High throughput microscopy of mechanism-based reporters in druginduced liver injury

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Chapter 1

General introduction and scope of this thesis

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Partly adapted from '*Quantitative High Content Imaging of Cellular Adaptive Stress Response Pathways in Toxicity for Chemical Safety Assessment*. Steven Wink *et al*. Chem Res Toxicol. 2014 Mar 17;27(3):338-55.

1. Drug-Induced liver injury is a major problem in the clinic and in drug development

1.1 The Liver

The liver is a critical organ in our body, as it has an essential role in overall systemic metabolic homeostasis, bile production and xenobiotic biotransformation and excretion. To enable lipid digestion in the intestines the liver produces bile salts, which split lipid droplets to enable breakdown of lipids to fatty acids. Also, the liver plays a key role to maintain blood glucose levels, as it is able to store glucose in the form of glycogen and produce glucose from glycogen and other sources in times of fasting. Furthermore, the liver is the major site for drug metabolism. Three subgroups of liver proteins are responsible for the metabolism process: phase I and II enzymes and phase III transporters. Phase I metabolizing enzymes belong to the cytochrome P450 (CYP) superfamily and are involved in catalysis of drugs by oxidation, reduction, hydrolysis. Phase II enzymes are involved in conjugation steps by glucuronidation, sulphation, acetylation and methylation. Phase I and II enzymes contribute to transform chemicals and drugs to less toxic water soluble molecules, which can be excreted in the blood, bile or urine. Phase III transporters like the multi resistance-associated proteins (MRPs) and organic anion transporting polypeptides (OATPs) are involved in transport of chemicals and drugs in and out of the hepatocytes. Approximately, 70% of all drugs are metabolized by CYP enzymes in the liver[1,2]. 80% of these drugs are metabolized by four CYPs: CYP3A4, CYP2D6, CYP2C9 and CYP2C19[2,3]. During drug metabolism, toxic reactive metabolites can be formed, which can damage the liver.

The smallest functional unit in the liver, the classical structural hexagon, is called the hepatic lobule (figure 1A). At each corner three ducts are situated: the hepatic artery, the portal vein and the bile duct. In the center of the hepatic lobule the central vein is located. The main cell type of the hepatic lobule are the hepatocytes. Hepatocytes execute main liver functions like glycogen storage, bile salt production and metabolism of chemicals. The hepatic artery transfers oxygen rich blood via the hepatocytes to the central vein. The hepatocytes use the oxygen, thus there is a gradient of oxygen which reduces towards the central vein. Produced bile salts are transported by bile canaliculi and flow towards the bile duct, where it is transferred to the gallbladder. Thus there is also a gradient of bile salts present. In addition, much metabolizing enzymes like CYP enzymes are also present in a gradient, however, in the opposite direction (figure 1B)[4]. Next to hepatocytes, the liver consists of a variety of other cell types of which stellate cells and Kupffer cells are the most prominent ones in the context of hepatotoxicity.

1.2 Drug-Induced Liver Injury

Adverse drug reactions (ADRs) form a major problem in the clinic and in the drug-industry, as 5% of hospitalized patients and 5% of hospital admissions are related to adverse drug reactions[5,6]. In total, ADRs account for €79 billion

