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SUPPLEMENTARY INFORMATION

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Managing the challenge of drug-induced liver injury: a roadmap for the development and deployment of preclinical predictive models

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1. Importance of dose, concentration and physicochemical properties

Evidence from retrospective analyses of large datasets supports a connection between lipophilicity (as measured by cLogP) and toxicity that can be explained by the increased promiscuity of high cLogP compounds[1,2]. In a retrospective study by Chen et al[3] using a database of 164 well-studied oral drugs, high daily doses (≥ 100 mg per day) and high lipophilicity (cLogP ≥ 3) are associated with an increased risk of human DILI ("rule of two"). However, the scientific literature is not completely consistent regarding the actual importance of lipophilicity with other studies pointing to only a minor association[4,5]. A recent study shows that in addition to a high daily dose and high lipophilicity, oral drugs associated with DILI have lower carbon bond saturation[6], among several other physicochemical properties including ADME, can impact on the biological fate of a drug, and both extent and severity of DILI in human[6-10].

Consistent with the importance of physicochemical properties and metabolites in DILI, the Biopharmaceutics Drug Disposition Classification System (BDDCS), which classifies drugs according to membrane permeability rate and aqueous solubility, appears to support use in the identification of compounds with a higher risk of DILI[11]. Specifically, BDDCS class 2 drugs, which have low solubility, high *in vitro* permeability and extensive metabolism, have a higher risk for DILI compared to the other three classes[11]. Interestingly, the most potent BSEP inhibitors are BDDCS class 2 drugs, which implies screening for BSEP inhibition *per se* is not predictive of hepatotoxic drugs[12]. The use of physicochemical properties of compounds to complement high-throughput screening assays with use of solubility, permeability, lipophilicity or microsomal stability measurements constitutes important tier 1 assays to facilitate ranking and prioritization of compound series. However, many biochemical and physicochemical parameters are interdependently related; solubility measures will depend on dose, formulation of the active drug substance and formulation such as salt or solid form selection. Whilst partitioning data into categories ('ADME features of drugs') such as BDDCS helps

simplify data handling, it may also exaggerate the strength of identified trends often referred to as "correlation inflation")[13]. However, despite the possible shortcomings of correlations to associate certain physicochemical properties with drug liabilities, these analyses support their use in optimizing the druggability of chemistry with drug potency toward the intended target, desired target selectivity, and optimal physicochemical and ADME properties. Medicinal chemists can apply such parameters for use in screening compounds, and thus enable a holistic multi-parametric optimization in drug design.

The importance of daily dose when assessing DILI risk for small molecules is relevant to the interpretation of available data on the identification of reactive metabolites and their potential to covalently bind cellular macromolecules[14-16]. However, there is questionable utility for the use of *in vitro* primary screens to interrogate the formation of reactive metabolites to guide selection where only certain drugs can be readily identified[17]. Moreover, because there is a requirement for the use of radiolabelled compounds, studies are typically only performed close to candidate nomination to identify potential hazards related to tissue retention of drug and/or drug metabolites, especially in the liver. The identified retention of drug-related material may in turn trigger further mechanistic and/or quantitative studies on a project-by-project basis.

Besides the use of *in silico* tools to predict hepatotoxic drugs, mechanism-based dynamic pathway models provide novel approaches to help stratify compounds according to their potential to elicit DILI. The construction of dynamic pathway models necessitates the study of specific pathways and the determination of concentration-time effect with drugs that can be employed as 'reagents' to probe pathway response(s). For example, tumour necrosis factor (TNF)alpha amplifies hepatotoxicity of diclofenac (DCF)[18], and with a combined time-resolved quantitative approach enables measurement of TNFalpha-induced NFkappaB signal transduction to quantitatively describe the signal perturbations caused by diclofenac (DCF) on the dynamics of TNFalpha-induced signal transduction. Such models may enable the development of future calibrated mathematical models

to identify systematically by sensitivity analysis, the model parameters most influenced by DCF. Such studies have revealed that DCF alters several steps in the TNFalpha signalling pathway by which multiple measurements are then integrated in order to identify the threshold above which hepatotoxicity would occur. Whilst these studies are exploratory and not yet ready for routine deployment in drug discovery, further research in dynamic pathway modelling to quantify the impact of test compounds acting on other putative pathways in TNFalpha-mediated signal transduction is ongoing. Future efforts in this direction are very likely to help elucidate mechanisms, concentration-effect and temporal relationship of signalling pathways essential to furthering our understanding of human DILI and more broadly the challenges to de-risk and predict drug safety in human[19].

2. Assessment and prediction of the role of transporters in bile transport

2.1. Bile acid synthesis, intrahepatic hepatobiliary transport and hepatocellular accumulation

BAs are amphipathic planar molecules synthesized within hepatocytes from cholesterol by two multistep pathways, as cholic acid (CA) and chenodeoxycholic acid (CDCA). These two primary BAs are then conjugated to glycine or taurine, giving rise to taurocholic acid (TCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), and taurochenodeoxycholic acid (TCDCA). Conjugated (amidated) BAs may also be metabolized by different liver enzymes, in particular glucuronosyl- and sulfo-transferases[20]. After secretion into bile via canalicular transporters, mainly the bile salt efflux pump (BSEP), conjugated BAs are released into the duodenum, where they facilitate the absorption of dietary lipids and liposoluble vitamins. The gut microbiota catalyzes 7α-dehydroxylation of CDCA and CA to generate the secondary BAs, namely lithocholic (LCA) and deoxycholic (DCA) acids. Around 95% of both primary and secondary BAs can be reabsorbed and returned back to the liver *via* the enterohepatic recirculation[20,21]. Intrahepatic accumulation of hydrophobic BAs (CDCA, DCA, LCA and glycochenodeoxycholate) is considered as the hallmark of cholestasis. Indeed, the toxicity of BAs is highly dependent on their hydrophobicity[22]. The localization of membrane

transporters, expression of family and sub-family members (BOX 1), and polarity of transporter proteins convey the highly regulated physiological and pharmacological role in the hepatobiliary transport of endogenous and exogenous substances.

In many cases, intrahepatic cholestasis results from functional changes in the hepatobiliary transporter system. The mechanisms by which drugs induce intra-hepatic cholestasis and the adaptive responses of the hepatocyte to disturbances in bile formation are broad and include changes in membrane transporter expression, peri-canalicular cytoskeletal modifications, and BA intracellular trafficking (indirect effects)[23]. A role for oxidative stress as a primary causal agent and/or as an aggravating factor has been shown in extra-hepatic cholestasis induced by bile duct ligation, but it remains poorly documented in intrahepatic cholestasis. In addition, genetic mutations in genes encoding transporters may increase the susceptibility to cholestasis and drug-induced liver injury[24-26].

2.2. Hepatocyte uptake transport proteins.

The sodium taurocholate co-transporting peptide (NTCP/ SLC10A1) enables sodium-dependent uptake of BA salts and conjugates, and some drugs from the space of Disse into hepatocytes. Human NTCP accepts most physiological bile salts and conjugates and some organic anions. NTCP-mediated transport accounts for most BA uptake with the remaining BAs transported by sodium-independent uptake by members of the OATP (SLCO) family[27]. Recent studies show selectivity and specificity against several quantitatively important BAs in human preference for the transport of efficient uptake of GCDC, TC and 3S-GLC by human NTCP. Human NTCP transports GCDC with greater efficiency than TCA and as effectively for 3S-GLC and TC[28]. Glyco- and tauro-conjugates are preferred to unconjugated BAs as substrates for OATP1B1 and OATP1B3[29]. Sulfated BAs are also transported more efficiently by OATP1B1[30].

