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

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# General discussion

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Nephrotoxicity and hepatotoxicity are the two major reasons of safety-related post-market drug withdrawal. To limit this unwanted drug withdrawal, a better understanding of the mechanisms involved in the toxicity of newly developed drugs as well as the development of new *in vitro* pre-clinical tests incorporating this knowledge is therefore necessary.

The research described in this thesis was designed to identify new signalling pathways involved in drug-induced organ toxicity. The role of inflammation and in particular the role of the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) was investigated in both drug-induced kidney and liver cell injury. A combination of innovative RNA interference live cell fluorescence microscopy-based screens and gene expression array analysis resulted in identification of mechanisms underlying nephro- and hepatotoxicity, which is discussed in this chapter.

## 1. The role of TNF–α in drug–induced organ toxicity

The importance of the contribution of the immune system to drug-induced kidney and liver toxicity has been well recognized over the past years. Several nephrotoxicants and hepatotoxicants have been shown to induce an inflammatory response, which participated in the organ injury (1-8). It is believed that during kidney and liver toxicity, the initial insult by the toxicant results in tissue damage, which leads to generation of inflammatory mediators by the injured cells as well as by immune cells. Subsequently, these inflammatory mediators induce migration and infiltration of leukocytes into the injured organs and aggravate the primary injury induced by the toxicant (4, 9). For both liver and kidneys, the pro-inflammatory cytokine TNF-α is the main orchestrator of this inflammatory response and in several cases has been shown to aggravate the toxicant-induced pathophysiological responses (4, 10-24). Although the contribution of TNF-α in drug-induced kidney and liver injury *in vivo* is clear, the mechanisms of enhanced toxicity combined drug/cytokine exposure are not known. Therefore, in order to understand the underlying mechanisms, an *in vitro* assay mimicking this *in vivo* physiological response should be designed.

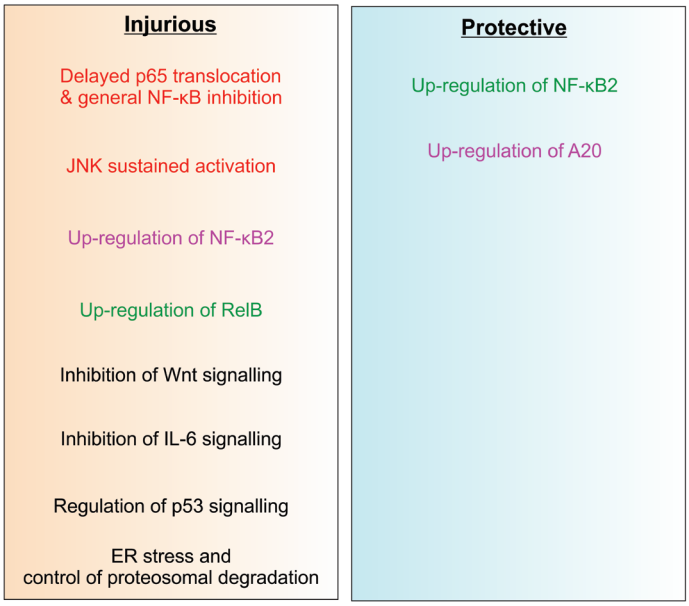
To identify mechanism underlying TNF-α mediated drug-induced toxicity, we therefore set-up *in vitro* models for nephrotoxicity and hepatotoxicity that mimick the immune-related drug response *in vivo* (**chapter 3** and **6**) using proximal tubular epithelial cells (PTECs) and HepG2 cells respectively. We showed that exposure to TNF-α in combination with the neprotoxicants cisplatin, cyclosporine A, tacrolimus or azidothymidine significantly enhanced the cytotoxicity of these drugs in the *in vitro*

model for nephrotoxicity (**chapters 3-4**). Furthermore, TNF-α enhanced HepG2 cell death induced by diclofenac and carbamazepine ((10), **chapters 6-7**). Transcriptomic analysis of cisplatin-treated PTECs revealed that the gene expression of numerous kidney injury markers combined with activation of inflammatory mediators involved in cisplatin-induced nephrotoxicity *in vivo* were enhanced by cisplatin/TNF-α treatment (**chapter 4**). Furthermore, pathway analysis of the hits identified in our siRNA-based apoptosis screen in HepG2 cells showed that mainly hits regulating the diclofenac/TNF-α-induced apoptosis were involved in toxicity pathways related to liver injury (**chapter 6**). Therefore, in both *in vitro* systems, exposure of the cells with a combination of toxicant and TNF-α proved to be a good model for studying the detailed mechanisms involved in the aggravation of toxicant-induced injury by inflammation. To unravel the mechanisms involved in the synergistic apoptosis induced by the combination drug/TNF-α in these *in vitro* systems, we used cisplatin and diclofenac as model compounds for nephrotoxicity and hepatotoxicity respectively.

## 2. Mechanisms involved in the TNF–α aggravation of drug–induced organ toxicity

In order to decipher new toxicity pathways, transcriptomics analysis as well as siRNA-mediated screens were combined with high content imaging in an innovative way. These techniques are introduced in **chapter 2**. In both siRNA screens performed, all known kinases, (de)-ubiquitinases as well immune components were knocked-down with siRNAs (**chapters 6-7**) and toxicity was measured with a live apoptosis assay developed by our laboratory (25). This method providing both quantitative and kinetic information on the apoptosis was also used for the other studies performed in this thesis. In addition to toxicity measurements, the siRNA screen performed in **chapter 7** associated high content confocal laser scan microscopy in combination with multiparametric image analysis to follow the NF-κB oscillation in ~300 individual cells per condition simultaneously. This innovative siRNA screening approach allowed, for the first time, measurement of the population dynamics upon knockdown of candidate genes.

Using the innovative combination of techniques described above, we were able to unravel several signalling pathways involved in TNF-α enhancement of drug-induced toxicity. These included the regulation of the NF-κB pathway, the activation of c-Jun N-terminal kinase (JNK), the up-regulation of A20 levels, the remodelling of the actin cytoskeleton and cellular adhesions which will be discussed below and are summarized




Legend: red: mechanisms common to both kidney and liver cells, pink: mechanisms specific to liver cells, green: mechanisms specific to kidney cells, black: potentially involved pathways

**Figure 1. Summary of the pathways identified as participating in the drug/TNF-α-induced synergistic apoptosis of kidney and liver cells.**

in Fig. 1.

