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8.1. Summary

Drug-induced liver injury (DILI) continues to be a major public health issue as it is among the primary reasons for drug attrition during preclinical drug development and clinical trials. Moreover, DILI is the leading cause of post-market safety actions and acute liver failure (1). The majority of DILI cases concern intrinsic toxicity (usually acetaminophen overdose), meaning that the drug causes dose-dependent and predictable hepatotoxic effects. Most drugs are safe at normal usage, some drugs, however, cause idiosyncratic DILI (IDILI). The underlying mechanisms of IDILI are largely unknown and its onset can therefore not (yet) be predicted based on pre-clinical and clinical studies. IDILI is thought to result from a combination of factors, which are drug related, but also related to the individual (2). Altered drug metabolism, in particular the formation of chemically reactive metabolites (CRMs), is proposed as an important factor (3). The role of drug metabolizing enzymes in (I)DILI is elaborated in **chapter 1**. To this end, hepatic drug metabolizing enzymes (phase I and phase II) and transporters (phase III) are discussed. The role of CRMs in the onset of liver injury and the formation of CRMs by phase I or phase II metabolism is described using examples from literature. Importantly, but sometimes scarcely taken into consideration, CRMs are enzymatically detoxified as well. The final cellular exposure to CRMs is therefore dependent on the balance between bioactivation and detoxification. Alteration of this balance can increase or decrease the susceptibility of an individual to develop (I)DILI. A brief overview is therefore given on genetic- and non-genetic factors conferring inter-individual variability in drug metabolism and side effects. Data from *in vitro* models considerably contribute to the mechanistic understanding of (I)DILI and have a great potential to ultimately aid in minimizing (I)DILI risk of drug candidates early in the drug development process (4). A brief summary of current non-cellular and cellular *in vitro* models which are used to study the drug metabolism and resulting toxicity is provided. The chapter is finalized by a brief discussion regarding the usefulness of *in vitro* data to identify a susceptible patient/drug combination.

The research described in this thesis, carried out in the context of the IMI-supported MIP-DILI project, had the primary aim to investigate the involvement of phase I and phase II metabolism in the outcome of *in vitro* cytotoxicity assays. The first part of this thesis (**chapters 2-3**) focusses on primary human hepatocytes (PHH), which are considered the gold standard in hepatic drug metabolism and resulting toxicity in *in vitro* research. Specifically, the alterations in metabolic phase I and phase II activities in response to culture conditions were assessed. The second part (**chapters 4-7**) is aimed at the evaluation of inter-individual variability in drug metabolism as a risk factor for CRM-related drug toxicity.

The phase I enzyme family of cytochrome P450s (CYPs) and the phase II enzyme families UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) are most often implicated in drug metabolism. All three enzyme families are active in isolated PHH, although activities are highly dependent on culture conditions. PHH are typically used in suspension (for short incubation times, usually up to 4 hours) or in monolayer (for longer incubation times, usually 1-7 days) (5). In **chapter 2** three model hepatotoxic drugs, chosen as training compounds in the MIP-DILI project (acetaminophen, diclofenac and tolcapone), and a general UGT/SULT substrate (7-hydroxycoumarin) were used to compare CYP, UGT and SULT activities in suspended and plated PHHs. Donor-dependent effects of culture conditions were incorporated in this chapter by evaluating the metabolism in PHHs derived from five donors. As expected from literature, CYP activities were significantly lower in plated PHH for all substrates and all donors. The

fold decrease in CYP activity was highly dependent on the substrate and, to a lesser extent, on the donor. Similarly, UGT activities were for some donor/substrate combinations comparable between culture conditions, but in most cases lowered in plated PHH, although the drop in activity was overall less compared to CYP activities. Furthermore, SULT activities showed opposite results when comparisons were made of culture conditions for specific substrate/donor combinations. Together, these data show a substrate- and donor-dependent alteration in the phase I/phase II metabolism ratio in plated PHH, and emphasized the need of proper characterization of relevant metabolic activities in cellular *in vitro* models for correct interpretation of readouts.

