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Discussion and conclusion

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The occurrence of adverse drug reactions (ADRs) is a significant problem both for society and for the drug-industry. A few decades ago, the biggest challenge during drug development was poor bioavailability and pharmacokinetics. This has now shifted towards issues related to ADRs, of which idiosyncratic drug-induced liver injury (iDILI) is the most common.

To tackle the ADR problems in drug development, there is need to better understand the underlying molecular mechanisms of ADRs. An improved mechanistic foundation of iDILI would open doors to establish new pre-clinical *in vitro* and *in vivo* tests that represent the most important programs that underlie iDILI. These would allow the removal of candidate drugs with a high likelihood of iDILI-inducing potential from further development at an early stage. In addition, such mechanistic insight could allow individualization of drug therapy by avoiding certain drug therapies for susceptible individuals, while maintaining access of these same drugs for patients that benefit from its actions without any demonstration of ADR. Finally, such a mechanistic understanding could help patients already suffering from ADRs, by providing candidate therapeutic strategies for protection, adaption or recovery.

In this thesis I have investigated the involvement of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα), in the toxicity of drugs that are known to cause iDILI in humans. I have given special attention to the non-steroidal anti-inflammatory drug diclofenac (DCF) and the anticonvulsant carbamazepine (CBZ). A variety of techniques including high content imaging of cell toxicity, cell based fluorescent reporters of drug-induced and inflammatory stress, and toxicogenomics, allowed me to present in depth understanding of the mechanisms that could be involved in iDILI.

**The role of TNFα-signaling in DILI**

The immune system is known to play a major role in the development of adverse drug reactions, and especially of DILI. In relation to this, both the innate and the adaptive immune system have been shown to play an important role. However, irrespective of which part of the immune system gets activated, the main mediators of their activity are cytokines.

The role of inflammatory stress in iDILI has been most extensively studied in the group of Patricia Ganey and Robert Roth. They have developed an animal model in which the severity of human iDILI has been replicated the best thus far (1). In this model, non-toxic doses of drugs like diclofenac, sulindac, trovafloxacin and amiodarone have been rendered toxic by the addition of a non-toxic dose of the bacterial endotoxin lipopolysaccharide (LPS). Although LPS, when absorbed from the intestines to the circulation, and thereby reaching the liver, causes activation of the liver stationary macrophages (Kupffer cells), the main source of injury in this model is infiltrating neutrophils (2-4). However, importantly, TNFα, a cytokine secreted by both macrophages and neutrophils, has been shown to be indispensable for the liver injury seen in these models (5,6). In chapter 2, for the first time, I show that this cytokine can significantly
enhance the toxicity of diclofenac in an *in vitro* model using HepG2 cells, providing a good cell system for the study of the detailed mechanism(s) behind the role of inflammatory stress in DILI.

**The unique role of the TNF receptor in the decision between life and death**

The TNF receptor 1 (TNFR1) is unique among other cytokine receptors in the sense that it can induce both pro-survival and pro-apoptotic signaling. In chapter 2, I demonstrated that diclofenac and TNFα co-exposure leads to an enhanced amount of apoptosis, compared to exposure to the drug or the cytokine alone. Next, I showed that this apoptosis is strictly dependent on caspase-8 activity. This suggests that diclofenac is sensitizing the cells to TNFα-induced apoptosis rather than the other way around. Necroptosis is a novel mechanism by which TNFα can induce cell killing in a caspase independent manner (7). However, I show that the DCF/TNFα-induced cytotoxicity, in these cells, is solely dependent on apoptosis and seems not to involve necroptosis, since z-VAD-fmk, a pan-caspase inhibitor, could completely inhibit the cytotoxicity induced.

In chapter 2, I also show an important role for c-Jun N-terminal kinase (JNK) in the induction of DCF/TNFα-induced apoptosis. JNK is known to induce apoptotic signaling after prolonged activation by the disruption of mitochondrial membrane integrity. Since the apoptosis is completely caspase-8 dependent, I propose that the pro-apoptotic role of JNK most likely occurs upstream of the mitochondria by controlling proteins at the proximity of caspase-8 activity control. Indeed, JNK regulates the expression of cellular FLICE-like inhibitory protein (c-FLIP), the endogenous inhibitor of caspase-8, by phosphorylation of the E3 ubiquitin ligase Itch (8). Furthermore, more recently, TRAF7 was described as an inducer of JNK activity downstream of the TNFR1 and additionally as a regulator of c-FLIP by inducing its degradation (9). This provides another common factor between the stress kinase JNK and the anti-apoptotic protein c-FLIP. Interestingly, the gene encoding the c-FLIP protein (CFLAR), was down-regulated after DCF and CBZ exposure of HepG2 cells (chapter 4), further supporting the potential role for c-FLIP in the survival of drug/TNFα co-exposed cells. Additionally, siRNA-mediated knockdown of CFLAR led to induction of DCF/TNFα-induced apoptosis, as shown in chapter 2. Recently, a mouse model with a conditional liver specific c-FLIP deletion was described (10). This mouse presented enhanced liver injury upon exposure to D-galactosamine and LPS, which induces liver cell death by activation of TNFR1. The cell death was also dependent of JNK activity, which suggests a bidirectional regulation of JNK and c-FLIP. A role for c-FLIP expression levels in regulating DILI under in vivo conditions needs further investigation.

