Chapter 7

Summary, conclusions and perspectives
Summary

Idiosyncratic Adverse Drug Reactions (iADRs), and specifically Idiosyncratic Drug-Induced Liver Injury (iDILI) remain a significant problem for drug therapy. Since these types of side effects are difficult to detect during drug development they are often only discovered after marketing [1]. For many drugs, biotransformation has been shown to be a key-factor in cases where the formation of toxic metabolites leads to hepatotoxicity. Toxic metabolites can be Chemically Reactive Metabolites (CRMs) as is the case with Nevirapine, Ticlopidine and Clozapine; however, this is not required as is the case with Flucloxacillin [2–5]. In the case of CRMs, cellular macromolecules can be covalently modified leading to a disruption of their function or even an immune response towards the modified peptides [6]. Mechanisms of hepatotoxicity via stable metabolites are exemplified by the inhibition of the Bile Salt Efflux Pump (BSEP) or other transport proteins [7]. In both cases the intracellular concentration of metabolites determines the outcome of the hepatotoxicity and this concentration is partly regulated by Drug Metabolizing Enzymes (DMEs), such as the bio-activating Cytochrome P450s (CYPs) and the bio-inactivating Glutathione S-Transferases (GSTs). Since both families of enzymes are variable and shown to be influenced by genetic and environmental factors, the balance between bio-activation and bio-inactivation will strongly differ between patients [8, 9]. This inter-individual variation causes certain patients, with high bio-activation and low bio-inactivation, to be more susceptible to iDILI than other patients, with low bio-activation and high bio-inactivation. However, to predict the cellular exposure to the toxic metabolite, the DMEs involved in the biotransformation of drugs need to be characterized. Without this knowledge, it is not possible to understand or model the susceptibility of patients to DILI. However, at the start of the research described in this thesis, there were still important gaps in the knowledge of the DMEs involved in the balance between bio-activation and bio-inactivation of Nevirapine, Ticlopidine, Clozapine and Flucloxacillin.

The aim of the research presented in this thesis was therefore to study the DMEs involved in the balance between bio-activation and bio-inactivation of Nevirapine, Ticlopidine, Clozapine and Flucloxacillin, thereby completing our knowledge of related DILI or IDILI.

For Nevirapine, Ticlopidine and Clozapine the focus was on identifying the DMEs involved in bio-inactivation in chapter 2, chapter 3 and chapter 4. Combined with already known knowledge on the bio-activation pathways, these data should provide us
a better insight into the bio-activation/bio-inactivation ratio for these drugs in more or less susceptible patients. For Flucloxacillin, the formation of its stable metabolite 5’-hydroxymethyl-Flucloxacillin was investigated in chapter 5, since this metabolite was shown to be toxic to biliary epithelial cells. In chapter 6, bacterial CYPs were investigated to find a bio-catalyst able to form 5’-hydroxymethyl-Flucloxacillin.

