Evaluation of the zebrafish embryo as an alternative model for hepatotoxicity testing

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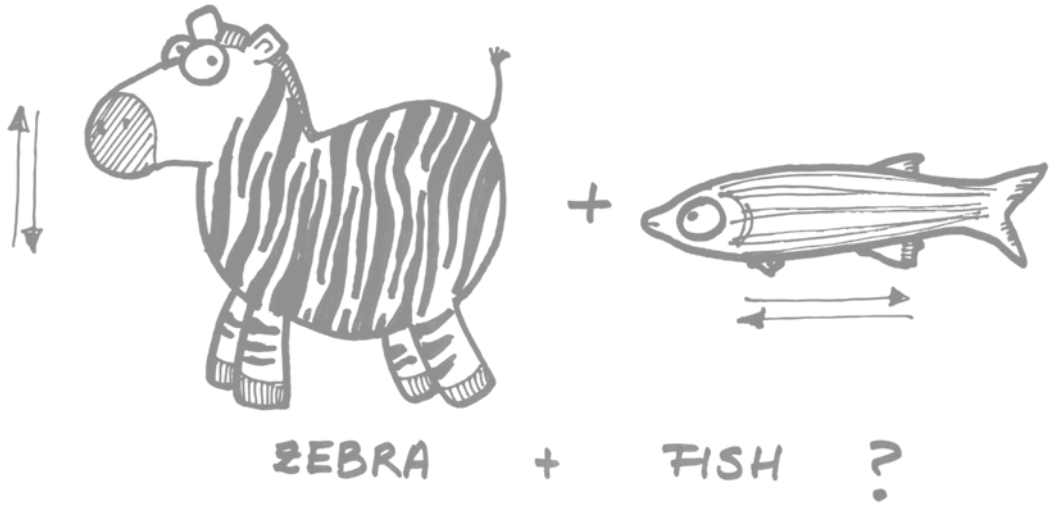

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General Introduction

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Hepatotoxicity screening on *in vitro* models and the role of Omics

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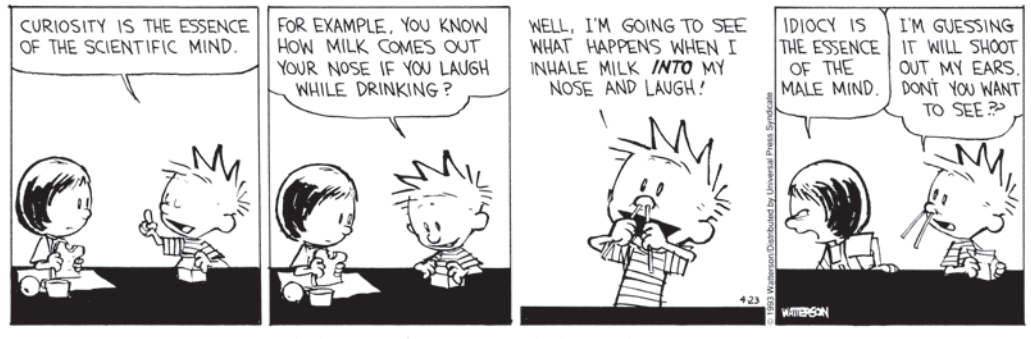

Humans are exposed throughout their entire life to multitude of diverse xenobiotic compounds, such as industrial chemicals and pharmaceuticals. This exposure is either intended, *e.g.* when prescribed as drug, or unintended, *e.g.* as contaminants in food or the environment. Xenobiotic exposure may pose health risks; when a hazardous concentration is reached adverse biological effects may occur. Toxicological research aims to identify these adverse health effects (hazard) and compare hazard threshold levels with real-life exposure levels to estimate the risk associated with actual exposure. The liver is one of the major targets of toxicological effects, because it is the first organ to be encountered after absorption from the gastro-intestinal tract, and it is therefore important to assess hepatotoxic properties of xenobiotics that humans are exposed to[1]. Traditionally hepatotoxicity is studied mainly in animal studies, but observations in animal exposure studies appeared to be not fully predictive for the effects in humans[2]. The predictivity of animal studies is limited mainly by physiological differences between species, leading to differences in toxicokinetic fate, *i.e.* absorption, distribution, metabolism, and excretion of the xenobiotic, and differences in sensitivity of target cells[2]. These differences are the cause of uncertainties associated with extrapolation from animal to man and from short-term to long-term exposure. Thus, effects that occur in humans may remain undetected in animals. Therefore, there is an imperative need to improve toxicological testing, and develop strategies that do not rely on traditional testing in rodents. Development of new screening methods should build on new *in silico* and *in vitro* methods, which can also help to implement the 3R strategy to Replace, Reduce and Refine the use of animals. An approach is to analyze the molecular mechanisms that underlay toxicological effects facilitating comparison across models and species, through the use of 'omics-technologies'. A promising alternative testing model is the zebrafish embryo, which we evaluate here for its application in hepatotoxicity testing through the use of several toxicogenomics tools.