### 2.1. A central role for NF-κB signalling in TNF-related drug-induced cell death

The role of the pro-survival NF-κB signalling in the drug/TNF-α synergistic response was shown at several levels in this thesis. The strength, duration and type of oscillatory pattern of NF-κB nuclear translocation determine the set of NF-κB target genes that will be transcribed after TNF receptor 1 (TNFR1) activation, resulting in either pro- or anti-apoptotic signalling. Transcriptomic analysis of our PTECs revealed that the inhibition of the typical TNF-α-induced nuclear translocation response of NF-κB by cisplatin coincided with decreased expression of several NF-κB-regulated anti-apoptotic genes, including c-IAP2, Bcl-XL, Bruce and Bcl2 (**chapter 4**). These anti-apoptotic proteins prevent apoptosis at different levels: Bcl-XL and Bcl2 prevent the release of cytochrome c from the mitochondria which would lead to caspase activation (26) and c-IAP2 and

Bruce interfere with the activation of caspases (27, 28). To better understand the role of these proteins in cisplatin/TNF-α-induced synergistic apoptosis, knock-down experiments should be carried out in the future. In addition, overexpression in an *in vivo* model could also rescue the kidneys from cisplatin-induced nephrotoxicity. Such rescue was observed by overexpressing Bcl-2 in a renal ischemia/reperfusion model *in vivo* (29). However, such therapeutic strategy should only be applied in the case of cisplatin in a targeted way since cancer cells could take advantage of overexpression of anti-apoptotic proteins to acquire survival and metastatic properties. These anti-apoptotic proteins seem, in the case of diclofenac-induced liver toxicity, not to play a role in the synergistic apoptosis since their knock-down didn't affect the apoptosis outcome (10).

NF-κB inhibition was also shown to play an important role in diclofenac/TNF-α-induced apoptosis in HepG2 cells (10). In addition, siRNA-mediated knock-down of kinases, (de)ubiquitinases and immune components in HepG2 cells followed by exposure to diclofenac, TNF-α and diclofenac/TNF-α combined treatment led to the identification of several proteins known to be involved in the regulation of the NF-κB pathway (**chapter 6**). Together these data implicated an important role for NF-κB signalling in synergistic cell death of both kidney and liver cells.

NF-κB consists of five members: RelA (or p65), RelB, NF-κB1, NF-κB2 and c-Rel (30). Out of these five family members, I showed in this thesis that the family members NF-κB2 and p65 were involved in both nephro- and hepatotoxicity, while RelB was only associated with nephrotoxicity. p65 was identified as regulator of the synergistic apoptosis in both PTECs and HepG2 cells. Delay of p65 nuclear translocation accompanied by general inhibition of NF-κB transcription led to an enhancement of the toxicant-induced cell death by TNF-α both in PTECs (**chapter 4**) and in HepG2 cells (**chapter 7** and (10)). Furthermore, knock-down of p65 sensitized both cell lines to drug/TNF-α-induced apoptosis (**chapter 4** and (10)).

The NF-κB member NF-κB2 was also identified in the regulation of the synergistic apoptosis of both PTECs and HepG2 cells. Gene expression of NF-κB2 was up-regulated upon exposure of cells to TNF-α, drug alone or a combination of both. Yet, knock-down of NF-κB2 in HepG2-p65-GFP cells led to a protection against diclofenac/TNF-α-induced apoptosis (**chapter 6**), while it enhanced both cisplatin and cisplatin/TNF-α-induced apoptosis in PTECs (**chapter 5**). These observations indicated that NF-κB2 was an inducer of the synergistic apoptotic response in HepG2 cells, but a protector in PTEC cells. Previous work in other cell types showed that NF-κB2 recruits p53 after DNA damage on multiple target genes and promotes cell cycle arrest and cell death

(31). We therefore speculated that NF-κB2 was inducing enhanced apoptosis in HepG2 cells after diclofenac/TNF-α treatment via induction of p53-dependent apoptosis. In addition to its role in activating p53-dependent apoptosis, NF-κB2 was also shown to repress c-myc (31). Therefore, it could well be that in PTECs, the main role of NF-κB2 is the repression of c-myc, whereas in HepG2 cells, it is mainly involved in p53 regulation. Possibly this difference is related to the transformed status of HepG2 cells. Further detailed investigations in primary renal and liver cells as well as *in vivo* analysis are necessary to confirm these findings.

In addition to an increase in NF-κB2 expression in PTEC cells, exposure of the cells to TNF-α led to an increase in the expression of RelB both at gene and protein levels in PTECs, as well as in HepG2 cells. However, stable shRNA-based knock-down of RelB abrogated the synergistic apoptosis induced by cisplatin/TNF-α treatment in PTECs (**chapter 5**), while siRNA-based knock-down of RelB did not affect diclofenac/TNF-α-induced HepG2 apoptosis (**chapter 6**). The protection of renal cells was conferred via an epithelial to mesenchymal transition (EMT)-like switch leading to the inhibition of the morphological changes induced by TNF-α in the cells.

It is well known that the NF-κB pathway integrates a variety of stimuli into a cell-type and context-specific response. In this thesis, I demonstrated that differences exist in the contribution of the different NF-κB family members in the TNF-α-mediated aggravation of drug-induced kidney and liver cells. However, delay in p65 translocation accompanied with general NF-κB inhibition is a common mechanism between these two cell types. Furthermore, this role of NF-κB signalling in the synergistic cell death could be common for all compounds inducing synergistic cell death as three other nephrotoxicants displaying synergistic apoptosis with TNF-α - namely cyclosporine A, tacrolimus and azidothymidine - were shown to inhibit TNF-α-induced NF-κB activation in renal cells (32) and Epstein-Barr virus-positive Burkitt lymphoma lines (33) (**chapter 3**). This needs to be investigated further using our panel of NF-κB family member shRNA-based knocked-down PTEC cell lines.

### 2.2. NF-κB signalling as a regulator of JNK activation

JNK phosphorylation and thereby activation status balances the cells between cell death and survival (34). Several nephrotoxic and hepatotoxic drugs have been shown to induce JNK activation and inhibition or knock-down of JNK protected from drug-induced organ injury (10, 35-40). Moreover, NF-κB is known to control the activation of JNK (41-43), while in turn JNK is known to inhibit NF-κB activation (44-46).