2.3. Hepatocyte efflux transport proteins

The efflux transporters involved in bile formation and drug clearance belong to the superfamily of ATP-binding cassette (ABC) proteins (BOX 1). These comprise members of the multidrug resistance family, e.g. MDR1 (ABCB1) and MDR3 (ABCB4) and the bile salt export pump (BSEP/ABCB11); the multidrug resistance-associated protein (MRP2) (ABCC2); the breast cancer resistance protein (BCRP1) (ABCG2) and the heterodimeric transporter ABCG5/8 on canalicular/apical membranes, and MRP3 (ABCC3), MRP4 (ABCC4) and MRP6 (ABCC6) on sinusoidal membranes. Bile formation begins with the uptake of bile constituents at the basolateral plasma membrane of hepatocytes and secretion of cholephilic compounds across the apical membrane. Excretion of cholephilic substances such as bile salts, phospholipids and cholesterol involves highly specialized canalicular proteins. BSEP is the major canalicular monovalent BA efflux transporter. MRP2 mainly excretes bilirubin–glucuronides and glutathione conjugates, but also exports divalent sulfo-conjugated BAs into the bile. Other canalicular transporters include MDR3 (ABCB4) that mediates excretion of phospholipids, in particular phosphatidylcholine and ABCG5/8 that is responsible for the efflux of cholesterol and requires the involvement of MDR3[31]. MDR1/P-gp is thought to have a minor contribution to bile flow but ensures elimination of various cationic drugs and natural products. BCRP has a large drug substrate specificity; it is involved in elimination of organic anions, particularly sulfo-conjugates[32]. Both MDR1 and BCRP have also been implicated in BA transport under cholestatic conditions[33], yet to be demonstrated in human. The basolateral (sinusoidal) membrane localizes members of the alternative basolateral BA efflux system, mainly the constitutively expressed MRP3, and the inducible MRP4 and heterodimeric organic solute transporter OSTα/OSTβ (SLC51a/SLC51b)[32,34,35]. MRP3, MRP4 and OSTα/OSTβ play an important role where a functional reduction of bile secretory processes can lead to decreased BA secretion, arrest of bile formation, flow and subsequent

cholestatic liver disease, suggesting that they have a role in protecting hepatocytes from BA-mediated toxicity[32,34].

3. Drug induced steatosis and steatohepatitis

Drug-induced steatosis (DIS) is a form of DILI characterised by aberrant fat accumulation, predominantly triglycerides and free fatty acids (FFA) in the liver and has been the subject of a number of reviews[36-38]. Histopathological assessment can further sub-divide their occurrence based upon the size and disposition of lipid droplets in the liver into macrovesicular or microvesicular steatosis. Primarily, macrovesicular steatosis presents usually one large lipid droplet in the cytoplasm of a hepatocyte, and has been associated with drugs including methotrexate, glucocorticoids and tamoxifen. Although macrovesicular steatosis is often reversible, it can persist over longer periods of months/years and potentially develop in to more severe damage; steatohepatitis or cirrhosis. Microvesicular steatosis is a more severe form of steatosis. It presents as the distribution of small lipid droplets throughout the hepatocyte and is often associated with drug-induced mitochondrial dysfunction, for example sodium valproate, nucleoside and non-nucleoside-based antiretroviral therapies and tetracycline. Both micro and macro vesicular steatosis can be associated with certain drugs. In keeping with such differences in histological presentation it is known that several molecular mechanisms can contribute to an endpoint of lipid accumulation in hepatocytes. In terms of drug-effects, there are three ways in which drugs can induce a steatotic response: by increasing lipogenesis; dysregulating beta-oxidation and/or fatty-acid metabolism or by increasing the hepatic uptake of free fatty acids. The onset of fatty liver and its progression from simple fatty liver build up, steatosis, to more serious forms of non-alcoholic fatty liver disease (NAFLD), including steatohepatitis, fibrosis and cirrhosis occurs in only a minority (2 – 3 %) of patients with steatosis[39]. Steatohepatitis, has complex molecular origins with underlying individual factors controlling aspects of predisposition and severity. Most recent understanding suggests that the presence of FFA and triglycerides deposits in the hepatocyte can overwhelm lipid homeostatic

mechanisms leading to the induction of downstream pathways including endoplasmic reticulum stress, oxidant injury and an inflammatory response that can ultimately produce the phenotype of steatohepatitis[40].

Steatosis can be induced with fatty acid overload and steatotic drugs in various human liver cell models, including primary human hepatocytes, liver cell lines, and hepatocyte-like cells derived from stem cells[41-45]. Exposure to monounsaturated or polyunsaturated fatty acids results in rapid accumulation of triglycerides and lipid droplets[46]. However, addition of oleic acid with palmitic acid (a saturated fatty acid) is also used and accumulation of lipid droplets has been described with a variety of steatotic drugs known to induce steatosis by different mechanisms[43,47]. Culture duration to obtain accumulation of lipids droplets with steatotic drugs depends on the liver cell model and culture conditions`, in particular fatty acid and drug concentrations. HepaRG cells are more sensitive than HepG2 cells to lipid accumulation`, likely because of higher expression of certain functions`, particularly those related to lipid and drug metabolism`, as well as particular nuclear receptors (PPARs`, LXR `, PXR)[43,47]. A high content screening assay can be used to analyze lipid content and other parameters such as ROS generation and mitochondrial damage[44].

There are currently no drugs approved to cure NASH[48]. Since in vitro liver cell models can rapidly accumulate lipid droplets, they represent potential suitable models to evaluate anti-steatotic drugs as well as the influence of steatosis on the toxicity of chemicals. Some studies have recently demonstrated that PPAR agonists and farnesol can reduce steatosis following oleic acid-overloading[49,50]. Lipid vesicles stained by Oil-Red O and triglyceride accumulation caused by oleic acid overload, were decreased, by up to 50%, while fatty acid oxidation was induced after 2-week co-treatment with PPAR agonists. Co-exposure of steatotic hepatocytes to benzo[a]pyrene and ethanol was found to increase cytotoxicity and expression of inflammatory markers[51]. If two-dimensional in vitro liver cell models are practical, economical and pertinent in steatotic and anti-steatotic drug screening, the use of three-dimensional models containing hepatocytes and other

non-hepatocyte cell types should be more valuable to analyze drug-induced steatohepatitis[52]. In particular if stellate cells and macrophages are used with or without an integrated microfluidic system.

Drugs such as valproic acid and tamoxifen can serve as positive reference compounds for drug-induced steatosis. In the context of the Roadmap, an assessment of drug-induced steatosis can be routinely determined by use of TIER 1 assays. Moreover, the panel of TIER 1 tests also facilitates initial mechanistic insight by the use of multiple endpoints such as functional mitochondrial assays (see section 3). The use of model steatogenic compounds currently identifies a diverse number of mechanisms linked through a common key event – lipid accumulation.