Chapter 1 is a general introduction in the concept of drug metabolism and related variation, Adverse Drug Reactions (ADRs) and more specifically iDRs and iDILI. Several mechanisms leading to iDILI are discussed, for instance involving DMEs, such as CYPs and GSTs, how these enzymes are responsible for the formation of, or protection against, CRMs and the mechanisms by which the activity of these enzymes can vary between patients. Next it is explained how this variation in DMEs can lead to more and less susceptible patients. Furthermore, several other mechanisms of iDILI are introduced, such as the involvement of the immune system, mitochondrial toxicity and lysosomal impairment. The IMI project ‘Mechanism-Based Integrated Systems for the Prediction of Drug Induced Liver Injury (MIP-DILI, grant 115536)’ which aimed for the development of better predictive models to detect and prevent idiosyncratic drug induced liver injury is introduced and how this thesis fits into the scope of this consortium [10]. Finally, the drugs studied in this thesis, namely Nevirapine, Ticlopidine, Clozapine and Flucloxacillin, are introduced. Nevirapine is a non-nucleoside reverse transcriptase inhibitor widely used to protect against the transmission of HIV1 from mother to child in third-world countries. SULT1A1 and CYP3A4 can bio-activate Nevirapine to various CRMs, namely 12-sulfoxo-Nevirapine, a quinone methide and epoxides and these CRMs can trigger an immune response in humans [11]. However, the GSTs involved in the bio-inactivation of these CRMs were not characterized yet. Ticlopidine is an ADP-receptor antagonist that can form a chemically reactive S-oxide and a chemically reactive epoxide [12–14]. This Ticlopidine CRMs can covalently bind to proteins, such as CYP2B6. Correlation studies with specific HLA-types have shown that the immune system is involved in the toxicity as well. However, the characterization of bio-activating CYPs is not complete, nor have the GSTs involved in bio-inactivation of the CRMs been characterized. Clozapine is an antipsychotic agent functioning as an antagonist for the serotonin and dopamine receptors. Clozapine can be bio-activated by CYPs to a chemically reactive nitrenium ion [15]. This nitrenium ion can be bio-inactivated by GSTs [16]. Although it has been shown that GSTs are involved in the bio-inactivation of the nitrenium ions, the variation in this pathway between patients remains unknown. Finally, Flucloxacillin is a β-lactam antibiotic agent shown to be able to covalently modify proteins. It causes liver injury
which has one of the highest correlations ever reported with HLA*B 57:01. However, this HLA-type still does not fully explain the human susceptibility to the toxicity [17]. Since a metabolite of Flucloxacillin, 5’-hydroxymethyl Flucloxacillin, is toxic to biliary epithelial cells, it is believed that the formation of this metabolite might be a risk factor [5]. Although the formation of this metabolite can be key to identification of susceptible individuals, only a limited number of CYPs have been investigated in an attempt to elucidate the formation of 5’-hydroxymethyl Flucloxacillin [5].

**Chapter 2** focusses on the characterization of GSTs involved in the bio-inactivation of Nevirapine CRMs. Oxidative metabolism of Nevirapine was believed to lead to the formation of a quinone methide [18]. However, by deuterating Nevirapine we were able to show that the chemically reactive metabolite leading to the Glutathione (GSH)-conjugate at position 3 is actually an epoxide. Furthermore, we found that the bio-inactivation of this epoxide to the corresponding GSH-conjugate can be catalyzed by GSTP1-1. Additionally, we were able to synthesize 12-sulfoxy Nevirapine and characterize the GSTs involved in the conjugation of this metabolite to GSH. It turns out that the conjugation to GSH can be catalyzed by GSTM1-1, GSTA1-1 and GSTA3-3. However, the relatively low $V_{\text{max}}$ of $2.0 \pm 0.27$ nmol/min/μmol GST for GSTM1-1, $0.23 \pm 0.021$ nmol/min/μmol GST for GSTA1-1 and $1.07 \pm 0.06$ nmol/min/μmol GST for GSTA3-3, combined with the incomplete trapping of 12-sulfoxy Nevirapine, make it unlikely that GSTs can completely protect against this CRM. This is in contrast with association studies implying that GSTM1-1 has a protective effect against Nevirapine induced hepatotoxicity [19]. Thus, **null** genotypes for GSTM1-1 would not make a significant differences in the ratio between bio-activation and bio-inactivation, but GSTM1-1 might protect against Nevirapine hepatotoxicity via other mechanisms

**Chapter 3** characterizes the DMEs involved in the bio-activation and bio-inactivation of Ticlopidine. Here we detected six GSH-conjugates of which two are formed via a chemically reactive S-oxide. The S-oxide bio-inactivation could be catalyzed by GSTM1, leading to a 40-fold increase of GSH-conjugate formed. Interestingly, a 40-fold variation in the amount of GSH-conjugate formed was detected when using cytosol from different human liver donors. However, to our surprise this effect did not correlate well to GSTM1, indicating that other GSTs or bio-inactivating enzymes might also affect the bio-inactivation of Ticlopidine. When studying the effects of GSTs on the formation of GSH-conjugates of the respective chemically reactive epoxides smaller effects were seen. The same was observed for the variation between bio-inactivation in cytosols from individual liver donors, in which smaller differences were detected for the epoxide than
for the S-oxide. The amount of bio-activation at 100 µM of Ticlopidine only varied 4-fold, CYP1A2, CYP2B6, CYP2C19 and CYP2D6 being the most active enzymes. Because the bio-activation capacity varied less than the bio-inactivation, clear differences were observed, when plotting these two parameters versus each other. Therefore, it is likely that patients with low Ticlopidine S-oxide bio-inactivation capacity, e.g. by GSTs, could be more susceptible to the DILI.

