Role of TNF-α and the NF-κB pathway in drug-induced organ injuries

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Aim and scope of the thesis

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Adverse drug reactions (ADRs) remain a major health issue. They are the cause of 5% of the hospital admissions in Europe (source: Annex 2 of the report on the impact assessment of strengthening and rationalizing EU Pharmacovigilance, 2008) and markedly increased the costs of drug development. The two major targets of ADRs are the liver and the kidneys. This is due to their exposure to high levels of toxicants, their drug metabolizing capacities (with higher capacities in liver cells), and their high consumption of oxygen. Furthermore, inflammation plays an important role in drug-induced toxicity for both liver and kidneys. Several nephrotoxicants and hepatotoxicants are known to induce an inflammatory response which contributes to the organ injury (1-8). During kidney and liver toxicity, the toxicant induces first mild tissue damage resulting in the generation of inflammatory mediators by the injured cells as well as by immune cells. Subsequently, leukocytes, attracted by the immune mediators secreted, migrate and infiltrate into the injured organs and aggravate the primary injury induced by the toxicant (4,9).

For both liver and kidneys, the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) is the main orchestrator of this inflammatory response. Elevated levels of TNF-α are found in liver and kidneys following exposure to many toxic agents (10-18) and inhibition of TNF-α prevents drug-induced toxicity from occurring (12-14,19-24). TNF-α is particularly interesting in drug-induced cytotoxicity context due to its dual role in regulating both cell survival and cell death. Binding to the TNF receptors TNFR1 and TNFR2 leads to recruitment of one of two complexes. Complex II, which is assembled only when TNFR1-associated death domain protein (TRADD) and receptor-interacting protein 1 (RIP1) are ubiquitinated, activates caspase-8 and -10 leading to the induction of apoptosis (25). Complex I activates the transcription factor nuclear factor κB (NF-κB), leading to transcription of inflammatory and survival genes (26).

The pleiotropic transcription factor NF-κB consists of 5 subunits: RelA (p65), c-Rel, RelB, NF-κB1/p50 and NF-κB2/p52 forming 15 potential homo- and heterodimers. The different NF-κB dimeric complexes formed allows the integration of the diverse stimuli to achieve cell type- and stimulus-specific responses. The RelA/p50 dimer is the most abundantly expressed and best characterized member of this family (26). Activation of NF-κB results in nuclear translocation and can proceed either through classical/canonical and alternative/non-canonical NF-κB pathways (Fig. 1). They are distinguished by two multiprotein IκB kinase (IKK) complexes that regulate the stimulus responsive degradation of IκB proteins. The canonical IKK complex contains the IKK2 (IKKβ) protein and is regulated by the scaffold protein NF-κB essential modulator (NEMO, also known as IKKγ), whereas the non-canonical IKK complex

