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Intracellular signaling in drug-induced liver injury

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1. ADVERSE DRUG REACTIONS

Adverse drug reactions (ADRs) constitute an important issue both in clinic and for the drug-industry. 5% of the hospital admissions in Europe are due to adverse drug reactions and in 2008 this was estimated to cost the society a total of €79 billion (source: Annex 2 of the report on the impact assessment of strengthening and rationalizing EU Pharmacovigilance, 2008).

Some decades ago, the major reasons for drug attrition were based on poor pharmacokinetic and bioavailability. While these drug development problems were tackled, 10 years ago this had shifted towards increased issues with clinical safety and toxicology, which was the underlying reason for ~30% of the compound withdrawals (1).

The development of a new drug is estimated to cost about 1 billion dollars (2), the cost for the society is significant if adverse drug reactions lead to hospitalization and, even more importantly, the ADRs cause a lot of suffering for the individuals affected. Therefore it is of outmost importance to improve our understanding of the underlying mechanisms of adverse drug reactions and integrate this knowledge in pre-clinical safety testing strategies of new medicines.

The most common type of adverse drug reactions, and the most common cause of drug withdrawal is drug-induced liver injury (DILI) (3,4). Moreover, DILI is the leading cause of acute liver failure in the Unites States with paracetamol (acetaminophen) accounting for about 50% of those cases and other adverse drug reactions (mostly idiosyncratic; see below) accounting for another 10% (5). This thesis will focus on mechanisms of DILI.

2. DRUG-INDUCED LIVER INJURY (DILI)

The reason why the liver is a critical target organ for adverse drug reactions is not surprising. The liver has the highest exposure to oxygen and nutrient rich blood, it is equipped with highest drug metabolizing capacity in the body to enhance excretion of xenobiotics. The first-pass effect ensures that the liver is the first organ exposed to anything that is ingested.

In addition to the drug metabolism capacity, the liver is an essential organ for clearance of circulating pathogens and products thereof. Thus, the liver contains the largest amount of resident macrophages (in the liver called Kupffer cells) and natural killer cells, which upon activation can produce large amounts of cytokines and chemokines (6) to activate the systemic immune systems. As a consequence the liver cells may also be exposed to not only toxic drug metabolites but also inflammatory cytokines, which potentially could act synergistically to increase the risk for the DILI.

DILI can roughly be classified into two types, intrinsic and idiosyncratic. Intrinsic DILI is generally dose-dependent, predictable and related to the pharmacological action of the drug, with an example being hepatotoxicity after paracetamol overdose. But only

a minority of the clinically associated DILIs is dose-dependent and predictable. In a pharmacological and toxicological context ''idiosyncratic'' DILI (iDILI) means that the adverse reaction to a drug is particular to a certain individual, involving risk factors such as gender, age, concomitant disease and genetic background (4). In concrete terms this means that the adverse drug reaction in question is unexplainable, rare (about 1 in 10,000-100,000 people who take the drug), occurring with variable times of onset and that it is typically non-related to the dose, although this can be discussed since drugs with a dosing of more than 50 mg/day is related to a higher incidence of iDILI (7-9). For the drug-industry iDILI is extremely difficult to foresee and prevent. The rarity and the unpredictability often lead to the adverse reactions only being recognized after the release of the drug to the market when many patients have been exposed. This commonly results in the withdrawal of the product or a restricted use. This causes both financial harm to the industry and unmet needs for patients that would otherwise benefit from this medication (7). Examples of drugs that induce iDILI are carbamazepine, diclofenac, ketoconazole and nefazodone (3,10). The mechanisms behind idiosyncratic DILI are incompletely understood but there are however, several hypotheses behind their incidence.

3. MECHANISTIC WORKING MODELS FOR IDILI

The formation of reactive intermediates during drug metabolism on the one hand and the activation of the immune system (innate and/or adaptive) on the other, are thought to represent the two most critical events in the pathogenesis of DILI (4,9,11). The hapten, danger and pharmacologic interaction (p-i) hypothesis has long been described as the primary working models for iDILI. These working models for iDILI will be discussed below also taking additional cellular stress responses and inflammatory stress conditions into consideration.

3.1 Adaptive immunity – allergic hepatotoxicity

3.1.1 Hapten hypothesis

Small molecules with a mass of less than 1,000 daltons are unable to induce an immune response unless they are bound to macromolecules, including proteins. Landsteiner already proposed this in 1935 (12) and it forms the basis of the hapten hypothesis to describe the occurrence of iDILI. This hypothesis also has its foundation in the fact that some DILIs are presented with an allergic response including fever, rash and the presence of circulating autoantibodies (3).

In the context of DILI, reactive drug metabolites formed in the liver can bind to cellular proteins that can subsequently act as haptens causing initiation of an adaptive immune response. In a first exposure, the immune system recognizes such a hapten and becomes sensitized towards it. Upon re-challenge, the hapten-exposing tissue is attacked by the immune system involving a strong sterile inflammation reaction and exposure

of target cells to for example reactive oxygen species (ROS), cytotoxic cytokines and proteases, thereby causing severe tissue damage (11). A classical example is the allergic reaction to penicillin. The penicillin molecule contains a reactive group that can bind to proteins and in some patients this result in an immune response against the penicillin-protein complex (11,13).

Idiosyncratic DILIs, such as the one caused by diclofenac and carbamazepine, have also been associated with T-cell activation and production of antibodies against drug-adducted proteins, which would support the role of haptenization and activation of the adaptive immune system in iDILI (11,14-16). As individuals may vary in the way they develop an adaptive immune response to a specific antigen this could explain the rarity of iDILI, and make the adaptive immune system an attractive target to understand iDILI.

3.1.2 P-i concept

The p-i concept (direct pharmacological interaction of drugs with immune receptors) is an alternative to the hapten hypothesis. According to this theory, iDILI is the result of a drug's ability to stimulate the T-cell receptor without hapten-protein formation and subsequent antigen presentation by antigen presenting cells (APCs) (17). The p-i concept was first developed after the observation that T-cells from sulfamethoxazole-sensitive patients, recognized the drug itself and not a protein adducted by the drug (18). Nowadays more drugs have been associated with this hypothesis, including carbamazepine (19). In some cases, the immunological evidence can not be explained by drug metabolism and the hapten model (20). The p-i concept then favors a iDILI working model whereby drugs do not require drug metabolism followed by covalent modification of target proteins to cause an immune response.

3.1.3 Danger hypothesis

The danger hypothesis is a modified version of the hapten hypothesis and it was first described by Matzinger in 1994 (21). It proposes that the major determinant whether an immune response is elicited against a drug or not is if this drug induces some type of cell damage by itself already (22). The fundamental step leading to an adaptive immune response is recognition by T-cells of processed antigens presented on the major histocompatibility complex (MHC; in humans human leukocyte antigen [HLA]) of APCs, for example macrophages. In addition to this first signal, a second one is needed for a fulminant immune response. If this is not presented, the result is tolerance to the presented antigen.

