverse drug reactions have a relative high activity of bioactivation and/or a deficiency in protective mechanisms, such as hGST-deficiency (*36*). Although recently it was demonstrated that bioactivation of CLZ in incubations with HLM from 100 individuals showed an 8-fold difference, so far no information is available on the variability of bioactivation *in vivo* in CLZ-treated patients. The only study in which urinary thioethers derived from GSH-conjugates of CLZ were

identified in urine of CLZ-treated patients, reported three thiomethyl-conjugates and one methylsulfone conjugate (*11*). This study did not provide information on the variability in excretion. In two more recent studies which aimed at the identification of the full spectrum of CLZ-metabolites, 18 different CLZ-metabolites were identified (*13*,*16*). However, no GSH-related metabolites could be identified by the analytical methods used, indicating that these are excreted in low amounts. In the only study in which interindividual variability of urinary metabolites was reported, urines of only three CLZ-patients were analyzed (*16*), and therefore provide little information on the variability of urinary metabolites. Additionally, metabolism of CLZ treated human PCLS has also been described, identifying phase I and II metabolites and suggesting that hPCLS could be a good translational model for CLZ metabolite profile between *in vitro* HLM incubations and *in vivo* urine sample analysis (*39*). Synergistic toxicity was found when mouse and hPCLS were co-incubated with lipopolysaccharide (LPS) and IDILI-associated drugs, having clozapine as an example *(39, 40).* The amounts of bioactivation pathway conjugates, namely glutathione and cysteine conjugates, were significantly lower among the responders (synergistic toxicity observed) in hPCLS incubated with LPS when compared to the ones without LPS but not among the nonresponders (synergistic toxicity observed). Depletion of GSH in the liver, resulting in lower levels of GSH and cysteine conjugates of CLZ is a possible underlying mechanism causing higher toxicity that might be due to the covalent binding of CLZ reactive metabolites to proteins.

The aim of the present study was, therefore, to investigate the variability in CLZ-metabolism by analyzing urine samples of 34 CLZ-treated patients and by incubating CLZ with hPCLS of 14 individuals.

As shown in Table 2, all 23 urinary CLZ-metabolites which have been reported previously (*11-15*), could be identified in the urine samples and/or slice incubations analyzed in the present study. Although not all urine was collected and quantification was based on peak areas of EICs, the present study shows that next to DMCLZ and CLZ-NO, the 7- and 8-hydroxylated metabolites of CLZ and DMCLZ and their corresponding glucuronides and sulfate conjugates represent major metabolites in urine of all patients. In all urine samples, 8-OH-DMCLZ was the most abundant metabolite, consistent with previous studies (*13,16*). Although the major urinary metabolites DMCLZ and CLZ-NO were also identified as major metabolites in the 24 h incubations of CLZ with human PCLS, Figure 2, only small amounts of the 7- and 8-hydroxylated metabolites of CLZ and DMCLZ were formed *in vitro* and could only be detected by LC-MS/MS. These metabolites were also formed in trace amounts in incubations with HLM and recombinant human CYPs (*10*), suggesting that the formation of the 7- and 8-hydroxylated metabolites mainly originate from extrahepatic metabolism by as yet to be identified enzymes.

In contrast to the previous studies of Dain et al, (*13*) and Schaber et al. (*16*), in the present study eleven different metabolites were identified in urine which are related to the bioactivation pathways of CLZ. Next to the four thiomethyl-conjugates previously identified by Stock et al., seven additional metabolites were found which originate from GSH-conjugates of CLZ and its major metabolites DMCLZ and CLZ-NO. The positive identification of these metabolites in the present study might be explained by the availability of reference metabolites, which were produced synthetically and by incubations of CLZ in human slices. This enabled selective screening for the expected metabolites by exact mass measurements using highly sensitive LC-MS/MS-equipment.

It has been shown previously that GSH-conjugates of drugs are often excreted as N-acetylcysteine conjugates in urine (*41-44*). They are therefore often used as biomarker for internal exposure to potentially toxic electrophiles (*45*). However, in the present study no N-acetylcysteine conjugates of CLZ could be detected. Previously, it has been shown that formed N-acetylcysteine conjugates can be deacetylated again by hepatic and renal acylases (*26*). The balance of N-acetylation and deacetylation, which is substrate-dependent, therefore, determines the excretion profile of GSH-conjugates. In case, N-deacetylation is high, cysteine S-conjugates can be converted by cysteine S-conjugates beta-lyase enzymes, producing thiol-metabolites which are subject to subsequent methylation reactions (*21*). The fact that seven of the eleven urinary GSH-related metabolites in the present study represent thiomethyl-conjugates of CLZ and DMCLZ, is consistent with the preference for the beta-elimination/S-methylation pathway above N-acetylation of the cysteine S-conjugates of CLZ.

When analyzing the profiles of GSH-related metabolites in the 34 urine samples, a very large variability in the ratio of the eleven products were observed. When comparing profiles between any combination of two individuals or each individual profile with the average profile, only very poor correlations were observed, as shown in Figure 5. In comparison, the profiles of the major metabolites showed much smaller variability. The explanation for the very large variability in profiles of the GSH-related metabolites might be the fact that much more enzymes are involved in their production than the major metabolites which can be produced in two to three steps. To produce thiomethyl-conjugates at least six steps are required, involving CYPs, GST, gamma-glutamyltranspeptidase, dipeptidase, beta-lyase and S-methyltransferases (*21*), each of which might some interindividual differences due to drug-drug interactions or genetic polymorphisms. Furthermore, GSH-conjugates of CLZ have been shown to be eliminated in the bile of rats and mice (*7*). In humans, 40% of radiolabeled CLZ-metabolites was excreted in the feces (*13*), which also points to extensive biliary excretion in human. Therefore, metabolism of GSH-conjugates of CLZ also might occur in the intestinal microflora, which can result in excretion of GSH-related CLZ-metabolites in feces and which might contribute to urinary metabolites by entohepatic circulation.

Previously it was shown that several hGST were able to catalyze conjugation of GSH to the CLZ-nitrenium ion, producing multiple regioisomer GSH-conjugates (*10*). Therefore, genetic polymorphism at the level of hGST was speculated to be a possible risk factor for the adverse effects of CLZ. Three of the GSH-related metabolites identified in urine are derived from the chlorine-substitution pathway, which only occurs in presence of hGSTs (*10*). Although for 28 patients the genotype of four hGSTs were determined, no association was found between a certain genotype and a certain profile of GSH-related metabolites, due to the wide variability in profiles. For the same reason, the urinary profiles of the two patients with a history of CLZ-induced agranulocytosis did not display a unique profile.

In conclusion, in the present study multiple GSH-related metabolites could be identified in urine of CLZ-treated patients that reflect the bioactivation pathway of CLZ. However, because of the very wide variability in amounts and profiles, excretion of GSH-related metabolites does not appear to be related to specific genotypes of hGST. Analysis of these products in urine does not appear to be useful for quantitative biomonitoring of internal exposure to reactive CLZ-metabolites, which might be useful to support association studies aiming at the identification of risk factors for CLZ-toxicity. The

preliminary association study presented in this study for the first time suggest that the double null-genotype of GSTM1 and GSTT1 might be a risk factor for CLZ-induced agranulocytosis. However, because of the relatively small number of cases and controls, this association remains to be validated in a large-scale association study. Because urinary GSH-related metabolites show very wide variability and also reflect extensive metabolism in non-target tissues, alternative biomarkers, for example protein adducts in neutrophils, are required to investigate the role of hGST-polymorphisms on exposure to reactive nitrenium ions.