Progression of steatosis to steatohepatitis, albeit rare, is poorly understood and largely extends from the study of NAFLD in animal models[53]. Steatohepatitis is believed to arise through progressive mitochondrial dysfunction, increased drug-induced uncoupling of β-oxidation, phosphorylation and production of ROS. In part, the CYP 2E1, which is induced by and metabolizes fatty acids can contribute to the pool of highly reactive carbonyl free radicals. The induction of CYP2E1 and contribution to ROS can be assessed as part of the panel of TIER 1 assays. The presence of ROS determines the progressive formation of lipid peroxidation, nuclear translocation of factor-κb (NFκb) which in turn upregulates inflammatory cytokines, TNF-α, TGF-β. The inflammatory signals stimulate hepatic stellate cell proliferation and fibrosis and presence of NFκb promotes apoptotic cell death through synthetase FAS-mediated CASP9 activation along with other caspases. To capture these multi-cellular effects requires use of TIER 2 test systems, such as spheroids or organ-on-chip solutions. Co-cultures of primary cell cultures or immortalized cell lines have been used to develop multi-cellular models comprising Kupffer cells, stellate cells or sinusoidal endothelial cells to emulate aspects of the physiological and pharmacological relevance of *in vivo* human liver tissue[54-56]. The limitations of 2D cell-based cultures and spheroids for their routine application in Drug Discovery safety screens often render these models impractical when requiring reproducible fidelity that

reflects the complex multicellular architecture of *in vivo* hepatic tissue. The more advanced microphysiological tissue-based systems (MPS) may permit detection and identification of steatohepatitis events. The extrapolation of any such results from TIER 2 studies will still require further work to understand individual subject susceptibilities and rare occurrence steatohepatitis in patients (see above).

TIER 3 animal models for the detection of steatosis and steatohepatitis currently rely on the histopathological examination of liver tissue as the 'gold standard', which are time-consuming single sample analyses. On-going efforts to identify specific serum or plasma clinical biomarkers[41,57,58] to detect liver changes could provide additional value to complement existing TIER 1 and TIER 2 biomarkers for use in Drug Discovery and Clinical Safety. In order to increase predictability and translation to humans, mechanistic knowledge has been captured as adverse-outcome pathways (AOPs) for steatosis through a combination of TIER 1 assays, in such a way as to help report on each of the key events defined within the AOPs[59].

In summary, steatosis is a benign cellular event, but individual susceptibilities to drug treatments may predispose certain individuals to develop the more serious condition, steatohepatitis. In the absence of validated test systems for the detection of risk to develop steatohepatitis, the identification of drug-induced steatosis during preclinical testing of drug candidates for further development is avoided.

Supplementary Box 1: Transporters in drug and bile acid (BA)-induced liver injury

Transporter Proteins

- ***Membrane transporters*** play an important role in the hepatobiliary function of the liver and are broadly grouped into two major families comprising of the ATP-binding cassette (ABC) and soluble carrier (SLC) proteins[60].
- Membrane transporters facilitate uptake and efflux of endogenous substances and drugs between the basolateral, sinusoidal and apical (bile canalicular) membranes of the hepatocyte.

Transport Function

- ***Vectorial uptake and efflux*** of BAs is predominantly mediated by ABC transporter proteins located on the basolateral and canalicular membranes of hepatocytes: MRP2 (ABCC2); MRP3 (ABCC3); MRP4 (ABCC4), MDR1 (ABCB1); MDR2/3 ABCB4); BCRP (ABCG2); BSEP (ABCB11). Other transporters include NTCP (SLC10A1); OATP1B1 (SLCO1B1); OATP1B3 (SLCO1B3) and the organic soluble transporter OSTα/OSTβ (SLC51a/b); MATE1 (SLC47A1) the multidrug and toxin extrusion1 that is a H+/organic cation (OC) exchanger. Their functions are described in detail (Supplementary Section 2).
- Among the efflux transporters, BSEP is physiologically the most important for the efflux of mBAs.
- ***Bi-directional transporters*** are involved only in the transport of some drugs**:**
  - Examples are SLC29 transporters that are sodium-independent equilibrative nucleoside transporters (ENT1/2) and SLC28 transporters that are sodium-dependent concentrative nucleoside transporters (CNT1/2).
  - These transporters can mediate bidirectional transport of purine and pyrimidine nucleosides (e.g. ribavirin); they can be present on sinusoidal and canalicular membranes.

Drug interactions

- Many drugs can interact directly and indirectly with membrane transporters.
- Drugs assigned to the hepatobiliary transporters include a representative selection of compounds for the study of human DILI mechanisms[61].
- Selectivity of transporters for the different monovalent, divalent and conjugated forms of BAs across the basolateral and apical membranes illustrates the multiplicity of transporters involved in bile uptake and efflux.
- Many drugs, such as troglitazone, cyclosporine and tolcapone among many others are inhibitors of BSEP activity.
- MRP3 and MRP4 return BAs back into systemic circulation if BSEP activity is impaired. Some amphiphilic BAs passively diffuse across the basolateral membrane[60,62-73]
- In general, for BA transporter inhibition by drugs, the mechanism of inhibition appears to be important[74].

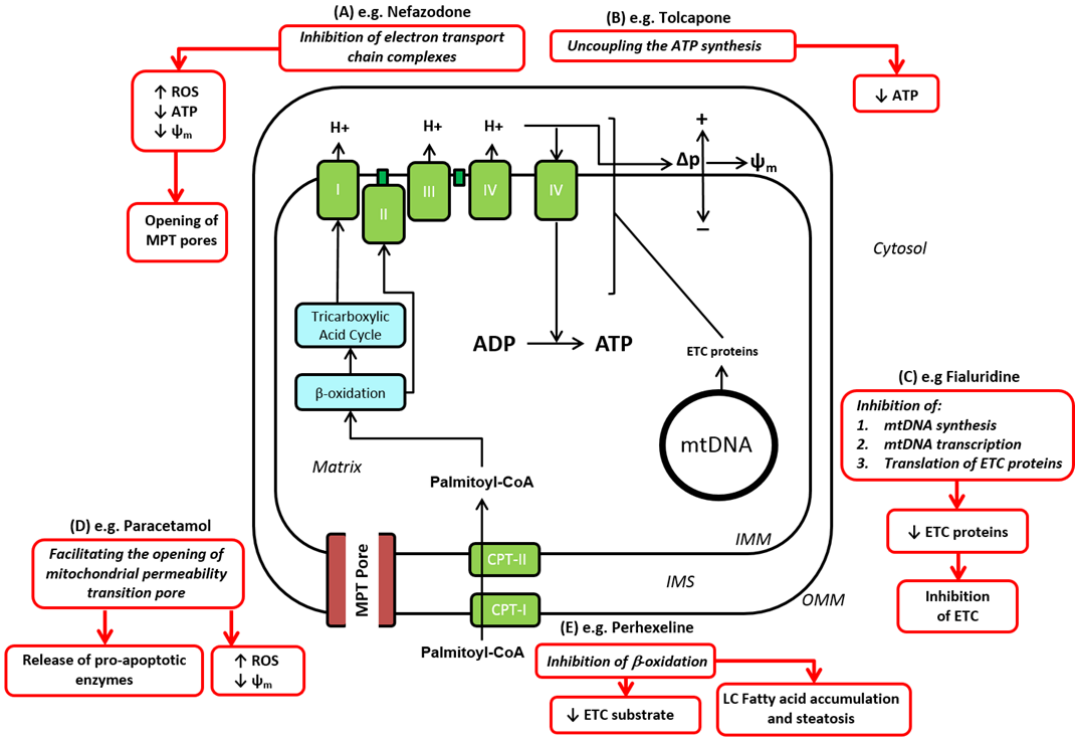

Figure 1. Mechanisms of mitochondrial dysfunction in drug-induced liver injury

The mitochondrion exhibit several key processes that are potential targets for drug toxicity; these include the electron transport chain (ETC) and oxidative phosphorylation; the permeability transition pore; fatty acid oxidation and mitochondrial DNA. The interaction of a drug with any of these processes can lead to mitochondrial dysfunction via a cascade of events, including loss of mitochondrial membrane potential, generation of reactive oxygen species and inhibition of ATP production, which are the subject of a review[75].