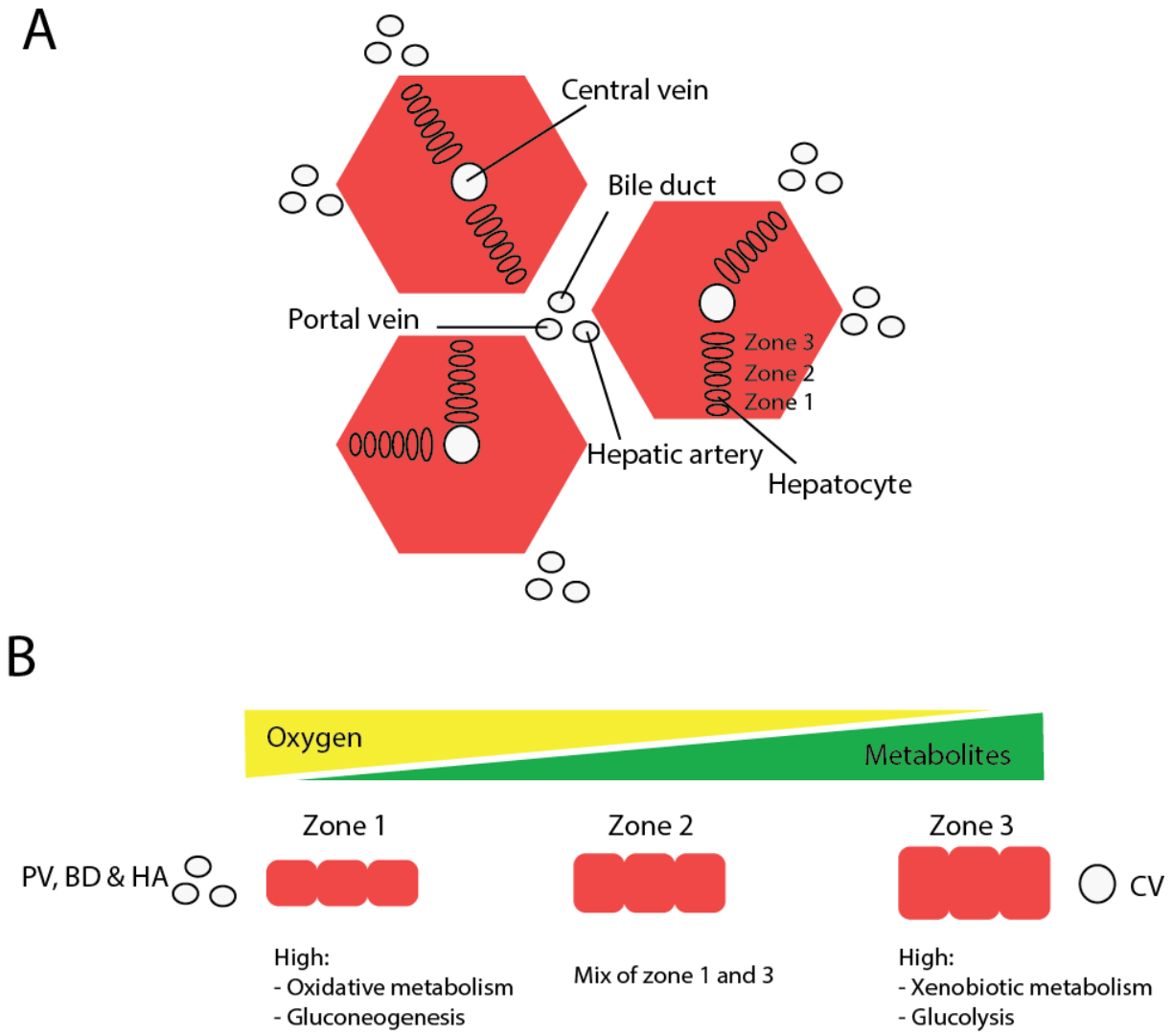

Figure 1. Liver physiology and function. A) Liver lobules; at each corner a portal vein, a bile duct and a hepatic artery are situated. The central vein is located in the center of a liver lobule. B) Zonal distribution of hepatocytes.

society costs in the EU (European Commission pharmacovigilance report, 2008). Furthermore, ADRs are responsible for an estimated 200,000 deaths each year in the EU[5]. Moreover, ADRs are an important cause for preclinical trial termination and drug market withdrawal. The liver has a prominent role in ADRs as it is the first organ reached by everything what is ingested and it is the major site for drug metabolism. Therefore, drug-induced liver injury (DILI) is a major issue both in the clinic and in drug development.

DILI can present in various different pathologies; e.g. cholestasis (accumulation of bile), steatosis (accumulation of fatty acids) or phospholipidosis (accumulation of phospholipids). Mostly, these are mild elevations, which will be counteracted by our body. However, DILI can also lead to acute liver failure due to a necrotic liver; when fulminant, this may require liver transplantation or death. Of all liver failures presented in the clinic, 50% are caused by drugs[7]. A large amount of these liver failures is caused by an overdose of acetaminophen. However, still

13% of all liver failures are caused by drugs taken on other prescribed dose regimens[7]. In most cases, these adverse reactions are called idiosyncratic reactions; idiosyncratic DILI (iDILI) does occur in rare cases and has a variable latency time. These features are the main reason these drugs are missed during preclinical safety testing and that liver injury is the leading cause for drug market withdrawal[8].

2. Current status in pharmaceutical toxicology safety testing

2.1 Current status of safety testing in drug development

The past decades pressure on drug development is increasing with a decreasing amount of successful marketed drugs and increasing costs. On average drug development takes 10-15 years and costs between 800 million and 1.8 billion dollars[9,10]. A key strategy to turn this negative trend around seems to be implementation of novel methods to screen for toxicity and efficacy in pre-clinical drug development. In addition, the 3R principle (reduction, refinement and replacement) urges to use minimal amount of animal testing[11]. Therefore, there is an urging need for new *in vitro* and *in silico* methods to screen for toxicity and efficacy. The past decades optimization of the screening protocols has led to enhanced prediction[12]. Even up to 70% of all toxicity observed in clinical trials were already predicted in preclinical studies[13]. Unfortunately, this does not account for iDILI.