CFLAR is one of the anti-apoptotic target genes of nuclear factor kappaB (NF-κB), the most important transcription factor induced down-stream of the TNFR1 upon TNFα exposure. In chapter 2 I described how diclofenac pre-exposure leads to a delay in the cytosol-to-nuclear translocation pattern of NF-κB using high content imaging of a GFP-tagged canonical NF-κB subunit, p65 (RelA). The strength, duration and type
of oscillatory pattern of NF-κB determines the set of NF-κB target genes that will be transcribed after TNFR1 activation (11-13). Thus, a delay in the NF-κB translocation response may very well lead to a differential regulation of the crucial target genes and thereby affect the activation of the pro-apoptotic pathway downstream of TNFR1. This provides a potential explanation to why pre-exposure to certain drugs result in a greater sensitivity to TNFα; however it needs further detailed investigations.

A shift in the NF-κB translocation response upon drug exposure does not per se result in enhanced apoptosis by any hepatotoxicant. This was observed in chapter 3, where three different high content imaging methods were used to assess apoptotic synergism with TNFα, induction of oxidative stress and ability to cause a shift in the TNFα-induced NF-κB nuclear translocation response. A panel of 15 drugs, including several iDILI-inducing compounds, was used. Interestingly, the majority of the compounds that were able to induce an oxidative stress response as monitored by the Nrf2-dependent expression of Srxn1, also caused an inhibition of NF-κB activation. This is in agreement with other reports (14,15). Yet, a conclusive link between oxidative stress induction, NF-κB nuclear translocation shift and TNFα apoptotic synergism could not be made for all compounds.

Translation initiation and mRNA processing - crucial determinants of TNFα-signaling

The lack of a direct link between oxidative stress-induction and increased sensitivity to TNFα calls for an alternative mechanism. Glutathione depletion (especially mitochondrial) also causes sensitization to TNFα-induced apoptosis (16,17). In this thesis I did not assess (mitochondrial) GSH after drug exposure, which could be an explanation for the differences in the TNFα-synergism. However, to explain the DCF/TNF and CBZ/TNF synergism, in chapter 4 we applied a toxicogenomics approach and determined the gene transcription profiles after exposure to synergizing drugs DCF and CBZ compared to the hepatotoxicants ketoconazole, nefazodone and the far less toxic methotrexate. For the first time, I in this chapter describe translation initiation as a crucial process for the control of TNFα-induced hepatocyte-toxicity. The translation initiation factor EIF4A1 was selectively up-regulated after diclofenac and carbamazepine exposure. Intriguingly, knockdown of this RNA helicase using transient siRNA gene silencing led to a marked protection against drug/TNFα-induced apoptosis. Also the transcription of other translation initiation factors was affected, including a close family member of EIF4A1, EIF4A2. Yet, knockdown of EIF4A2 did not affect apoptosis. Only until recently, there was no difference in action described between EIF4A1 and EIF4A2 and they were presumed to have redundant functions. However, interestingly, selective knockdown of EIF4A1 can apparently increase the expression of EIF4A2. Yet, this did not completely compensate for the loss of EIF4A1, possibly due to differences in enzymatic activity or substrate specificity (18). This could also form the basis for the discrepancy between the two isoforms in our hands.
Inhibition of EIF4A in general (without isoform specificity) using the pharmacological inhibitor hippuristanol, has been described to result in selective inhibition of translation of injurious proteins, while translation of protective proteins remained intact (19). A similar scenario could be set in our case, where selective inhibition of EIF4A1 by knockdown, would result in decreased translation of crucial apoptotic proteins including CHOP, as was described in chapter 4, while translation of essential anti-apoptotic proteins would remain intact or even enhanced, due to increased activity of EIF4A2 upon EIF4A1 inhibition. The role for translation initiation in drug-induced liver injury is an unexplored area. My data indicate that this clearly deserves more attention both in the context of DILI but also in relation to other liver pathological conditions.