Activation of APCs leads to up-regulation of co-stimulatory molecules that would induce this second, activating signal. The activation of APCs can occur via several mechanisms but the most attractive one, in this context, is indeed that injured cells (by drugs and drug metabolites) provide the APCs a "danger signal". This danger signal could include the secretion of heatshock proteins, the nuclear protein high mobility group box 1 (HMGB1) and the calcium binding S100 proteins (23-25). However, other examples of danger signals, non-related to cellular damage, could be concomitant bacterial or viral

infections, or secreted cytokines (26). This type of allergic hepatotoxicity is for example more common in HIV patients (27) and especially in patients with concomitant hepatitis B or C infection (28), which would further support the need for a danger signal in the induction of DILI as well as generally supporting the hypothesis that an activated immune system might affect the drug toxicity (9).

Due to frequent exposure to circulating and absorbed antigens in the liver, and the large amount of immune cells present in this organ, adaptation is crucial to avoid a hyper-activated immune system. Importantly, the response in the liver is usually adaptation (4,6). Yet, failure of an adaptation response could be another susceptibility factor for the development of iDILI. It is likely that cytokines play an important role in the balance of either promoting a tolerogenic or a pathogenic response to drug-protein adducts (26).

3.2 Mitochondrial dysfunction hypothesis

The mitochondria can be the direct intracellular target of drug toxicity as well as the reason for patient susceptibility to DILI (29). Gradual accumulating mitochondrial damage could explain the temporal onset of iDILI; it might happen faster in certain individuals than in others due to genetic or acquired mitochondrial abnormalities (30). Moreover, mutations in mitochondrial DNA seem associated with DILI caused by certain drugs (9,29).

As an example, superoxide dismutase (SOD2) is a protein that is located in the mitochondria where it plays a critical role in detoxifying superoxide anion radicals that are constantly being formed during electron transport in the respiratory chain, an essential step for cellular energy production. Heterozygous $SOD2^{+/-}$ mice exhibit a similar phenotype to susceptible humans in the sense that the “mutation” goes unnoticed until there is a drug exposure leading to mitochondrial damage by enhanced mitochondrial oxidative stress. Troglitazone-induced hepatotoxicity, which goes typically unnoticed in “normal” animal models, has been shown to involve mitochondrial damage in this mouse-model and drug exposure led to a late onset of hepatic necrosis, mimicking a human iDILI process (31).

For mitochondria to get fatally damaged, certain thresholds need to be reached at multiple levels. For example inhibition of the different complexes in the electron transport chain needs to reach more than 50% to affect the ATP production, and about 60% of the mitochondrial DNA must be deleted before this has an overt effect (30). This is called the mitochondrial threshold effect and might also explain the lag time of iDILI since it will likely take a long time for the critical thresholds to be reached after drug exposure. The results obtained with the $SOD^{+/-}$ mice described above represents an interesting model for studying the role of the threshold effect in iDILI.

3.3 Inflammatory stress hypothesis

Inflammatory infiltrates are common in patients suffering from iDILI. This raised the possibility that some idiosyncratic reactions might be explained by episodes of modest

inflammation (32,33). Conditions associated with inflammation include arthritis, atherosclerosis, infection by bacteria and virus, immune responses to certain antigens etc. However, activation of the innate immune response could also be elicited by apoptotic and necrotic hepatocytes and their release of so-called damage-associated molecular pattern molecules such as heat shock proteins (4), as described above in the section about the danger hypothesis, and thereby lead to inflammatory stress.

The underlying mechanism of sensitization to DILI by viral infections remains unknown. Possibly the mechanism includes activation of the innate immune system, whereby secreted cytokines and chemokines are important components of an anti-viral response (34). These cytokines might then modulate the extent of inflammation and, thereby, control the levels of injury (26).

The balance between the secretion of protective cytokines like IL-6 and IL-10 and injurious ones like TNFα, FasL, IL-1β and IFN-γ have also been implicated in the development of iDILI and genetic circumstances that could make the balance tip one way or the other could be detrimental (3,4).

Another source of innate immune system activation are bacterial endotoxins released from the intestines into the circulation. The magnitude of such endotoxin release may vary depending on different factors such as diet and alcohol consumption (33). This could also explain the random temporal onset of iDILI as well as the rare incidence. Animal models have been developed to simulate this scenario using the Gram-negative bacterial endotoxin lipopolysaccharide (LPS). Injection of LPS to animals in this model has rendered an otherwise non-toxic dose of a drug known to cause iDILI in humans, toxic (7). This LPS co-exposure model is also the first animal model to mimic the pronounced liver injury seen in patients with iDILI (7). Examples of drugs that have been used and proven toxic in such a model include diclofenac, amiodarone, sulindac and trovafloxacin (35-38). Importantly, the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) has been determined the most important mediator of the toxicities observed. Inhibition of TNFα transcription significantly reduced trovafloxacin/LPS induced liver injury indicating that the hepatotoxicity is largely TNFα dependent (38) and the same was shown for sulindac using a soluble TNFα-receptor to inactivate the cytokine, etanercept (37), emphasizing the important role for cytokines and cytokine-induced signaling in iDILI.

3.4 Multiple-determinant hypothesis

The above hypotheses are not mutually exclusive. It is more likely that they are all individual parts of a complex puzzle that provides a complete mechanistic understanding of human iDILI. A combination of many factors would support the sporadic occurrence of iDILI, and provide a condition whereby the risk of developing iDILI would be the product of all the different mechanistic risk factors taken together (3,34). As a summary of this we foresee an overall working model whereby the main susceptibility determinants for the development of iDILI include the biochemical properties of the drug, environmental factors, genetic factors, and immune system components.

Potential genetic factors include for example polymorphisms in drug-metabolizing enzymes and mutations that determine sensitivity to mitochondrial damage and to inflammatory stresses. Environmental factors could include exposures to inducers of drug metabolism and viral/bacterial products (7).

Because of their rarity and unpredictability, iDILIs are thought to depend largely on genetic variation (9). The most apparent example is provided by the highly variable human leukocyte antigen (HLA) system. This plays a key role in delayed immune-mediated adverse drug reactions, including DILI, since it is involved in the T-cell recognition of the drug-induced antigen (9,39,40). For some reactions, for example abacavir hypersensitivity, one single gene locus (HLA-B*5701) can provide adequate predictive accuracy to allow the gene to be used in a clinical setting as a biomarker for the risk of idiosyncratic adverse drug reactions (41,42). A correlation with HLA polymorphism has also been found for more drugs, including the antibiotics amoxicillin-clavulanate and flucloxacillin (40). For most other drugs there are likely correlations between polymorphisms in single gene and specific adverse drug reactions. Yet, it seems more plausible that such individual gene polymorphism are insufficient to significantly increase the susceptibility for ADR, but rather would act in combination with other genes (43).