In **chapter 4**, we demonstrated that inhibition of NF-κB by cisplatin in PTECs led to an enhanced and prolonged JNK activation, which was directly linked to the enhancement of cisplatin-induced apoptosis by TNF-α. Furthermore, we also demonstrated that sustained activation of JNK further inhibited NF-κB signalling via a feedback loop mechanism. Although not directly demonstrated in this thesis, our laboratory showed in a previous publication that NF-κB inhibition also led to sustained JNK activation in HepG2 cells (10), suggesting a common role for JNK signalling in TNF-α synergy in chemical-induced cytotoxicity. The effect of JNK on the regulation of pro-apoptotic signalling in the cells was directly shown by the JNK-mediated up-regulation of X-linked inhibitor of apoptosis protein associated factor 1 (XAF1) in PTECs with cisplatin/TNF-α treatment (**chapter 4**). XAF1 has been shown to act as an antagonist of XIAP anti-caspase activity and therefore its up-regulation leads to enhanced apoptosis of the cells. Interestingly, a recent study showed that XAF1 transcript and protein were expressed at low levels in several hepatoma cell lines, including HepG2 cells (47). Therefore, it could well be that XAF1 up-regulation plays an important role in JNK-mediated cell death in HepG2 cells as well. In addition, the siRNA screen performed in **chapter 6** led to the identification of the TRAF2 and NCK interacting kinase (TNIK) as inducer of diclofenac/TNF-α-induced apoptosis, which is known to mediate TNF-α-mediated activation of both JNK1 and 2 in cancer cells (48). Its role in its capacity to activate JNK1 and 2 in HepG2 cells as well as in PTECs needs to be further investigated.

### 2.3. A controversial role for the NF-κB target gene A20

Using RNA interference screening we identified several proteins for which knock-down led to an almost complete inhibition of p65 nuclear translocation response. For most of the genes, this correlated with an inhibition of drug/TNF-α-induced apoptosis in HepG2 cells (**chapter 7**), including CDK12, UFD1L, TRIM8 and RNF126. This result could seem contradictory to our other findings in which inhibition of NF-κB signalling was associated with enhanced apoptosis (**chapters 3**, **4** and **7**), but in this case, p65 nuclear oscillation was not only delayed but completely inhibited. We showed that knock-down of the proteins that inhibited both NF-κB translocation and apoptosis led to up-regulation of the levels of the NF-κB target gene A20 (**chapter 7**), which is known to lead to NF-κB inhibition via a feedback-loop mechanism (49). A20 knock-down itself sensitized cells to apoptosis and double knock-down of those proteins and A20 rescued the synergistic apoptosis. Indeed, A20 has also a critical role in regulating the apoptotic response after TNF-α exposure as demonstrated in an A20-deficient mouse model

(50). Our data demonstrate that up-regulation of A20 levels may lead to inhibition of apoptosis in HepG2 cells. Possibly this is related to blocking TNFR signalling altogether and preventing the formation of a pro-apoptotic signalling complex downstream from receptor activation, but further research is ongoing to clarify its exact role. Interestingly, in contrast to HepG2 cells, a decrease in A20 levels after cisplatin/TNF-α treatment in comparison to TNF-α treatment was observed in PTECs (**chapter 4**). The role of A20 was not investigated in these cells, but could be another mechanism responsible for the TNF-α-enhancement of cisplatin-induced apoptosis of PTECs. Clearly, a full understanding of the regulatory systems that control pro-apoptotic and survival signalling downstream of the TNFR in different organ systems will be essential to further extend our understanding of the interplay of chemical stress and cytokine signalling in target organ toxicities.

### 2.4. A novel role for NF-κB in drug-induced remodelling of the actin cytoskeleton and cellular adhesions

It is known that disruption of the actin cytoskeleton of the PTECs is an early event, leading to loss of adhesion of cells from the extracellular matrix (ECM) as well as neighbouring cells, ultimately resulting in pro-apoptotic signalling and cell death (6, 51-56). In **chapter 5**, we studied the involvement of TNF-α and downstream NF-κB signalling in the regulation of cisplatin toxicity through the control of actin cytoskeletal changes during cisplatin cytotoxicity. We demonstrated for the first time that TNF-α aggravated the cisplatin-induced disruption of actin stress fibres, reduction of focal adhesions and loss of cell-cell contacts. TNF-α exposure resulted in up-regulation of the NF-κB family member RelB and knock-down of RelB inhibited the morphological changes induced by TNF-α which led to protection of the cells against cisplatin/TNF-α-induced apoptosis. Several recent studies indicate that reorganization of the cytoskeleton modulates the activity of NF-κB and particularly of the p65/p50 heterodimer. Disruption of the microtubule network by a variety of agents leads to rapid induction of p65/p50 DNA binding and subsequent NF-κB-dependent gene expression (57-59). The mechanisms on how NF-κB is activated by the cytoskeleton disruption are not yet known and are likely involving the degradation of the NF-κB inhibitor IκB. In our PTECs, we could observe a slight increase in IκB phosphorylation and degradation with the combined cisplatin/TNF-α treatment in comparison to TNF-α treatment alone (**chapter 4**). This increase in IκB turnover could therefore be explained by the enhanced microtubule network disruption observed with cisplatin/TNF-α

treatment (**chapter 5**). However this increased turnover was associated with decreased p65 nuclear translocation and transcription indicating that cisplatin inhibited the NF-κB pathway via blocking p65 nuclear entry.

The association between RelB activation and the cytoskeleton was to our knowledge not previously directly demonstrated. However, the expression of RelB was shown to correlate with dendritic cell development and maturation (60) as well as to invasiveness in oestrogen receptor alpha (ERα)-negative breast cancer cells (61). For both maturation and invasiveness processes, the cells need to undergo morphological changes, indicating that RelB directly or indirectly controls the morphology of the cells. In our PTECs, the inhibition of the cytoskeletal changes in RelB knocked-down cells was done via a snail2-controlled EMT-like switch in association with RhoA activation, since RhoA inhibition abrogated the synergistic apoptosis induced by cisplatin/TNF-α treatment. This observation was in contradiction with the results of Wang et al. in breast cancer cells where RelB knock-down induced decreased expression of Snail and fibronectin and increased the levels of E-cadherin and γ-catenin expression indicating that RelB is required for maintenance of the mesenchymal phenotype of the cells (61). However in this study, Bcl-2 was shown to be a critical mediator of the invasive properties induced by RelB since ectopic Bcl-2 expression in RelB knocked-down cells decreased E-cadherin and γ-catenin expression in association with a restoration of the invasive phenotype of the cells. Furthermore, the status of RhoA activation was not determined in these cells. In our PTECs, Bcl-2 expression was not modified by RelB knock-down (**chapter 5**). It seems therefore that the unique combination of Snail2 and RhoA activation controlled by RelB is driving the phenotypic switch and protection of the cells.

Although RelB did not seem to play a role in HepG2 synergistic apoptosis, the RhoA pathway was also shown to be involved in drug-induced liver toxicity. In HepG2 cells, RhoA inactivation and to a lesser extent Rho-kinase inhibition were implicated in the hepatotoxicity induced by lipophilic statins (62). Furthermore, Rho-kinase inhibitor was reported to prevent carbon tetrachloride (CCl4)- or dimethyl nitrosamine-induced liver fibrosis and hepatic ischemia-reperfusion injury in rats (63-65) as well as hepatocyte damage in CCl4-induced acute liver injury (66). Although, in this thesis, we did not study the role of RhoA in diclofenac/TNF-α-induced apoptosis of HepG2 cells, it could be another mechanism of toxicity to explore in the future.