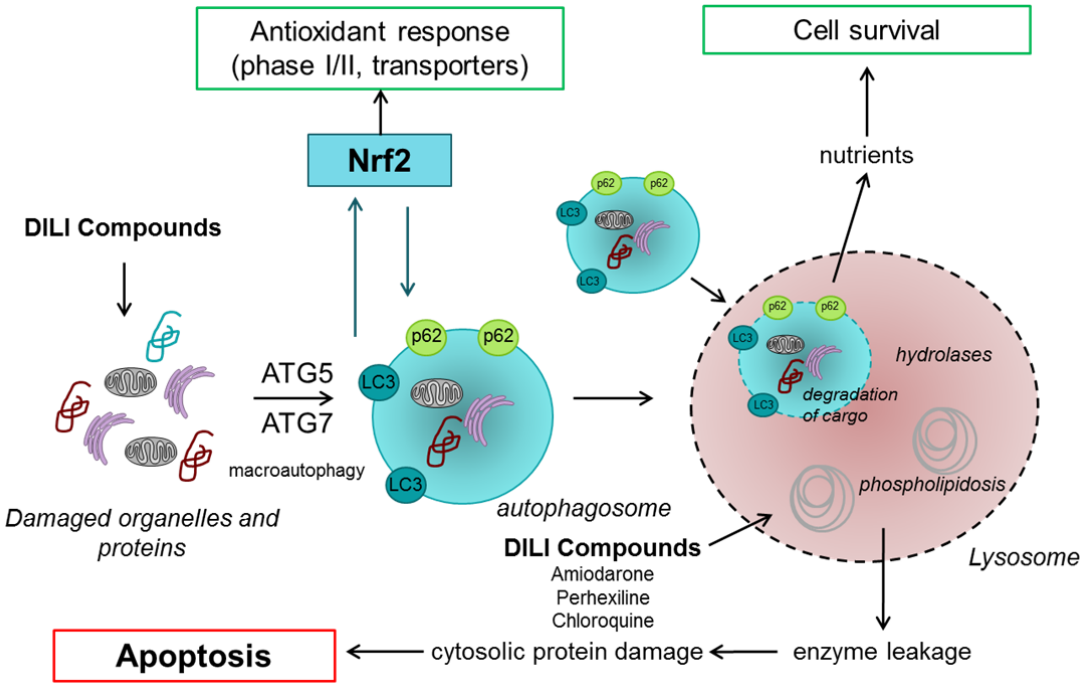

Figure 2. The role of lysosomal impairment in drug induced liver injury.

Drugs have been predicted to influence the function of the lysosomal pathway through two distinct mechanisms (reviewed recently in Woldemichael[76]): 1) damage of cellular proteins through covalent binding leading to enhanced autophagy, cross-talk with antioxidant response pathways, and an increase in cellular energy through breakdown of proteins and organelles in the lysosome; 2) cationic amphiphilic drugs have been demonstrated to cause accumulation of phospholipids within the lysosome, leading to phospholipidosis[77]. This in turn can lead to the breakdown of the lysosome and release of proteolytic hydrolases into the cytoplasm, causing a cascade of protein damage leading to cell death.

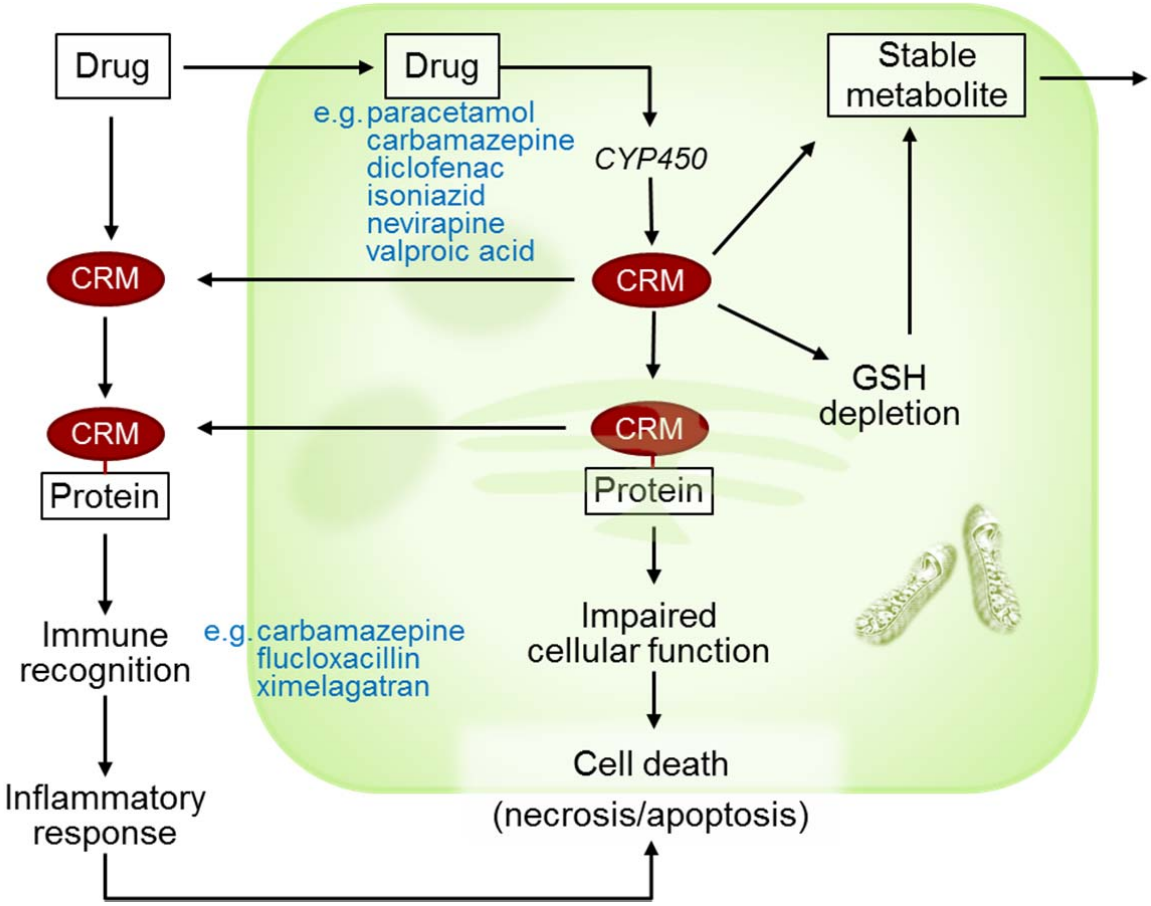

Figure 3. The role of chemically reactive metabolites in drug-induced liver injury.

The work of a large number of groups has demonstrated that during the metabolism of drugs, *chemically reactive metabolites* are often produced, and that many pharmaceuticals known to cause DILI are also converted to reactive metabolites by drug metabolising enzymes, in particular by the cytochromes P450 (CYP450)[61,78-80]. The liver has an abundance of protective mechanisms to remove these metabolites, e.g. through conjugation to glutathione and upregulation of Nrf2, nevertheless these are not always sufficient to prevent damage[81,82]. Whilst it is mechanistically difficult to unequivocally link as cause and effect the generation of reactive metabolites with DILI, it is generally accepted that these reactive species will bind proteins and other reactive biomolecules, and that this may lead to immune cell recognition of altered protein antigens and an inflammatory immune response. Concurrently, depending on the nature and abundance of the reactive species, certain function(s) of the cell will be impaired, and where this becomes overwhelming, liver cells may die, either through apoptosis or necrosis; thereafter, this may lead to a further inflammatory exacerbation and prolongation of tissue injury.