The liver as critical target organ for toxicity

The liver is an important target for adverse effects of xenobiotics, as it is the first organ to pass after enteric absorption, positioned between the gastro-intestinal tract and systemic circulation. The liver has metabolic activity, which allows deactivation or detoxication of xenobiotics, thereby facilitating their excretion. This process is called biotransformation, which is a multistep process including Phase I and Phase II metabolism. Phase I metabolism can be divided into by three steps including modification, oxidation and reduction preparing the xenobiotics for Phase II metabolism. In Phase II metabolism, the xenobiotics are conjugated resulting in clearance[3]. Due to the position of the liver and its metabolic function, it serves as a 'first pass' organ resulting in relatively high concentrations of xenobiotics that depend on oral uptake. This may lead to toxic activity of the xenobiotic and thereby to the induction of liver injury[1,4–6].

The liver consists of several cell types. Hepatocytes are the most predominant cell type, covering approximately 60% of the liver and carrying out most of the metabolic functions.

Furthermore, they express a wide scale of circulating plasma proteins including albumin[1,3,7]. Less than 40% of the liver is comprised of sinusoid endothelial cells, Kuppfer cells, and stellate cells[1,3,7]. Sinusoidal endothelial cells are the largest group of non-parenchymal cells compromising around 19% of the total liver cell mass. These cells are a physical barrier for blood circulation allowing the interchange of nutrients, toxicants, hormones and other molecules from the plasma to the hepatotocytes. In addition, these cells play an important role in the inflammatory reactions[7,8]. Kuppfer cells compromise 15% of the total liver mass and in healthy situations these cells are present as resting macrophages. However, upon activation, for example due to liver damage, these cells will excrete pro-inflammatory cytokines potentially aggravating the damage in the hepatocytes and promoting the pro-apoptotic responses[7,9,10]. Stellate cells are the smallest non-parenchymal cells in the liver, only compromising 6% of the total liver mass. Their function in the liver is to store vitamin A, however, under toxic stress these cells have various other functions including the secretion of chemokines and activation of natural killer cells[7]. This shows that hepatotoxicity may occur through interaction of a xenobiotic with various cell types within the liver. Depending on the toxic functions and pathways that are activated, xenobiotic interaction may lead to a variety of liver pathologies including cholestasis (intra- or extracellular bile accumulation), steatosis (fatty liver), and necrosis (cell death)[1,4,6,11]. These specific toxic phenotypes are explained below in more detail, together with actions of some reference toxicants.

Cholestasis. Cholestasis is one of the main xenobiotic-induced liver pathologies resulting from either impairment in bile secretion or obstruction in bile flow[12–14]. Bile and bile acids are not only important for the digestion of fat from the diet, but also for the clearance of xenobiotics from the liver[1]. Cholestasis occurs when there is either an impaired bile transport or an inhibition of bile transporters[12]. Several drugs are known to induce cholestasis via different molecular mechanisms.

First, 17α-ethinylestradiol (EE2), a synthetic estrogen derivative, is used in combined oral contraceptive pills, which reduces bile flow by inhibiting bile acid active transporters at the hepatic canalicular membrane. EE2 also increases the permeability of cells in the bile ductules elevating the reabsorption of bile components[1,15].