4. INTRACELLULAR STRESS SIGNALING IN IDILI

Since both drug metabolism-dependent cell injury responses as well as immune-mediated responses are critical components of iDILI, a cross-talk between drug-induced and cytokine-induced intracellular signaling events that enhances the drug-toxicity in certain individuals is likely to occur. In the following paragraphs these two responses and their cellular consequences will be described.

4.1 Drug-induced intracellular stress signaling

4.1.1 The role of drug-metabolism

Drugs are generally designed as hydrophobic molecules to allow proper absorption into the body after oral intake. Biotransformation reactions are essential to metabolize these molecules into more hydrophilic compounds that can easily be eliminated via the urine or the bile. Most of this xenobiotic metabolism occurs in the liver. The primary biotransformation of drugs involves the cytochrome P450 (CYP450) oxidase system, consisting of over 100 different family members (44). Typically, drug metabolism leads to their inactivation and ultimate elimination. But alternatively it leads to the formation of reactive moieties that can then covalently bind to cellular macromolecules, and as a consequence disturb the intracellular homeostasis or induce oxidative stress and thereby cause cellular damage (3,45).

Drugs that are being metabolized have a significantly higher risk of causing iDILI (46) and the formation of harmful reactive metabolites is an anticipated main mechanism behind DILI (4,11,45,47). In addition, polymorphisms in drug metabolizing and detoxifying

enzymes are related to increased incidence of this type of adverse drug reactions (9,48). Further support for biotransformation as a mechanism is based on the association of iDILI with dosages higher than 50 mg/day, which would result in higher absolute levels of metabolites formed than with a lower daily dosing. However, importantly, inability to metabolize can also lead to toxicity as this most likely leads to decreased elimination, and thereby the possibility for enhanced, toxic, levels of the parent drug (47).

Not only oxidative metabolism by CYP450s has been shown to form reactive intermediates, but also conjugation reactions (phase II metabolism). Thus, acyl-glucuronide formation has been linked to DILI (45,49). Diclofenac is a good example here since the acyl-glucuronides and not the CYP produced hydroxyl-metabolites are linked to diclofenac-induced iDILI (15,49). In addition, a genetic variant of the enzyme that produces the diclofenac acyl-glucuronide, uridine diphosphate glucuronosyl-transferase 2B7 (UGT2B7), has been shown to significantly contribute to the risk of developing diclofenac-induced DILI (50).

Many drugs are being metabolized into reactive species, however, clearly not all of them cause hepatotoxicity. The reason for this is the tight association between bioactivation and detoxifying enzyme systems. Examples of inactivation systems are glutathione *S*-transferases (GSTs) and their conjugation of glutathione (GSH) to reactive molecules as well as other reactive oxygen species (ROS) involved in downstream hepatotoxic mechanisms. Interestingly, double mutations in two relevant GSTs (GSTT1 and GSTM1) have been linked to troglitazone hepatotoxicity in Japanese patients (51). In the case of reactive metabolite formation cytotoxicity will only occur when the (adaptive) detoxifying defense systems are saturated or fail (45).

Formation of protein adducts is implicated in the toxicity of many drugs including paracetamol. Moreover, protein adduct formation seems correlated with higher incidence of iDILI (52). If the adducts are formed on proteins critical for certain cellular functions and signaling pathways, it increases the likelihood of inhibiting/activating critical cellular functions eventually resulting in cytotoxicity (45,53). Additionally, if adduct formation occurs on proteins that are subsequently presented to the immune system, an adaptive immune response can be elicited (see above). However, protein adduction does not *per se* mean enhanced toxicity. A good example of this is the difference observed between paracetamol and its regioisomer 3'-hydroxyacetanilide (AMAP). Parecetamol is liver toxic while AMAP is not, but interestingly, both drugs cause similar levels of total cellular protein adduction. However, paracetamol causes mitochondrial protein adduct formation, while AMAP does not, which possibly explains their difference in toxicity (54). This underlines the critical requirement of gaining more mechanistic information about the exact cellular targets modified by covalent binding (45) and the resulting cellular injury such as mitochondrial damage or oxidative stress (4). Although, it might be tempting to completely abandon the chemical entities in (candidate) drugs that show covalent binding, this will not always offer a solution since some drugs, like penicillins and omeprazole, are dependent on covalent binding for their efficacy (45).

New techniques involving mass spectrometry and glutathione trapping are

fruitful in identifying the structures of reactive intermediates that are formed in the course of drug metabolism and their intrinsic potential to cause hepatotoxicity (55). In addition, identifying critical residues within proteins that become adducted and that leads to protein inactivation, as well as which proteins become adducted, adds another importance to these types of studies (45,56,57).

4.1.2 Mitochondrial damage

The role of mitochondria in hepatocellular death can be either direct by drug accumulation, inhibition of electron transport or depletion of antioxidant defense mechanisms, or indirect by the activation of different signaling pathway that affect the mitochondrial integrity and, thereby, the cell survival outcome (30).

Hepatocytes have many mitochondria that besides taking care of the energy supply of the cell ensuring cell survival, play a role in the control of cell death. Mitochondria are unique organelles involved in the control of both apoptosis and necrosis. For example high pH, pro-oxidants and activation of the pro-apoptotic Bcl-2 family member Bax can lead a so-called mitochondrial membrane permeability transition (mPT) (30).

The induction of mPT leads to loss of the transmembrane potential, which is essential for ATP production. Moreover, mPT makes the mitochondria release a large pool of mitochondrial free calcium resulting in cellular calcium-overload and related cytotoxicity (58). The direct toxic targeting of all mitochondria followed by the induction of mPT is in general believed to lead to necrosis as a result of loss of cellular energy. On the contrary, more subtle mitochondrial perturbation, as described in the following paragraph, would most likely favor apoptosis (3).

Selective permeabilization of the outer mitochondrial membrane, MOMP, is induced by translocation and binding of the two pro-apoptotic Bcl-2 family members Bax and Bak to the membrane pore, leading to the release of pro-apoptotic factors that are normally located in the inter-membrane space, such as cytochrome c, apoptosis-inducing factor (AIF) and Smac/Diablo, (29,59-61). The release of cytochrome c from the mitochondrial inner membrane space leads to activation of pro-caspase-9 via formation of the so called apoptosome, which includes cytochrome c and apoptosis protease-activating factor 1 (APAF1) (62). Caspase-9 subsequently activates the effector capase-3 which cleaves several cytosolic and nuclear proteins to trigger apoptosis (63). Importantly, for the cell to undergo apoptosis the mitochondrial membrane potential needs to be retained thus ensuring enough energy for the active apoptotic process to proceed. The two factors that determine if the cell will undergo apoptosis or necrosis, is the amount of mitochondria affected and the extent of ATP depletion.

