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Chapter 1

Introduction, aim and scope of thesis

Highlights

- Idiosyncratic drug-induced liver injury remains a major problem in drug discovery, safety assessment and clinical practice
- Host factors, including immune system activation, play an increasingly recognized role in the etiology of idiosyncratic drug-induced liver injury
- In this thesis, we assess hepatocyte proinflammatory TNFα signaling mechanistically and in applied in vitro testing systems
Idiosyncratic drug-induced liver injury (DILI) remains one of the major challenges in drug development, safety assessment and clinical practice. Increased mechanistic understanding and improved toxicity testing strategies are essential to predict DILI hazard for specific compounds and patients. In this thesis, we studied possible mechanisms of drug-induced liver toxicity, with a focus on TNFα signaling in hepatocytes. Furthermore, we developed a model to monitor drug-induced cellular stress responses and predicted DILI hazard of a set of compounds. Here we will introduce the liver, liver specific immune regulation and the role of TNFα in DILI. Furthermore, we will discuss how immune signaling currently can be assessed with recent liver toxicity models.

The liver
The liver is an essential organ in maintaining both metabolic and immunological homeostasis in the human body. In agreement with this diverse function, the structure of the liver is very complex. The functional units of the liver are the hexagonal liver lobules (Fig. 1A). Oxygen-rich blood enters the liver via the hepatic artery. Nutrient-rich blood arrives via the portal vein, together with the bile duct situated in the portal triad. The portal vein collects blood from both spleen and intestines, making the blood rich in antigens from food, toxic substances and pathogens. Blood from the artery and vein mix inside the sinusoids, and is relatively long exposed to and detoxified by hepatocytes. Due to this process the amount of specific molecules, including metabolites, toxic substances and oxygen, differ over the length of the sinusoidal axis (Fig. 1C). Among other mechanisms, oxygen responsive transcription factors mediate the expression of specific metabolic enzymes, thereby regulating the metabolic zonation. Toxicity is most seen in the periportal region, warranting a different classification of liver regions in periportal, pericentral and midlobular zones (Fig. 1A)\textsuperscript{1,2}.

In general, fenestral liver sinusoidal cells (LSEC) surrounded by stellate cells line up between the sinusoid and the hepatocytes, forming the perisinusoidal space (Fig. 1B). The diffusion rate into the perisinusoidal space is regulated by the hepatic stellate cells, which also store the body's main stock of vitamin A\textsuperscript{3}. To facilitate the clearance and efficient transport of specific molecules to the hepatocytes, LSECs are specialized in endocytosis and transcytosis\textsuperscript{4}. The liver resident macrophages, Kupffer cells (KCs), travel through the liver sinusoids and eliminate large antigens. Together with a relatively large fraction of natural killer cells they form the resident liver immune cell population. When activated, the Kupffer cells can differentiate into the proinflammatory M1 phenotype or in the alternative M2 phenotype. In contrast to the M2 phenotype, M1 macrophages express high proinflammatory cytokines, including TNFα, and low IL10 levels. This differentiation process depends on the activation signal, the environmental context and the genetic background of the individual\textsuperscript{5}. However, not only the immune cells affect inflammation. Also the stellate and LSEC have recognized immunomodulatory functions, which have recently been reviewed\textsuperscript{3,6}. Hepatocytes are the most abundant cell type in the liver, responsible for most metabolic and homeostatic functions. These parenchymal cells produce bile to aid in the digestion, which is transported via the bile canaliculi and collected in bile ducts, lined by
cholangiocytes. Logically, liver failure is in the end always a result of malfunctioning hepatocytes. However, the interplay between hepatocytes and non-parenchymal cell populations plays a very important and increasingly recognized role on the path to hepatotoxicity.

Fig 1. Structure of the liver A) The liver lobule is schematically represented. The different zones are indicated. The portal triad consists of a bile duct, hepatic vein and a hepatic artery. The bile duct and bile canaliculi are indicated in green, the arterial blood flow is indicated in red and the venal blood flow in blue. B) An enlargement of A, the sinusoid lined with hepatocytes. The different cell types are indicated and the bile canaliculi are visualized in green. C) Part of B, the metabolic zonation. Oxygen and xenobiotic levels are indicated by the relative height of the triangles.
Drug-induced liver injury and inflammatory stress

Drug-induced hepatotoxicity is a common adverse drug reaction, raising problems in drug development and clinical practice. The unpredictability and low incidence of idiosyncratic DILI (iDILI) makes it difficult to detect in expensive clinical trials. Furthermore, the risk on iDILI for few patients leads to black box warnings and inhibits prescription of an, for most patients, effective drug therapy.