Before a potential new drug can be tested in preclinical testing stages, different steps have to be performed. Firstly, the discovery of novel targets and their association with disease have to be defined during the process called target identification. Secondly, during the stage of target validation numerous studies ensure that the drug target can be linked to the diseased state. In addition, the studies need to validate that a biological effect can be achieved by hitting the target. Thirdly, a synthetic chemical has to be identified, which is able to target the biological target with a certain degree of specificity. This phase is called lead identification[14–16]. During lead identification hundreds of synthetic structures, also called new molecular entities (NMEs), are generated. Next, the NMEs are tested during the process called lead optimization, to find the most optimal structure based on safety and efficacy. NMEs are screened with different *in silico*, *in vitro* and *in vivo* tools to determine the clinical potency. The *in silico* tool computer-aided drug discovery (CADD) selects chemical structures as putative NMEs. CADD encompasses structure and ligand-based drug design, binding site identification, docking and scoring, pharmacophore modeling and quantitative structural-activity relationship identification[16]. *In vitro* tests are used to determine the no observed adverse effect levels (NOAEL)[12]. This is a measurement for the ideal concentration in terms of efficacy and safety. To this end, e.g. EC50 values for pharmacological effects, and safety testing for e.g. mitochondrial activity perturbations, onset of phospholipidosis and several other cytotoxicity tests are conducted. The battery of *in vitro* tests performed differ between pharmaceutical companies. Next, several regulatory required animal tests are carried out to determine the effect *in vivo*. Altogether, lead

optimization and preclinical testing lead to one NME which will be tested in clinical trials. During the three phases of clinical trials the NME is tested on human volunteers to determine safety and dosing (phase I and II) and on patients to test the efficacy and safety (phase III).

2.2 Cell lines used in preclinical liver toxicity screening

As mentioned before, preclinical data does not predict the clinical outcome in iDILI. Furthermore, clinical trials are not sufficient to determine iDILI hazard on the market, which can lead to market withdrawal. Preclinical *in vitro* screening is performed with different cell lines, all with their own advantages and disadvantages.

Primary hepatocytes: Primary hepatocytes are isolated from human or experimental animal liver tissue, typically from rat, and are widely used in pharmaceutical toxicity screening. Freshly isolated primary hepatocytes are viewed as the golden standard for *in vitro* hepatotoxicity screening[4]. However, isolation of primary human hepatocytes is a complex process, which can result in poor retention of liver enzyme activity. Moreover, once plated, primary hepatocytes rapidly lose their enzyme activity and liver specific function[17]. Recent techniques of cryopreservation show the possibility to retain the differentiation status without damaging hepatocyte function[18,19]. In addition, culturing primary hepatocytes in sandwich culture (either between two layers of collagen or one layer of collagen and one of matrigel) results in improved liver function[20,21]. Despite the recent advantages on improved primary hepatocyte culturing, disadvantages for high throughput toxicity screening remain pronounced. Complex handling, high costs, donor-donor variation and limited lifespan remain unmet issues limiting the suitability for hepatotoxicity screening.

HepaRG: The HepaRG cell line is derived from hepatic differentiated grade 1 Edmonson tumor and is a bi-potent liver progenitor cell. Upon presence of DMSO HepaRG differentiate towards biliary and hepatocyte-like cells[22]. The hepatocyte like cells exhibit high levels of metabolic enzymes, transporters and nuclear receptors[23]. A drawback of the HepaRG is the high concentration of DMSO in the culture medium, which may affect cell behavior and might artificially enhance metabolic enzyme activity as CYP3A4 is not inducible with rifampicin[23,24].

HepG2: HepG2 is an immortalized hepato-carcinoma cell line originating from an 15 year old American Caucasian boy. HepG2 cells exhibit expression of metabolizing enzymes, although at very low levels compared to primary hepatocytes and HepaRG[25]. Despite the fact that HepG2 cells are a carcinoma cell line, they express functional active p53 protein[4]. In addition, HepG2 cells show expression of Nrf2, which is responsible for detoxification upon oxidative stress and upregulation of various liver metabolizing enzymes[26]. HepG2 cells are widely used in toxicological and pharmacological screening, in particular due to the low costs, the robustness in culturing and unlimited lifespan. However, the major disadvantages are the low expression levels of liver specific metabolizing enzymes and liver like properties as bile salt production and glycogen storage; this is however improved by culturing HepG2 as 3D microtissue spheroids[27].