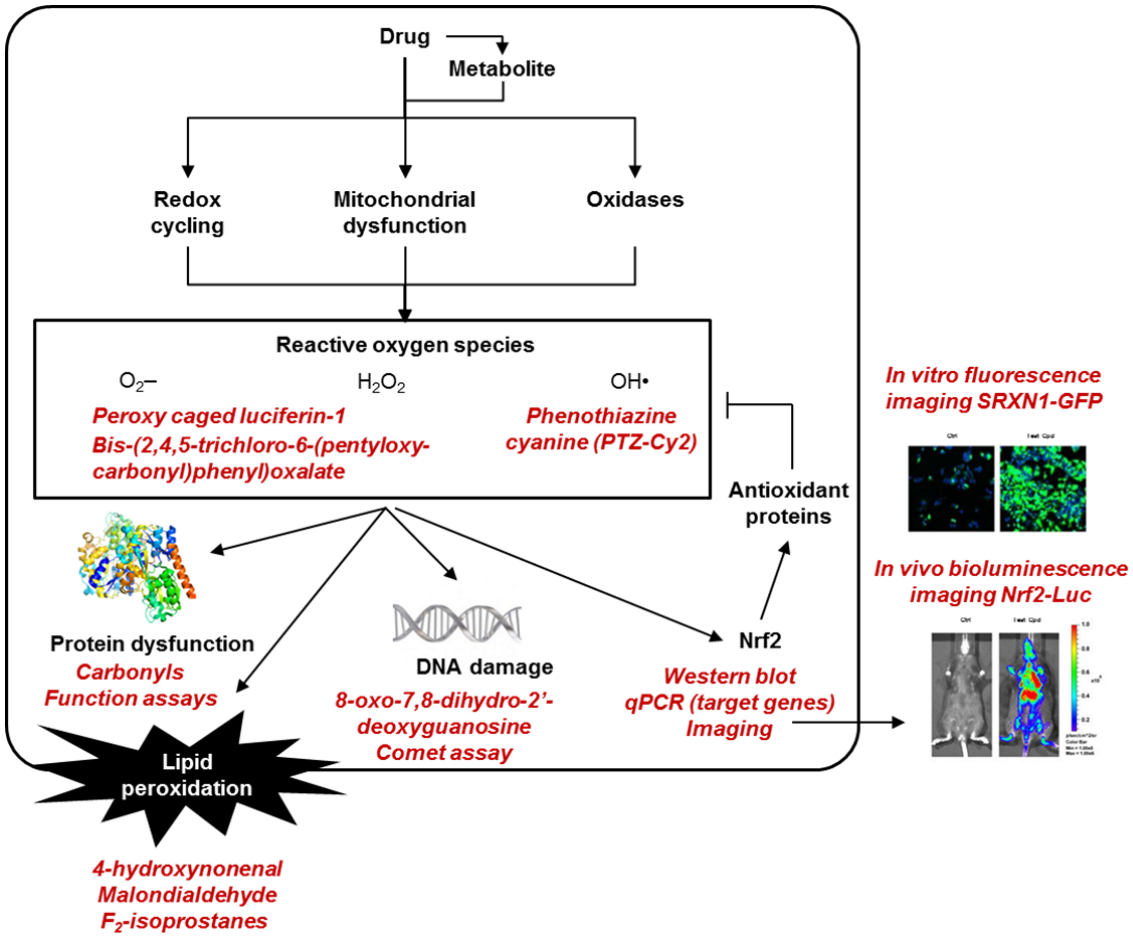

Figure 4. The role and detection of oxidative stress in drug-induced liver injury.

Drugs and/or their metabolites can provoke oxidative stress via inhibition of mitochondrial function, perturbation of cellular oxidase enzymes or through redox cycling. If left unchecked, oxidative stress can cause the dysregulation of critical proteins and cellular processes, leading to cell death. Indeed, oxidative stress has been associated with the hepatotoxicity of several drugs (for a review, see[83]). Selected ROS can be detected with chemical probes (indicated in red) in focussed experiments or as part of high content screens. The downstream cellular consequences of ROS accumulation can be measured with assays of carbonyl formation, lipid peroxidation or DNA damage. Cellular adaptive responses to oxidative stress, primarily mediated by the transcription factor Nrf2, can be assessed through standard molecular techniques or with engineered cell lines and mouse models that enable the stress response to be imaged in real-time.

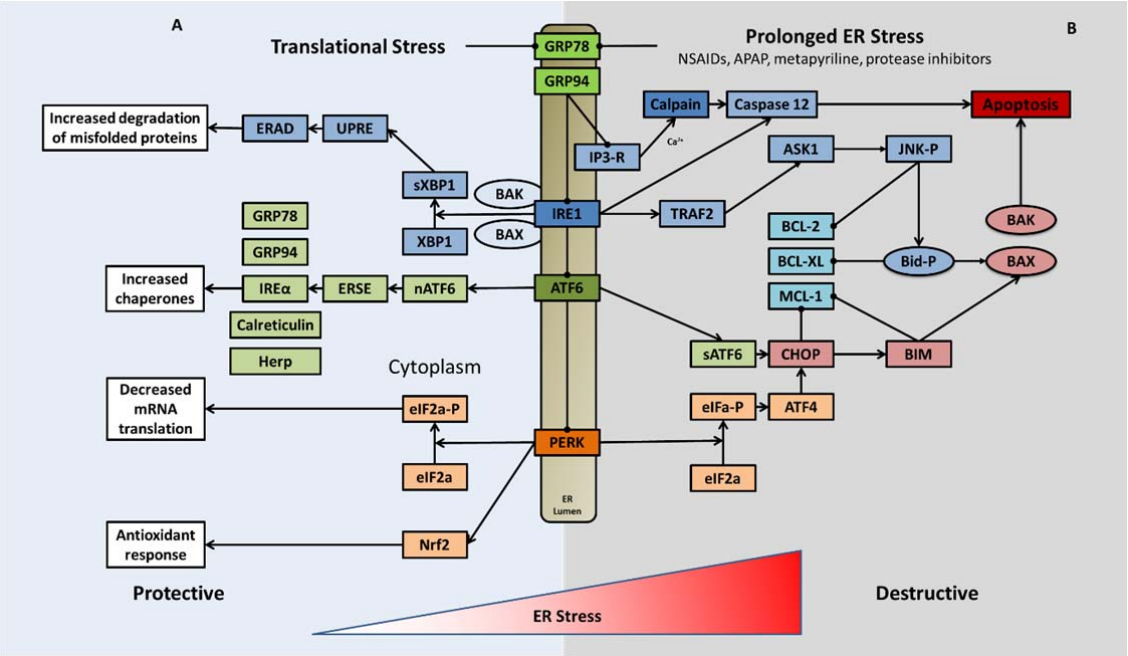

Figure 5. The role of endoplasmic reticulum stress in drug-induced liver injury.