Fig 2. Etiology of idiosyncratic drug-induced liver injury in hepatocytes

Different processes and factors are represented in this overview of risk factors for idiosyncratic drug-induced liver injury. Host factors are visualized in the inner circle and cellular processes in hepatocytes in the outer circle. Going clockwise, drug exposure will lead to cellular stress. Host factors as pharmacokinetics and drug metabolisms can diverge between individuals due to genetic variability, and thereby modulate DILI liability in a specific individual. Upon cellular stress, immune activation and mitochondrial impairment can be regulated by genetic and environmental factors (including concurrent therapies and diseases). Combined, these processes can lead to apoptosis or necrosis of the hepatocyte. The liver can restore after some injury, called clinical adaptation. However, in some cases hepatotoxicity levels will increase and lead to liver failure. In this thesis, cytokine secretion is mimicked by manual addition of proinflammatory cytokines.
To predict which drugs are going to induce iDILI in specific people from \textit{in vitro} and \textit{in vivo} studies, we have to understand the interaction of drug properties with both general and drug-induced cellular signaling. One reoccurring host factor involved in DILI is activation of the innate immune system (Fig. 2).

\textit{In vivo}, mimicking viral or bacterial infection, for example by poly I:C or LPS exposure, concurrent to exposure of a non-toxic dose of certain drugs lead to synergistically increased hepatotoxicity\cite{9,10,11}. For trovafloxacin toxicity upon LPS exposure, this synergism has been proven to be dependent on TNF receptor and TNF\(\alpha\) expression\cite{12}. TNF\(\alpha\) is a proinflammatory cytokine, in the liver mainly produced by KCs or invading immune cells upon damage or inflammation-induced chemotaxis. Drug-induced signaling in the KC itself can lead to proinflammatory cytokine production, including TNF\(\alpha\) production\cite{13}. However, recognition of pathogen-associated molecular patterns or damage-associated molecular patterns secreted by drug-treated liver cells can evoke KC activation and cytokine production as well. TNF\(\alpha\) produced by KCs can signal to hepatocytes via the TNF receptor (TNFR). Healthy hepatocytes are not sensitive for TNF\(\alpha\)-induced cell death. However, excessive and long-term hepatic inflammation characterized by chronic high levels of TNF\(\alpha\), can lead to massive hepatotoxicity and is associated with several hepatic diseases\cite{13}. Currently, it is unknown how TNF\(\alpha\) stimulation together with drug exposure can induce synergistic cell death in hepatocytes in only few individuals. The role of MAP kinases and cellular stress response pathways have been studied before\cite{14,15,16}. In chapter 5 we detect several known regulators of the TNF receptor signaling pathway that play important roles in drug/TNF\(\alpha\) synergistic apoptosis.

\textbf{TNF\(\alpha\) signaling pathway}

Upon binding of TNF\(\alpha\), trimerization of the TNFR leads to recruitment of the TNFR signaling complex. The TNFR signaling complex activates the IKK kinase complex and subsequently induces NF-\(\kappa\)B transcriptional activity. NF-\(\kappa\)B activation protects hepatocytes from TNF\(\alpha\)-induced cytotoxicity\cite{17}. Besides NF-\(\kappa\)B activation, also MAPK pathways JNK and p38 are activated via the TNFR signaling complex. The induction of antioxidant proteins via NF-\(\kappa\)B transcriptional activity prevents prolonged JNK activation and cell death\cite{18,19}. Furthermore, NF-\(\kappa\)B induces general anti-apoptotic protein expression and these dissociate the TNFR signaling complex. Without NF-\(\kappa\)B activity induced anti-apoptotic protein expression, the TNFR signaling complex progresses into complex II, inducing cytotoxicity\cite{17}. TNF\(\alpha\)-induced apoptosis is characterized by caspase 8 activation and necroptosis by RIPK3 activation. The exact TNF\(\alpha\)-induced cell death mechanisms have recently been reviewed by Brenner et al.\cite{20}.