## 2.5. Possible other mechanisms underlying the control of TNF-mediated cell death

In addition to the regulation of NF-κB, JNK and A20, the different "omics" techniques used in this thesis led to the identification of novel genes of interest. These genes were involved in the control of apoptosis at the receptor, transcription, mRNA processing and endoplasmic reticulum levels.

### *2.5.1. Receptor level*

At the receptor level, genes involved in the regulation of the wnt and interleukin 6 (IL-6) signallings were identified. The wnt signalling pathway regulates apoptosis via a variety of mechanisms including through β-catenin, GSK3β-NF-κB signalling, JNK and NEMO (67). It was previously shown that the Wnt/β-catenin signalling transduction pathway is activated with aberrant expression of Wnt1 in HepG2 cells (68) and ZNRF3, known to associate with the Wnt receptor complex leading to its inhibition (69), was identified as a protector of diclofenac-induced apoptosis in HepG2 cells (**chapter 6**). Further investigations need to be done in order to determine if ZNRF3 could indeed inhibit the wnt signalling in HepG2 cells. Nevertheless, other evidences point out toward the involvement of the wnt signalling in the control of diclofenac-induced apoptosis in HepG2 cells: diclofenac down-regulates by three-fold the gene expression of Dickkopf-1 (Dkk-1), a known inhibitor of the wnt signalling (70) and up-regulates the gene expression of frizzled-7, a receptor for wnt signalling (71) by three-fold and of lymphoid enhancer factor 1 (LEF-1), a known nuclear effector of the wnt signalling (72), by two-fold. Wnt signalling has been shown to be important for repair and regeneration of the kidney after acute kidney injury (73). In PTECS cells, ZNRF3 was down-regulated at the gene expression level with cisplatin/TNF-α treatment suggesting that this protein might also play a role in cisplatin/TNF-α-induced synergistic apoptosis of renal cells.

The cytokine IL-6 has been shown to protect hepatocytes from transforming growth factor β (TGF-β)-induced apoptosis (74) and demonstrated its protective role in several liver injury models including drug-induced hepatoxicity (75). In our siRNA screen, we identified SOCS5 as an inducer of diclofenac/TNF-α-mediated apoptosis (**chapter 6**) and overexpression of SOCS5 has been shown to partially inhibit IL-6 signalling *in vitro* (76). We therefore hypothesized that upon diclofenac/TNF-α treatment SOCS5-mediated inhibition of IL-6 would lead to enhanced apoptosis; however this still needs to be proven. Interestingly, in PTECs, the gene expression of SOCS5 was up-regulated

with cisplatin/TNF-α treatment. In the context of acute kidney injury, IL-6 has been shown to have a dual role: it promotes kidney injury through the promotion of an injurious inflammatory response and it protects the kidney from further injury through a mechanism of trans-signalling (77). In IM-PTECs, exposure to IL-6 led to a protection against cisplatin/TNF-α treatment. However, in order to know which role SOCS5 might play in the synergistic apoptosis induced by cisplatin/TNF-α, knock-down of this protein and further investigation on IL-6 levels would be needed.

### *2.5.2. Transcriptional level*

At the transcriptional level, regulation of the transcription factor p53 seemed to play a role in the TNF-mediated enhancement of diclofenac-induced apoptosis. The transcription factor p53 is a well known inducer of apoptosis in all type of cells including PTECs and hepatocytes (78). p53 is known to transcribe several pro-apoptotic proteins and to inhibit the anti-apoptotic protein Bcl2 (79). In several cancer cells, TNF-α has been shown to activate p53 (80-84) and diclofenac up-regulates p53 gene expression in HepG2 cells (Fredriksson, L. et al., data submitted). These previous studies indicate that the p53 pathway might play a role in TNF-α, diclofenac or diclofenac/TNF-α-induced apoptosis in HepG2 cells. Indeed, the two hits PRPF19 and NF-κB2 both known to regulate p53-induced apoptosis (31, 85) were identified as protectors of TNF-α-induced apoptosis and diclofenac/TNF-α-induced apoptosis respectively (**chapter 6**). In order to confirm that these two proteins are protecting the cells via interfering with the p53 pathway, further investigations need to be done. In the case of cisplatin/TNF-α-induced synergistic apoptosis in PTECs, p53 signalling was shown not to be involved since the phosphorylation of p53 was not changed between cisplatin and cisplatin/TNF-α treatments (**chapter 4**).

### *2.5.3. mRNA processing level*

A large number of apoptotic factors are regulated via alternative splicing, a process that allows for the production of different protein isoforms with often distinct functions from a common mRNA precursor. The two siRNA screens described in **chapters 6** and **7** identified three hits involved in mRNA processing and knock-down of two of them, PRPF19 and PHF5A (86, 87), enhanced the apoptosis induced by TNF-α (**chapters 6-7**). This suggests aberrant mRNA splicing as a mechanism for induction of apoptosis after TNF-α exposure. The effect of PRPF19 on TNF-α-induced apoptosis could be explained

by its effect on the regulation of p21Cip1 mRNA splicing which counteracts p53-mediated apoptosis (85). The role of PHF5A in controlling apoptosis remains unknown. Since we showed that knock-down of this protein led to a delayed p65 translocation similar to the one observed with diclofenac treatment, it seems that PHF5A controls activator(s) of NF-κB upstream via mRNA processing. However, it is not sure whether the delayed p65 translocation induced by PHF5A knock-down is responsible for the enhanced apoptosis observed since other proteins delaying p65 translocation upon knock-down did not affect the apoptosis of the cells. Further experiments should be done to investigate if known inducers of apoptosis with diclofenac are affected by PHF5A knock-down, such as c-FLIP.