Second, cyclosporine A (CsA) is widely used as an immunosuppressant in organ transplantation to avoid rejection. In humans, it induces cholestasis by interacting with and inhibiting the bile transporter, also known as the bile salt export pump (BSEP)[12].

Third, chlorpromazine (CPZ) is used to treat acute and chronic psychoses[16]. CPZ induces cholestasis by directly acting at the intracellular membrane thereby decreasing bile acid-dependent bile flow resulting in a loss of bile acids via defective bile canaliculi. In addition, the Na^+/K^+ pump and hydroxylation of cholesterol to bile acids are inhibited by CPZ[16].

Furthermore, all of these compounds are frequently used in *in vivo* and *in vitro* studies to induce cholestasis[1,12,15–17].

Several studies have shown that these model compounds are able to induce cholestasis in a wide variety of models including *in vivo* mouse and rats, and also *in vitro* mouse liver slices, primary hepatocytes and many more.

Steatosis. Fat metabolism is another important function of the liver, specifically through fatty acid synthesis and promoting lipid circulation by lipoprotein synthesis[1,3]. Changes in fat metabolism may result from an increase in uptake of lipids, elevation of the *de novo* synthesis of fatty acids, an impairment of lipoprotein synthesis/secretion, and/or a reduction in fatty acid oxidation[19]. The accumulation of cytoplasmic fatty acids in the form of lipid droplets is known as steatosis[11,19]. Disrupted fat metabolism is manifested as

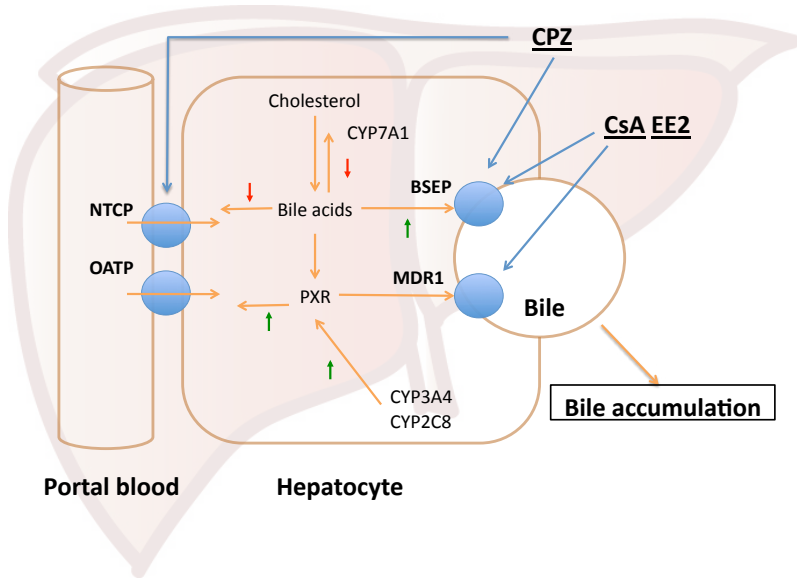

Figure 1 Toxicity pathway of cholestasis.
Cyclosporine A (CsA) and 17α-ethinylestradiol (EE2) block the transporters regulating bile flow from hepatocyte to bile duct (bile salt pump (BSEP) and multidrug resistance protein 1 (MDR1)), resulting in accumulation of bile in the hepatocyte. Chlorpromazine (CPZ) and EE2 act on the uptake transporters Na^+-dependent taurocholic cotransporting polypeptide (NTCP) and organic anion-transporting polypeptide (OATP), but also inhibit the excretion transporters BSEP and MDR1. This inhibition of bile acid excretion leads to accumulation of intracellular bile acids. Cholesterol is known to be converted to bile acids by cytochrome P450 CYP7A1 into bile acids, which in turn activate pregnane X receptor (PXR) leading to an increase in expression of MDR1 and OATP (Adapted from Jansen *et al.,* and Zimmerman[1,18])

either microvesicular (multiple small vacuoles in the hepatocytes) or macrovesicular steatosis (larger vacuoles that dislocate the nucleus from the center of the cell). Several drugs and chemicals are known to induce steatosis in humans including amiodarone (AM), tetracycline (TET), and valproic acid (VPA).