Human induced pluripotent stem cell derived hepatocyte like cells: Recent progress in generating human pluripotent stem cells from somatic cells brought stem cells research in fast-forward[28]. Pluripotent stem cells can be differentiated in all types of human tissue, including liver cells. The past years different groups made progress in establishing advanced hepatocyte like cells[29–32]. Despite the recent advancements, stem cell-derived hepatocyte-like cells are still more comparable to fetal hepatocytes than to adult hepatocytes[31,33,34]. To obtain adult hepatocytes in a robust and reproducible way will probably take additional years. When this is achieved the applications in disease modeling, drug screening and cell therapy are within reach. However, the disadvantage for the high throughput application in drug toxicity screening remains four weeks of differentiation. In addition, the differentiation protocol is costly and time consuming, making it likely less suitable for a primary screening platform for liver toxicity.

3D culturing efforts: A key factor to obtain a human relevant liver model is extracellular matrix covering the cells. This can not be achieved in conventional 2D monolayer culturing. Therefore, different 3D models were developed to gain a more liver relevant model. Commonly used models are the hanging drop method[35], bioreactors[36], microtissue cultures[37], micro-pattern systems[38], organ-on-a-chip[39], microfluidic systems[40] and collagen and matrigel cultures[27,41]. Most of these methods are tested for primary hepatocytes, HepaRG and HepG2. In most of the cases the 3D models show enhanced liver properties, making them a more advanced liver model. When the goal is to design a system which mimics the human liver the best, 3D systems perform better. However 3D platforms are also more costly, which makes them less suitable for high throughput screening[39].

In summary, the past years a large variety of approaches were taken to develop novel *in vitro* liver models to mimic the liver situation. All models are valuable in different stages of drug development. For primary screening, an easy and cheap model like HepG2 seems the right decision as a first tier approach. However, more advanced models as second tier steps can aid in determining which of the NMEs will be tested in clinical trials. As for the different cell lines available, primary human hepatocytes still count as the golden standard. The promise of HepaRG is slightly dampened by reports about limited sensitivity to DILI drugs compared to primary human hepatocytes and HepG2 cells[42]. Differentiated induced pluripotent hepatocyte like cells do not show all features of adult hepatocytes yet, but holds great promise for future toxicity testing. HepG2 cells seem to have their own niche in toxicity screening, with unique features as easy to culture, cheap and unlimited lifespan. All efforts in developing novel models will aid to achieve the goal of safe preclinical testing. However, no current *in vitro* model will be able to mimic the complexity of idiosyncratic liver injury occurring in the human body. Idiosyncratic DILI is a complex multifaceted problem, which only occurs in susceptible humans. Therefore, the only model which will be able to predict whether a patient will develop DILI, is that particular patient himself. This does not mean that the *in vitro* models that are used cannot provide useful information concerning dosing, early cell state changes and cytotoxicity.

This can give clear indications on how a newly developed drug will manifest in the average human. Since iDILI does not occur in the average human, *in vitro* models will never be able to mimic iDILI. However, by using the models in the appropriate way, by knowing its advantages and limitations, we can extract as much information as possible to reduce the risk that an NME, which will cause DILI, will slip through to the market.

2.3 Current efforts taken to solve the iDILI problem

The past decades several different large collaborative project have studied the multifaceted problem of iDILI. An important step was the onset of a registry of iDILI patients to be able to monitor the genetic, environmental and immunological risk factors. This initiative is called the DILI network (DILIN)[43]. DILIN contributes to the understanding which individuals are susceptible to DILI drugs under what circumstances. Obviously, this will take several years before clear hinds can be identified.

Another initiative is the Tox21 approach. Tox21 was initiated by the National Research Council (NRC) in 2007 with the article '*Toxicity testing in the 21st century: a vision and strategy*'. Tox21 was not meant for (i)DILI in particular, but for toxicity testing in general[44]. As part of Tox21, the ToxCast program was initiated by the US Environmental Protection's Agency (EPA). Within ToxCast research groups from different disciplines in toxicology did gather quantitative toxicity data using >700 different high throughput *in vitro* assays. This has resulted in an extensive library of data generated in concentration dependent manner. The data can now be used to identify toxicity profiles for an individual chemical as well as for assessment responses of structural similarity[45]. The FDA has already included a large panel of DILI compounds in the Tox21 phase, assessing the responses of ~10,000 compounds in ~30 assays. These assays allow the identification of biological key responses of iDILI compounds in these *in vitro* assays.

Not only *in vitro* assays are important in preclinical safety testing, but also *in silico* methods are becoming more and more pronounced. To this end, the DILIsim approach tries to link physiology to the preclinical data generated during safety screening[46]. The aim of DILIsim is to establish so-called physiological-based pharmacokinetic models for liver toxicity. When NMEs are being tested in preclinical testing DILIsim could aid in the prediction of clinical DILI outcome using this in silico approach.