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Chapter 9

SUMMARY, CONCLUSIONS AND PERSPECTIVES

1. Summary

Adverse drug reactions (ADRs) still remained to be the second cause of failure in drug development between 2007-2010 even though extensive research has been performed. This fact underlines the importance of ADRs, on the one hand as potential risk for patients and on the other hand as financial threats for pharmaceutical industry (*1*). The unpredictable and barely understood idiosyncratic drug reactions (IDRs) are of special concern. Several hypothetical mechanisms underlying IDRs have been proposed but none of these theories is robust for all cases nor are fully confirmed thus far. Generally, the hypotheses do propose an important role for bioactivation and subsequent reactions of chemically reactive metabolites (CRMs) with cellular macromolecules (especially proteins), and the protective mechanisms that prevent these reactions (Figure 1). Bioactivation is collectively proposed to be required in the mechanism leading to IDRs, but is thought to be not sufficient on its own. In most people (non-susceptible individuals), the formation of CRMs is counterbalanced by detoxification mechanisms (bioinactivation). It is considered that these CRMs are able to elicit toxicity once the key detoxification pathway (i.e. glutathione (GSH) conjugation) has been overwhelmed. Risk factors, which might be genetic or host factors such as age, enzyme induction, and disease, may perturb favourable balance between bioactivation and bioinactivation.

The main focus of the research described in this thesis was to get deeper insights into the role of CRMs, and the balance between bioactivation and protections processes in mechanisms underlying IDRs and risk factors for the individual patients for the occurrence of these rare but severe toxicities. More specifically, **Chapters 2** to **5** describe *in vitro* studies performed to investigate the role of polymorphic glutathione S-transferases (GSTs) in the detoxification of drug reactive metabolites, while **Chapters 6** to **8** aimed at the development of novel strategies for discovering the risk factors and leading to a better understanding of the development of IDRs, taking clozapine as a typical model drug. Clozapine is very effective atypical antipsychotic, which causes severe idiosyncratic agranulocytosis in approximately 1% of the patients as well as hepatotoxicity. Bioactivation to reactive nitrenium ion is presumed to be responsible for these adverse events.

In **Chapter 1,** a general introduction on ADRs with the emphasis on IDRs is given. Although the exact mechanism for the occurrence of IDRs is not known, proposed mechanisms are including the formation of CRMs and subsequent reactions with cellular components, especially proteins (Figure 1). Idiosyncratic toxicity can occur when a convergence of risk factors tips the risk-benefit balance away from benefit and toward risk (*3*). Therefore, drug- and patient-related risk factors for the occurrence of IDRs are also summarized here. As prediction and the unraveling of mechanisms of IDRs are very important, current applications of *in vitro* and *in vivo* techniques to screen for the CRMs formation are also reviewed. Finally, the aim and scope of the thesis are formulated.

Glutathione S-transferases (GSTs) are major enzymes involved in the detoxification of xenobiotics by catalyzing conjugation reactions to GSH. Thus, polymorphisms (especially deletions) of human GST genes could cause increased susceptibility of patients to idiosyncratic drug-induced toxicity, if these enzymes would play a role in the detoxification of CRMs. Hence, **Chapter 2** presents an overview of the polymorphisms of GSTs, association studies that show correlations between polymorphisms and idiosyncratic toxicities as well as *in vitro* and *in vivo* studies where

the role of these enzymes in the detoxification of reactive drug metabolites was investigated.

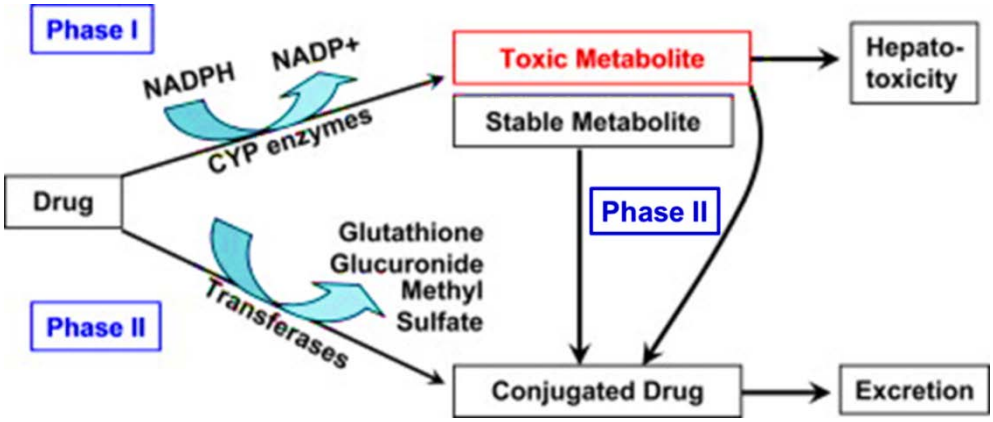

Figure 1. Metabolic processes and drug fate in the human body. Adapted from (*2*).

Chapter 3 is about the role of (polymorphic) hGSTs on the inactivation of reactive drug metabolites of clozapine. In this *in vitro* study we investigated the ability of four major recombinant human GSTs (GSTA1-1, GSTM1-1, GSTP1-1, and GSTT1-1) on the detoxification of clozapine reactive intermediates and formation of GSH adducts. Human and rat liver microsomes and a highly active drug-metabolising P450 BM3 mutant M11his were used for the bioactivation of clozapine and formation of reactive nitrenium ion. In presence of three of the tested hGSTs, namely hGSTP1-1, hGSTM1-1 and hGSTA1-1, GSH conjugation was strongly increased while the polymorphic hGSTT1-1 did not show any activity. Major changes in the regioselectivity of GSH conjugation also occurred, possibly due to the different active side geometries of hGST isoenzymes. Two GSH conjugates, previously only found in *in vivo* animal studies (*4*) were completely dependent on the presence of hGSTs, which explains their absence in *in vitro* studies without GSTs. With this, we have shown for the first time that addition of GSTs in *in vitro* systems is needed to obtain adequate reflections of GSH conjugation in *in vivo systems.*

The effects of human GSTs on the GSH adduct formation of diclofenac were also studied and described in **Chapter 4**. First, a single BM3 mutant showing the best metabolism of diclofenac, the double mutant CYP102A1 M11H Phe87, was selected for diclofenac bioactivation. The effects of the four major recombinant human GSTs, hGSTA1-1, hGSTM1-1, hGSTP1-1 and hGSTT1-1, on the formation of GSH conjugates of diclofenac were studied. Addition of hGSTA1-1, hGSTM1-1, and hGSTP1-1 increased GSH conjugation, with hGSTP1-1 showing the highest activity and hGSTA1-1 the lowest. hGSTs catalyzed the formation of GSH conjugates from all four described bioactivation pathways. hGSTP1-1 showed the highest activity towards the formation of GSH conjugates from 5-

OH diclofenac which leads to a different conjugate profile than the other hGSTs. This might be an important role as the 5-OH diclofenac is less stable than 4'-OH diclofenac, i.e., more prone to further oxidation to reactive quinone imine. hGSTP1-1 is highly expressed in the epithelia of the gastrointestinal tract and, based on the observed results, could play a crucial role in protection against gastrointestinal toxicity of diclofenac. On the other hand, high increases of 4'-OH diclofenac conjugates, the major diclofenac bioactivation pathway, in the presence of hGSTM1-1 imply that deficiency of hGSTM1-1 might be a risk factor for diclofenac induced hepatotoxicity. This is particularly important in conditions when cellular GSH becomes depleted and inactivation of reactive diclofenac metabolites will be more dependent on GST-catalyzed GSH-conjugation. Further investigation is warranted to confirm if GSTP1 and GSTM1 polymorphisms contribute significantly to these diclofenac-induced toxicities.