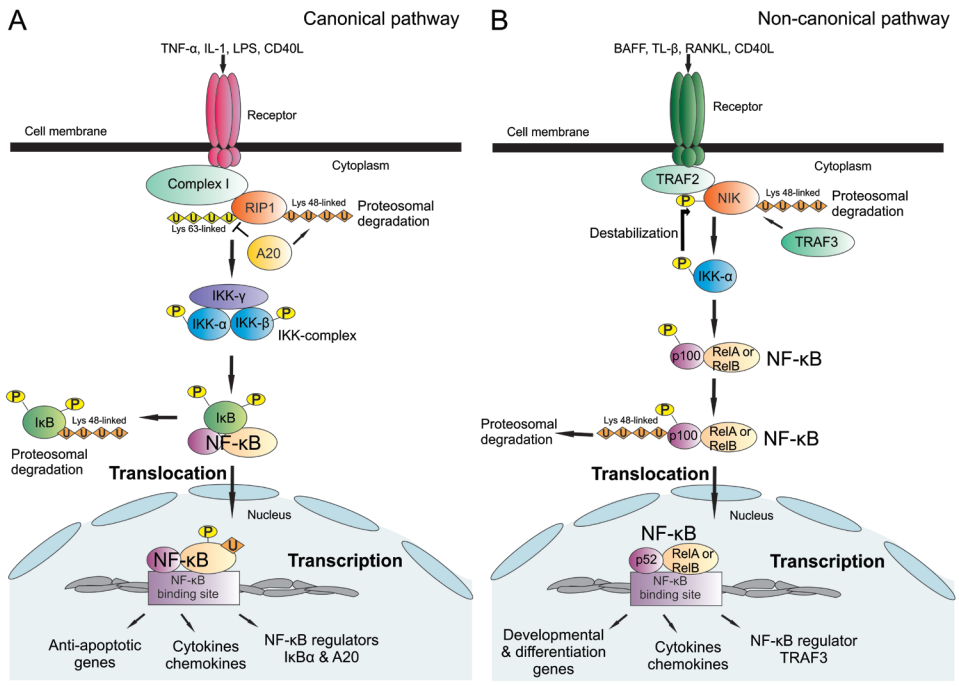

Figure 1. Canonical and non-canonical pathways of NF-κB activation. (A) Canonical pathway. Under resting conditions, NF-κB dimers are sequestered in the cytoplasm by their inhibitory IκB proteins. Upon stimulation, IKK-complex phosphorylates IκB proteins leading to their subsequent ubiquitination and proteasomal degradation. The NF-κB dimers are then released so they can translocate to the nucleus and transcribe anti-apoptotic and inflammatory genes, as well as the two inhibitors of NF-κB signalling, IκBα and A20. (B) Non-canonical pathway. Upon stimulation, NIK phosphorylates and activates IKKα leading to the phosphorylation of p100 associated with RelB. p100 is then partially processed to generate the transcriptionally active p52-RelB complexes transcribing genes involved in development and differentiation, inflammatory genes as well as the NF-κB signalling regulator TRAF3. IKKα also regulates NIK via phosphorylation at certain residues.

consists solely of IKK1 (IKKα) homodimer (26). Classical NF-κB activation is a rapid and transient response to a wide range of stimuli, such as TNF-α, interleukin 1 (IL-1), CD40 ligand and lipopolysaccharide (LPS) (27). Upon inflammatory stimulation, the canonical IκB proteins are phosphorylated by the canonical IKK complex, tagging it for Lys 48–linked polyubiquitination and proteasomal degradation. NF-κB, mainly p65/p50, which was sequestered by IκB in the cytoplasm, is then free to translocate to the nucleus and to bind and activate the transcription of its target genes (Fig. 1A). The activation of the canonical NF-κB pathway is tightly regulated by different post-translational modification steps but is also regulated by transcriptionally mediated feedback mechanisms. NF-κB–driven IκBα re-synthesis contributes to a fast turn-off

response (28). In addition, the NF-κB target gene A20 inhibits NF-κB activation by de-ubiquitinating the Lys 63-linked activating ubiquitin chains on the kinase RIP1 and promoting its Lys 48-linked polyubiquitination and proteosomal degradation (29, 30).

Non canonical signalling is induced by specific members of the TNF cytokine family, such as CD40 ligand, B cell activating factor (BAFF), lymphotoxin-β and receptor activator of NF-κB ligand (RANKL) (27). It involves slow activation of the RelA/p52 and RelB/p52 heterodimers leading to prolonged activation of NF-κB target genes. The sequence of molecular events that result in the induction of these dimers remains poorly understood. In a simplified model, p100 functions as an IκB protein by sequestering both RelA/p50 and RelB/p50 complexes in resting cells. Stimulation of IKKα activity leads to processing of p100 to p52 via NF-κB–inducing kinase (NIK), which dimerizes with both RelB and RelA to ensure a long-lasting activity of both RelA- and RelB-containing dimers (Fig. 1B). Unlike the canonical NF-κB pathway, there are few negative feedback controls of the non-canonical pathway. NF-κB-inducible expression of TNF receptor-associated factor 3 (TRAF3) may impede RelB activation by degrading NIK and phosphorylation of NIK at certain serine residues by IKKα destabilizes NIK providing a way to fine-tune non-canonical signalling (31).