Lastly, the European Union organization IMI set up the Mechanism based Integrated systems for Prediction of Drug Induced Liver Injury (MIP DILI) consortium. In this initiative pharmaceutical companies and academic institutes are gathered to identify the underlying mechanisms responsible for DILI. In addition, identification of novel mechanisms should also provide novel assays to prevent DILI in the clinic. The aim is to establish a panel of robust mechanism-based testing strategies that are fit-for-purpose in DILI assessment, with a focus on mitochondrial toxicity and immune-mediated DILI. The systematic physiological, pharmacological and toxicological evaluation of different in vitro systems is central in this project.

3. Importance of adaptive stress response pathways in toxicological screening

The past decade the field of toxicology has realized that important measurements were lacking in the current toxicological screening approaches. Most *in vitro* assays have focused on cytotoxicity or energy homeostasis (ATP levels). In the above mentioned NRC report from 2007 ('*Toxicity testing in the 21st century: a vision and strategy*') the importance of quantitative assessment of early cell state changes was emphasized. Adaptive stress response pathways are the key event in the switch between adaptation and cell death, which makes the assessment of the activation of these pathways essential in toxicity evaluation. The different adaptive stress response pathways which the cell uses to defend itself against environmental or toxicological insult have been summarized[47–49]. Since these reports different groups set out to measure the activation of the adaptive stress response pathways. Most groups followed a genome wide transcriptomic approach[50–52]. This led to a bulk of data typically only covering one chemical-concentration-time point combination. While this is valuable information to identify pathway activation for different individual conditions, this approach is less suitable for high throughput toxicological screening, since a clear dose-matrix is missing. Other groups have used microscopy-based approaches as this allows time dynamic monitoring of multiple drug concentrations[53,54]. Both O'Brien *et al*. and Xu *et al*. applied fluorescent dyes and screened with drugs important in DILI. However, the fluorescent dyes used so far primarily stain for mitochondrial activity and reactive oxygen species formation and not for adaptive stress response pathway activation. The importance of adaptive stress response pathways are known in toxicology in general, but also in DILI[49].

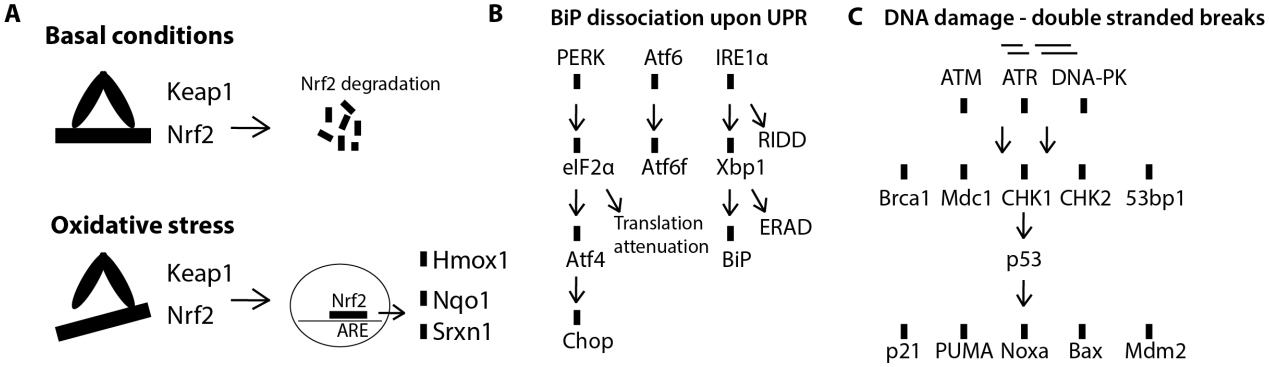

Figure 2. Overview of stress response signaling pathways. A) Nrf2 anti-oxidant response signaling. B) ER unfolded protein response signaling. C) DNA damage signaling.

Below three highly essential adaptive stress response pathways are summarized:

The Nrf2 anti-oxidant response pathway: When a cell suffers from oxidative stress caused by reactive oxygen species (ROS), detoxification is achieved via activation of nuclear factor erythroid 2-related factor 2 (*NFE2L2*/Nrf2). Under basal conditions Nrf2 is targeted for degradation by Kelch-like ECH-associated protein 1 (*KEAP1*/Keap1). Upon oxidative stress,

cysteine residues of Keap1 are modified by ROS, which results in a conformational change of Keap1 and inhibition of Nrf2 degradation. Newly assembled Nrf2 cannot bind Keap1 and translocates to the nucleus. In the nucleus, Nrf2 forms a heterodimer with masculoaoneurotic fibrosarcoma (MAFs) and subsequently binds to the Anti-Response Element (ARE) (figure 2A). Binding the ARE results in expression of a large variety of target genes, ranging from detoxification enzymes to nuclear hormone receptors and some CYP enzymes[55]. The Nrf2 response pathway also plays a key role in DILI: Nrf2 is activated by a wide variety of drugs inducing liver injury[26]. In addition, Nrf2 activation is shown to be essential in the *in vivo* protection of the liver. Nrf2 knock-out mice are much more susceptible for a number of hepatotoxicants[56]. These data emphasize a protective role for Nrf2 in DILI. Therefore, an improved understanding of the signaling programs that control Nrf2 signaling is important. This is discussed in detail in chapter 6.