The integrity of the mitochondrial membrane is thus in large parts determined by the Bcl-2 family members. This family of proteins is composed of both pro- and anti-apoptotic members, with the former group including the already introduced Bax and Bak, and the latter for example Bcl-2 itself and Bcl-xL. The anti-apoptotic Bcl-2 family members act by binding to the pro-apoptotic ones and thereby inhibiting the release of pro-apoptotic factors (61). The Bcl-2 family members are consequently very important for

the control of life or death.

Oxidant stress is a crucial regulator of mitochondria-mediated cell death. Under normal conditions the large amount of antioxidant systems in the mitochondria will prevent any damage, but if this protective system is compromised due to genetic defects or for example drug exposure, the cellular organelles and substructures will get damaged (29). Oxidant stress can also lead to damage of the mitochondrial DNA. Since this DNA encodes specific subunits of the electron transport chain, long term problems here may involve further increased production of ROS and additional cellular damage, thus causing a vicious cycle (29). In addition, mutations in mitochondrial DNA are not uncommon in the population, although this normally does not result in damage since there are so many mitochondria present in one cell. However, under particular stress conditions, as after drug exposure, this might change if the drug of interest also targets the mitochondria (29).

Many hepatotoxic drugs, or the metabolites thereof, can interfere with the mitochondrial function (58) and this is also common with drugs associated with iDILI. Interestingly, in a study by Xu and colleagues (64), 50-60% of the 300 drugs associated with iDILI showed mitochondrial changes while 0-5% of the negative controls did not. Moreover, drugs like carbamazepine, known to cause iDILI, interferes with the mitochondrial respiration, mitochondrial membrane potential and ATP synthesis (10). Diclofenac on the other hand, is known to cause mitochondrial damage by for example the uncoupling of the oxidative phosphorylation and inhibition of complex I/III which leads to an increased mitochondrial-derived oxidative stress due to enhanced superoxide formation (29,65,66)

Mitochondrial inhibition is most likely not by itself the cause of cellular injury, however it might increase the susceptibility to other damaging factors. This includes for example depletion of the reduced glutathione (GSH) storage (34) or by stress kinases increased susceptibility of the mitochondria to induction of cell death (30). It is likely that underlying defects in mitochondrial function, possibly genetic, amplify the risk of iDILI development in certain susceptible individuals.

4.1.3 Oxidative stress

Formation of reactive oxygen species (ROS) is one of the most commonly cited cell death mechanisms in organ toxicity, including DILI (59). The most apparent sources of intracellular ROS are the electron transport chain in the mitochondria, the drug metabolizing CYP450 system, and intracellular oxidases (59). In addition, reactive metabolites originating from drug metabolism can by themselves induce oxidative stress (45). This either directly through redox cycling, or indirectly through glutathione depletion, increases the amount of reactive oxygen species as observed with APAP and diclofenac exposure (45,49,67).

Extensive ROS formation bares a problem to the cells since it increases the damage to macromolecules through protein oxidation, lipid peroxidation and DNA damage (4). Superoxides are the most reactive ROS and their main source is the mitochondrial

electron transport chain. Alternatively, drug metabolism can result in superoxide formation through the development of unstable radicals by P450 reductases, which then reacts with molecular oxygen (30).

Because of the damage that can be caused by oxidative stress, the cell has a well-developed system to deal with ROS. Superoxide dismutases (SOD1 and SOD2) are responsible for detoxifying superoxides, and glutathione peroxidases (GPXs) take care of reducing hydrogen peroxides, where the isoform GPX4 is specialized in reducing fatty acid hydroperoxides (59). In addition, small antioxidant molecules such as glutathione (GSH) constitute a very important anti-oxidant defense system by scavenging different types of ROS (59). Emphasizing the importance of this small molecule, the toxicity of drugs such as paracetamol and nevirapine can be diminished by supplementing with the glutathione precursor N-acetyl-cysteine (68). The mitochondria have their own separate GSH pool and especially depletion of this one upon drug exposure has been linked to enhanced toxicity (69). The reason for this is enhanced reactive metabolite-mediated impairment of the electron transport chain, or by other means enhanced ROS generation by mitochondria since GSH is especially important in hydrogen peroxide detoxification as a conjugation molecule for GPX (70).

Another consequence of reduced GSH levels is alterations of the protein redox status, possibly leading to altered protein function (71). However, such post-translational redox modifications of proteins can also be an important mechanism for activating or inactivating signaling pathways in hepatocytes following drug exposure. As an example, ROS can lead to inhibition of JNK phosphatases crucial for the inactivation of this pro-apoptotic stress-kinase (72). Yet, ROS also activates the anti-oxidant adaptive response through the activation of the transcription factor nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2).

Factors that influence drug metabolism and detoxification are likely all critical components in iDILI. A major pathway regulating both of these processes is Nrf2-signaling. Nrf2 initiates transcription of both drug metabolizing enzymes, detoxifying enzymes and molecules, such as sulfiredoxin (SRXN1), GSTs and GSH (73,74), and is as such the most important transcription factor in the anti-oxidant response system. Under non-stressed conditions, Nrf2 is kept in the cytosol by its endogenous inhibitor Kelch-like ECH-associated protein 1 (Keap1) and thereby guided to proteasomal degradation as Keap1 acts as a substrate adaptor for the ubiquitination complex responsible for the polyubiquitination of Nrf2 (75). Many reactive drug metabolites and intermediates as well as ROS activate the Nrf2 response by reacting with the many critical cysteine residues on Keap1 (74,76-78) and thereby allowing newly synthesized Nrf2 to translocate to the nucleus. In the nucleus Nrf2 heterodimerizes with small Maf proteins and subsequently binds with high affinity to the so-called antioxidant response elements (ARE) in the promoter region of antioxidant genes (79). In support of the Keap1/Nrf2 system in the control of DILI, $Nrf2^{-/-}$ mice have been shown to be more susceptible towards paracetamol toxicity (80) while liver specific Keap1 knockout resulted in resistance against the organ damage (73).

Although overwhelming oxidative stress and mitochondrial dysfunction due to reactive metabolite formation can lead to hepatocyte death, ROS can also initiate cell injury by (in)activation of intracellular (stress) signaling (30,81,82). Whether the hepatocyte survives or dies, either by controlled apoptosis or by necrosis, is in great part determined by the balance between pro-death and pro-survival signaling pathways activated (81).

ROS has been shown to induce JNK activation, and when sustained, this activity causes cell death (82,83). Inhibition or knockdown of JNK showed protection against paracetamol-induced hepatotoxicity in mice (84) and troglitazone-induced apoptosis in cultured hepatocytes (HepG2) (85). JNK can actively promote hepatocyte cell death by translocating to the mitochondria and there promote membrane permeabilization resulting in the release of pro-apoptotic factors (30,81,86) (see above). However, active JNK does not *per se* induce mitochondrial permeabilization and cell death; most likely the mitochondria must first suffer redox-related damage (30,81). In addition, the exact mechanism by which JNK induces mitochondrial membrane permeabilisation has not been determined, but JNK has been shown to translocate to the mitochondria, and there anti-apoptotic Bcl-xL and pro-apoptotic Bax are known downstream targets of JNK (30,84,87).