Human GSTP1-1 gene is polymorphic in human populations. Four allelic variants of hGSTP1-1 have been identified (*5*). These variants result from Ile105Val and Ala114Val substitutions. hGSTP1-1 polymorphisms are becoming increasingly relevant since previous studies suggested variations among individuals in regards to enzyme activity (*6*–*9*). In **Chapter 5** we studied the ability of four allelic variants of hGSTP1-1, namely hGSTP1*A (Ile105/Ala114), hGSTP1*B (Val105/Ala114), hGSTP1*C (Val105/Val114) and hGSTP1*D (Ile105/Val114), to catalyze the GSH conjugation of the reactive metabolites of diclofenac, clozapine, and paracetamol. Reactive metabolites were generated *in vitro* by human liver microsomes and drug metabolizing P450 BM3 mutants. Differences in activity between the proteins could not be attributed to a general decrease in catalytic efficiency. Rather, the differences reflected the effect of residue 105 and 114 on events specific for given substrates. Single substitutions at residue 105 or 114 did affect the ability to catalyze GSH conjugation. However, when both residue 105 and 114 were substituted the effect could be enhanced or diminished. Based on the results in this chapter, we suggest that the binding orientation of substrates in the active site of P450 BM3 mutants is changed and has effect on GSH conjugation.

Last three chapters are more closely dedicated to clozapine, its bioactivation to reactive nitrenium ion and possible risk factors that might lead to idiosyncratic toxicities, agranulocytosis and hepatotoxicity. **Chapter 6** describes the application of P450 BM3 mutants for clozapine bioactivation and structural characterization of the GSH conjugates formed. A saturation mutagenesis study was performed in which the active-site residue at position 87 was mutated to all 20 possible amino acids. In BM3 M11 the residue at this position is Val87, introduced at an early stage of the mutagenesis process, to expand the substrate selectivity to drugs and drug-like molecules (*10*). In the saturation mutagenesis studies, it was demonstrated that the type of amino acid at position 87 has a strong effect on substrate selectivity when comparing a series of alkoxyresorufins and on the activity and regioselectivity of testosterone hydroxylation (*11*). We also proved the importance of the residue at position 87 on the regioselectivity of clozapine metabolism. In particular, we showed that physical properties of the side chain of aminoacid in position 87 are very critical for the total activity of the enzyme. The mutant with phenylalanine at position 87 was very selective for the bioactivation of clozapine and was therefore chosen for large scale production of the GSH conjugates. Five major GSH adducts of clozapine, four having the same mass and three of them synthesized for the first time, were produced in high levels, purified and structural elucidation was done by ^{1}H-NMR. This study confirmed the

utility of highly active and selective P450 BM3 mutants as tool to characterize human-relevant metabolites, applied here for the first time for formation of CRMs.

Chapter 7 is describing involvement of individual human CYPs in the bioactivation of clozapine and formation of reactive intermediates. Fourteen different recombinant human CYPs were used for the complete metabolic studies of clozapine, and more specifically to elucidate enzymes responsible for its bioactivation. Also, inhibition of reactive metabolite formation (measured as GSH conjugates) by addition of selective inhibitors of individual CYP enzymes to human liver microsomes incubations was investigated. Six out of fourteen recombinant human P450s were able to bioactivate clozapine, with CYP3A4 and CYP2D6 showing the highest specific activity. To establish the importance of CYP2D6 in the bioactivation of clozapine, collaboration with prof. Magnus Ingelman-Sundberg's group was set up. Individual liver microsomes prepared from 100 different human livers could thus also be used to study the contribution of CYP3A4 and CYP2D6 in clozapine bioactivation *in vitro* and to evaluate the role of polymorphic CYP2D6. No significant inhibition by quinidine, inhibitor of CYP2D6, occurred in any of 100 individual incubations, suggesting that CYP2D6 polymorphism is not an important factor in determining susceptibility to hepatotoxicity of clozapine. It was also observed that the bioactivation of clozapine to reactive nitrenium ion contributes equally to metabolism of clozapine as major biotransformation pathways, i.e. demethylation and N-oxidation, do. There were 2 out of the 100 individuals with significantly higher formation of the reactive metabolites (Figure 7, **Chapter 7**) compared to the others. Based on these results, it was finally concluded that CYP3A4 is the major enzyme responsible for clozapine bioactivation in the liver and that drug-drug interactions and induction at the level of CYP3A4, more than genetic variability, might be factors determining exposure of hepatic tissue to reactive clozapine metabolites.

The most recent and major studies to translate previous results into humans and human patients treated with clozapine are described in **Chapter 8.** This was done by measuring urine samples from schizophrenic patients treated with clozapine, by performing human precision-cut liver slice (hPCLS) incubations, as well as by analyzing the association of GST polymorphisms with the occurrence of agranulocythosis. Metabolic profiles based on urine samples from clozapine treated patients corresponded to previously described metabolic profiles (*12*–*14*). Bioactivation of clozapine was identified by measuring the formation of GSH related conjugates. Surprisingly, cysteine conjugates were measured rather than the expected N-acetyl cysteine conjugates in human urine. Previously described clozapine thiomethyl conjugates found in human urine (*12*) were also measured. In correspondence with measurements in patients urine, in human liver slice incubations cysteine and thiomethyl conjugates were also found as well as all other phase I and phase II stable metabolites. With this, we could show that hPCLS are a good model for predicting human metabolic profiles of clozapine *in vivo*. The exact structures of the identified GSH related conjugates were determined using reference standards, produced by enzymatic and chemical syntheses from corresponding GSH conjugates and described in **Chapter 6**. Both, chemical and enzymatically catalyzed (GST-dependent) GSH related conjugates were observed. The GSH conjugate structures, reflecting the involvement of CRMs of clozapine, the importance of polymorphic human GSTs for their formation (as described in **Chapter 3**), and the occurrence of agranulocythosis were correlated with genotyping results for the human GSTs. Due to the extremely large variability in amounts and profiles of GSH-related metabolites, no correlation was

observed with the polymorphic alleles of hGSTM1, GSTT1, GSTP1 and GSTA1. Remarkably, however, three out of seven patients that developed agranulocytosis had double null genotypes for GSTM1-1 and GSTT1-1 while within control group only one out of thirty one patients was a carrier of the double-null genotype. Larger number of adequate samples would be necessary to confirm this most interesting and relevant observation.

2. Conclusions and perspectives

The research described in this thesis was a part of a wider interdisciplinary project "Towards Novel Translational Biomarkers for Adverse Drug Reactions (ADRs)" financed by the Dutch Top-Institute Pharma (grant D3-201), notably involving the formation of CRMs. Several industrial and academic partners participated in this project that led to several publications and translational strategies to better predict drug safety early in the drug discovery and development processes (Figure 2).