ER stress response: The endoplasmatic reticulum (ER) is the major cellular organelle involved in protein folding[57]. Cells have evolved an adaptive protective mechanism to cope with perturbations in the protein processing capacity of the ER called the unfolded protein response (UPR). The UPR pathway is separated into three branches: the inositol requiring enzyme 1α (*ERN1*/IRE1α) branch, the activating transcription factor 6 (*ATF6*/Atf6) branch and the protein kinase RNA-like ER kinase (*EIF2AK3*/PERK) branch[58]. These three branches all have one sensing molecule in common: the ER-resident chaperone BiP. Under normal conditions, BiP binds IRE1α, Atf6 and PERK on the ER luminal membrane. When unfolded proteins start to accumulate, BiP dissociates from IRE1α, ATF6 and PERK and binds the unfolded proteins. Dissociation of BiP triggers PERK to homodimerize and autophosphorylate[59,60]. Activated PERK phosphorylates eukaryotic translation initiator factor 2α (eIF2α). This leads to an attenuation of general translation; however, it also leads to a specific activation of mRNA that encodes the transcription factor activating transcription factor 4 (*ATF4*/Atf4). Atf4 in turn activates genes involved in amino acid metabolism, redox balance, protein folding and autophagy[61,62]. IRE1α is also activated via homodimerization and autophosphorylation triggered by BiP dissociation. The activated ribonuclease domain of IRE1α catalyzes excision of a 26 nucleotide intron from ubiquitously expressed *XBP1* mRNA, which causes a frame shift in the *XBP1* coding sequence resulting in its translation. X-box binding protein 1 (XBP1) then translocates to the nucleus and induces transcription of ER-associated degradation (ERAD), phospholipidosis to promote ER-membrane expansion and protein folding by expression of chaparones like p58, ERdj4 and BiP[63]. In addition, activated IRE1α programs including regulated IRE1 dependent decay (RIDD; selective degradation of mRNA of proteins located in the ER), macroautophagy and inhibition of translocation of proteins into the ER-lumen[64–66]. Following BiP dissociation Atf6 translocates to the golgi apparatus where it is cleaved into the transcriptionally active form (Atf6f)[67]. Atf6f subsequently activates genes involved in ERAD and protein folding[68]. Thus, all three UPR axes (PERK, IRE1α and ATF6) initially contribute to the adaptation of the cell to overcome the

overload of unfolded proteins (Figure 2B). However, when the amount of unfolded proteins keeps accumulating during sustained stress conditions, the UPR switches to pro-apoptotic mechanisms. A key transcription factor in this switch is C/EBP-homologous protein (CHOP, also known as GADD153). CHOP is mainly activated via the PERK-ATF4 axis[69], however, there is also evidence for an a-specific activation via (one of) the other two branches[70,71]. CHOP regulates transcription of a variety of pro-apoptotic genes including Death Receptor TRAIL receptor 2 (DR5)[72] and Bcl-2 family member Bim[73], thereby sensitizing cells to apoptosis. In addition, CHOP also deattenuates general translation by inducing expression of GADD34, which dephosphorylates eIF2α. Deattenuation of general translation can result in an accumulation of premature proteins in the ER, which is shown to induce accumulation of reactive oxygen species (ROS) and subsequent mitochondrial damage and apoptosis[74]. Recent publications demonstrate a crucial role for ER-stress in hepatosteatosis, cholestasis and hepatotoxicity. Elevated levels of Atf4 and spliced Xbp1 were observed in fatty liver samples compared to normal and steatotic liver samples[75]. Also, *in vivo* evidence for a role of UPR in cholestasis was recently observed where CHOP-null mutants developed much less liver fibrosis compared to wildtype livers[76]. In addition, Chop knock-out mice are less susceptible to acetaminophen-induced liver injury[77]. Moreover, knock down of Chop resulted in protection of HepG2 cells against lipotoxicity, indicating an important role for Chop in non-alcoholic steatohepatitus[78]. Altogether there is clear evidence for different UPR/ER stress programs in liver injury responses.