#### *2.5.4. ER stress level*

Disturbances in the normal functions of the ER lead to a cell stress response called the unfolded protein response (UPR), which can trigger cell death if ER dysfunction is severe or prolonged. We identified in our siRNA screen the protein UBE2G2 as an inducer of diclofenac/TNF-α-induced apoptosis and this protein is an essential component of the endoplasmic reticulum-associated degradation (ERAD) pathway which targets misfolded, unassembled or tightly regulated proteins of the ER for poly-ubiquitination and ultimately proteasomal degradation (88). We therefore hypothesized that UBE2G2 regulates the degradation of some anti-apoptotic proteins involved in ER stress. One of the anti-apoptotic molecules known to be degraded during ER stress-induced apoptosis is Bcl-2 (89). Our laboratory demonstrated that diclofenac induced ER stress which led to the activation of protein kinase R-like ER kinase (PERK) and the subsequent induction of C/EBP homologous protein (CHOP) expression and knock-down of PERK and CHOP protected the cells against diclofenac/TNF-α-induced apoptosis (Fredriksson L. et al., data submitted). It would be therefore interesting to investigate whether knock-down of UBE2G2 would reduce PERK activation and CHOP expression and whether it would prevent Bcl-2 degradation. In PTECs, the expression of UBE2G2 was up-regulated with TNF-α, cisplatin and cisplatin/TNF-α treatments. ER stress is known to be induced by cisplatin (90, 91), therefore it would not be surprising if UBE2G2 would also play a role in cisplatin/TNF-α-induced synergistic apoptosis.

### 2.6. p65 as the common dominator of all these pathways

Although all the above mentioned pathways individually contribute to TNF-α-

mediated enhancement of drug-induced apoptosis of kidney and liver cells, the NF-κB family member p65 seems to be the common dominator. As described earlier, JNK and p65 reciprocally control each other (41-46). RelB has been shown to sequester p65 in transcriptionally inactive p65/RelB complexes and prevent p65-regulated transcription (92). Wnt1 was shown to induce p65 translocation in microglial cells (93) and NF-κB, mainly p65/p50 heterodimers, was shown to be required for the transcription of IL-6 (94). Numerous studies provided evidence that p53 and NF-κB pathways reciprocally regulate each other by interactions at key nodal intersections and these studies implicated the heterodimer p65/p52 (95). Alternative splicing events have been described at every level of NF-κB signalling, including p65, and serve to regulate proper NF-κB signalling (96). ER stress was shown to activate p65/p50 heterodimers via inositol-requiring enzyme 1 (IRE1) (97). All these observations were done in different cell lines, therefore further experiments would need to be done in HepG2 and PTECs in order to confirm the involvement of p65 in all these pathways/processes.

## 3. Conclusion and perspectives

Current biomarkers and pre-clinical tests lack sensitivity and predictability. It is therefore necessary to tackle the molecular mechanisms underlying nephro- and hepatotoxicity to improve these bio-markers and pre-clinical tests. Furthermore, *in vivo*, a combination of several signalling pathways, inflammatory processes and morphological events is activated altogether leading to both injury and regeneration processes. The balance between these factors defines the severity of the injury caused by a drug. It is therefore necessary to create new pre-clinical *in vitro* tests better mimicking the *in vivo* situation. The new *in vitro* nephrotoxicity screen performed in **chapter 3** allowed an improvement in the detection of nephrotoxicity by incorporating the pro-inflammatory cytokine TNF-α in the assay. However, several nephrotoxicants were still not detected. Since we showed that the NF-κB pathway plays an important role in drug/cytokine synergistic apoptosis, we suggest that p65 translocation in combination with NF-κB activation read-outs should be incorporated with the fluorescence-based cell death read-out already used. This could be done via generating bacterial artificial chromosome (BAC)- GFP-p65 and NF-κB luciferase reporter cells. This would allow us to determine whether the drugs depicting synergistic apoptosis with TNF-α also have an inhibitory effect on the NF-κB signalling. Alternatively, other BAC-GFP reporter cells could be generated with some of the kidney injury markers that were shown to be up-regulated with cisplatin/TNF-α treatment in IM-PTECs in **chapter 4** as well as with

RelB. This could allow the identification of the compounds that did not induce toxicity in IM-PTECs in **chapter 3** via the detection of an increase in the BAC-GFP-marker levels and therefore improve the sensitivity of the screening system. Such BAC-GFP reporter cell lines could also be generated with HepG2 cells with some of the hits identified in **chapters 6** and **7**. Furthermore other cytokines known to be secreted during drug-induced organ toxicity should be incorporated in these *in vitro* assays in a cytokine cocktail manner in which the stoichiometry of these cytokines observed *in vivo* would be reproduced. Similarly, regeneration factors known to be involved *in vivo* could be introduced, such as epidermal growth factor (EGF), hepatocyte growth factor (HGF) and transforming growth factor β (TGF-β). In addition, morphological parameters of the cells should also be taken into consideration since a new drug could not induce cellular apoptosis in the *in vitro* assay but could affect the morphology of the cells and therefore their ability to perform their physiological tasks in an *in vivo* environment leading to organ dysfunction. In conclusion, we suggest that an ideal future *in vitro* toxicity assay for detecting both nephro- and hepatotoxicity would consist of exposing the cells to the drugs in combination with a cytokine/regeneration factors cocktail and perform a multi-parametric analysis with a combination of apoptotic, GFP-based stress reporters and morphological read-outs.

In addition to the improvement of pre-clinical tests, the work in this thesis led to the identification of several new toxicity pathways and potential biomarkers. The role of A20, the Wnt signalling pathway, mRNA processing, the ER stress and all the hits identified in HepG2 cells are candidate regulators of TNF/toxicant synergy in IM-PTECs. Systematic knock-down of these candidate genes in PTECs using lentiviral-based knock-downs of the proteins of interests as well as inhibitors of the pathways of interest should be performed. Vice versa, the role of XAF1 and RhoA should also be investigated in HepG2 cells in a similar way as described for the cisplatin/TNF-α studies in PTECs (**chapters 4-5**). Furthermore, the potential biomarkers identified in HepG2 cells in **chapters 6** and **7** should be further investigated with other hepatotoxicants as well as *in vivo* in order to confirm their sensitivity for the detection of hepatotoxicity. In addition, material from patients suffering from drug-induced hepatotoxicity could be used in order to establish whether these potential biomarkers are up-regulated.

In conclusion, the investigation performed in this thesis allowed the identification of new, detailed mechanisms involved in drug/TNF-α-induced organ toxicity, the development of a new *in vitro* nephrotoxicity screening system and the identification of new potential toxicity biomarkers.

