Summary

The liver is the primary organ in drug metabolism, and consequently drug induced liver injury (DILI) is an adverse event commonly linked with exposure to chemically reactive drug metabolites (CRMs). The adduction of cellular macromolecules by CRMs is linked to the onset of this adverse drug reaction [1]. Because of its severe clinical outcome and the financial risks for the drug discovery and development process, idiosyncratic drug induced liver injury (IDILI) is of special interest. These reactions are currently mechanistically poorly understood and therefore unpredictable in pre-clinical phases of drug development. In addition, IDILI has a low incidence and delayed onset, properties which make it generally difficult to detect these adverse events in clinical stages in drug development. IDILI is therefore often only identified after marketing of a drug. Typically IDILI has major impact including black box warnings and/or drug withdrawal, patient morbidity and mortality and financial loses for the pharmaceutical industry [2,3]. Although detailed mechanistic insights are still lacking, it is postulated for many IDILI associated drugs that CRMs initiate IDILI by cellular macromolecule haptanization which are consequently recognized as non-self and excessively activate the immune system [4,5]. In this thesis inter-individual variation in CRM formation is assessed, which provides additional insight in factors contributing towards IDILI sensitivity.

Chapter 1 provides an introduction in IDILI, which is by definition the result of properties of the specific drug as well as of the individual patient (idiosyncratic is particular for an individual) and thus multi-factorial in nature. Inter-individual variability in biotransformation is usually a major contributor to susceptibility towards (I)DILI. Chapter 1 describes the sources of variation in hepatic exposure to CRMs. In particular, the balance in bioactivation (CRM formation) and detoxification (CRM inactivation) is introduced conceptually. The variability in the major enzymes involved in bioactivation (cytochrome P<sub>450</sub>s (CYPs)) and detoxification (glutathione S-transferases (GSTs) and quinone oxidoreductases (NQOs)) is considerable and is reflected on genetic, mRNA, protein and activity levels. CRM identification and quantification, which is a challenging but essential experimental aspect, is evaluated, specifically the existing methodologies for the identification and quantification of GSH trapped CRMs. Lastly, some cellular in vitro models are described in which the relative expression levels of bioactivation and detoxification enzymes are modified. This allows the investigation of the effect of alterations in the bioactivation/detoxification balance on the outcome in cellular toxicity.
In order to understand the role of CRMs and CRM exposure in individual susceptibility towards (I)DILI, detailed insights in metabolic pathways of the corresponding drugs is required. Therefore, the first aim of this thesis was contributed specifically to the molecular mechanistic knowledge regarding the metabolic pathways responsible for the bioactivation to and detoxification of CRMs resulting from biotransformation of (I)DILI associated drugs. The second aim of this thesis explored the degree of heterogenicity in activities of the major phase I (CYPs and NQOs) and phase II drug metabolizing enzymes (GST, UDP-glucuronosyltransferase (UGT), sulfotransferases (SULT)), which is essential to define inter-individual variability in drug metabolism. The work in this thesis was performed as part of the IMI project ‘Mechanism-Based Integrated Systems for the Prediction of Drug-Induced Liver Injury (MIP-DILI; Grant 115336)’, which aimed for the development of better predictive models for (I)DILI in human [6].

Since the basis for accurate determination of bioactivation in toxicological in vitro and in vivo models is the identification of CRMs and the quantitative assessment of CRM formation, chapter 2 introduces a generic method for absolute quantification of CRMs after conjugation to GSH. Although for detection of GSH-conjugates sensitive LC-MS(/MS) methodologies are available, the absolute quantification relies on the availability of authentic reference solutions. Nuclear magnetic resonance (NMR) is the method of choice for quantification of these isolated (bio)chemically generated metabolites but is restricted by sensitivity [7], which is important for GSH conjugates which are mostly formed in low quantities. Our alternative method described in chapter 2 relies on hydrolysis of the peptide bonds in the GSH-moiety, resulting the release of glutamic acid. Quantification of this amino acid, after o-phthalodialdehyde (OPA) derivatization, which is representative for quantification of the GSH-conjugate, can be performed using calibration curves of glutamic acid and was shown to be 100-fold more sensitive compared to the conventional NMR methodology. The OPA-method was validated with commercial alkyl-GSH conjugates and applied to GSH-conjugates of the hepatotoxic drugs acetaminophen, diclofenac and clozapine. For the respective GSH-conjugates a methodological comparison was made with NMR and relative LC-UV quantification.