DNA damage response: Cells in our body are exposed to exogenous and endogenous sources of DNA damage inducing factors, e.g. UV light, genotoxic substances and metabolic processes causing single or double strand breaks, purine base modification or intra or inter-strand crosslinks[79]. A highly conserved cell cycle check point and DNA damage repair system has evolved enabling cells to first repair the various types of DNA lesions before dividing with the risk of mutational accumulations[80]. The signaling involved in sensing the types of DNA damage, halting cell division at the cell-cycle check point and repairing the damaged DNA is fitted to the various types of lesions. Excessive DNA damage can lead to senescence, differentiation or apoptosis[81]. The DNA damage is detected by specific damage sensing mechanisms and by enzymes involved in DNA replication and transcription[82]. Crucial early regulators in the DNA damage response are the PI3-K-related protein kinases ataxia-telangiectasia mutated (ATM), RAD3 related (ATR) and DNA-dependent protein kinase (DNA-PK)[83]. From these proteins the DNA damage signal is thought to be transmitted via CHK and CHK2 (check point kinase 1 and 2, respectively), aided by scaffold proteins such as MDC1 (mediator of DNA-damage checkpoint 1), 53bp1 (p53-binding protein 1) and BRCA1 (breast cancer 1 early-onset)[84–86]. Among others, ATM and ATR can activate p53 by phosphorylation of p53 or its inhibitor - the E3 ubiquitin ligase Mdm2 (Figure 2C)[87,88].

p53 is mainly known as a tumor suppressor, but numerous additional roles have been reported. At least 129 direct transcriptional targets of p53 exist[89]. Under conditions of severe stress, p53 tumor suppression activity leads to irreversible apoptosis programs by activating extrinsic and intrinsic apoptosis targets including *BAX*, *FAS*, *NOXA* and *PUMA*[90]. The best-studied pro-apoptotic protein required for apoptosis induction by p53 is *PUMA*, a p53 target gene that is required to release cytoplasmic p53 from the antiapoptotic protein Bcl-XL, followed by mitochondria outer membrane permeabilization[91]. Alternatively, under conditions of low-level stress, p53 mediates its tumor suppression function via cellular growth arrest by activating the expression of cyclin-dependent kinase inhibitor (*CDKN1A*/p21), giving individual cells the possibility to repair DNA damage[92]. In this way, the p53 response pathway either aids to adapt to the induced DNA damage to bring the cell back to homeostasis or programs the cell to die in an organized way to reduce stress load on neighboring cells, depending on the severity of the DNA damage.

4. Overall summary

The role of adaptive stress responses in toxicity has been underestimated for decades. Current approaches of the past decade demonstrate the overall incentive to evaluate stress response activation. Also in drug-induced liver injury, information of stress response activation can provide more mechanistic insight in how DILI develops. Nrf2, UPR and DNA damage signaling are all involved in liver injury and cytotoxicity, as key components as Chop or Nrf2 knock down results in liver injury and cytotoxicity. Therefore, if we would be able to monitor adaptive stress signaling pathway activation, more insight in mechanisms of DILI can be obtained. Hence, we tagged different components of Nrf2, p53 and UPR signaling with Green Fluorescent Protein (GFP) to be able to follow stress response activation dynamics in a high throughput confocal microscopy approach. We used HepG2 cells as these cells are mainly used in primary preclinical pharmaceutical screening. The GFP reporter platform can be used, as an addition to the already used preclinical toolbox, to obtain information on early cell state changes in DILI. During the decision making stages of lead identification and optimization, information on adaptive stress response activation can be pivotal to make the decision.

5. Aim and scope of this thesis

5.1 From adaptive stress response pathways to a high throughput screening platform

The classical biomarker for liver injury in the clinic is alanine or aspartate aminotransferase (ALT/AST) and bilirubin increase in the blood. Unfortunately, ALT/AST and bilirubin levels are not always predictive for disease progression and are not exclusively elevated in DILI[93]. Also, preclinical toxicity testing with liver cell lines turned out to be not sufficient for making the distinction between high and low iDILI hazard drugs. iDILI does not occur for every drug and only occurs in susceptible individuals. This means that there is a drug intrinsic factor, a genetic