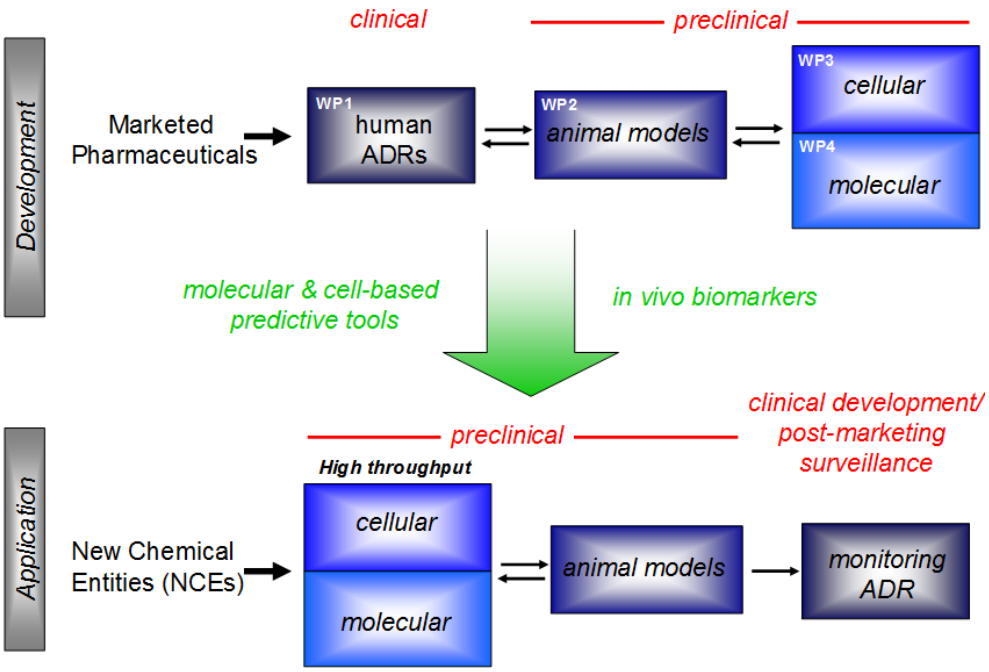

Figure 2. Strategy and approaches towards novel translational safety biomarkers for adverse drug toxicity (TI-Pharma D-301 project).

IDRs are usually rare, not evident in animal species, but can be serious and even fatal in humans and lead to withdrawal of otherwise effective therapeutic agents. The fact that IDRs will mostly occur only at the post-approval stage, when such problems typically first become evident, is a major impediment to drug development. When the research described in this thesis started, there were no validated methods for the identification of

drugs that may cause hypersensitivity or idiosyncratic drug reactions in humans during preclinical drug evaluation.

It is well established that most IDRs result from the bioactivation of drugs to CRMs (*15*). The first step towards developing a valid methodology would be the testing of molecules for their ability to form reactive metabolites. However, there is no simple correlation between drug bioactivation *in vitro* and ADRs in the clinic. Even though bioactivation is collectively proposed to be required in mechanisms leading to IDRs, not all drugs that undergoing bioactivation by drug-metabolizing enzymes are associated with IDRs in the clinic. In particular, very little is known about the relationship between the chemical properties of CRMs and the mechanisms underlying clinical IDRs. Studies on how these properties contribute to toxicity (in preclinical species and in humans) appeared to be key for future research. The main aim of this thesis was therefore to develop *in vitro* techniques for the bioactivation of drugs and detection and characterization of stable and, more importantly, chemically reactive metabolites.

However, it is impossible to predict an individual's susceptibility to IDRs due to drugs only on bioactivation. Second aim of this thesis was investigate which other factors determine interindividual susceptibility to drug toxicity. The most common step following bioactivation is bioinactivation or detoxification (Figure 3). The efficiency of detoxification of the chemically reactive intermediates, often via GSH conjugation, might be a crucial risk factor for the occurrence of IDRs. The balance between bioactivation and bioinactivation pathways in the metabolism of drugs could be the critical factor that determines individual susceptibility for IDRs (Figure 3). In susceptible people the usually favourable balance between bioactivation and bioinactivation may be perturbed by either genetic or host factors, allowing the toxic metabolites to escape detoxification. Chemical properties of the drug, daily doses, drug metabolism, drug-drug interactions, and other factors such as age, sex, nutritional factors, and underlying disease states might mediate the development of IDRs. Under these circumstances, the toxic metabolites may bind covalently to various cellular macromolecules and cause toxicity. With most drugs, however, the factors which cause this imbalance are unknown, which explains why such reactions continue to occur. Genetic susceptibility, however, is one of the most important risk factors, although the precise genetic bases is still poorly understood for most drugs with documented IDRs (*16*). Therefore, our aim was to identify if polymorphic enzymes, primarily hCYPs and hGSTs, are involved in biotransformation and bioinactivation processes of model drugs causing IDRs. More specifically, we investigated in *in vitro* studies if genetically polymorphic enzymes are involved in bioactivation and detoxification of the reactive nitrenium ion of clozapine, drug that causes idiosyncratic agranulocytosis and hepatotoxicity. Finally, we tried to correlate the importance of these polymorphisms with the *in vivo* data obtained from the patients on clozapine treatment.

2.1. Role of (polymorphic) hGSTs in the detoxification of chemically reactive drug metabolites

In the first part of this thesis, we demonstrated that (polymorphic) human GSTs might play a significant role in the inactivation of reactive drug metabolites (**Chapter 3** to **Chapter 5**). We have shown that hGSTs are able to catalyze the GSH conjugation of CRMs, resulting in different regioisomeric GSH conjugates of clozapine and showing selectivity for different bioactivation pathways for diclofenac. This data indicated the possible

importance of polymorphic GSTs, namely hGSTM1-1 and hGSTP1-1, as risk factors for the occurrence of idiosyncratic toxicity. Several clinical studies demonstrated an increased susceptibility to idiosyncratic drug-induced liver injury by a combined GSTM1-T1 double-null genotype (*17*, *18*). A reduced ability to detoxify electrophilic reactive metabolites, which is expected among individuals with GSTM1-1 null genotypes, might play a role in determining or predicting the risk for clozapine and/or diclofenac related toxicities. Inter-individual differences in hGSTP1-1 enzymes derived from polymorphisms that could also lead to greater exposure to reactive metabolites may also be a possible explanation for a varying susceptibility to drug-induced ADRs. Remarkably, our GSTs genotyping study in clozapine treated schizophrenic patients showed that there might be a correlation between GSTM1-T1double-null genotype and occurrence of agranylocytosis. In addition to reported *in vitro* studies, specifically designed case-control studies are required to investigate whether genetic polymorphisms of hGSTP1-1 and hGSTM1-1 causally contribute to the inter-individual differences in susceptibility to these drug-induced ADRs. We have developed and validated a strategy for investigation of the role of hGSTs in the detoxification of CRMs and formation of GSH adduct. More importantly, we proved that the addition of GSTs is required for the formation of all human relevant GSH conjugates.