In chapter 3, the bioactivation of the NSAID diclofenac to reactive quinone imines was investigated in more detail. Bioactivation of diclofenac is preceded by hydroxylation, followed by oxidation to quinone imines and is considered to be related to diclofenac induced IDILI. The CYP isoforms responsible for the initial hydroxylation were already well characterized. However, the subsequent dehydrogenation reactions resulting in the
respective CRMs were poorly defined. The results indicate that the formation of 4'-hydroxy
diclofenac and the subsequent bioactivation to the related 1',4'-quinone imine appeared to
be selectively catalyzed by CYP2C9, with an approximately 2-fold higher intrinsic clearance
compared to the first oxidative step. For 5-hydroxy diclofenac, however, CYP3A4 is the major
enzyme for the initial hydroxylation, whereas CYP2C9 (followed by CYP2C19) is the most
active enzyme for the subsequent bioactivating dehydrogenation step. The second oxidation
step has a 14-fold higher intrinsic clearance. For determination of the variability in CRM
formation, all these metabolic steps had to be considered, and indicated the involvement of
isoforms of the enzymes for which activities are highly variable within the human
population.

Chapter 4 continues with the elucidation of enzymes for which altered activities may be risk
factors of diclofenac-induced IDILI and describes GST-catalyzed detoxification of diclofenac-
derived quinone imines. Ten recombinant GST-isoforms were screened for their capability to
inactivate the respective CRMs. Importantly, significant inhibition of several GST-isoforms
by diclofenac was observed. Incubation conditions were therefore optimized compared to
previous work [8]and high catalytic activity was observed for several GST-isoforms,
including the highly abundant and differentially expressed hepatic isoforms GSTA1-1, A2-2
and M1-1. In addition, the highest activity was observed for GSTP1-1, which is not expressed
in hepatocytes but probably in the bile duct, which is distant from the site of bioactivation.
The capacity of the highly active isoforms GSTP1-1 and M1-1 to protect against (protein) thiol
adduction by diclofenac quinone imines was assessed in vitro by application of N-acetyl
cysteine as probe thiol for adduct formation. Addition of GSTs significantly reduced the N-
acetyl cysteine adduct formation, which was the result of an enzymatic process as confirmed
by ethacrynic acid (EA) inhibition of the GSTs. Lastly, the specific activities of the
recombinant enzymes with diclofenac quinone imines, obtained for each GST-isoform, were
used to predict the variability in diclofenac detoxification in a small population, using
hepatic GST expression levels obtained from literature.

In Chapter 5, both CYP- and GST-isoenzyme activities are investigated for the bioactivation
and detoxification of mefenamic acid, which has been associated with severe liver injury in
mice [9]. Although reactive mefenamic acid acyl-glucuronides are well investigated and
associated with toxicity, the CYP-catalyzed bioactivation was previously poorly
characterized. For this purpose, recombinant CYP-isoenzyme and human liver microsomal
incubations were performed. Besides a non-toxic 3'-hydroxy-methyl mefenamic acid
metabolite, 4'- and 5-hydroxy metabolites were identified which, as previously described for
diclofenac, have the potential to be converted to reactive quinone imine metabolites. Indeed, three resulting GSH-conjugates of mefenamic acid were detected and identified, thus confirming the formation of the respective CRMs. Formation of the GSH-conjugates was shown to be dependent mainly on CYP1A2, 3A4 and 2C9 activity. Importantly, the formation of GSH-conjugates was found to be increased by addition of GSTs, mainly GSTP1-1, A2-2 and M1-1. So apparently, the chemical conjugation is not sufficient to fully trap the formed CRMs and GST catalyzed conjugation contributes to the protection against these harmful metabolites.