Amiodarone (2-butyl-3-benzofuranyl 4-[2-(diethylamino)etoxcy]-3,5-diiodophenyl ketone; AM) is a frequently prescribed antiarrythmic agent, to prevent atrial fibrillation. AM induces steatosis in humans, and also in rodent models, probably through inhibiting mitochondrial β-oxidation of long-, medium-, and short-chain fatty acids. Furthermore, this compound inhibits carnitine palmitoyltransferase-I (CPTI)-dependent transport of long-chain fatty acids across the mitochondrial membrane [20] resulting in fatty acid accumulation in hepatocytes.

Tetracycline is a frequently used antibiotic, to fight infections with both gram-positive and gram-negative bacteria. Tetracycline induces microvesicular steatosis in the liver by down-

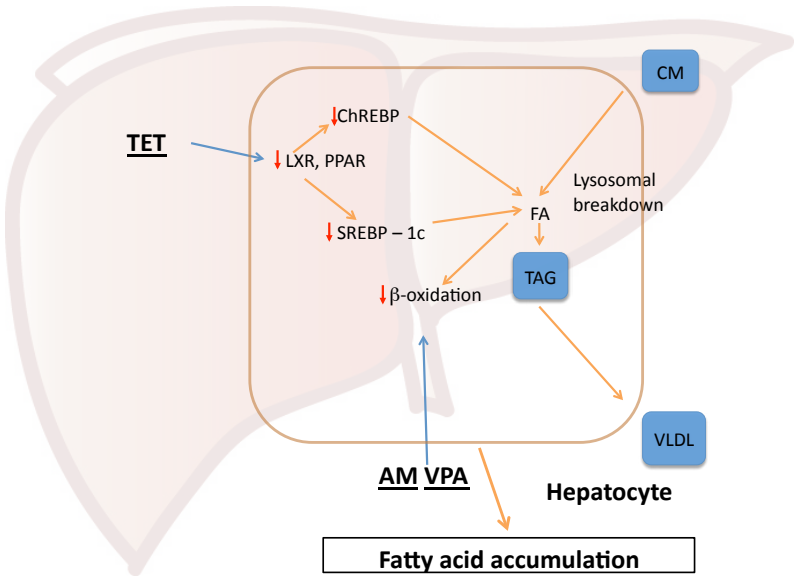

Figure 2 Toxicity pathway of steatosis.
Sources of fatty acids (FA) are triacylglycerol (TAG) and chylomicrons (CM). TAG is exported out of the hepatocytes in the form of very low density lipoproteins (VLDL). Tetracycline (TET) induces steatosis by downregulation of nuclear receptors including liver X receptor (LXR) and peroxisome proliferator receptors (PPARs) and their target genes sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate response element-binding protein (chREBP); this in turn leads to an increase in fatty acids (FA) level. Valproic acid (VPA) and amiodarone (AM) inhibit the β-oxidation cycle resulting in decreased metabolism of fatty acids, which then accumulate in the hepatocyte (Adapted from Matsuzaka and Shimano (2011)[26])

regulating the peroxisome proliferator activated receptors (PPARs), which are involved in the development of steatosis through their important role in lipid deposition[21,22].

Valproic acid (VPA) is an antiepileptic agent, used to treat specific types of seizures. VPA hepatotoxicity is believed to be mediated by either an inhibitory effect on the mitochondrial β-oxidation pathway, which gives rise to microvesicular steatosis, or by metabolic effects which promote weight gain and insulin resistance giving rise to macrovesicular steatosis and steatohepatitis[23].

Studies have shown that VPA and TET induce steatosis in mice through an increase in cholesterol and triglyceride biosynthesis and a decrease in fatty acid oxidation[22,24,25]. Furthermore, AMD has been found to promote steatosis in mice through altering lipid homeostasis in the liver[20].

Necrosis. Necrosis, or cell death, is caused by severe metabolic perturbations resulting in ATP depletion. Diminished levels of ATP breakdown and cytoskeleton integrity, which results in the characteristic morphology of cell swelling accompanied by membrane blebbing[11]. Furthermore, mitochondrial permeability transition (MPT) plays an important role in necrosis. MPT is known to lead to either necrosis or apoptosis after mitochondrial oxidative stress[7,27]. Known drugs or compounds that induce necrosis are acetaminophen (APAP), paraquat (PQ) and thioacetamide (TAA).