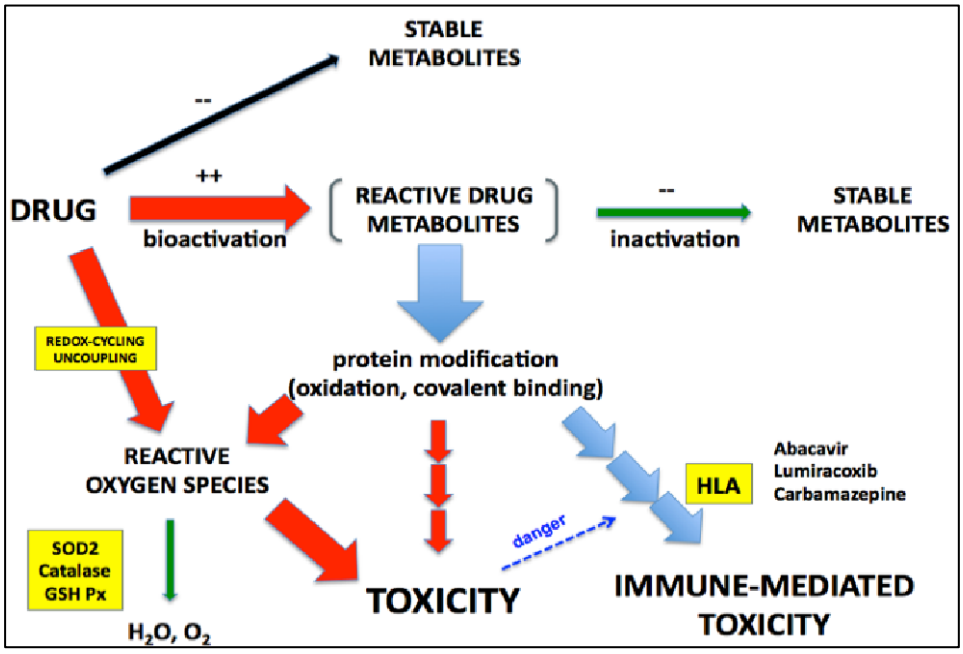

Figure 3. Schematic representation of the main focuses of this thesis. Individuals, susceptible for the expression of IDRs show a disturbed balance between increased bioactivation (i.e. CRMs formation) and decreased detoxification (i.e. GSH conjugation).

2.2. Bioactivation of clozapine and risk factors for its idiosyncratic toxicity

Measuring the potential of drugs and drug candidates to be bioactivated to CRMs in early drug discovery is often hampered by the difficulties in detecting and characterizing low levels of CRMs. When this research started, Damsten *et al.* already showed that BM3 mutants were useful to produce reactive metabolites from the drugs clozapine, diclofenac and acetominophen (*19*). We have been able to identify novel human relevant GSH conjugates of clozapine by discovering that hGSTs had to be added and by using NMR for unequivocal structural elucidation. This study confirms the high potential of P450 BM3 mutants as tool to produce and characterize human-relevant and/or CRMs for the development of safer drugs.

The involvement of individual hCYPs in the hepatic metabolism of clozapine to its major metabolites N-desmethyl clozapine and clozapine N-oxide has been well characterized previously (*20*–*22*). Using several *in vitro* approaches, we showed for the first time that CYP3A4 is the major enzyme responsible for clozapine bioactivation. Interindividual differences and drug-drug interactions at the level of CYP3A4, often occurring (*23*, *24*), may therefore well add to differences in susceptibility of patients to clozapine IDRs. We have also shown that hGSTs, specifically polymorphic hGSTM1-1, play a significant role in of the protection against the CRMs of clozapine formed by cytochrome P450s (**Chapter 3**). Therefore, a high activity of bioactivation by CYP3A4 in combination with reduced activity of protective hGSTs, and specifically polymorphic hGSTM1-1, might explain high susceptibility of part of the patients to hepatotoxic effects of CLZ.

Finally, in **Chapter 8**, we described an approach leading to the discovery of stable urinary metabolite biomarkers, namely cysteine and thiomethyl conjugates, indicative for clozapine bioactivation in clozapine treated patients. Translational biomarkers such as these, first time discovered and validated in *in vivo* studies, can be used to explore associations between metabolic activation and the incidence and risk factors of ADRs in patients. The importance of polymorphic hGSTs for the formation and urinary excretion of detected thioether conjugates was also discussed. Due to the extremely large variability in amounts and profiles of GSH-related metabolites, no correlation was found with polymorphic alleles of hGSTM1, GSTT1, GSTP1 and GSTA1. Urinary GSH-related metabolites of CLZ, therefore, do not seem useful biomarkers for quantitative biomonitoring of internal exposure to reactive CLZ-metabolites. In the same study, we have also shown that hPCLS, containing both phase I and phase II enzymes, are an excellent model for patient-relevant metabolic profile characterization. The here developed strategy, involving *in vitro* approach for drug metabolism by more complex system (hPCLS) and urine analysis from the chronically treated patients, forms a unique and translational bridge between *in vitro* studies and clinical studies in patients. Interestingly, we could also demonstrate for the first time the relevance of a combined GSTM1-T1 double-null genotype in the investigated clozapine-treated patients for the occurrence of clozapine-induced agranulocytosis.

In conclusion, associations between the generation of chemically reactive metabolites (CRMs) during the drug metabolism and various drug toxicities is generally well-established. However, considerable uncertainty is still surrounding the predicitvity of reactive metabolite formation with regard to risks for ADRs or IDRs in humans. Although recent developments in molecular toxicology have increased our understanding of how drug metabolism may contribute to drug bioactivation and bioinactivation and to

possible drug–related ADRs or IDRs, it is not yet possible to predict these toxicities based on chemical structures alone. There remains a need for mechanistic drug safety related research to be better equipped to informing both medicinal chemists and clinicians about risk and hazard identification due to drug exposure. Recent developments have provided new strategies that have greatly improved our basic understanding of the role of drug metabolism in ADRs. However, much remains to be done to fully understand the basic molecular and cellular mechanisms, and to enable the translation of this knowledge and methods to better predict drug safety. We have developed methods which will help to explore the molecular mechanisms in order to: (1) determine the functional group(s) within molecules and metabolic reactions that might be responsible for the toxicity; and (2) identify (biological) factors that may determine cell-directed toxicity and predispose individuals for ADRs or IDRs. As such, the results presented in this thesis will contribute significantly to the development of novel, translational technologies and methodologies, which can moreover serve as the interesting starting points for future research in drug safety sciences.

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Appendices

Nederlandse samenvatting

Schadelijke bijwerkingen, in het engels 'adverse drug reactions' (ADR), waren tussen 2007 en 2010 de op een na belangrijkste reden waarom kandidaat geneesmiddelen tijdens de ontwikkeling moesten worden teruggetrokken. Dit ondanks het feit dat er al decennia veel onderzoek wordt verricht naar de oorzaak van deze ongewenste reacties. Vooral de zeldzame 'idiosyncratische drug reacties' (IDR), die meestal pas aan het licht komen nadat een geneesmiddel op de markt is geïntroduceerd, zijn een bron van zorg vanwege hun onvoorspelbare en zeer ernstige aard. Tot nu toe zijn verschillende hypotheses geformuleerd met betrekking tot de oorzaak van deze vaak fatale reacties. Geen van deze hypotheses is tot nu toe voldoende gevalideerd. Over het algemeen wordt enzymatische bioactivatie van geneesmiddelen tot hoog-reactieve metabolieten die met cellulaire macromoleculen kunnen reageren, in het bijzonder eiwitten, beschouwd als een belangrijke risicofactor in het ontstaan van IDR. Hoewel cellen verschillende beschermingsmechanismen bevatten tegen reactieve metabolieten, zoals inactivatie door middel van glutathion (GSH)-conjugatie, kan in sommige situaties de vorming van reactieve metabolieten te hoog zijn om nog effectief te kunnen inactiveren. Factoren die de balans van bioactivering en inactivering negatief kunnen beïnvloeden zijn genetische afwijkingen op het niveau van de biotransformatie enzymen of transporteiwitten, enzyminductie of enzymremming door andere geneesmiddelen of voedingscomponenten, leeftijd en de ziekte waaraan een patiënt lijdt.