The NF-\(\kappa\)B transcription factor family consists of 5 proteins: RelA (p65), RelB, cREL, p50/p105 and p52/p100. All proteins share a Rel homology domain for dimerization and DNA binding, but only RelA, RelB and cREL have a c-terminal transactivation domain. RelB forms the core of the non-canonical NF-\(\kappa\)B pathway. This pathway is activated by cytokines including CD40 and lymphotoxin beta, and characterized by stabilization of NIK (MAP3K14) and IKK\(\alpha\) activity\cite{21}. Precursors p105 and p100 need processing, before taking
their active DNA binding form. For p100, this process is regulated by phosphorylation by active IKKα, while p105 is constantly processed. Several different homo- and hetero-dimers can be formed. Homodimers from p50 and p52 are thought to be transcriptionally inactive and inhibit active NF-κB dimer DNA binding by competitive binding\(^22\). The best-characterized NF-κB signaling heterodimer is RelA-p50, which becomes transcriptionally active upon TNFα signaling.

Upon TNFα stimulation, NF-κB translocates into the nucleus and transcribes its own inhibitors IκBα and A20 (TNFAIP3). By this negative feedback loop NF-κB itself regulates its oscillatory nuclear phenotype upon stimulation\(^23,24\). The reason behind and physiological result of this oscillatory phenotype is largely unknown. Currently, the major belief is that this oscillatory phenotype translates into differentially regulated functional gene transcription patterns\(^25–27\). However, the function of this relation between transcription factor oscillations and subsequent transcription profiles is not well understood. NF-κB regulates the transcription of around 600 target genes. These genes are expressed in many functional and three temporally differentiated groups; early, middle and late\(^28\). NF-κB target genes function in many cellular processes, including inflammation, cell growth, cell cycle and cell survival\(^29\). In chapter 3 and chapter 4, we studied the regulation of TNFα-induced NF-κB nuclear oscillation and the effect of drug exposure on its target gene induction, respectively.

**Drug toxicity hepatocyte models**

Cell death of hepatocytes is the hallmark feature of hepatotoxicity. Therefore, most hepatic drug safety testing systems include hepatocytes at the core of their model. However, as all models do, they limit the complexity of the human liver. For example, metabolic zonation of the liver forms a factor not often reflected. Interactions between hepatocytes themselves, the different cell types and the extracellular matrix and blood stream is another feature that is difficult to capture in in vitro models. The attempts to reflect all these layers of complexity in 3D models and artificial liver ‘on a chip’ approaches have been extensively reviewed elsewhere\(^2,30\).

Reliable co-culture, or co-exposure methods of hepatocytes and KCs have recently been developed and increase the predictive power for specific immune related toxicants\(^12,31,32\). However, the need for glucocorticoids to stabilize CYP expression in the hepatocytes has a clear anti-inflammatory effect on the Kupffer cell\(^31\). Besides some of these technical drawbacks, co-culture models using donor-specific Kupffer cells and hepatocytes can add major mechanistic insight into the role of the innate immune system and the individual susceptibility of certain patients herein. Furthermore, once optimized and validated, they can form a testing platform for early detection of possible immune-related DILI. Trovafloxacin-mediated toxicity can be detected in Kupffer cell and hepatocyte co-culture\(^31\). Interestingly, this increased hepatocyte toxicity can also be detected in a hepatocyte only model with the manual addition of TNFα\(^33\).

Separate hepatocyte and immune cell culture methods can provide mechanistic understanding, distinguishing between hepatocyte and Kupffer cell signaling in the process.
of developing DILI. For example, IkBα phosphorylation in 7 day old mice is regulated by TRAF2 expression in liver and spleen, but not in lung and muscle\textsuperscript{34}. Also, ketoconazole treatment did lead to NF-κB pathway activation in HepG2 cells, but not in the macrophage cell line THP-1\textsuperscript{35}. These studies underline that the specific cellular background is of major importance for mechanistic research and the design of fit-for-purpose studies, especially concerning immune signaling.

The golden standard in hepatocyte toxicity screening are currently freshly isolated primary human hepatocytes (PHH). The variety of PHH models, including 3D culture, co-culture and precision-cut liver slices have been reviewed extensively by Godoy et al.\textsuperscript{1}. As fresh hepatocytes are difficult to use due to their unpredictable availability, cryopreservation is a good alternative. None of the hepatocyte cell lines HepG2, Upcyte or HepaRG, are currently able to mimic PHHs in metabolic enzyme expression and activity\textsuperscript{36}. However, also PHHs quickly lose their metabolic capacity upon isolation and culturing\textsuperscript{37}. Adjusting the culture methods to low glucose medium, 3D cultures or co-culture systems can improve hepatic function\textsuperscript{1}.