factor involved, as well as environmental and life style factors. As mentioned before, 13% of all liver failures in the United States are caused by iDILI drugs[7]. However, mild liver injury is much more common. Studies in France and Iceland demonstrated 13.9 and 19.1 cases of DILI per 100,000 habitants respectively[94,95]. This indicates DILI drugs are altering liver homeostasis in many patients. Furthermore, these numbers are most likely an underestimation as not every patient with mild forms of DILI will notice this. This implicates that at the cellular level adaptive stress response pathways are likely activated in most patients administrating drugs that have iDILI liabilities. Therefore, adaptive stress response activation could be indicative for DILI and useful as a measurement in preclinical safety tests. To be able to follow the activation of adaptive stress response over time, we tagged different components of the pathways with GFP. Confocal microscopy enables us to monitor live cell dynamics in a high throughput fashion. GFP was tagged with the bacterial artificial chromosome (BAC) recombineering technique. BACs are large plasmids (~150-200 kB) able to carry a whole eukaryotic locus including introns and promotor regions. In 2008 Poser *et al.* proposed a method to apply BACs in a high throughput way[96]. We used this method to tag different components of adaptive stress responses with GFP. The BAC technique enables incorporation of large pieces of DNA in the host genome, ensuring control of the gene by the endogenous promotor region and other regulatory sequences. Endogenous gene expression is a prerequisite for safety toxicity testing as overexpression of central adaptive stress response signaling components could decrease sensitivity due to the inherent enhanced protection against cellular injury as well as improved adaptive stress response activation.

5.2 Establishment and characterization of a BAC-GFP reporter platform to screen for DILI liability

In **chapter 2** we established and characterized eleven BAC-GFP reporters for three adaptive stress response pathways: DNA damage, Nrf2 signaling and ER stress. For each pathway a sensor, the key transcription factor and a specific target was chosen. For DNA damage 53bp1 (sensor), p53 (transcription factor) and p21 (target) were tagged with GFP. In Nrf2 signaling Keap1 (sensor), Nrf2 (transcription factor) and Srxn1 (target) were chosen to represent Nrf2 activation. For ER stress no upstream sensor was chosen, since these are the kinases IRE1α and PERK, but two branches were tagged with GFP (Atf4-Chop and Xbp1-BiP). Chapter 2 demonstrates the characterization steps and zooms in on the usability of these GFP reporters to screen for drugs inducing DILI.

5.3 Adaptive stress response activation dynamics after treatment with DILI compounds

Chapter 3 focusses on the screen of 176 compounds, of which 146 have DILI liability based on FDA drug labels, conducted with GFP reporters of the targets of the three adaptive stress response pathways characterized in chapter 2. The screen reveals time dynamics of Srxn1, Chop and p21 after treatment with DILI drugs. The drugs that were screened can be divided in three different categories: drugs with most liver injury concern, drugs with less liver injury concern

and drugs with no liver injury concern. An overrepresentation of most-DILI-concern drugs was observed for those compounds that did activate the different stress response reporters.

5.4 An advanced 3D HepG2 BAC-GFP reporter system to screen for stress response activation after treatment with DILI compounds

To encounter the minimal metabolizing capacity of HepG2 cells, the GFP reporter system was also evaluated in 3D HepG2 spheroids. We showed previously that wildtype 3D HepG2 cells demonstrate enhanced liver-like properties when grown in matrigel[27]. **Chapter 4** combines 3D HepG2 culturing with the GFP reporter platform as six different GFP reporter cell lines (two targets of each stress response pathway) were tested for their ability to be induced in 3D cultures. In addition, 33 drugs with known DILI liability were selected and assayed in these six different adaptive stress response reporters in 3D spheroid systems.

5.5 Evaluation of crosstalk between the Nrf2 and p53 response pathways

As mentioned before, DILI is not only dependent on the intrinsic drug features, but also on the genetics of susceptible individuals. Therefore, full understanding of the underlying mechanisms of adaptive stress response activation is necessary to tackle the complexity of the DILI problem. An important aspect of adaptive stress response activation concerns crosstalk between different stress responses. In literature, ambiguous observations are presented in the crosstalk between the Nrf2 and the p53 pathway (further discussed in **chapter 5**). Therefore, we set out to determine crosstalk between the Nrf2 and p53 stress response pathways in our platform, thereby contributing to a more elaborate understanding of stress response pathway activation.

5.6 RNAi based knock down screening to identify novel regulators of Nrf2 activation in a DILI related system

The Nrf2 pathway has a central role in cellular homeostasis in preventing cells from oxidative injury. To obtain full mechanistic understanding of Nrf2 signaling an RNAi-based study was conducted to identify novel regulators of Nrf2 activation in **chapter 6**. 3027 siRNAs smartpools were tested for their effect on CDDO-me induced Nrf2 activation. This systemic screen allowed the identification of 58 validated suppressors and 19 validated activators of Nrf2 activation. These novel regulators were shown to also affect the onset of oxidative stress-induced cytotoxicity. Therefore, we anticipate the discovery of potential important novel factors that contribute to the individual susceptibility towards DILI.

5.7 General discussion and conclusion

Finally, **chapter 7** provides a summary and discussion of the conclusions and implications of the work performed in this thesis.