I searched for novel regulators of NF-κB nuclear translocation in chapter 5. Interestingly, two of the candidate genes whose knockdown had a significant effect on the TNFα-induced nuclear translocation of NF-κB are involved in mRNA splicing: splicing factor PHF5A and the cyclin dependent kinase CDK12 (20,21). Despite the fact that CDK12 has also been implicated in gene-set specific transcription by its phosphorylation of RNA polymerase II (22), this further emphasizes the important role for post-transcriptional regulation of protein expression in a situation of cellular stress. Interestingly, depletion PHF5A or CDK12 led to a “delayed” translocation phenotype under DMSO conditions, much like the one seen by diclofenac pre-treatment. Additionally, knockdown of PHF5A led to a slight enhancement of apoptosis induced by TNFα under DMSO conditions (observation not shown). This suggests aberrant mRNA splicing as a mechanism for diclofenac-induced inhibition of NF-κB translocation as well as induction of apoptosis after TNFα exposure. The description of similar substrate specificity between EIF4A1 initiated translation and PHF5A and CDK12 mediated mRNA splicing, would provide further mechanistic insight to how certain drugs can sensitize towards TNFα-induced apoptosis and thereby induce DILI. It would be relevant to investigate the expression of c-FLIP after knockdown of EIF4A1, PHF5A and CDK12.

A central role for A20 in regulating cell death and survival in TNFα-signaling

I demonstrated that perturbations in NF-κB signaling are critical in drug-induced hepatocyte cell death. NF-κB is well known for its involvement in the transcription of genes involved in for example inflammation, survival and proliferation. However, inappropriate activity of NF-κB signaling has been linked to the development of (liver) cancer, but also of autoimmune and inflammatory diseases (23). Therefore, the identification of novel regulators of the NF-κB signaling would provide improved insight for both hepatotoxicity as well as (liver) carcinogenesis. We focused on the NF-κB translocation response for our siRNA screen. Interestingly, most of our hits from the screen inhibited the NF-κB translocation response (described as “no oscillation”). Additionally, we were able to link this “no oscillation” phenotype to inhibition of drug/TNFα-induced apoptosis. “No oscillation” could be explained by inhibition of TNFR1 activation, which would also result in inhibition of TNFR1-mediated apoptosis. This suggests that the knockdown of these
candidate genes would affect the assembly of TNFR1 downstream signaling complexes, thereby preventing the pro-survival NF-κB signaling, but also preventing the pro-apoptotic caspase-8 activation. We showed that a common denominator for the knockdowns that inhibited both processes was the expression of the protein A20.

A20, or TNFAIP3, is a target gene of NF-κB and constitutes, together with IκBα (NFKBIA), the most important transcriptionally regulated feedback mechanisms to down-regulate NF-κB activation (24). A20 inhibits NF-κB signaling by deubiquitinating receptor-interacting protein 1 (RIP1) and subsequently targeting this protein for proteasomal degradation by ubiquitin ligation (25). However, apart from its role in terminating the NF-κB signaling, A20 has a crucial role in regulating the apoptotic response after TNFα exposure as demonstrated in an A20-deficient mouse model (26). The inhibition of apoptosis has been shown to occur via deubiquitination of caspase-8 downstream of the death receptor 4 and 5 (27). Further research in this topic is however needed to clarify the exact role of A20 in repression of the pro-apoptotic signaling downstream of the TNFR1. One possibility is that A20 is involved in the direct switching from anti-apoptotic complex I formation, to pro-apoptotic complex II initiation, and thereby regulating the apoptosis induced by TFNα.

Because of its dual role in the regulation of TNFα and other immune-mediated signaling, A20 has been thoroughly investigated as a key mediator in cancer and chronic as well as autoimmune disease. Moreover this gene has been described as a susceptibility locus for the development of such diseases (28). Together with its protecting role in drug/TNFα-induced liver cell death, further studies on the regulation of A20 expression in DILI is needed. In chapter 5 I describe four novel regulators of A20 expression, CDK12, UFD1L, TRIM8 and RNF126. Importantly, knockdown of these genes resulted in an enhanced expression of A20 leading to the inhibition of TNFα-induced NF-κB nuclear translocation, as well as apoptosis under drug pre-exposure conditions. Further investigation on how this regulation occurs is relevant to exploit this pathway to treat chronic inflammatory and autoimmune diseases as well as cancer, in which A20 has been shown to play an important role (28).