## References


1. Araujo LP, Truzzi RR, Mendes GE, Luz MA, Burdmann EA, Oliani SM. Annexin A1 protein attenuates cyclosporine-induced renal hemodynamics changes and macrophage infiltration in rats. Inflamm Res 2012;61:189-196.
2. Jaeschke H, editor. toxic responses of the liver: The McGraw-Hill Companies, Inc.; 2010.
3. Kaplowitz N. Biochemical and cellular mechanisms of toxic liver injury. Semin Liver Dis 2002;22:137-144.
4. Luster MI, Simeonova PP, Gallucci RM, Bruccoleri A, Blazka ME, Yucesoy B. Role of inflammation in chemical-induced hepatotoxicity. Toxicology letters 2001;120:317-321.
5. Naughton CA. Drug-induced nephrotoxicity. Am Fam Physician 2008;78:743-750.
6. Pabla N, Dong Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. Kidney international 2008;73:994-1007.
7. Quiros Y, Vicente-Vicente L, Morales AI, Lopez-Novoa JM, Lopez-Hernandez FJ. An integrative overview on the mechanisms underlying the renal tubular cytotoxicity of gentamicin. Toxicol Sci 2011;119:245-256.
8. Yalavarthy R, Edelstein CL. Therapeutic and predictive targets of AKI. Clin Nephrol 2008;70:453-463.
9. Akcay A, Nguyen Q, Edelstein CL. Mediators of inflammation in acute kidney injury. Mediators Inflamm 2009;2009:137072.
10. Fredriksson L, Herpers B, Benedetti G, Matadin Q, Puigvert JC, de Bont H, Dragovic S, et al. Diclofenac inhibits tumor necrosis factor-alpha-induced nuclear factor-kappaB activation causing synergistic hepatocyte apoptosis. Hepatology 2011;53:2027-2041.
11. Shaw PJ, Hopfensperger MJ, Ganey PE, Roth RA. Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. Toxicol Sci 2007;100:259-266.
12. Tukov FF, Luyendyk JP, Ganey PE, Roth RA. The role of tumor necrosis factor alpha in lipopolysaccharide/ranitidine-induced inflammatory liver injury. Toxicol Sci 2007;100:267-280.
13. Zou W, Beggs KM, Sparkenbaugh EM, Jones AD, Younis HS, Roth RA, Ganey PE. Sulindac metabolism and synergy with tumor necrosis factor-alpha in a drug-inflammation interaction model of idiosyncratic liver injury. J Pharmacol Exp Ther 2009;331:114-121.
14. Ramesh G, Brian Reeves W. Cisplatin increases TNF-alpha mRNA stability in kidney proximal tubule cells. Renal failure 2006;28:583-592.
15. Ramesh G, Kimball SR, Jefferson LS, Reeves WB. Endotoxin and cisplatin synergistically stimulate TNF-alpha production by renal epithelial cells. American journal of physiology. Renal physiology 2007;292:F812-819.
16. Ramesh G, Reeves WB. TNF-alpha mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. The Journal of clinical investigation 2002;110:835-842.
17. Ramesh G, Reeves WB. TNFR2-mediated apoptosis and necrosis in cisplatin-induced acute renal failure. American journal of physiology. Renal physiology 2003;285:F610-618.
18. Liu M, Chien CC, Burne-Taney M, Molls RR, Racusen LC, Colvin RB, Rabb H. A pathophysiologic role for T lymphocytes in murine acute cisplatin nephrotoxicity. Journal of the American Society of Nephrology : JASN 2006;17:765-774.
19. Asvadi I, Hajipour B, Asvadi A, Asl NA, Roshangar L, Khodadadi A. Protective effect of pentoxyfilline in renal toxicity after methotrexate administration. European review for medical and pharmacological sciences 2011;15:1003-1009.
20. Ghosh J, Das J, Manna P, Sil PC. Acetaminophen induced renal injury via oxidative stress and

TNF-alpha production: therapeutic potential of arjunolic acid. Toxicology 2010;268:8-18.

21. Helal GK, Aleisa AM, Helal OK, Al-Rejaie SS, Al-Yahya AA, Al-Majed AA, Al-Shabanah OA. Metallothionein induction reduces caspase-3 activity and TNFalpha levels with preservation of cognitive function and intact hippocampal neurons in carmustine-treated rats. Oxidative medicine and cellular longevity 2009;2:26-35.

22. Piao RL, Liu YY, Tian D, Ma ZH, Zhang M, Zhao C, Niu JQ. Adefovir dipivoxil modulates cytokine expression in Th1/Th2 cells in patients with chronic hepatitis B. Molecular medicine reports 2012;5:184-189.

23. Pogrebniak HW, Matthews W, Pass HI. Chemotherapy amplifies production of tumor necrosis factor. Surgery 1991;110:231-237.

24. Weimer R, Melk A, Daniel V, Friemann S, Padberg W, Opelz G. Switch from cyclosporine A to tacrolimus in renal transplant recipients: impact on Th1, Th2, and monokine responses. Human immunology 2000;61:884-897.

25. Puigvert JC, de Bont H, van de Water B, Danen EH. High-throughput live cell imaging of apoptosis. Curr Protoc Cell Biol 2010;Chapter 18:Unit 18 10 11-13.

26. Zamzami N, Brenner C, Marzo I, Susin SA, Kroemer G. Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. Oncogene 1998;16:2265-2282.

27. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 1997;275:1129-1132.

28. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. The EMBO journal 1997;16:6914-6925.

29. Chien CT, Chang TC, Tsai CY, Shyue SK, Lai MK. Adenovirus-mediated bcl-2 gene transfer inhibits renal ischemia/reperfusion induced tubular oxidative stress and apoptosis. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2005;5:1194-1203.

30. Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signalling. Immunol Rev 2006;210:171-186.

31. Barre B, Coqueret O, Perkins ND. Regulation of activity and function of the p52 NF-kappaB subunit following DNA damage. Cell cycle 2010;9:4795-4804.

32. Du S, Hiramatsu N, Hayakawa K, Kasai A, Okamura M, Huang T, Yao J, et al. Suppression of NF-kappaB by cyclosporin a and tacrolimus (FK506) via induction of the C/EBP family: implication for unfolded protein response. Journal of immunology 2009;182:7201-7211.

33. Kurokawa M, Ghosh SK, Ramos JC, Mian AM, Toomey NL, Cabral L, Whitby D, et al. Azidothymidine inhibits NF-kappaB and induces Epstein-Barr virus gene expression in Burkitt lymphoma. Blood 2005;106:235-240.

34. Wullaert A, Heyninck K, Beyaert R. Mechanisms of crosstalk between TNF-induced NF-kappaB and JNK activation in hepatocytes. Biochemical pharmacology 2006;72:1090-1101.

35. Jeon SH, Park HM, Kim SJ, Lee MY, Kim GB, Rahman MM, Woo JN, et al. Taurine reduces FK506-induced generation of ROS and activation of JNK and Bax in Madin Darby canine kidney cells. Human & experimental toxicology 2010;29:627-633.

36. Pallet N, Thervet E, Anglicheau D. c-Jun-N-Terminal Kinase Signalling Is Involved in Cyclosporine-Induced Epithelial Phenotypic Changes. Journal of transplantation 2012;2012:348604.

37. Gunawan BK, Liu ZX, Han D, Hanawa N, Gaarde WA, Kaplowitz N. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. Gastroenterology 2006;131:165-178.