Het belangrijkste doel van het onderzoek dat in dit proefschrift wordt beschreven was om meer inzicht te krijgen in de rol van reactieve geneesmiddel metabolieten, en de rol van erfelijkheid in de balans van bioactiverende- en beschermende mechanismen die bepalend kan zijn voor het risico de zeldzame IDRs. Daartoe werden *in vitro* methoden ontwikkeld waarmee zowel de stabiele als reactieve metabolieten van geneesmiddelen kunnen worden gedetecteerd en gekarakteriseerd. Ook werd onderzocht welke factoren de interindividuele verschillen in de gevoeligheid voor ADR zouden kunnen verklaren. Daartoe werd onderzocht of erfelijk bepaalde cytochroom P450s en glutathion S-transferases (GSTs) betrokken zijn bij het metabolisme van geneesmiddelen die zelfzame IDR veroorzaken. Cytochroom P450s zijn vaak verantwoordelijk voor de vorming van reactieve metabolieten, terwijl GSTs betrokken kunnen zijn bij de enzymatisch GSH-conjugatie, en daardoor de detoxificatie van reactieve metabolieten. Uit de uitgevoerde *in vitro* studies kwam naar voren dat enkele genetisch bepaalde glutathion S-transferases betrokken zijn bij de inactivatie van de reactieve metabolieten van de geneesmiddelen clozapine en diclofenac. Om het mogelijke verband tussen deficiëntie van glutathion transferases en het risico op ADR aan te tonen *in vivo* werd het metabolisme van clozapine onderzocht in patiënten die wel en niet gevoelig zijn voor de toxiciteit van dit geneesmiddel. Het in dit proefschrift beschreven onderzoek was onderdeel van een breder interdisciplinair onderzoeksprojects, getiteld "Towards Novel Translational Biomarkers for Adverse Drug Reactions (ADRs)", dat werd gefinancieerd door het Topinstituut Pharma (projectnummer D3-201). Aan dit project namen verschillende industriële en academische partners deel.

In **Hoofdstuk 1** wordt een algemene introductie gegeven over ADR, met speciale nadruk op de zelfzame IDRs. Hoewel het exacte mechanisme van IDRs nog niet bekend is, wordt vorming van reactieve metabolieten die vervolgens reageren met cellulaire macromoleculen, zoals eiwitten, als een belangrijke eerste stap beschouwd.

Idiosyncratische toxiciteit treed hoogstwaarschijnlijk alleen op als meerdere risicofactoren samenvallen in een patiënt. De verschillende risicofactoren die gerelateerd kunnen zijn aan het geneesmiddel of aan de patiënt worden in dit hoofdstuk samengevat. Omdat reactieve metabolieten waarschijnlijk een belangrijke rol spelen worden de verschillende experimentele benaderingen waarmee de vorming van reactieve metabolieten kunnen worden aangetoond in hoofdstuk 1 ook behandeld.

Glutathione S-transferases (GSTs) kunnen soms een belangrijke rol spelen in de ontgifting van electrofiele chemicaliën door ze te koppelen aan het tripeptide glutathion (GSH). Genetisch polymorfismen van GSTs kunnen daarom een bepalende factor zijn in de gevoeligheid voor IDRs als erfelijk bepaalde GSTs een belangrijke rol spelen in de inactivatie van reactieve geneesmiddelmetabolieten. In **Hoofdstuk 2** wordt een overzicht gegeven van de genetische polymorfismes van GST die in de bevolking zijn aangetoond. Verschillende associatiestudies worden beschreven waarin het verband tussen deze polymorfismes en het risico op toxiciteit van geneesmiddelen in patiënten populaties is onderzocht. Daarnaast worden de *in vitro* en *in vivo* benaderingen beschreven waarmee de beschermende rol van GSTs kon worden aangetoond.

In het onderzoek dat wordt beschreven in de hoofdstukken 3 tot en met 5 werd bestudeerd of erfelijk bepaalde menselijke GSTs een rol spelen in de inactivatie van de reactieve metabolieten van clozapine en diclofenac. Clozapine is een zeer effectief geneesmiddel in de behandeling van psychiatrische patiënten. Echter, in ongeveer 1% van de met clozapine behandelde patiënten treedt ernstige, levensbedreigende agranulocytose op. Daarnaast veroorzaakt clozapine in een deel van de patiënten een asymptomatische verhoging van plasma transaminase levels; slechts bij een zeer klein aantal patiënten kan ernstige leverschade optreden. Bioactivatie van clozapine tot een hoog-reactief nitrenium ion door myeloperoxidase en P450s wordt beschouwd als de oorzaak voor deze toxische bijwerkingen. In **Hoofdstuk 3** wordt beschreven dat drie van de onderzochte GSTs, namelijk hGSTP1-1, hGSTM1-1 en hGSTA1-1, in staat waren de koppeling van het reactieve nitrenium ion aan GSH te katalyseren. Het genetisch polymorfe GSTT1-1 vertoonde geen katalytische werking. Tussen de actieve GSTs bleken grote verschillen in regioselectiviteit in GSH-conjugatie aan het nitrenium ion te bestaan, hetgeen mogelijk kan worden verklaard door de verschillende geometrie van de substraat bindingsplaatsen van deze enzymen. Twee van de gevonden GSH-conjugaten, die voorheen alleen in *in vivo* proefdier studies waren aangetoond bleken alleen in aanwezigheid van GSTs te kunnen worden gevormd, hetgeen verklaart waarom deze conjugaten tot nu toe nooit in *in vitro* experimenten konden worden aangetoond. Dit laat zien dat toevoegen van GSTs aan *in vitro* incubaties noodzakelijk is om een adequate weerspiegeling te krijgen van het profiel van GSH conjugaten zoals die in vivo kunnen worden gevormd.