In chapter 4, the bio-inactivation of Clozapine in in vitro incubations, in patient urine and in human liver slices was investigated. The in vitro incubations with recombinant DMEs showed that GSTP1 is the most active enzyme in the bio-inactivation to the Clozapine nitrenium ion. This is mostly because of this GST-isoenzyme's high capability of forming the GSH conjugate CG-6. Next, the metabolites found in precision cut human liver slices and urine have been identified by LC-MS and their relative abundance has been determined as well. Here it is seen that more than 40 metabolites are identified, but their relative abundance differed highly between patients. The same was found for eleven GSH-derived metabolites. However, because of the wide variability in amounts and profiles, excretion of GSH-related metabolites did not clearly appear to be related to specific genotypes of GSTs. Analysis of the GSH-related products in urine did neither appear to be useful for quantitative biomonitoring of internal exposure to chemically reactive Clozapine metabolites.

Chapter 5 and 6 focus on the metabolism of Flucloxacillin to 5'-hydroxymethyl Flucloxacillin by human and bacterial CYPs. In chapter 5, CYP3A4 and CYP3A7, and to a lesser extent CYP2C9 and CYP2C8 are identified as the most active human enzymes metabolizing Flucloxacillin, e.g. by incubations with recombinant enzymes, inhibition studies and correlation studies. Furthermore, it was shown that 5'-hydroxylation of Flucloxacillin by human liver microsomes from different human liver donors can vary approximately 6-fold between the donors. Since 5'-hydroxymethyl Flucloxacillin is reported to cause toxicity in biliary epithelial cells this variation could be a possible risk factor for Flucloxacillin-related DILI. However, as indicated by the low Pearson R in correlation studies, multiple enzymes seemed to be involved. This indicates that the variability in 5'-hydroxymethyl Flucloxacillin concentrations between patients might is not likely due to polymorphisms in a single enzyme. Remarkably, Sulfaphenzole was able to inhibit the CYP3A-family from 5'-hydroxylating Flucloxacillin. This could indicate that unexpected drug-drug interactions could occur between the CYP3A-family and Flucloxacillin metabolism, which would not be expected based on the isoenzyme specificity of the perpetrating drug. Since both recombinant CYP3A4 and CYP3A7 were
inhibited by sulfaphenazole and since sulfaphenazole has also been shown to inhibit CYP2C9, these types of drug-drug interactions can strongly affect the intracellular exposure to the 5'-hydroxymethyl Flucloxacillin. **Chapter 6** focusses on finding a selective and active bio-catalyst capable of producing 5'-hydroxymethyl Flucloxacillin. Here, we report that the BM3 mutant M11 L437E to be able to regioselectively 5'-hydroxylate Flucloxacillin with relatively high activity. LC-MS and NMR confirmed the identity and purity of this metabolite. Molecular Dynamics simulations moreover suggested that M11 L437E is more likely to bind Flucloxacillin in a catalytically active pose over other binding poses, compared to other mutants. This finding was supported by incubation with Oxacillin, which lacks the halogens of Flucloxacillin. Oxacillin can be hydroxylated by BM3 at the aromatic ring as well and the ratio between the 5'-hydroxylation and hydroxylation at this ring is higher for BM3 M11 L437E than for other mutants. We therefore propose BM3 M11 L437E as a suitable bio-catalyst for the production of 5'-hydroxymethyl Flucloxacillin and an adequate alternative to rat liver microsomes.

**References**