38. Francescato HD, Costa RS, Junior FB, Coimbra TM. Effect of JNK inhibition on cisplatin-induced renal damage. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 2007;22:2138-2148.

39. Bae MA, Song BJ. Critical role of c-Jun N-terminal protein kinase activation in troglitazone-

induced apoptosis of human HepG2 hepatoma cells. Molecular pharmacology 2003;63:401-408.

40. Kluwe J, Pradere JP, Gwak GY, Mencin A, De Minicis S, Osterreicher CH, Colmenero J, et al. Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition. Gastroenterology 2010;138:347-359.

41. Bubici C, Papa S, Pham CG, Zazzeroni F, Franzoso G. NF-kappaB and JNK: an intricate affair. Cell cycle 2004;3:1524-1529.

42. De Smaele E, Zazzeroni F, Papa S, Nguyen DU, Jin R, Jones J, Cong R, et al. Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. Nature 2001;414:308-313.

43. Tang G, Minemoto Y, Dibling B, Purcell NH, Li Z, Karin M, Lin A. Inhibition of JNK activation through NF-kappaB target genes. Nature 2001;414:313-317.

44. Polk WW, Ellis ME, Kushleika JV, Simmonds PL, Woods JS. RhoA regulation of NF-kappaB activation is mediated by COX-2-dependent feedback inhibition of IKK in kidney epithelial cells. American journal of physiology. Cell physiology 2007;293:C1160-1170.

45. Lee YH, Schiemann WP. Fibromodulin suppresses nuclear factor-kappaB activity by inducing the delayed degradation of IKBA via a JNK-dependent pathway coupled to fibroblast apoptosis. The Journal of biological chemistry 2011;286:6414-6422.

46. Kesanakurti D, Chetty C, Bhoopathi P, Lakka SS, Gorantla B, Tsung AJ, Rao JS. Suppression of MMP-2 attenuates TNF-alpha induced NF-kappaB activation and leads to JNK mediated cell death in glioma. PLoS One 2011;6:e19341.

47. Zhang F, Wu LM, Zhou L, Chen QX, Xie HY, Feng XW, Zheng SS. Predictive value of expression and promoter hypermethylation of XAF1 in hepatitis B virus-associated hepatocellular carcinoma treated with transplantation. Annals of surgical oncology 2008;15:3494-3502.

48. Gui J, Yang B, Wu J, Zhou X. Enormous influence of TNIK knockdown on intracellular signals and cell survival. Human cell 2011;24:121-126.

49. Renner F, Schmitz ML. Autoregulatory feedback loops terminating the NF-kappaB response. Trends in biochemical sciences 2009;34:128-135.

50. Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP, Ma A. Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science 2000;289:2350-2354.

51. Cordes N. Integrin-mediated cell-matrix interactions for prosurvival and antiapoptotic signalling after genotoxic injury. Cancer letters 2006;242:11-19.

52. Gailit J, Colflesh D, Rabiner I, Simone J, Goligorsky MS. Redistribution and dysfunction of integrins in cultured renal epithelial cells exposed to oxidative stress. The American journal of physiology 1993;264:F149-157.

53. Kruidering M, van de Water B, Zhan Y, Baelde JJ, Heer E, Mulder GJ, Stevens JL, et al. Cisplatin effects on F-actin and matrix proteins precede renal tubular cell detachment and apoptosis *in vitro*. Cell death and differentiation 1998;5:601-614.

54. Qin Y, Alderliesten MC, Stokman G, Pennekamp P, Bonventre JV, de Heer E, Ichimura T, et al. Focal adhesion kinase signalling mediates acute renal injury induced by ischemia/reperfusion. The American journal of pathology 2011;179:2766-2778.

55. Van de Water B, Jaspers JJ, Maasdam DH, Mulder GJ, Nagelkerke JF. *In vivo* and *in vitro* detachment of proximal tubular cells and F-actin damage: consequences for renal function. The American journal of physiology 1994;267:F888-899.

56. Zuk A, Bonventre JV, Brown D, Matlin KS. Polarity, integrin, and extracellular matrix dynamics in the postischemic rat kidney. The American journal of physiology 1998;275:C711-731.

57. Bourgarel-Rey V, Vallee S, Rimet O, Champion S, Braguer D, Desobry A, Briand C, et al. Involvement of nuclear factor kappaB in c-Myc induction by tubulin polymerization inhibitors. Molecular pharmacology 2001;59:1165-1170.

58. Nemeth ZH, Deitch EA, Davidson MT, Szabo C, Vizi ES, Hasko G. Disruption of the actin

cytoskeleton results in nuclear factor-kappaB activation and inflammatory mediator production in cultured human intestinal epithelial cells. Journal of cellular physiology 2004;200:71-81.

59. Rosette C, Karin M. Cytoskeletal control of gene expression: depolymerization of microtubules activates NF-kappa B. The Journal of cell biology 1995;128:1111-1119.

60. Clark GJ, Gunningham S, Troy A, Vuckovic S, Hart DN. Expression of the RelB transcription factor correlates with the activation of human dendritic cells. Immunology 1999;98:189-196.

61. Wang X, Belguise K, Kersual N, Kirsch KH, Mineva ND, Galtier F, Chalbos D, et al. Oestrogen signalling inhibits invasive phenotype by repressing RelB and its target BCL2. Nature cell biology 2007;9:470-478.

62. Maeda A, Yano T, Itoh Y, Kakumori M, Kubota T, Egashira N, Oishi R. Down-regulation of RhoA is involved in the cytotoxic action of lipophilic statins in HepG2 cells. Atherosclerosis 2010;208:112-118.

63. Murata T, Arii S, Mori A, Imamura M. Therapeutic significance of Y-27632, a Rho-kinase inhibitor, on the established liver fibrosis. The Journal of surgical research 2003;114:64-71.

64. Murata T, Arii S, Nakamura T, Mori A, Kaido T, Furuyama H, Furumoto K, et al. Inhibitory effect of Y-27632, a ROCK inhibitor, on progression of rat liver fibrosis in association with inactivation of hepatic stellate cells. Journal of hepatology 2001;35:474-481.

65. Tada S, Iwamoto H, Nakamuta M, Sugimoto R, Enjoji M, Nakashima Y, Nawata H. A selective ROCK inhibitor, Y27632, prevents dimethylnitrosamine-induced hepatic fibrosis in rats. Journal of hepatology 2001;34:529-536.

66. Ikeda H, Kume Y, Tejima K, Tomiya T, Nishikawa T, Watanabe N, Ohtomo N, et al. Rho-kinase inhibitor prevents hepatocyte damage in acute liver injury induced by carbon tetrachloride in rats. American journal of physiology. Gastrointestinal and liver physiology 2007;293:G911-917.