Determination of activities of the major CYP isoforms in intact cells *in vitro* is well described, however, similar approaches have not been optimized for UGTs. Therefore, **chapter 3** describes the development of an assay to phenotype activities of the major hepatic UGT isoforms (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 and UGT2B7) in intact PHH. For this purpose, isoform-selective probe substrates were chosen from literature describing UGT-isoform phenotyping in human liver microsomes (HLM). The use of a cocktail of substrates, which is generally applied for CYP substrates, was limited because of cytotoxicity and interference of probe reactions by other substrates, which were simultaneously present in the PHH incubations. Subsequent incubations were therefore performed with single substrates. UGT activities were determined in intact suspended and plated PHHs from five donors, and were compared to activities in HLM from respective donors. Assessment of major the hepatic CYP activities (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A) was performed analogously. Donor-variability in UGT and CYP activities were comparable between PHH and HLM, indicating that metabolite formation in PHH primarily reflected enzyme activity. In agreement with **chapter 2**, UGT activities of most isoforms were in similar range in suspended and plated PHH, while the majority of CYP isoforms showed a drop in activity upon plating. An important additional finding was the substantial loss of CYP2B6 and CYP2C19 activities in HLM, which further emphasizes that the contribution of specific CYP isoforms to total metabolism is dependent on the choice of *in vitro* assay. In summary, **chapter 3** describes a convenient standard assay for UGT and CYP activity profiling which can be applied to reaction phenotype intact cells. Importantly, **chapters 2** and **3** underscore the fact that PHH represent a good *in vitro* model to investigate inter-individual variability of phase I and II related drug metabolism.

From **chapter 4** onwards, the focus of this thesis shifts from the differences in metabolic activities between *in vitro* assays to inter-individual variability in hepatic drug metabolism. It is well acknowledged that variability in drug metabolizing enzyme activities results in heterogeneity of drug efficacy and safety within the population. Although variability in CYP levels/activities, and to a certain extent also in UGT levels, is well investigated, data is only limited for remaining major hepatic drug metabolizing enzymes, like SULTs and CRM detoxification enzymes, like quinone reductases and glutathione S-transferases (GSTs). The aim of chapter 4 was therefore to assess isoform-specific activities of CYPs, UGTs, quinone reductases, GSTT1 and GSTT2, and non-specific activities of SULT and GSTs in liver homogenates of a population of 20 individuals. The work is complemented by quantification of cytosolic GST isoforms (other than GSTT1 and GSTT2) at the protein level. Variability of CYP and UGT isoforms was within reported ranges. Both members of the quinone reductase family (NAD(P)H:quinone oxidoreductase 1, NQO1 and NRH:quinone oxidoreductase 2, NQO2), which are structurally highly similar (6), showed divergent variability, NQO1 being highly variable and NQO2 more conserved. Similarly for the GST family, GSTA1

levels were among the more conserved enzymes, while GSTM3 levels showed highest variability of all enzymes assessed. The comprehensive parallel assessment of activities of all major drug metabolizing enzymes additionally allowed analysis of correlations (mostly found within/between UGT and CYP isoforms). These are especially important for *in silico* pharmacokinetic predictions.