Supportive for the role of extensive ROS production in iDILI is the genotypic variation in SOD2 and GPX1 in humans suffering from this drug-induced damage (48,88). This would lead to enhanced production/reduced detoxification of ROS within the mitochondria upon drug exposure leading to enhanced mitochondrial damage. In addition to this, combined deficiency of GSTT1 and GSTM1, enzymes important for catalyzing the conjugation of GSH to ROS and reactive metabolites, has been linked to troglitazone hepatotoxicity and other iDILI-inducing compounds in humans (51,89,90).

In addition to mitochondrial effects of oxidative stress, the activity of heat shock proteins (HSPs) is also enhanced after this type of stress induction. Pre-treatment with hyperthermia, which up-regulates heat shock proteins, has been shown to protect against paracetamol-induced hepatotoxicity while HSP70 knockout induced it (91), illustrating the role of these proteins in drug-induced toxicity. Heat shock proteins are chaperones that ensure proper folding of proteins, which is likely altered as a consequence of covalent binding and redox changes after drug exposure. A central organelle in which protein folding is continuously taking place is the endoplasmic reticulum.

4.1.4 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is mostly recognized for its role in protein synthesis and folding and as intracellular calcium storage. However the endoplasmic reticulum is also involved in many processes that are important in drug-induced toxicity. It is in the membrane of this organelle that the P450 enzymes, the UDP-glucuronosyltransferases (UGTs) and some GSTs that are so important in drug metabolism reside (92,93). Furthermore, the ER is now recognized as a target of reactive intermediate-mediated damage through covalent binding and the induction of oxidative stress. In addition, it is

involved in the signaling induced after such damage determining the fate of the affected cell (94).

The ER lumen has two unique properties that are relevant to drug toxicity (94). Firstly, it has an oxidizing environment relative to the cytosol, something that is critical in the oxidative protein folding but that can also contribute to the generation of oxidative stress (95). Secondly, the ER contains a much higher concentration of calcium and thereby serves as a storage for calcium which is needed for intracellular signaling (96). Severe ER stress can lead to calcium release, thereby increasing cytosolic calcium levels which enters the mitochondria to trigger mPT and MOMP and thereby cell death (97).

The ER lumen also contains proteins that are involved in ER function. The most prominent protein, which is also important in the sensing of ER stress, is the glucose-regulated protein 78 (GRP78; a heat shock protein family member also known as BiP) (98,99). This stress protein is expressed under normal conditions but the expression is enhanced by insults that disrupts ER-function and causes accumulation of unfolded proteins. GRP78 plays a crucial role in initiation of the so called unfolded protein response (UPR) in ER stress (99).

The UPR functions to counteract the ER stress in three major ways: 1) by decreasing protein synthesis to decrease the protein load in the ER (100); 2) by up-regulating chaperones (such as GRP78) to enhance the protein folding capacity (100); and 3) by increasing the activity of ER-associated degradation pathways to remove unfolded proteins (101). The initiating step to the UPR is the binding of GRP78 to the unfolded proteins in the lumen of the ER. This releases and thereby activates the signaling molecules that then transmit the intracellular signals of the UPR (102).

The UPR signaling is transduced by three ER resident proteins, which are inhibited by GRP78 in a non-stressed state; protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1-alpha (IRE-1α) and activating transcription factor 6 (ATF6). The reduction in protein synthesis is mediated by PERK-induced eIF2α phosphorylation (103). This attenuates global translation but favors translation of activating transcription factor 4 (ATF4) that induces production of proteins involved in amino acid transport and protection against oxidative stress, but most importantly, also C/EBP-homologous protein (CHOP; also known as GADD153 and DDIT3) which is an important transcription factor in ER-stress dependent apoptosis (104). The transcriptional up-regulation of genes involved in the protein processing is mediated by activation of the other ER-resident kinase and endonuclease, IRE-1α (105). Once activated IRE-1α splices X-box binding protein 1 (XBP1) mRNA yielding a mature mRNA that encodes the required transcription factor (106). The last ER stress signal transducing protein that resides in the ER membrane is ATF6. After translocating to the Golgi apparatus where it gets cleaved, ATF6 induces genes involved in for example quality control in the ER (107). These three pathways of the UPR are critical for the cell to be able to withstand disruption of normal ER homeostasis. However, if the stress is too immense the apoptotic program will be initiated.

The mitochondrial death pathway mediates ER stress-induced apoptosis, and a central modulator of this pathway is the transcription factor CHOP (104). Importantly,

CHOP overexpression by itself does not cause apoptosis (108). However, CHOP sensitizes mitochondria to pro-apoptotic signals by inhibiting the transcription of anti-apoptotic Bcl-2 (108), by transcribing pro-apoptotic Bim (109) and by disturbing the cellular redox state including depletion of cellular GSH (108). The activity of IRE-1α has also been implicated in the apoptosis induction downstream of ER-stress-induction. However, the importance of this pathway is less clear than that of CHOP (110), although it has been shown to involve the activation of JNK which results in apoptosis (111). More importantly, XBP1 splicing and protein levels decline with time of ER stress, which is associated with enhanced apoptosis, and reconstitution of IRE-1α protects against cytotoxicity (112). Also ATF6 was shown to have a protective effect, while prolonged activation of PERK including CHOP expression is what caused the ER-stress-induced apoptosis (112).

Since drugs are being metabolized and potentially bioactivated by P450 enzymes in the ER, the ER-resident proteins also serve a high risk of getting covalently adducted by these metabolites. This has been reported for both CYP-enzymes themselves, but also for other ER-related proteins such as UGTs (94,113). This binding has also been shown associated to the formation of antibodies targeting ER proteins suggesting a resulting immune related response (94).

4.2. Cytokine-induced intracellular stress signaling

Cytokines are small soluble messenger molecules that can be secreted by all types of cells in the body, although they are mainly used by the immune system and the main function of the cytokines is to regulate inflammatory responses (26). The liver can produce both hepatotoxic and -protective cytokines in response to injury and it is believed that the balance between these is what affects an individual's susceptibility to DILI. The secretion of cytokines has even been proposed as potential biomarkers of DILI, although an increased understanding of their role in the actual damage induction is required before this can be implemented (114,115).

4.2.1 The role of TNFα in DILI

TNFα is a cytokine that is mainly secreted by monocytes and activated macrophages but also T-cells (26), and in hepatic inflammation, TNFα release is one of the earliest events observed. Moreover, TNFα is secreted by the liver stationary macrophages, Kupffer cells, after contact with bacterial factors absorbed via the gastrointestinal tract to activate an immunological response as a defense mechanism (6). Interestingly, polymorphisms within the promoter region of the gene encoding this cytokine has been linked to the severity of inflammatory reactions in humans (116) although this has so far not been shown to correlate with the risk of developing DILI (117).