67. Pecina-Slaus N. Wnt signal transduction pathway and apoptosis: a review. Cancer cell international 2010;10:22.

68. Wang QM, Jia LQ, Zhou HY, Li YS. [Study on the role of Wnt/beta-catenin signalling transduction pathway in hepatocellular carcinoma cell line HepG2 and L02 cell line]. Xi bao yu fen zi mian yi xue za zhi = Chinese journal of cellular and molecular immunology 2007;23:926-928.

69. Hao HX, Xie Y, Zhang Y, Charlat O, Oster E, Avello M, Lei H, et al. ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. Nature 2012;485:195-200.

70. Fedi P, Bafico A, Nieto Soria A, Burgess WH, Miki T, Bottaro DP, Kraus MH, et al. Isolation and biochemical characterization of the human Dkk-1 homologue, a novel inhibitor of mammalian Wnt signalling. The Journal of biological chemistry 1999;274:19465-19472.

71. Tanaka S, Akiyoshi T, Mori M, Wands JR, Sugimachi K. A novel frizzled gene identified in human esophageal carcinoma mediates APC/beta-catenin signals. Proceedings of the National Academy of Sciences of the United States of America 1998;95:10164-10169.

72. Eastman Q, Grosschedl R. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Current opinion in cell biology 1999;11:233-240.

73. Kawakami T, Ren S, Duffield JS. Wnt signalling in kidney diseases: dual roles in renal injury and repair. The Journal of pathology 2012.

74. Chen RH, Chang MC, Su YH, Tsai YT, Kuo ML. Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways. The Journal of biological chemistry 1999;274:23013-23019.

75. Streetz KL, Luedde T, Manns MP, Trautwein C. Interleukin 6 and liver regeneration. Gut 2000;47:309-312.

76. Nicholson SE, Willson TA, Farley A, Starr R, Zhang JG, Baca M, Alexander WS, et al. Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction. The EMBO journal 1999;18:375-385.

77. Nechemia-Arbely Y, Barkan D, Pizov G, Shriki A, Rose-John S, Galun E, Axelrod JH. IL-6/IL-6R axis plays a critical role in acute kidney injury. Journal of the American Society of Nephrology : JASN 2008;19:1106-1115.

78. Amaral JD, Xavier JM, Steer CJ, Rodrigues CM. The role of p53 in apoptosis. Discovery medicine 2010;9:145-152.

79. Hemann MT, Lowe SW. The p53-Bcl-2 connection. Cell death and differentiation 2006;13:1256-1259.

80. Donato NJ, Perez M. Tumor necrosis factor-induced apoptosis stimulates p53 accumulation and p21WAF1 proteolysis in ME-180 cells. The Journal of biological chemistry 1998;273:5067-5072.

81. Gotlieb WH, Watson JM, Rezai A, Johnson M, Martinez-Maza O, Berek JS. Cytokine-induced modulation of tumor suppressor gene expression in ovarian cancer cells: up-regulation of p53 gene expression and induction of apoptosis by tumor necrosis factor-alpha. American journal of obstetrics and gynecology 1994;170:1121-1128; discussion 1128-1130.

82. Jeoung DI, Tang B, Sonenberg M. Effects of tumor necrosis factor-alpha on antimitogenicity and cell cycle-related proteins in MCF-7 cells. The Journal of biological chemistry 1995;270:18367-18373.

83. Yin D, Kondo S, Barnett GH, Morimura T, Takeuchi J. Tumor necrosis factor-alpha induces p53-dependent apoptosis in rat glioma cells. Neurosurgery 1995;37:758-762; discussion 762-753.

84. Rokhlin OW, Gudkov AV, Kwek S, Glover RA, Gewies AS, Cohen MB. p53 is involved in tumor necrosis factor-alpha-induced apoptosis in the human prostatic carcinoma cell line LNCaP. Oncogene 2000;19:1959-1968.

85. Chen Y, Zhang L, Jones KA. SKIP counteracts p53-mediated apoptosis via selective regulation of p21Cip1 mRNA splicing. Genes & development 2011;25:701-716.

86. Rzymski T, Grzmil P, Meinhardt A, Wolf S, Burfeind P. PHF5A represents a bridge protein between splicing proteins and ATP-dependent helicases and is differentially expressed during mouse spermatogenesis. Cytogenetic and genome research 2008;121:232-244.

87. David CJ, Boyne AR, Millhouse SR, Manley JL. The RNA polymerase II C-terminal domain promotes splicing activation through recruitment of a U2AF65-Prp19 complex. Genes & development 2011;25:972-983.

88. Chen B, Mariano J, Tsai YC, Chan AH, Cohen M, Weissman AM. The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. Proceedings of the National Academy of Sciences of the United States of America 2006;103:341-346.

89. Egger L, Madden DT, Rheme C, Rao RV, Bredesen DE. Endoplasmic reticulum stress-induced cell death mediated by the proteasome. Cell death and differentiation 2007;14:1172-1180.

90. Mandic A, Hansson J, Linder S, Shoshan MC. Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signalling. The Journal of biological chemistry 2003;278:9100-9106.

91. Peyrou M, Hanna PE, Cribb AE. Cisplatin, gentamicin, and p-aminophenol induce markers of endoplasmic reticulum stress in the rat kidneys. Toxicological sciences : an official journal of the Society of Toxicology 2007;99:346-353.

92. Marienfeld R, May MJ, Berberich I, Serfling E, Ghosh S, Neumann M. RelB forms transcriptionally inactive complexes with RelA/p65. The Journal of biological chemistry 2003;278:19852-19860.

93. Shang YC, Chong ZZ, Hou J, Maiese K. Wnt1, FoxO3a, and NF-kappaB oversee microglial integrity and activation during oxidant stress. Cellular signalling 2010;22:1317-1329.

94. Xiao W, Hodge DR, Wang L, Yang X, Zhang X, Farrar WL. NF-kappaB activates IL-6 expression through cooperation with c-Jun and IL6-AP1 site, but is independent of its IL6-NFkappaB regulatory site in autocrine human multiple myeloma cells. Cancer biology & therapy 2004;3:1007-1017.

95. Ak P, Levine AJ. p53 and NF-kappaB: different strategies for responding to stress lead to a functional antagonism. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2010;24:3643-3652.

96. Leeman JR, Gilmore TD. Alternative splicing in the NF-kappaB signalling pathway. Gene 2008;423:97-107.

97. Kaneko M, Niinuma Y, Nomura Y. Activation signal of nuclear factor-kappa B in response to endoplasmic reticulum stress is transduced via IRE1 and tumor necrosis factor receptor-associated factor 2. Biological & pharmaceutical bulletin 2003;26:931-935.

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