Chapter 5 is dedicated to the quinone reductase family. NQO1 and NQO2 can both reduce quinone-like structures to corresponding hydroquinones and are therefore considered detoxifying enzymes (7). NQO1-catalyzed reduction of drug-derived quinone imines was previously shown, while no such information was available for NQO2. Hepatic protein concentrations of NQO1 and NQO2, and consequently their possible participation in hepatic drug metabolism, were unknown as well. Therefore, during analysis of NQO1 and NQO2 activities in the set of 20 human liver homogenates (as described in **chapter 4**), calibration curves of recombinant NQO1 and NQO2 were included to quantify native NQO1 and NQO2 protein concentrations. Hepatic NQO1 concentrations ranged from 10 nM to 213 nM, while hepatic NQO2 concentrations were much higher, ranging from 2.4 μM to 30.6 μM. Using recombinant protein, we further demonstrated that, like NQO1, NQO2 can reduce quinone-like drug metabolites (i.e. from acetaminophen, clozapine, diclofenac and mefenamic acid). Additionally, incubations with purified quinone-like metabolites derived from amodiaquine and carbamazepine showed that low nM or μM concentrations, from NQO1 or NQO2 respectively, are sufficient to fully reduce these quinone-imines, thus suggesting that hepatic levels of both quinone reductases are sufficient to contribute in detoxification of quinone-like CRMs.

GSTs adopt a central role in **chapter 6**. Results on inter-individual variability in hepatic GST expression described in **chapter 4** were further elaborated by discussing the results on absolute hepatic abundances of GST isoforms (GSTA1 > GSTA2 > GSTM1 > GSTP1 > GSTT1 > GSTM3) and incidences of polymorphisms in the population of 20 human liver donors. Individual mixtures of native GSTs (GSTA1, GSTA2, GSTM2, GSTM3 and GSTP1), which were isolated from each of the 20 donors, were incubated with the model hepatotoxic drugs clozapine and diclofenac. Both drugs are bioactivated by CYPs, resulting in a reactive nitrenium ion (clozapine) or two quinone imines (diclofenac). Incubations were composed such that the native GST concentrations resembled cytosolic levels, and results therefore closely mirrored inter-individual variability of hepatic GST activities in detoxification of clozapine and diclofenac CRMs. Inter-individual variability in formation of clozapine and diclofenac CRMs was studied in separate incubations containing HLM from each individual donor. The balance of bioactivation/detoxification was significantly affected by GSTs, both for clozapine and diclofenac. This contribution was dependent on GSH-concentration (clozapine) and on the intrinsic characteristics of the CRM (diclofenac).

The role of GSTs as a detoxifying enzymes is further evaluated in **chapter 7**. *In vitro* studies previously showed that GSTP1 catalyzes GSH conjugation of quinone imines from the hepatotoxic drug amodiaquine. However, the physiological protective role of GST enzymes is to a certain extent ambiguous as it also regulates cell signaling pathways, thereby determining cell proliferation and survival (8). The protective capacity of GSTP1 was investigated *in vitro* by exposing GSTP1-overexpressing HepG2 cells to purified amodiaquine quinone imines. Metabolism studies showed an increase in GSH-conjugate formation in GSTP1 expressing cells, confirming the catalytic activity of GSTP1. Furthermore, treatment with quinone imines decreased cell viability of GSTP1-expressing cells significantly less compared to mock-transfected cells. Similarly, caspase 3 activity, which is a marker for apoptosis, was less induced following quinone

imine treatment in GSTP1-expressing cells. By means of adaptive stress response HepG2 reporters (9), it was additionally shown that amodiaquine quinone imines activate the ER-CHOP stress response. Intriguingly, activation of this stress response did not significantly differ in GSTP1- and mock-transfected cells. These results imply that GSTP1 protects HepG2 cells against amodiaquine quinone imine cytotoxicity in two distinct ways. Apart from the catalytic activity of GSTP1, protection via modulation of cell signaling pathways by GSTP1 was implied.

8.2. Conclusions and perspectives

The objective of this thesis was to evaluate the importance of phase I and phase II enzyme activities and variation in interpretation of *in vitro* results, with the ultimate aim to improve (I)DILI risk assessment. Two main objectives were assessed: 1) the importance of characterization of phase I and phase II enzyme activities in *in vitro* cellular assays and 2) the significance of incorporating inter-individual variability in drug metabolizing enzyme activities in the risk assessment of (I)DILI.