TNFα has been implicated in liver injuries induced by several types of drugs including paracetamol (118), trovafloxacin (38), ranitidine (119) and sulindac (37) and its role in DILI has been demonstrated using neutralizing antibodies (38,119). Other cytokines including IFNγ, also seem to play a role in DILI since neutralizing antibodies

against IFNγ as well as IFNγ$^{-/-}$ mice protected against paracetamol-induced toxicity (120). However, the proposed mechanism underlying this observation was down-regulation of other cytokines including TNFα, which further emphasizes the role of the latter cytokine in DILI.

TNFα is especially interesting in the context of toxicity due to the nature of its receptor, which can induce both direct pro-apoptotic signaling via its death domain and pro-survival signaling via activation of the nuclear factor kappa B (NF-κB) pathway. These two signaling pathways will be further discussed in the following paragraphs.

4.2.2 NF-κB signaling

Hepatocytes, like most cells are resistant to TNFα exposure due to their activation of NF-κB. NF-κB is an important transcription factor that promotes expression of anti-apoptotic genes such as Bcl-xL, cellular FLICE inhibitory protein (cFLIP) and inhibitor of apoptosis proteins (IAPs), although it is mostly recognized as a master regulator of the immune response due to its transcription of for example cytokines and adhesion molecules (121).

The NF-κB proteins are dimeric transcription factors composed of five different subunits, p65 (RelA), RelB, cRel, p50 and p52 (121,122). Under normal conditions the transcription factor is kept in the cytoplasm through masking of its nuclear localization sequence by the inhibitor of kappa B (IκB) proteins. There are two routes through which NF-κB can be activated, the classical or canonical activation route and the alternative, non-canonical route. After TNFα binding to its receptor, it is the canonical pathway that gets activated, and therefore this pathway will be in focus here. An overview of the signaling pathway can be seen in Figure 1.

After TNFα binding to its receptor, the adaptor protein TNF receptor-associated death domain (TRADD) connects with the cytoplasmic tail of the receptor, and so does the kinase receptor-interacting protein 1 (RIP1) (122,123). This leads to the recruitment of the TNF receptor associated factor 2 (TRAF2) adaptor protein (124), which in turn promotes cellular (c) IAP1 and cIAP2 association to the complex. This is an essential event for the IκB kinase (IKK) activation since this promotes the K63-linked ubiquitination of RIP1 (125) needed for IKK and TGF-β-activated kinase-1 (TAK1)/ TAK1 binding protein (TAB) complex recruitment (126). In the canonical pathway, the IKK complex consist of the catalytic subunits IKKβ (IKK2 or IKBKB), IKKα (IKK1 or CHUK) and the regulatory component IKKγ (NEMO or IKBKG). The latter is important for the binding to the K63 ubiquitin chain on RIP1 needed for activation (127). This subsequently leads to the rate-limiting and crucial step of (IKK) complex activation (128) via TAK1-mediated or auto-phosphorylation of IKK (126). The activated IKK complex then phosphorylates IκB proteins, for the canonical p65/p50 dimer the IκB-protein IκBα, resulting in its K48-linked polyubiquitination and proteasomal degradation, which subsequently leads to the nuclear translocation of NF-κB (126).

The activation of the NF-κB pathway is clearly tightly regulated by different post-translational modification steps, as illustrated above. However, this pathway is also regulated by transcriptionally mediated feedback mechanisms. IκBα is one of the

target genes of NF-κB and constitutes the most important inhibitory protein of NF-κB signaling: the newly synthesized IκBα protein transports NF-κB from the nucleus back into the cytoplasm (129). A20 is another NF-κB target gene and an important regulator of NF-κB activation by its effect on RIP1 ubiquitination (130). A20 deubiquitinates the K63-linked activating ubiquitin chains on RIP1 and it also promotes K48-linked poly-ubiquitinations that targets the protein for proteasomal degradation, resulting in inhibited NF-κB activation (131).

NF-κB and the proteins involved in NF-κB activation all contain critical cysteine residues that are important for proper function. Non-hepatotoxic doses of APAP alter the redox environment of hepatocytes which lead to the inhibition of NF-κB activation, sensitizing primary hepatocytes to TNFα-induced apoptosis (132). Also other compounds, such as hydrogen peroxide and antimycin, that affect the redox status of cells, cause hepatocyte sensitization to TNFα (133). Additionally, glutathione depletion has been linked to inhibition of NF-κB activation (134,135).

The reason why inhibition of NF-κB renders TNFα-exposure cytotoxic is due to the dual role of the TNF receptor 1. The pro-apoptotic role of this receptor will be introduced in the following section.

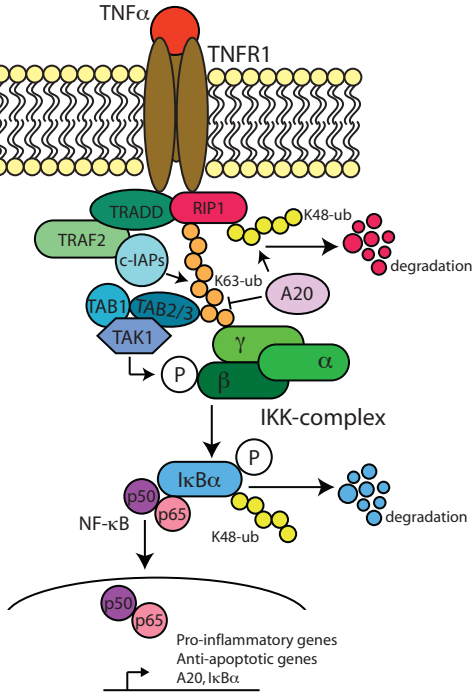

Figure 1. *NF-κB signaling downstream of TNFR1.* Upon TNFα binding to its receptor a complex sequence of protein recruitment and posttranslational modification events is initiated, ultimately leading to the rate-limiting step of IKK-complex activation by phosphorylation. The activated IKKβ then phosphorylates the inhibitor of NF-κB, IκBα, which is followed by its polyubiquitination and proteasomal degradation. This unmasks the nuclear localization signal in NF-κB, allowing nuclear translocation of the transcription factor and transcription of genes involved in for example inflammation, suppression of apoptosis and, importantly, the inhibitors of NF-signaling, IκBα and A20.

4.2.2 Pro-apoptotic death receptor signaling

There are six TNF receptor (TNFR) family death receptors, including TNFR1, and they are characterized by the presence of a death domain (DD) in their cytosolic segments (136). Under normal conditions, the pro-apoptotic signaling is not induced by TNFα exposure as the NF-κB-mediated activation of gene transcription leads to production of proteins that inhibit this pathway, such as cIAP1/2 and cFLIP (121).

RIP1 is essential for the activation of NF-κB and it is hypothesized that when RIP1 gets degraded, or not ubiquitinated by cIAP1/2 (137), this results in activation of the pro-apoptotic pathway. However, this hypothesis has not fully been proven and it is still a mystery how and when TNFα-induced signaling switches from pro-survival to pro-apoptotic (138).