Additional enzymatic detoxification potential of CRM-forming (I)DILI drugs is further assessed in chapter 6. In this chapter, we determine hepatic expression levels of NAD(P)H:quinone oxidoreductase 1 (NQO1) and NADH-quinone oxidoreductase 2 (NQO2) in 20 human liver donors. In addition, recombinant NQOs were used to assess the catalytic activity for reduction of quinones derived from the (I)DILI drugs acetaminophen, diclofenac, mefenamic acid, clozapine, amodiaquine and carbamazepine, as a follow up of a previous study concerning NQO1 only [10]. Hepatic expression levels of NQO1 were low (nM range) and strongly variable between the human donors. NQO2 levels were higher (μM range) and less variable. The low hepatic concentrations of NQO1 were shown to be sufficient for efficient inactivation of CRMs derived from amodiaquine and carbamazepine. NQO2, for which no information was available regarding catalytic activity towards CRMs, appeared to be less active for most CRMs when compared to NQO1, or not active (5-hydroxy diclofenac derived quinone-imine). However, for the clozapine nitrenium ion, NQO2 but no NQO1 activity was observed. This is an interesting finding since NQO2 genotypes are associated with idiosyncratic clozapine toxicity [11]. Besides the enzymatic reduction of CRMs, catalyzed by NQO2, non-enzymatic CRM scavenging by the NQO2 protein was observed as well. On the in vivo relevance of NQO1 and NQO2 as detoxifying proteins we speculate that NQO1, which is highly inducible, becomes especially important either after induction or when competing protective routes are exhausted (e.g. GST). For NQO2, the in vivo relevance remains to be established since the required co-factor (dihydronicotinamide riboside (NRH)) has very low physiological levels in in vivo models. However, the non-enzymatic CRM scavenging by the NQO2 protein may also significantly protect essential cellular targets due to the high hepatic NQO2 expression levels (up to 30 μM in human).

In chapter 7, the variability in major drug metabolizing enzymes was simultaneously assessed in homogenates of 20 human liver donors. Variations in drug metabolizing enzymes are a major contributor to heterogeneity in drug clearance and safety in general, and may
lead to altered hepatic CRM exposure. Although inter-donor variations in expression and activity of CYP- and UDP-glucuronosyltransferases (UGTs) have been reported, simultaneous characterization with other important enzymes (e.g. sulfotransferases (SULTs), GSTs, NQO1 and NQO2) is lacking. In this chapter, the phenotyping of 20 human liver homogenates is described on activity levels. Since several GST isoforms are indistinguishable on activity level, additionally protein expression levels were determined. The highest variability was observed for the detoxifying enzymes NQO1 (activity level) and GSTM3 (protein level). Correlations between the enzymes were mainly found within and between members of the CYP- and UGT-family. The most significant correlations ($p < 0.001$) were observed for CYP2E1/CYP3A4 (Rs 0.78), UGT1A6/UGT1A9 (Rs 0.61), UGT1A3/UGT2B7 (Rs 0.74), CYP2A2/CYP2E1 (Rs 0.71), UGT1A1/UGT1A4 (Rs 0.42) and UGT1A4/CYP2C9 (Rs 0.70). The study provides the most comprehensive dataset on variations in drug metabolizing enzymes in a small cohort of human livers, and can serve as resource for in silico modeling of enzyme kinetics.

Lastly, in chapter 8, the previously described human liver homogenates are used to experimentally assess variability in the balance between bioactivation and detoxification of the IDILI associated drugs clozapine and diclofenac. Firstly, the GST protein content in cytosolic extracts (isolated by affinity chromatography) of the 20 human donors was quantified by LC-UV and was investigated by LC-MS for identification of GST-isoforms and genetic variants. In line with the (limited) human data available in literature, the hepatic abundance of GSTA1 was highest, followed by GSTA2, M1, P1, T1 and M3. For GSTA2, M1 and P1, genetic variants were identified as well. Subsequently, the GST extracts were incubated with diclofenac or clozapine in presence of a common bioactivation system created to mimic an averaged composition of human CYP activity, in order to assess the variability in GST activity in CRM detoxification. Furthermore, the variability in bioactivation was assessed by incubations with the individual liver microsomes from the 20 donors and quantification of the formed CRMs. The resulting balance between bioactivation and detoxification of both clozapine and diclofenac indicates that for most pathways variations in GST-activity significantly contribute to the heterogeneity, especially in presence of low GSH concentrations. In addition, comparison of native (isolated) GST activity with reported recombinant GST activities (chapter 4) showed that recombinant GSTs were well predictive for hepatic GST activities in detoxification of quinone imines derived from 5-hydroxy diclofenac, but not from 4'-hydroxy diclofenac.