Het antiontstekingsmiddel diclofenac veroorzaakt bij een zeer klein deel van de patiënten een zeer ernstige vorm van levertoxiciteit. Daarnaast kan diclofenac toxiciteit veroorzaken in het maagdarmkanaal. Aangetoond is dat diclofenac door zowel glucuronidering als oxidatie-reacties tot verschillende eiwit-reactieve metabolieten kan worden omgezet. In **Hoofdstuk 4** wordt het effect beschreven van GSTs op de ontgifting van vier verschillende reactieve metabolieten die door cytochroom P450s worden gevormd. Toevoeging van hGSTA1-1, hGSTM1-1 en hGST1-1 bleek de GSH-conjugatie van alle vier reactieve intermediairen te versnellen; de hoogste activiteit werd waargenomen met hGSTP1-1, terwijl hGSTA1-1 de laagste activiteit vertoonde. hGSTP1-1 bleek vooral

actief te zijn in de vorming van GSH-conjugaten die via 5-hydroxydiclofenac worden gevormd. 5-Hydroxydiclofenac is gevoelig voor autoxidatie tot een reactief quinonimine dat als het meest toxische metaboliet van diclofenac wordt beschouwd. hGSTP1-1 heeft een lage expressie in levercellen, maar een hoge expressie in de cellen van het maagdarmkanaal. Dit enzym speelt daarom waarschijnlijk een belangrijke rol in de bescherming tegen de darmtoxiciteit van diclofenac. 4'-Hydroxydiclofenac is een van de hoofdmetabolieten van diclofenac, en kan door cytochroom P450 verder worden geoxideerd tot een ander quinonimine. Het erfelijk bepaald hGSTM1-1 bleek zeer actief te zijn in de GSH-conjugatie van dit quinonimine. Deficiëntie van hGSTM1-1, welke ongeveer 45% van de bevolking voorkomt, kan de gevoeligheid voor levertoxiciteit van diclofenac mogelijk verhogen, vooral in situaties waarin de concentratie van GSH in de lever is verlaagd, en de inactivatie van de quinonimines sterk afhankelijk worden van GSTs.

Verschillende studies suggereren dat genetische polymorfismen van hGSTP1-1 ook tot interindividuele verschillen in enzymactiviteit kunnen leiden. In **Hoofdstuk 5** worden de enzymactiviteiten beschreven waarmee vier allelische varianten van hGSTP1-1 de reactieve metabolieten van paracetamol (acetaminophen), clozapine en diclofenac conjugeren aan GSH. De vier onderzochte varianten waren hGSTP1*A (Ile105/Ala114), hGSTP1*B (Val105/Ala114), hGSTP1*C (Val105/Val114) en hGSTP1*D (Ile105/Val114). De drie geneesmiddelen werden gebioactiveerd door menselijke levermicrosomen en het bacteriële P450 BM3. Uit de resultaten blijkt dat de allelische varianten slechts kleine verschillen vertoonden in de activiteit waarmee de reactieve metabolieten van deze geneesmiddelen werden gekoppeld aan GSH. Verschillen in enzymactiviteit tussen de GSTP1-1 mutanten verklaart dus waarschijnlijk niet de waargenomen verschillen in gevoeligheid voor toxiciteit. hGSTP1-1 heeft echter ook nog andere functies in de cel, zoals beïnvloeding van de werking van transcriptiefactoren (bijvoorbeeld jun-K) en de glutathionylering van eiwitten. Het is dus mogelijk dat het genetische polymorfisme van hGSTP1-1 meer invloed heeft op deze andere functies van dit eiwit.

In **Hoofdstuk 6** wordt beschreven dat mutanten van het bacteriële P450 BM3 waardevolle hulpmiddelen zijn bij het karakteriseren van GSH-conjugaten van geneesmiddelen. P450 BM3 is het meest actieve cytochroom P450 dat tot nu toe in de natuur is gevonden, en heeft daarom veel perspectief als katalysator in de biotechnologie. Door middel van mutagenese zijn in de groep Moleculaire Toxicologie mutanten ontwikkeld die is staat zijn geneesmiddel metabolieten op grote schaal te produceren. In Hoofdstuk 6 is een serie van mutanten onderzocht waarin het aminozuur op positie 87 is vervangen door elk van de andere aminozuren, ookwel 'saturation mutagenesis' genoemd. Het aminozuur op deze positie bevindt zich in de substraat bindingsplaats, zodat verwacht werd dat vervanging van het aminozuur tot verschillen in activiteit en/of regioselectiviteit kan leiden. Door deze mutanten te incuberen met clozapine, in aanwezigheid van GSH, bleek dat de onderzochte mutanten in staat waren de metabolieten te produceren die ook door menselijke P450s worden gevormd. Inderdaad bleken de 20 mutanten een grote variatie in activiteit en metaboliet-profiel vertoonden. De mutant met een fenylalanine op positie 87 bleek zeer selectief te zijn in de bioactivatie van clozapine tot het toxische nitrenium ion, en een hoge activiteit te bezitten. Met behulp van deze mutant konden vijf verschillende GSH-conjugaten van clozapine op grote schaal worden geproduceerd, zodat daarvan de absolute structuur met behulp van ^{1}H-NMR voor de eerste keer kon worden opgehelderd. Deze studie toont aan dat P450 BM3 mutanten

waardevolle hulpmiddelen kunnen zijn in toxicologisch onderzoek omdat ze ook de humaan-relevante reactieve metabolieten kunnen produceren.

Het onderzoek dat beschreven is in **Hoofdstuk 7** was erop gericht te identificeren welke menselijke cytochroom P450s betrokken zijn bij de bioactivatie van clozapine in de lever. Uit incubaties van clozapine met 14 verschillende recombinant humane P450s bleek dat zes van de P450s in staat waren clozapine te bioactiveren; CYP3A4 en CYP2D6 vertoonden de hoogste specifieke activiteit. De belangrijke rol van CYP3A4 werd bevestigd door remmingsexperimenten met gepoolde menselijke levermicrosomen; alleen de CYP3A4-specifieke remmer bleek een sterke verlaging van de GSH-conjugaten te vertonen. Remming van CYP2D6 in de gepoolde levermicrosomen bleek geen significate invloed op vorming van GSH-conjugaten te vertonen, hetgeen verklaard kan worden door het gemiddeld lage expressieniveau van dit P450. Omdat een klein percentage van de bevolking meerdere kopiën van het gen van CYP2D6 bezit, hetgeen tot sterk verhoogde CYP2D6 kan leiden, is, in samenwerking met prof. Magnus Ingelman-Sundberg (Karolinska Instituut, Stockholm) ook de bioactivatie van clozapine onderzocht met een panel van levermicrosomen van 100 verschillende individuën. Uit deze studie bleek dat er een grote interindividuele variabiliteit bestaat in de bioactivatie van clozapine, en dat de bioactivatieroute kwantitatief bijna even belangrijk is als de N-demethylering en N-oxidatie-route. Echter, toevoeging van de CYP2D6-specifieke remmer quinidine bleek bij geen van 100 levermicrosomen significante remming te vertonen op de bioactivatieroute, zodat genetisch polymorfisme van CYP2D6 waarschijnlijk geen risicofactor is voor de levertoxiciteit van clozapine.

Uit deze studie blijkt dat CYP3A4 het belangrijkste enzym is voor de bioactivatie van clozapine in de lever. Drug-drug interacties op het niveau van CYP3A4, zoals enzym inductie, spelen dus waarschijnlijk een belangrijke rol in de variabiliteit in bioactivatie van clozapine. Op basis van de resultaten die beschreven zijn in de Hoofdstukken 3 en 7 kan als hypothese worden geformuleerd dat een combinatie van enzyminductie van CYP3A4 in combinatie met deficiëntie van GSTs, zoals hGSTM1-1, tot verhoogde gevoeligheid voor clozapine-geïnduceerde levertoxiciteit kan leiden.