A - Under low levels of transcriptional/ER stress, the chaperones GRP78/94 release IRE-1, PERK and ATF6 to initiate downstream signalling events which result in reduced mRNA translation, increased antioxidant response through the Nrf2 pathway, and an increase in chaperone expression, as a negative feedback pathway[61,84-89] . This will have the effect of reducing the translational load on the ER in times of stress. **B -** After prolonged or severe ER stress, or damage due to covalent binding of reactive metabolites/drugs to ER proteins, the pathway can become destructive[90]. Briefly, damage to, or down-regulation of chaperones (GRP78/94) during severe ER stress leads to activation of the CHOP and TRAF2 pathways, which lead to the down-regulation of anti-apoptotic signalling mechanisms and the activation of the pro-apoptotic Bid, and Bax. Prolonged stress can also cause release of intraluminal calcium stores from the ER, through the IP3 receptor, which can initiate caspase-mediated apoptosis through a mitochondrial signalling route. This leads to increased oxidative stress, through release of ROS through the MTP, and an increase in inflammation through activation of NF-κB[91,92].

References

1 Leeson, P. D. & Springthorpe, B. The influence of drug-like concepts on decision-making in medicinal chemistry. *Nature reviews. Drug discovery* **6**, 881-890, doi:10.1038/nrd2445 (2007).

2 Wenlock, M. C. Designing safer oral drugs. *Medchemcomm* **8**, 571-577, doi:10.1039/c6md00706f (2017).

3 Chen, M., Borlak, J. & Tong, W. High lipophilicity and high daily dose of oral medications are associated with significant risk for drug-induced liver injury. *Hepatology* **58**, 388-396, doi:10.1002/hep.26208 (2013).

4 Yu, K. *et al.* High daily dose and being a substrate of cytochrome P450 enzymes are two important predictors of drug-induced liver injury. *Drug Metab Dispos* **42**, 744-750, doi:10.1124/dmd.113.056267 (2014).

5 Weng, Z., Wang, K., Li, H. & Shi, Q. A comprehensive study of the association between drug hepatotoxicity and daily dose, liver metabolism, and lipophilicity using 975 oral medications. *Oncotarget* **6**, 17031-17038, doi:10.18632/oncotarget.4400 (2015).

6 Leeson, P. D. Impact of Physicochemical Properties on Dose and Hepatotoxicity of Oral Drugs. *Chem Res Toxicol* **31**, 494-505, doi:10.1021/acs.chemrestox.8b00044 (2018).

7 Lovering, F., Bikker, J. & Humblet, C. Escape from flatland: increasing saturation as an approach to improving clinical success. *J Med Chem* **52**, 6752-6756, doi:10.1021/jm901241e (2009).

8 Regan, S. L. *et al.* Acyl glucuronides: the good, the bad and the ugly. *Biopharm Drug Dispos* **31**, 367-395, doi:10.1002/bdd.720 (2010).

9 Sawamura, R. *et al.* Predictability of idiosyncratic drug toxicity risk for carboxylic acid-containing drugs based on the chemical stability of acyl glucuronide. *Drug Metab Dispos* **38**, 1857-1864, doi:10.1124/dmd.110.034173 (2010).

10 Van Vleet, T. R., Liu, H., Lee, A. & Blomme, E. A. G. Acyl glucuronide metabolites: Implications for drug safety assessment. *Toxicology letters* **272**, 1-7, doi:10.1016/j.toxlet.2017.03.003 (2017).

11 Chan, R. & Benet, L. Z. Evaluation of DILI Predictive Hypotheses in Early Drug Development. *Chem Res Toxicol* **30**, 1017-1029, doi:10.1021/acs.chemrestox.7b00025 (2017).

12 Chan, R. & Benet, L. Z. Measures of BSEP Inhibition In Vitro Are Not Useful Predictors of DILI. *Toxicol Sci* **162**, 499-508, doi:10.1093/toxsci/kfx284 (2018).

13 Kenny, P. W. & Montanari, C. A. Inflation of correlation in the pursuit of drug-likeness. *J Comput Aided Mol Des* **27**, 1-13, doi:10.1007/s10822-012-9631-5 (2013).

14 Uetrecht, J. Prediction of a new drug's potential to cause idiosyncratic reactions. *Curr Opin Drug Discov Devel* **4**, 55-59 (2001).

15 Smith, D. A. & Obach, R. S. Seeing through the mist: abundance versus percentage. Commentary on metabolites in safety testing. *Drug Metab Dispos* **33**, 1409-1417, doi:10.1124/dmd.105.005041 (2005).

16 Stepan, A. F. *et al.* Structural alert/reactive metabolite concept as applied in medicinal chemistry to mitigate the risk of idiosyncratic drug toxicity: a perspective based on the critical examination of trends in the top 200 drugs marketed in the United States. *Chem Res Toxicol* **24**, 1345-1410, doi:10.1021/tx200168d (2011).

17 Nakayama, S. *et al.* A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. *Drug Metab Dispos* **37**, 1970-1977, doi:10.1124/dmd.109.027797 (2009).

18 Oppelt, A. *et al.* Model-based identification of TNFalpha-induced IKKbeta-mediated and IkappaBalpha-mediated regulation of NFkappaB signal transduction as a tool to quantify the impact of drug-induced liver injury compounds. *NPJ systems biology and applications* **4**, 23, doi:10.1038/s41540-018-0058-z (2018).

19 Weaver, R. J. & Valentin, J. P. Today's Challenges to De-Risk and Predict Drug Safety in Human "Mind-the-Gap". *Toxicol Sci* **167**, 307-321, doi:10.1093/toxsci/kfy270 (2019).
20 Chiang, J. Y. Bile acids: regulation of synthesis. *Journal of lipid research* **50**, 1955-1966, doi:10.1194/jlr.R900010-JLR200 (2009).
21 Fuchs, M. Bile acid regulation of hepatic physiology: III. Regulation of bile acid synthesis: past progress and future challenges. *American journal of physiology. Gastrointestinal and liver physiology* **284**, G551-557, doi:10.1152/ajpgi.00468.2002 (2003).
22 Hofmann, A. F. Detoxification of lithocholic acid, a toxic bile acid: relevance to drug hepatotoxicity. *Drug metabolism reviews* **36**, 703-722, doi:10.1081/DMR-200033475 (2004).
23 Roma, M. G. & Sanchez Pozzi, E. J. Oxidative stress: a radical way to stop making bile. *Ann Hepatol* **7**, 16-33 (2008).
24 Padda, M. S., Sanchez, M., Akhtar, A. J. & Boyer, J. L. Drug-induced cholestasis. *Hepatology* **53**, 1377-1387, doi:10.1002/hep.24229 (2011).
25 Yang, L. *et al.* Gene expression variability in human hepatic drug metabolizing enzymes and transporters. *PloS one* **8**, e60368, doi:10.1371/journal.pone.0060368 (2013).
26 Karpen, S. J. & Dawson, P. A. Not all (bile acids) who wander are lost: the first report of a patient with an isolated NTCP defect. *Hepatology* **61**, 24-27, doi:10.1002/hep.27294 (2015).
27 Boyer, J. L. Bile formation and secretion. *Comprehensive Physiology* **3**, 1035-1078, doi:10.1002/cphy.c120027 (2013).
28 Jani, M. *et al.* Kinetic characterization of bile salt transport by human NTCP (SLC10A1). *Toxicology in vitro : an international journal published in association with BIBRA* **46**, 189-193, doi:10.1016/j.tiv.2017.10.012 (2018).
29 Suga, T. *et al.* Preference of Conjugated Bile Acids over Unconjugated Bile Acids as Substrates for OATP1B1 and OATP1B3. *PloS one* **12**, e0169719, doi:10.1371/journal.pone.0169719 (2017).
30 Toth, B. *et al.* Human OATP1B1 (SLCO1B1) transports sulfated bile acids and bile salts with particular efficiency. *Toxicology in vitro : an international journal published in association with BIBRA* **52**, 189-194, doi:10.1016/j.tiv.2018.06.018 (2018).
31 Langheim, S. *et al.* ABCG5 and ABCG8 require MDR2 for secretion of cholesterol into bile. *Journal of lipid research* **46**, 1732-1738, doi:10.1194/jlr.M500115-JLR200 (2005).
32 Keppler, D. Progress in the Molecular Characterization of Hepatobiliary Transporters. *Dig Dis* **35**, 197-202, doi:10.1159/000450911 (2017).
33 Halilbasic, E., Claudel, T. & Trauner, M. Bile acid transporters and regulatory nuclear receptors in the liver and beyond. *Journal of hepatology* **58**, 155-168, doi:10.1016/j.jhep.2012.08.002 (2013).
34 Malinen, M. M. *et al.* Novel in Vitro Method Reveals Drugs That Inhibit Organic Solute Transporter Alpha/Beta (OSTalpha/beta). *Mol Pharm* **16**, 238-246, doi:10.1021/acs.molpharmaceut.8b00966 (2019).
35 Suga, T., Yamaguchi, H., Ogura, J. & Mano, N. Characterization of conjugated and unconjugated bile acid transport via human organic solute transporter alpha/beta. *Biochimica et biophysica acta. Biomembranes* **1861**, 1023-1029, doi:10.1016/j.bbamem.2019.03.003 (2019).
36 Amacher, D. E. & Chalasani, N. Drug-induced hepatic steatosis. *Semin Liver Dis* **34**, 205-214, doi:10.1055/s-0034-1375960 (2014).
37 Bessone, F., Razori, M. V. & Roma, M. G. Molecular pathways of nonalcoholic fatty liver disease development and progression. *Cell Mol Life Sci* **76**, 99-128, doi:10.1007/s00018-018-2947-0 (2019).
38 Rabinowich, L. & Shibolet, O. Drug Induced Steatohepatitis: An Uncommon Culprit of a Common Disease. *BioMed research international* **2015**, 168905, doi:10.1155/2015/168905 (2015).
39 Grieco, A. *et al.* Fatty liver and drugs. *Eur Rev Med Pharmacol Sci* **9**, 261-263 (2005).