What is known, though, is the basic compilation of proteins leading to the formation of the pro-apoptotic complex (see Fig. 2). Upon induction of pro-apoptotic TNFR1 signaling the TRADD adaptor protein, which is also essential for activation of the NF-κB pathway, recruits Fas-associated death domain (FADD) together with pro-caspase-8 to form the cytoplasmic pro-apoptotic complex. In the case of functional NF-κB signaling, cFLIP is also present in this complex inhibiting the activation of caspase-8 (139). Although caspase-8 can lead to direct activation of caspase-3 (140) the apoptotic signal usually needs amplification. This is achieved by caspase-8 mediated cleavage of the Bcl-2 family member Bid (141). tBid then promotes the disruption of the mitochondrial membrane integrity by Bax and Bak oligomerization (see above in the section about the role of mitochondria in DILI).

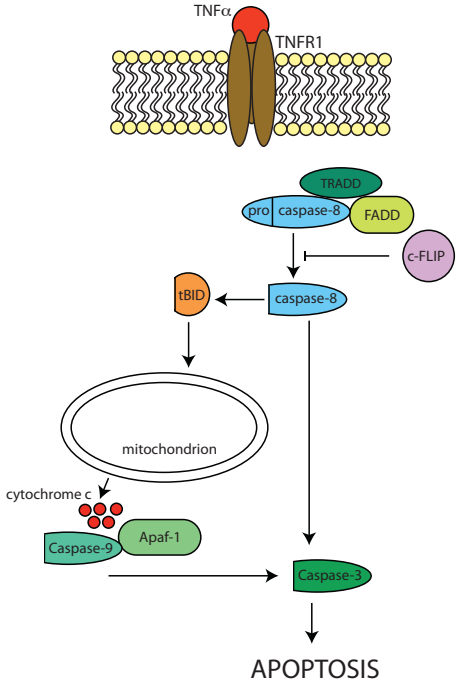

Figure 2. *Pro-apoptotic signaling downstream of TNFR1.* Unless inhibited by NF-κB transcribed genes, such as cIAPs and cFLIP, intracellular signaling induced by TNFα also leads to apoptosis. This event is initiated by TRADD dissociation from the receptor leading to FADD and pro-caspase-8 recruitment. This is followed by activation of caspase-8, which can then induce apoptosis either directly by caspase-3 cleavage or by involvement of the mitochondrial death pathway via activation of the Bcl2-family protein Bid.

5. METHODS TO STUDY CROSS-TALK BETWEEN DRUG- AND CYTOKINE-INDUCED SIGNALING IN LIVER INJURY

As already mentioned in the beginning of this chapter, it is of outmost importance to integrate a complete molecular mechanistic understanding of iDILI in advanced pre-clinical cell and animal models to identify candidate drugs that are at high risk to induce DILI. Of course relevant models to uncover these molecular mechanisms are essential to bring this project forward.

5.1 Existing *in vivo* and *in vitro* models

It has been suggested that only animal models should be able to model the complexities that iDILIs constitute (7). However, as iDILI is expected to be as rare in animal models as in humans, not any animal model would be sufficient to get a complete mechanistic understanding of these rare but serious adverse drug reactions. Most likely, a first step is to move away from healthy animal models towards more intrinsically stressed ones, especially since humans on drug treatment have at least one condition, their illness, that potentially makes them more susceptible to adverse drug reactions (142). Two animal models have received special attention in this aspect, the $SOD2^{+/-}$ heterozygous mice, used to study the role of latent mitochondrial susceptibility (143), and the LPS co-exposure model, to address the role of inflammatory stress in iDILI (7).

As animals cannot be used for drug safety screening campaigns, there is also a need for in vitro mechanism based high throughput tests that could be used in an even earlier preclinical toxicity testing phase (7). Cosgrove and colleagues presented such a model, which is analogous to the *in vivo* LPS co-exposure model developed by Roth and Ganey (144). By exposing different cell types (HepG2 cell line and primary rat as well as human hepatocytes) to known hepatotoxicants and their non-toxic counterparts, together with pro-inflammatory cytokines, they demonstrated that primarily drugs known to cause idiosyncratic DILI, displayed synergism in cytotoxicity when combined with cytokines. Important in the context of the studies presented in this thesis, TNFα was one of the cytokines that contributed most to the observed toxicity.

5.2 High content imaging

High content cellular imaging techniques provides an in vitro platform to investigate the toxicity-inducing potential of many drugs and it provides an outstanding method to determine the mechanisms behind such toxicity. The most beneficial aspects is the possibility to see what is happening to the cells in time after drug exposure in a non-invasive way that does not require many sample preparation steps which could result in the outcome deviating more than necessary to reality.

One example of non-invasive measurement of apoptosis is the use of AnnexinV-

mediated fluorescence staining in combination of automated imaging (145). This method does not only provide the amount of apoptosis induced as an end-point, but can also provide information on the kinetics of the apoptosis induced, something that could potentially provide a more mechanistic insight.

The use of fluorescent fusion proteins has opened up a whole new field of mechanistic research as it makes it possible to for example follow the translocation of crucial transcription factors from the cytoplasm to the nucleus in real time, or the induction of certain stresses by following the induction of typical target genes such as SRXN1 that is induced upon oxidative stress and the activation of Nrf2 (146).

Another way to follow the induction cytotoxic stress, such as glutathione depletion and mitochondrial damage proven important for the induction of idiosyncratic DILI (see above), is by the use of fluorescent probes indicative of the particular damage. Using this technique Xu and colleagues were able to develop an in vitro screening method, with a true-positive rate of 50-60% and a false-positive rate of 0-5%, for the identification of hepatotoxicants (64). This technique gives a good example of how high content imaging can prove very useful, not only for mechanistic insight, but also for the pre-clinical screening of novel compounds in pharmaceutical companies.

5.3 Pharmacogenetics

As genetic variations are important determining factors for the development of iDILI, pharmacogenetics constitutes an important method in the study of these adverse drug reactions.

The most common way of studying relationships between genes and adverse events is by a targeted candidate gene association studies (CGAS) (40). However as it, due to the low incidence of iDILI, is rather unlikely to find a one-gene-association, genome wide association studies (GWAS) is a better approach as they are more likely to identify a combination of genetic risk factors associated to one adverse drug reaction (40). However, important for both the CGAS and the GWAS approach, is a more mechanistic insights to the iDILI in question. It is a prerequisite for the targeted CGAS study but it has also been proven more fruitful for GWAS studies as this helps to narrow down the target genes by only focusing on certain functional areas (40). This approach was nicely demonstrated when identifying genetic variation of the glucuronidation enzyme UGT2B7 as a risk factor for diclofenac induced DILI (50).