So far, CYP activities have mostly been the primary focus of all drug metabolizing enzymes, mainly because of their high contribution in drug metabolism (10,11). Phase II activities, however, gain increasingly more interest because their contribution in drug metabolism is also relevant and likely to increase for new drugs (12,13). Furthermore, especially in the context of (I)DILI risk assessment, activities of (CRM) detoxifying enzymes are specifically of interest as the ultimate (hepatic) exposure to CRMs is dependent both on the formation of CRMs (enzymatic bioactivation of drugs) and the decrease of CRM levels (chemical or enzymatic scavenging, enzymatic reduction and competing pathways).

8.2.1. Characterization of enzyme activities in cellular in vitro models

It is well established that culture conditions of PHH considerably affect CYP activities (14,15). The choice between suspended and plated PHH is usually made based on incubation time, while the loss of CYP activities is taken into account in plated PHH (5). In **chapters 2** and **3**, we complemented existing biotransformation research by including phase II enzyme activities in the investigation of culture effects on enzyme activities. From these results, we concluded that the effects of PHH culture conditions on CYP activities cannot be extrapolated to UGT and SULT activities. In fact, the use of isoform specific probe substrates (**chapter 3**) further revealed that CYP and UGT isoform activities are differentially affected by plating of PHH. The results presented in the first part of this thesis provide evidence that the choice of culture condition strongly affects metabolic ratios, specifically the relative contribution of different enzymes, and by inference of the balance between formation and detoxification of CRMs. It is therefore highly recommendable to identify participating enzyme isoforms (both phase I and phase II) using recombinant proteins and/or liver homogenates and to use cellular models properly characterized for enzyme activities. The assays described in **chapter 3**, to characterize UGT isoform activities in intact cells, is highly beneficial for this purpose. This will aid in correct interpretation of the corresponding readouts and in extrapolation of *in vitro* data to the *in vivo* situation. In the meantime, the assay has been successfully applied to PHH, HepaRG cells, HepG2 cells and hepatocytes derived from Human Induced Pluripotent Stem Cells.

8.2.2. Inter-individual variability in drug metabolizing enzyme activities

The rare incidence of IDILI is related to the fact that (differential) combinations of causative factors are unique to an individual (2). Genetic studies have proposed polymorphisms in genes encoding drug metabolizing enzymes, transporters and in immunomodulatory genes as risk factors for IDILI caused by specific drugs (16). It is however still unknown to which extent the inter-individual variability in IDILI susceptibility arises from environmental versus genetic factors (17). Since CRM formation is considered an initiating event in IDILI for many drugs (3), special emphasis is placed on drug liabilities for CRM formation and inter-individual variability in drug metabolizing enzyme activities. The overview of drug metabolizing enzyme activities in a population of human liver donors provided in **chapter 4** is the first to compare simultaneously inter-individual variability in the large enzyme set of CYPs, UGTs, SULTs, quinone reductases and GSTs. Correlations which were present or absent between enzyme isoforms are especially valuable for *in silico* PK-PD modeling, which have the advantages of being rapid with relatively low costs and which can be used to generate virtual human populations (18,19). **Chapter 4** can serve as a basis for analogous future research. Indeed, gathering such information is critical for future research, as combinations of specific enzyme activities rather than single enzyme activities are more likely to alter the balance of bioactivation and detoxification to such an extent that liver toxicity in initiated.

Whilst the responsible enzymes in CRM formation and corresponding physiological relevance are well described for many drugs, data on enzymatic detoxification has been catching up only in recent years. For the quinone reductases, few reports describe *in vitro* NQO1 activity in reduction of drug-derived quinones (20–23). We have shown that NQO2 can catalyze reduction of specific drug-derived quinones as well (**chapter 5**). Moreover, human hepatic expression levels of both NQO1 and NQO2 were found to be in concentration ranges sufficient to efficiently reduce purified quinone imines *in vitro*. Expression of both NQO1 and NQO2 is highly variable due to genetic and environmental factors (24,25).

