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

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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 1H-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 agranulocytosis. 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 agranulocytosis 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-T1 double-null genotype and occurrence of agranulocytosis. In addition to reported \textit{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.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{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).}
\end{figure}
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 acetaminophen (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 IDR. We have also shown that hGSTs, specifically polymorphic hGSTM1-1, play a significant role in 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 predictivity of reactive metabolite formation with regard to risks for ADRs or IDR 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.

References