One of the difficulties in using PHHs for hepatocyte inflammatory signaling research is the possible contamination with a minority of immune cells during the isolation procedure. These few immune cells can excrete massive amounts of signaling mediators when activated, shifting the homeostasis within a “hepatocyte only” culture massively. However, even pure hepatocyte cultures seem to activate an inflammatory disease phenotype\textsuperscript{37}. In addition, the preferred PHH culture medium includes a glucocorticoid, both inducer of CYP enzyme expression and NF-κB inhibitor. This could inhibit the inflammatory response in primary hepatocyte cultures. However, as hormones as hydrocortisone are also present in the human body, it is unknown if these additives result in non-physiological behavior of hepatocytes. A final consideration on the use of PHH cultures is that donor differences can be major. As some donors are not plateable, we probably underestimate the donor variability based upon available data\textsuperscript{1,38}.

For early toxicity screening in the pharmaceutical industry, the HepG2 cell line is still routinely used. HepG2 cells have retained many hepatocyte specific functions, but have in general very low expression of drug and bile transporter proteins and phase I metabolic enzymes. These levels can differ between separate batches\textsuperscript{39}. Simple endpoint studies performed with the same clone at different companies showed stark differences in outcome, although HepG2 cells are generally viewed as a very stable cell line\textsuperscript{40}. Both in PHHs and HepG2 cells co-exposure of drug and cytokines, including TNFα, can lead to synergistic apoptosis\textsuperscript{33,41}. To increase the fit-for-purpose applicability of the easy-to-handle HepG2 cell in toxicity screening, their relation to primary human hepatocytes should be more precisely determined. Including cytokines as co-stimulation would also increase usability. In chapter 6, we predicted DILI liabilities of marketed compounds using a HepG2 stress response reporter platform, including TNFα-induced NF-κB target gene ICAM1.
**Aim and scope of this thesis**

In previous studies at our laboratory it was demonstrated that drug exposure of HepG2 cells can lead to an altered TNFα-induced NF-κB oscillatory phenotype, concurrent with a synergistically increased sensitivity for TNFα-induced apoptosis\(^{42}\). We have also shown that synergistic drug/ TNFα-induced cell death is dependent on concurrent cellular stress responses\(^{16,43}\). To monitor these stress responses, we have developed a fluorescent protein stress response reporter platform, feasible for high throughput approaches\(^{44}\). In this thesis, we focus on an *in vitro* HepG2 cell model, in which the addition of TNFα reflects inflammatory stress (Fig.2). We studied inflammatory signaling, both for use in improved *in vitro* testing approaches and to gain mechanistic understanding of TNFα-induced signaling in hepatocytes. In **Chapter 2**, the current knowledge on the role of TNFα signaling during iDILI and several models in which TNFα signaling could be reflected *in vitro* have been summarized. In **Chapter 3**, we used a siRNA-mediated screen approach, detecting regulators of the TNFα-induced RelA oscillatory phenotype. We studied their regulation of A20 expression and synergistic drug/TNFα cytotoxicity. We revealed CDK12 (CRK7) as a novel regulator of TNFα-induced RelA oscillation, A20 expression and synergism. In **Chapter 4**, we systematically evaluated drug effects on TNFα-induced NF-κB nuclear translocation and target gene induction. Interestingly, drug exposure by itself induced TNFα target gene induction, including RelB, CCL5 and IL8. RelB expression showed strong correlation with oxidative stress response induction and was independent of RelA transcriptional activity. The drug-induced expression of chemokines CCL5 and IL8 was RelA-dependent, but also negatively regulated by NF-κB family members RelB and cRel. In **Chapter 5**, we studied known TNF receptor pathway components and their role in carbamazepine/TNFα synergism towards apoptosis by a siRNA-mediated approach, revealing 24 candidate genes. We determined 3 out of 124 drugs that acted synergistically towards apoptosis with TNFα (colchicine, griseofulvin and valproic acid). The candidate genes consisted of both specific and generic drug/TNFα synergism regulators (e.g. A20, PHF5A) The effect of candidate gene knockdowns on drug/TNFα synergism were correlated with drug-induced cellular stress responses. We detected two separate branches of the oxidative stress and DNA damage response pathways that were strongly correlated with certain candidate gene effects. For **Chapter 6**, a HepG2 cell line harboring a fluorescent TNFα responsive ICAM1 gene has been generated and used as part of the stress response fluorescent reporter platform to predict DILI potential in humans. We predicted DILI hazard with a sensitivity of 60% and a specificity of 75%. Finally, in **Chapter 7** I summarize all findings in this thesis and provide an overview and future perspective on the implications of these studies.
Introduction, aim and scope of thesis