40 Friedman, S. L., Neuschwander-Tetri, B. A., Rinella, M. & Sanyal, A. J. Mechanisms of NAFLD development and therapeutic strategies. *Nat Med* **24**, 908-922, doi:10.1038/s41591-018-0104-9 (2018).
41 Amacher, D. E. Strategies for the early detection of drug-induced hepatic steatosis in preclinical drug safety evaluation studies. *Toxicology* **279**, 10-18, doi:10.1016/j.tox.2010.10.006 (2011).
42 Moya, M., Gomez-Lechon, M. J., Castell, J. V. & Jover, R. Enhanced steatosis by nuclear receptor ligands: a study in cultured human hepatocytes and hepatoma cells with a characterized nuclear receptor expression profile. *Chem Biol Interact* **184**, 376-387, doi:10.1016/j.cbi.2010.01.008 (2010).
43 Antherieu, S., Rogue, A., Fromenty, B., Guillouzo, A. & Robin, M. A. Induction of vesicular steatosis by amiodarone and tetracycline is associated with up-regulation of lipogenic genes in HepaRG cells. *Hepatology* **53**, 1895-1905, doi:10.1002/hep.24290 (2011).
44 Donato, M. T., Tolosa, L., Jimenez, N., Castell, J. V. & Gomez-Lechon, M. J. High-content imaging technology for the evaluation of drug-induced steatosis using a multiparametric cell-based assay. *J Biomol Screen* **17**, 394-400, doi:10.1177/1087057111427586 (2012).
45 Rodrigues, R. M. *et al.* In vitro assessment of drug-induced liver steatosis based on human dermal stem cell-derived hepatic cells. *Archives of toxicology* **90**, 677-689, doi:10.1007/s00204-015-1483-z (2016).
46 Madec, S. *et al.* CYP4F3B expression is associated with differentiation of HepaRG human hepatocytes and unaffected by fatty acid overload. *Drug Metab Dispos* **39**, 1987-1996, doi:10.1124/dmd.110.036848 (2011).
47 Tolosa, L. *et al.* Advantageous use of HepaRG cells for the screening and mechanistic study of drug-induced steatosis. *Toxicol Appl Pharmacol* **302**, 1-9, doi:10.1016/j.taap.2016.04.007 (2016).
48 Oseini, A. M. & Sanyal, A. J. Therapies in non-alcoholic steatohepatitis (NASH). *Liver Int* **37 Suppl 1**, 97-103, doi:10.1111/liv.13302 (2017).
49 Rogue, A. *et al.* PPAR agonists reduce steatosis in oleic acid-overloaded HepaRG cells. *Toxicol Appl Pharmacol* **276**, 73-81, doi:10.1016/j.taap.2014.02.001 (2014).
50 Pant, A., Rondini, E. A. & Kocarek, T. A. Farnesol induces fatty acid oxidation and decreases triglyceride accumulation in steatotic HepaRG cells. *Toxicol Appl Pharmacol* **365**, 61-70, doi:10.1016/j.taap.2019.01.003 (2019).
51 Bucher, S. *et al.* Co-exposure to benzo[a]pyrene and ethanol induces a pathological progression of liver steatosis in vitro and in vivo. *Scientific reports* **8**, 5963, doi:10.1038/s41598-018-24403-1 (2018).
52 Feaver, R. E. *et al.* Development of an in vitro human liver system for interrogating nonalcoholic steatohepatitis. *JCI Insight* **1**, e90954, doi:10.1172/jci.insight.90954 (2016).
53 Kanuri, G. & Bergheim, I. In vitro and in vivo models of non-alcoholic fatty liver disease (NAFLD). *International journal of molecular sciences* **14**, 11963-11980, doi:10.3390/ijms140611963 (2013).
54 Chavez-Tapia, N. C., Rosso, N. & Tiribelli, C. In vitro models for the study of non-alcoholic fatty liver disease. *Current medicinal chemistry* **18**, 1079-1084 (2011).
55 Dambach, D. M., Andrews, B. A. & Moulin, F. New technologies and screening strategies for hepatotoxicity: use of in vitro models. *Toxicologic pathology* **33**, 17-26, doi:10.1080/01926230590522284 (2005).
56 Wobser, H. *et al.* Lipid accumulation in hepatocytes induces fibrogenic activation of hepatic stellate cells. *Cell research* **19**, 996-1005, doi:10.1038/cr.2009.73 (2009).
57 Filozof, C., Goldstein, B. J., Williams, R. N. & Sanyal, A. Non-Alcoholic Steatohepatitis: Limited Available Treatment Options but Promising Drugs in Development and Recent Progress Towards a Regulatory Approval Pathway. *Drugs* **75**, 1373-1392, doi:10.1007/s40265-015-0437-3 (2015).