Aim and scope of this thesis

Although the role of inflammation and the cytokine TNF-α has been clearly demonstrated for both drug-induced kidney and liver toxicity, the signalling pathways activated synergistically by the toxicant and the immune system components leading to cellular death are still not very clear. Therefore, the general aim of the research presented in this thesis is to assess the molecular mechanisms underlying the enhanced toxic response induced by combined cytokine/drug treatment of kidney and liver cells. The focus is on the cytokine TNF-α since this is the main mediator of inflammation during toxicity for both kidney and liver.

Chapter 2 reviews the toxicogenomics studies performed in the last years in the attempt to obtain more insight into the molecular mechanisms of toxicity of nephro- and hepatotoxicants. These toxicogenomics studies include transcriptomics, proteomics, metabolomics as well as functional genomics and the role of inflammation in nephrotoxicity as well as in hepatotoxicity is emphasized.

To investigate whether we could reproduce the inflammation-mediated aggravation of nephrotoxicant-induced renal injury in an *in vitro* model, we evaluated the toxicity

of several known nephrotoxicants in combination or not with the main mediator of inflammation, TNF-α, in mouse immortalized PTECs using a sensitive fluorescence live cell imaging based assay (**chapter 3**). Four out of the 16 tested nephrotoxicants induced synergistic apoptosis with TNF-α, including the chemotherapeutic cisplatin. The molecular mechanisms underlying cisplatin/TNF-α-induced synergistic apoptosis were investigated in **chapters 4** and **5** using, amongst others, lentiviral-based knock-down and transcriptomic technologies. In **chapter 4**, we demonstrated that the TNF-α-mediated enhancement of cisplatin-induced apoptosis was due to an alteration of the NF-κB/JNK/c-Jun balance in these cells. This imbalance led to a pro-apoptotic and inflammatory gene expression pattern in cells exposed to a combination of TNF-α and cisplatin. In **chapter 5**, we showed that TNF-α also aggravated the cytoskeleton and cell adhesion disruptions induced by cisplatin. The NF-κB family member RelB was required for these TNF-α mediated disruptions as well as for the TNF-α induced aggravation of cisplatin mediated renal cell apoptosis by controlling a Rho kinase-dependent signalling network.

In addition to its effect on renal cell injury, TNF-α also aggravated the toxicity induced by the hepatotoxic drug diclofenac in HepG2 liver cells via its effect on the NF-κB/JNK/c-Jun balance. Detailed mechanistic insight was obtained in studies described in **chapters 6** and **7** using two distinct small interfering RNA screening approaches. In **chapter 6**, the mechanism behind the synergistic apoptotic response observed with diclofenac and TNF-α treatment was unravelled using siRNA screening in combination with fluorescence imaging-based toxicity assessment. This led to the identification of 12 genes that essentially control the apoptotic response under exposure to diclofenac, TNF-α or diclofenac in combination with TNF-α. Some of these genes could in the future be novel toxicity biomarkers. In **chapter 7**, the role of individual proteins known to be involved in post-translational modifications was determined in relation to NF-κB nuclear translocation since such translocation was found to be important for the outcome of diclofenac/TNF-α exposure. Here we used a siRNA screen in combination with high content imaging of green fluorescent protein (GFP)-tagged p65 nuclear translocation following exposure to TNF-α in combination or not with diclofenac. In total 46 genes were identified that significantly affected the typical TNF-α dependent NF-κB nuclear translocation response by either increasing, decreasing or completely blocking NF-κB nuclear translocation. We observed that gene knock-downs that resulted in a complete blockage of TNF-α-induced NF-κB nuclear translocation also resulted in protection against diclofenac/TNF-α-induced synergistic apoptosis. This was associated with elevated levels of the (de)ubiquitinase and critical component in

the NF-κB feedback loop, A20 in these knock-down cells.

Finally, **chapter 8** provides a general discussion on the results obtained in our studies and on the implications for future research. Here the focus is on the overlap and differences in signalling pathways underlying nephrotoxicity and hepatotoxicity.

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