Hoofdstuk 8 beschrijft de resultaten van de studies die zijn uitgevoerd om de klinische relevantie van de *in vitro* studies van clozapine met recombinant GSTs en P450s aan te tonen. Ten eerste werd het metabolisme van clozapine onderzocht met menselijke leverslices van 14 verschillende individuen. Daarnaast werd de uitscheiding van metabolieten van clozapine in urine van 28 psychiatrische patiënten onderzocht, waarvan sommigen clozapine-geïnduceerde agranulocytose ondervonden. Zowel in de incubaties van clozapine met leverslices, als in de urine-monsters konden meer dan 30 verschillende metabolieten van clozapine worden aangetoond die worden gevormd door fase 1 en fase 2 enzymen, hetgeen de waarde van leverslice-experimenten voor het voorspellen van geneesmiddel metabolisme bevestigd. Een aantal van de metabolieten kon worden toegeschreven aan de bioactivatie-route van clozapine. In de urine monsters van patiënten werden zowel cysteine- als thiomethyl-conjugaten van clozapine aangetoond. Deze thioethers ontstaan bij afbraak van GSH-conjugaten van clozapine, een proces dat in meerdere weefsels kan plaatsvinden. Aan de hand van de structuren kon worden bevestigd dat GSH-conjugatie van het reactieve clozapine metaboliet bij de mens ook door GSTs wordt gekatalyseerd. Van zowel de leverslices als de patiënten was het genotype van vier GSTs (hGSTM1-1, hGSTT1-1, hGSTP1-1 en hGSTA1-1) bepaald. Echter, er werd geen correlatie gevonden tussen het genotype van GSTs en het profiel van de

GSH-conjugaten en daarvan afgeleide producten, mede vanwege de zeer grote interindividuele verschillen in metaboliet-profielen in de slice-incubaties en urine monsters. Bij het vergelijken van het GST-genotype van patiënten met en zonder agranulocytose bleek dat drie van de zeven patiënten met agranulocytose zowel het gen voor hGSTM1-1 en hGSTT1-1 misten. Bij de dertig patiënten die géén agranulocytose ontwikkelden bleek slechts één patiënt beide genen te missen. Deze zeer interessante resultaten zouden erop kunnen wijzen dat het missen van beide genen een risicofactor voor agranulocytose kan zijn. Deze vinding dient echter te worden bevestigd in een grotere associatiestudie.

Concluderend, onderzoek in de laatste decennia heeft aangetoond dat reactieve metabolieten van geneesmiddelen waarschijnlijk een belangrijke rol spelen bij het ontstaan van verschillende vormen van ADRs. Echter, de voorspellende waarde van vorming van reactieve metabolieten in tot nu toe gebruikte *in vitro* modellen voor het optreden van ADRs, en de meer zeldzame IDRs, is nog steeds zeer beperkt. Er bestaat daarom grote behoefte aan betere, op mechanistisch onderzoek gebaseerde modelsystemen waarmee de risicoschatting voor de toxische bijwerkingen van geneesmiddelen kan worden verbeterd. Met de mechanistische kennis kunnen door farmacochemici veiliger geneesmiddelen worden ontwikkeld. Mechanistische studies verschaffen namelijk inzicht over welke functionele groepen en welke enzymatische reacties tot de vorming van reactieve, mogelijk toxische metabolieten kunnen leiden. Daarnaast wordt steeds meer duidelijk dat ook patiënt-specifieke biologische factoren, zoals genetische polymorfismen op het niveau van de biotransformatie-enzymen, transporters en het eiwitten van het immuunsysteem bepalend kunnen zijn voor het risico op een ADR. Indien deze erfelijke factoren bekend worden kan de arts beter voorspellen of een te behandelen patiënt gevoelig zal zijn voor toxiciteit. De resultaten van het in dit proefschrift beschreven translationele onderzoek, waarin zowel methodes zijn ontwikkeld om de vorming van reactieve metabolieten te detecteren en identificeren, als waarin de rol van erfelijke factoren op het niveau van beschermende GSTs is aangetoond, kunnen daarom een belangrijke bijdrage leveren aan de verbetering van de risicoschatting van geneesmiddeltoxiciteit in gevoelige patiënten.

List of abreviations

ADR	Adverse drug reaction
AMAP	3'-hydroxyacetanilide
APAP	Acetaminophen (paracetamol)
CDNB	1-chloro-2,4-dinitrobenzene
CLZ	Clozapine
CLZ-NO	Clozapine N-oxide
CN^-	Cyanide anion
CRM	Chemically reactive metabolites
CYPs	Cytochrome P450s
DF	Diclofenac
DMCLZ	Desmethylclozapine
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
GST	Glutathione S-transferase
hGST	Human glutathione S-transferase
$HClO_4$	Perchloric acid
HLM	Human liver microsomes
HPLC	High performance liquid chromatography
IDR	Idiosyncratyc drug reaction
KPi	Potassium phosphate
LC-MS	Liquid chromatography–mass spectrometry
NAC	N-acetyl cysteine
NMR	Nuclear Magnetic Resonance
NSAID	Nonsteroidal anti-inflammatory drug
PCLS	Precision-cut liver slices
hPCLS	Human precision-cut liver slices
P450	Cytochrome P450 monooxygenase
P450 BM3	Cytochrome P450 BM3
RI	Reactive Intermediate

List of publications

- Effect of human glutathione S-transferases on glutathione-dependent inactivation of cytochrome P450-dependent reactive intermediates of diclofenac. Dragovic S, Boerma JS, Vermeulen NP, Commandeur JN. Chem Res Toxicol. 2013 Oct.

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Curriculum Vitae

Sanja Dragovic was born on June 26th 1981 in Zajecar, Serbia. After graduating from highschool at the Gymnasium Zajecar, she started studying for the degree of pharmacist at the Faculty of Pharmacy, University of Belgrade, Serbia. In 2007, she was granted by Government of Serbia with a scholarship, "Fund for Young Talents", which supported her studies at the Laboratory for Pharmaceutical Analysis, KU Leuven in Belgium, under supervision of Prof. Dr. Jos Hoogmartens and Prof. Dr. Erwin Adams. During her studies, she performed a major research on development and validation of analytical methods for active pharmaceutical ingredients and pharmaceutical formulations applied in the International Pharmacopoeia. She was also involved in the development of a column classification system - characterisation and classification of stationary phases in reversed-phase liquid chromatography. After obtaining her second master's degree in September 2008, she started her PhD research under supervision of Prof. Dr. Nico Vermeulen and Dr. Jan Commandeur at VU University Amsterdam, The Netherlands. The research focused on better understanding of underlying mechanisms of the (idiosyncratic) adverse drug reactions and the identification of possible risk factors for individual susceptibility, contributing to the development of novel, translational technologies and methodologies, which can moreover serve as the interesting starting points for future research in drug safety sciences. To perform part of this research project, she visited the Pharmacogenetics group at the Karolinska Institute (Stockholm, Sweden) and performed a study under supervision of Prof. Dr. Magnus Ingelman-Sunberg. Since October 2012, Sanja joined the managing entity of two projects funded by IMI-JU (EC, European Commision and EFPIA, European Federation of Pharmaceutical Industries and Associations): SafeSciMET, an European Modular Education and Training Programme in Safety Sciences for Medicines, and MIP-DILI, The Mechanism-based Integrated Systems for the Prediction of Drug-induced Liver Injury, a research project which is closely reletad to the research described in her thesis.

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