58 Gorden, D. L. *et al.* Biomarkers of NAFLD progression: a lipidomics approach to an epidemic. *Journal of lipid research* **56**, 722-736, doi:10.1194/jlr.P056002 (2015).
59 Angrish, M. M. *et al.* Editor's Highlight: Mechanistic Toxicity Tests Based on an Adverse Outcome Pathway Network for Hepatic Steatosis. *Toxicol Sci* **159**, 159-169, doi:10.1093/toxsci/kfx121 (2017).
60 International Transporter, C. *et al.* Membrane transporters in drug development. *Nature reviews. Drug discovery* **9**, 215-236, doi:10.1038/nrd3028 (2010).
61 Dragovic, S. *et al.* Evidence-based selection of training compounds for use in the mechanism-based integrated prediction of drug-induced liver injury in man. *Archives of toxicology* **90**, 2979-3003, doi:10.1007/s00204-016-1845-1 (2016).
62 Giacomini, K. M. & Huang, S. M. Transporters in drug development and clinical pharmacology. *Clin Pharmacol Ther* **94**, 3-9, doi:10.1038/clpt.2013.86 (2013).
63 Obaidat, A., Roth, M. & Hagenbuch, B. The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. *Annu Rev Pharmacol Toxicol* **52**, 135-151, doi:10.1146/annurev-pharmtox-010510-100556 (2012).
64 Dawson, S., Stahl, S., Paul, N., Barber, J. & Kenna, J. G. In vitro inhibition of the bile salt export pump correlates with risk of cholestatic drug-induced liver injury in humans. *Drug Metab Dispos* **40**, 130-138, doi:10.1124/dmd.111.040758 (2012).
65 Pedersen, J. M. *et al.* Early identification of clinically relevant drug interactions with the human bile salt export pump (BSEP/ABCB11). *Toxicol Sci* **136**, 328-343, doi:10.1093/toxsci/kft197 (2013).
66 Mita, S. *et al.* Inhibition of bile acid transport across Na+/taurocholate cotransporting polypeptide (SLC10A1) and bile salt export pump (ABCB 11)-coexpressing LLC-PK1 cells by cholestasis-inducing drugs. *Drug Metab Dispos* **34**, 1575-1581, doi:10.1124/dmd.105.008748 (2006).
67 Kock, K. *et al.* Risk factors for development of cholestatic drug-induced liver injury: inhibition of hepatic basolateral bile acid transporters multidrug resistance-associated proteins 3 and 4. *Drug Metab Dispos* **42**, 665-674, doi:10.1124/dmd.113.054304 (2014).
68 Jemnitz, K., Veres, Z. & Vereczkey, L. Contribution of high basolateral bile salt efflux to the lack of hepatotoxicity in rat in response to drugs inducing cholestasis in human. *Toxicol Sci* **115**, 80-88, doi:10.1093/toxsci/kfq044 (2010).
69 Brouwer, K. L. *et al.* In vitro methods to support transporter evaluation in drug discovery and development. *Clin Pharmacol Ther* **94**, 95-112, doi:10.1038/clpt.2013.81 (2013).
70 Swift, B., Pfeifer, N. D. & Brouwer, K. L. Sandwich-cultured hepatocytes: an in vitro model to evaluate hepatobiliary transporter-based drug interactions and hepatotoxicity. *Drug metabolism reviews* **42**, 446-471, doi:10.3109/03602530903491881 (2010).
71 Trauner, M. & Boyer, J. L. Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev* **83**, 633-671, doi:10.1152/physrev.00027.2002 (2003).
72 Kostrubsky, S. E. *et al.* Inhibition of hepatobiliary transport as a predictive method for clinical hepatotoxicity of nefazodone. *Toxicol Sci* **90**, 451-459, doi:10.1093/toxsci/kfj095 (2006).
73 US Food and Drug Administration. *Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers*, <http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling> (2017).
74 Longo, D. M. *et al.* Quantitative Systems Toxicology Analysis of In Vitro Mechanistic Assays Reveals Importance of Bile Acid Accumulation and Mitochondrial Dysfunction in TAK-875-Induced Liver Injury. *Toxicol Sci* **167**, 458-467, doi:10.1093/toxsci/kfy253 (2019).
75 Labbe, G., Pessayre, D. & Fromenty, B. Drug-induced liver injury through mitochondrial dysfunction: mechanisms and detection during preclinical safety studies. *Fundamental & clinical pharmacology* **22**, 335-353, doi:10.1111/j.1472-8206.2008.00608.x (2008).

76 Woldemichael, T. & Rosania, G. R. The physiological determinants of drug-induced lysosomal stress resistance. *PloS one* **12**, e0187627, doi:10.1371/journal.pone.0187627 (2017).
77 Alakoskela, J. M., Vitovic, P. & Kinnunen, P. K. Screening for the drug-phospholipid interaction: correlation to phospholipidosis. *ChemMedChem* **4**, 1224-1251, doi:10.1002/cmdc.200900052 (2009).
78 Sakatis, M. Z. *et al.* Preclinical strategy to reduce clinical hepatotoxicity using in vitro bioactivation data for >200 compounds. *Chem Res Toxicol* **25**, 2067-2082, doi:10.1021/tx300075j (2012).
79 Schadt, S. *et al.* Minimizing DILI risk in drug discovery - A screening tool for drug candidates. *Toxicology in vitro : an international journal published in association with BIBRA* **30**, 429-437, doi:10.1016/j.tiv.2015.09.019 (2015).
80 Thompson, R. A. *et al.* In vitro approach to assess the potential for risk of idiosyncratic adverse reactions caused by candidate drugs. *Chem Res Toxicol* **25**, 1616-1632, doi:10.1021/tx300091x (2012).
81 Goldring, C. E. *et al.* Activation of hepatic Nrf2 in vivo by acetaminophen in CD-1 mice. *Hepatology* **39**, 1267-1276, doi:10.1002/hep.20183 (2004).
82 Copple, I. M. *et al.* The hepatotoxic metabolite of acetaminophen directly activates the Keap1-Nrf2 cell defense system. *Hepatology* **48**, 1292-1301, doi:10.1002/hep.22472 (2008).
83 Ramachandran, A. & Jaeschke, H. Oxidative Stress and Acute Hepatic Injury. *Curr Opin Toxicol* **7**, 17-21, doi:10.1016/j.cotox.2017.10.011 (2018).
84 Dara, L., Ji, C. & Kaplowitz, N. The contribution of endoplasmic reticulum stress to liver diseases. *Hepatology* **53**, 1752-1763, doi:10.1002/hep.24279 (2011).
85 Fontana, R. J. *et al.* Drug-Induced Liver Injury Network (DILIN) prospective study: rationale, design and conduct. *Drug safety* **32**, 55-68, doi:10.2165/00002018-200932010-00005 (2009).
86 Chalasani, N. *et al.* Features and Outcomes of 899 Patients With Drug-Induced Liver Injury: The DILIN Prospective Study. *Gastroenterology* **148**, 1340-1352 e1347, doi:10.1053/j.gastro.2015.03.006 (2015).
87 Nagy, Z. *et al.* The human SPT20-containing SAGA complex plays a direct role in the regulation of endoplasmic reticulum stress-induced genes. *Mol Cell Biol* **29**, 1649-1660, doi:10.1128/MCB.01076-08 (2009).
88 Cribb, A. E., Peyrou, M., Muruganandan, S. & Schneider, L. The endoplasmic reticulum in xenobiotic toxicity. *Drug metabolism reviews* **37**, 405-442, doi:10.1080/03602530500205135 (2005).
89 Tsutsumi, S. *et al.* Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. *Cell Death Differ* **11**, 1009-1016, doi:10.1038/sj.cdd.4401436 (2004).
90 Ji, C., Mehrian-Shai, R., Chan, C., Hsu, Y. H. & Kaplowitz, N. Role of CHOP in hepatic apoptosis in the murine model of intragastric ethanol feeding. *Alcohol Clin Exp Res* **29**, 1496-1503 (2005).
91 Li, J., Lee, B. & Lee, A. S. Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. *J Biol Chem* **281**, 7260-7270, doi:10.1074/jbc.M509868200 (2006).
92 Cullinan, S. B. & Diehl, J. A. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J Biol Chem* **279**, 20108-20117, doi:10.1074/jbc.M314219200 (2004).