For GSTs, *in vitro* data on conjugation of CRMs by recombinant GSTs is accumulating. We have significantly complemented this research by showing GSH conjugation of the well-studied CRMs from clozapine and diclofenac using native GSTs, which were isolated from liver cytosol from 20 human liver donors while remaining the relative hepatic concentrations of GST isoforms (**chapter 6**). Importantly, the results showed high inter-individual variability in total GST activity for detoxification capacity of CRMs. Furthermore, we employed a cell-based assay to show that GSTP1 overexpression protects HepG2 cells from amodiaquine quinone imine cytotoxicity (**chapter 7**). Based on these results, it can be reasoned that differential expression of quinone reductases and GSTs contribute to the balance in bioactivation and detoxification and can consequently affect an individual's risk for (I)DILI. Further research, however, is needed to discriminate the enzymatic activities of quinone reductases and GSTs from their roles in alterations in cell signaling pathways, particularly when it comes to the exact mechanisms of protecting hepatocytes from CRM induced toxicity.

8.2.3. Towards better in vitro models in (I)DILI risk assessment: future research

Although clinical research regarding human (I)DILI is indispensable, *in vitro* models hold great promise to elucidate the distinct underlying mechanisms which together lead to the manifestation of (I)DILI in susceptible patients. In recent years much effort has been made in the development of more complex *in*

vitro models with the intention to improve the detection of hepatotoxic compounds in preclinical drug development (26,27). These advancements are expected to increase considerably in the near future. Considerable examples include the emergence of complex 3D models which were shown to improve the detection of hepatotoxins (28,29), progression in the generation of human induced pluripotent stem cells (hiPSCs) which could be used to generate hepatocytes *in vitro* from susceptible patients (30) and innovative research using microfabrication techniques for liver tissue engineering *in vitro* (31). The work described in this thesis accentuates the importance of variability in drug metabolizing enzyme activities, partly known and partly unknown, particularly phase II enzymes, within *in vitro* models and within the human population. The methodologies described to assess the variability on the level of enzyme activities (CYP, UGT, SULT, quinone reductases and GSTs) or protein levels (GSTs) can be used in liver homogenate and cellular models to confirm and supplement data on inter-individual variability and to compare *in vitro* models. Also for complex models such as 3D cultures, it remains essential to assess cultures derived from various donors preparations as these can visualize inter-donor variability in sensitivity to hepatotoxic drugs (32). In particular the CYP and UGT, but also SULT and quinone reductase phenotyping procedures can be extended to existing and novel (complex) cellular models, to allow detailed interpretation of cytotoxicity or toxicogenomics results, by including activities of enzyme isoforms involved in the metabolism of a given drug.

In conclusion, inter-individual variability in drug metabolism, whether arising from genetic or environmental factors, should be correlated to inter-individual differences in susceptibility towards drug toxicities like (I)DILI. High inter-individual variability exist for all drug metabolizing enzymes, and it is therefore plausible that the (I)DILI risk of a susceptible individual is increased because of a high hepatic bioactivation and low detoxification capacity for a specific drug. To understand the variability of this balance, data is needed on inter-individual variability in and correlations between bioactivating and detoxifying enzyme activities. The results described in this thesis further support the hypothesis that quinone reductases and GSTs are important detoxifying enzymes which contribute to reduction of CRM levels in human liver. Importantly, *in vitro* assessment of the consequences of an altered balance in bioactivation/detoxification for cytotoxic potential of drugs require cellular models which are characterized for all relevant enzyme activities. Approaches to characterize phase I and phase II enzyme activities in cellular models for drug metabolism related toxicity and to assess related inter-individual variability in bioactivating and detoxifying enzymes are described in this dissertation, thereby providing a solid and partly new basis for future work elucidating the association between variability in drug bioactivation/inactivation and (I)DILI risk in humans. The work presented in this thesis, performed within the IMI-supported MIP-DILI project, contributes to the evaluation and harmonization of existing and novel models and provides further (mechanistic) insight in the formation and detoxification of CRMs.

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