Apart from the use of pharmacogenetics in identifying single or small groups of genes that can be linked to the development of iDILI, gene expression analysis is an important method to identify signaling pathways that are activated in the cellular stress response to a toxic insult (147). To achieve this, the development of dedicated and commercially available pathway analysis software such as Ingenuity Pathway Analysis® and MetacoreTM has been a major accomplishment (148,149).

Although informative in the search for transcriptionally regulated genes, transcriptomics provides little information about the functional role of the genes that are

differentially expressed. Furthermore, transcriptional up-or down-regulation does not per se mean a functional change relevant for the phenotype studied. Small interfering RNAs (siRNAs) were first discovered in the early 2000s and they constitute an outstanding method for targeted silencing of individual genes for the study of their function (150). siRNA screening is now widely used by both academia and drug-industry for the discovery of novel drug targets affecting a certain intracellular system or for example for fundamental studies of mechanisms behind certain cellular processes (151,152). In addition, siRNA technology comprises an invaluable resource for the study of the functional roles of differentially expressed genes identified in GWAS studies.

6. AIM AND SCOPE OF THIS THESIS

It has been anticipated that *in vitro* studies of idiosyncratic drug reactions cannot simply mimic all the complex interactions that occur *in vivo* (22). Rather, animal models could be better used to support hypothesis coming from clinical observations. However, in depth mechanistic *in vitro* studies at the molecular level are still essential to gain more detailed insight. Where appropriate the gathered knowledge could be integrated in improved pre-clinical *in vitro* toxicity screening.

In this thesis I used an *in vitro* approach for the mechanistic studies of DILI by investigating the hypothesis that cross–talk between drug (metabolite)-induced and cytokine-induced intracellular stress signaling is a likely critical event that leads to an enhanced toxic response in susceptible individuals (Fig. 3). This hypothesis does not exclude either the innate or the adaptive immune system activation, as cytokines are the mediators of both. The focus is here on TNFα since this is a major mediator of inflammation-induced toxicity.

Diclofenac-induced liver injury has been termed a "paradigm of idiosyncratic drug toxicity" (49). In **chapter 2** I first used this drug in combination with a non-toxic dose of TNFα to provide a proof-of-concept for our working hypothesis. The apoptotic mechanism behind the enhanced drug-induced toxicity seen with the addition of TNFα was further studied using siRNA-screening technology and high-content imaging of apoptosis as described above. Using this methodology, the enhanced apoptosis was shown to originate from the TNF receptor, suggesting that diclofenac enhances the pro-apoptotic properties of TNFα and not the other way around. Furthermore, I showed that this enhanced toxicity is related to diclofenac's ability to inhibit the oscillatory pattern of NF-κB translocation. This is in line with the observation that TNFα can only be cytotoxic in the case of NF-κB inhibition.

In **chapter 3** the concept of TNFα enhancing the apoptotic outcome of drugs associated with idiosyncratic DILI was further investigated using a panel of 15 drugs, involving drugs linked to iDILI, DILI without an inflammatory component as well as non-liver-toxic compounds. Here I show that the synergistic response with TNFα addition is not only linked to the inhibition of NF-κB as this could also be observed following the exposure to non-toxic compounds as well, but also dependent on induction of oxidative stress by the drug. The kinetics of oxidative stress induction was determined using high content imaging of Srxn1-GFP, a reporter for the activation of Nrf2-mediated oxidative stress response. I anticipate that the use of these three high content imaging methods can be used as a part of a toxicity-screening panel for the identification of compounds in a pre-clinical setting with a potential risk for human iDILI.

In **chapter 4** the mechanism behind the synergistic apoptotic response seen with certain drugs and TNFα addition was further investigated using a transcriptomics and subsequent functional genomics approach. In addition to diclofenac, carbamazepine was shown, both in this chapter and in chapter 2, to have a clear apoptotic synergism with TNFα. Using Ingenuity Pathway Analysis (IPA®), genes related to the death receptor, oxidative

stress and ER stress pathways were shown to be significantly regulated with diclofenac and carbamazepine, but not with the non-toxic drug methotrexate. The involvement of these pathways could be confirmed using RNA interference. Moreover, a critical role for translation initiation mediated by RNA helicase EIF4A1 was shown for diclofenac/TNFα- and carbamazepine/TNFα-induced apoptosis. Potentially this gene could be used as a susceptibility marker to identify individual with a higher risk of developing iDILI.

As the nuclear translocation of NF-κB was found important for the toxic outcome of drug/TNFα exposure, the role of individual proteins involved in post-translational modifications in this response was investigated in **chapter 5** using an siRNA screening approach and high content imaging of GFP-p65 translocation following TNFα exposure. Knockdown of genes that resulted in a faster, slower or blocked translocation response were identified. Further attention was given to the knockdowns that stopped the TNFα response and unexpectedly this was related to a protective response in a drug/TNFα exposure condition. Interestingly, both the translocation and the apoptosis outcomes were related to enhanced expression of the (de)ubiquitinase A20, a critical component in the NF-κB feedback loop, by the knockdowns themselves.

Finally, **chapter 6** provides a summary and a general discussion on the findings and implications of the work in this thesis.

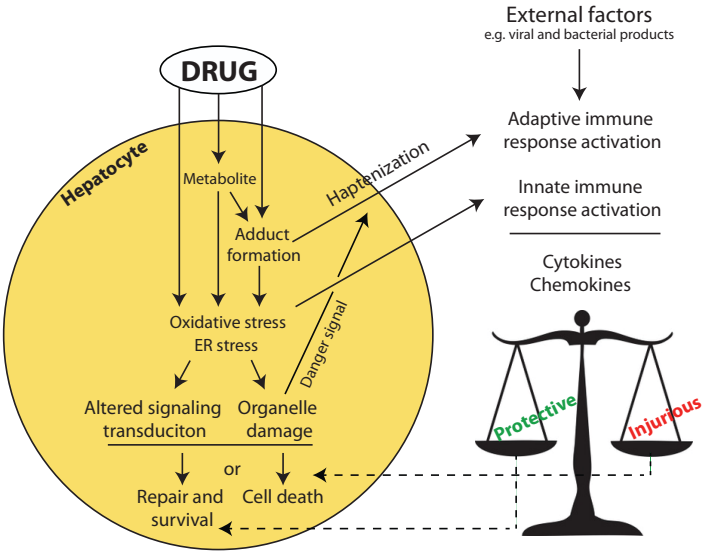

Figure 3. *Summary of the hypothesized interplay between drug- and cytokine-induced stress signaling in the hepatocyte.* A drug, when taken up by the hepatocyte, may induce intracellular stress either by itself or by the formation of reactive metabolites. This can alter the intracellular signaling pattern or cause organelle damage resulting in increased susceptibility of the cell. The hepatocyte damage, or external factors such as exposure to bacterial or vial products, can lead to activation of the innate and/or adaptive immune system. These exert their response by secretion of cytokines and chemokines, which either allow repair and survival of the already susceptible hepatocyte in the case of protective cytokines, or enhanced cellular injury and ultimately cell death in the case of secretion of injurious ones.

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