The future for in vitro hepatotoxicity assessment

A golden standard for human liver toxicity testing in a pre-clinical setting is the use of primary or cryo-preserved human hepatocytes. However, this cell system has been proven difficult to work with, as the batch-to-batch variability in the cellular responses is large, and as the cells go through rapid dedifferentiation. The use of HepG2 cells is then an attractive option since these cells, as an immortal cell line, show low variability in between experiments and allow detailed mechanistic research using techniques such as the functional genomics screening in this thesis. Granted an important drawback of using a transformed cell line such as HepG2 is its low drug-metabolizing capacity. Yet, while HepG2 cells show low phase I metabolizing activity (29), its phase II metabolism is more adequate, leaving them as an acceptable cellular system for the study of drug-toxicity
induced by certain drug-metabolites (30). Furthermore, and critically importantly for our research, the acyl-glucuronide metabolite for the induction of idiosyncratic diclofenac-mediated liver injury, could be readily identified in our culture system (chapter 2).

An additional advantage of the HepG2 cells is that they can be easily engineered to express for example GFP-tagged proteins as reporters for different types of intracellular stress. This was first introduced in chapter 2 using a GFP-p65 reporter cell line, and further expanded in more detail in chapter 3. Here it is nicely illustrated how the combination of a few different cellular assays, can be used to estimate the toxicity potential of novel drug candidates. Although in the end not proven to result in apoptosis in our cellular system, the capability of a drug to induce oxidative stress as detected by the Srxn1-GFP reporter, or its ability to inhibit the nuclear translocation response of NF-κB, can provide enough information from a toxicological perspective. Especially since both these events have been linked to enhanced drug toxicity in vivo (see chapter 1).

Multi-parametric analysis of cells stained with different dyes to assess different drugs’ hepatotoxic potential has recently been described as a high throughput method for fast assessment of (mode of) toxicity (31). However, such a method involves different sample preparation steps as well as the limitation of a fixed-time-point. Instead an extended panel of in vitro reporters of different types of cellular stresses known to be involved in drug-induced liver injury, similar to the stress reporters described in chapter 3, would constitute an important asset to the pharmaceutical safety assessment. These would provide minimal amount of wet-work, resulting in reduced variability. Since GFP-based reporter systems allow live cell imaging of stress responses, kinetic information about the toxicity onset on a cell-to-cell basis provides additional valuable information. In addition, different GFP stress reporter cell lines would provide important mechanistic information about the pathways of toxicity that are induced by drug exposure. When considering the results from chapter 4, I would additionally suggest reporters for CHOP and EIF4A1-induction, as a part of the pre-clinical in vitro tox-screening panel. These proteins were both up-regulated after liver-toxic drug exposure and functionally critical for the drug/TNFα-induced synergistic toxicity.

The use of induced pluripotent stem cells (iPSCs) that are later differentiated into hepatocytes is becoming an increasingly attractive alternative for in vitro toxicity testing. These newly differentiated cells seem more “hepatocyte-like” than HepG2 cells when it comes to for example drug metabolism capacity (32). In an ideal world, these could also be used for stress reporter construction, as this background would be even more relevant to the human situation compared to the HepG2 cells. Interestingly, considering the individual variances in drug responses, projects are underway to generate iPSCs, and subsequently hepatocytes, from patients experiencing iDILI (33). This would provide very important information on the hepatocyte-based molecular mechanisms underlying ADRs in highly susceptible patients.
CONCLUSION

In this thesis I used an in vitro model to study the role of inflammatory stress in idiosyncratic DILI. I have described in detail the interplay between hepatotoxicant- and cytokine-induced signaling in liver cell death. Two drugs, diclofenac and carbamazepine, which have been associated with inflammatory DILI in humans, showed an exceptionally strong synergistic response with the pro-inflammatory cytokine TNFα. This response was linked to the inhibition of NF-κB signaling (chapter 2 and 3) and the induction of oxidative as well as to onset of endoplasmic reticulum and translation-dependent stress (chapter 3 and 4). Finally in chapter 5, I described novel regulators of A20 expression and indirectly of NF-κB signaling as well as apoptosis induction. Potentially these genes could be used as markers for inflammatory diseases as well as susceptibility markers for the development of DILI. Moreover, I anticipate that more detailed studies of post-transcriptional regulation will provide further important information about the mechanisms behind drug-induced liver injury.

The findings reported in this thesis provide important contributions to the understanding of the mechanisms behind drug-induced liver-injury. This deeper understanding may aid in the development of advanced pre-clinical drug safety assessment models for novel candidate drugs. Furthermore, I anticipate that the novel findings regarding the regulation of NF-κB activation can contribute to areas beyond drug-induced liver injury, including inflammatory and autoimmune diseases, as well as cancer.
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