Acetaminophen (paracetamol; APAP) is an analgesic and antipyretic, which is considered safe at therapeutic doses. In this setting, APAP is inactivated by sulfation and glucuronidation. However, when therapeutic doses are exceeded, APAP causes hepatic damage and may eventually lead to liver failure. APAP toxicity is due to its active metabolic conversion by CYP2E1 to the highly toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). In normal situations, NAPQI is conjugated to reduced glutathione and is excreted the bile. However, when therapeutic doses are exceeded, NAPQI concentrations increase due to saturation of the sulfation and glucuronidation pathways. Hepatotoxicity occurs when NAPQI binds to macromolecules, for example in the mitochondria, which leads to mitochondrial dysfunction and DNA damage[11,28,29].

Paraquat (PQ), a contact herbicide, is a dehydrate and defoliant used for harvesting cotton, potatoes, and soy beans. PQ is a bipyridyl derivative, which can be taken up by the redox cycle leading to the release of active oxygen radicals, which in turn can induce damage to the hepatocyte[30].

Thioacetamide (TAA) was formerly used as a fungicide. The underlying mechanism of hepatotoxicity of this compound appears to be its metabolism via CYP2E1. TAA is metabolized to di-S-oxide (CH3-C(SO2)NH2), which is a reactive intermediate that covalently binds to hepatic macromolecules resulting in necrosis[31]

Hepatotoxicity testing

Traditionally, rodent studies are used to predict the hepatotoxic effects of xenobiotics. As introduced above, an important scientific argument against the use of rodent studies is that the obtained effects are not always fully predictive for the effects in humans[2]. In general, animal studies are helpful to identify human toxicants, but particularly in the case of hepatotoxicity, the identification remains low due to a poor correlation between human and animals[2,7,11]. Missed hepatotoxic effects in traditional screening assays is a major cause of the high attrition rate in drug development and therewith associated with high cost[1,4,6]. From the perspective of animal welfare it is unfavorable that animals need to be exposed to high doses of compounds for extended periods of time. Therefore, there is an imperative need to develop alternative models to assay the hepatotoxic potential of xenobiotics. Currently, multiple alternatives are available for hepatotoxicity testing, including single liver cell systems, liver slices and whole organisms[7,11].

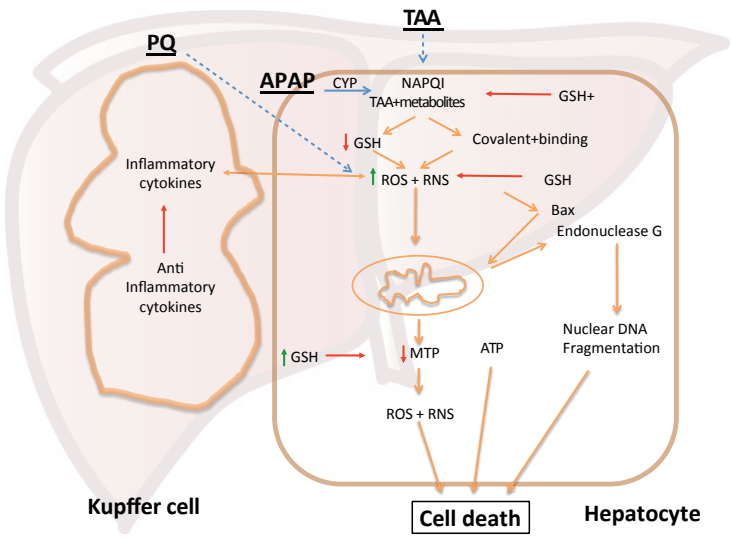

Figure 3 Toxicity pathway of hepatic necrosis.
Necrosis is the consequence of induction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which eventually leads to a decrease of glutathione (GSH). GSH depletion eventually leads excess of ROS/RNS, oxidative damage and hence to cell death. Acetaminophen (APAP) induces ROS/RNS through its toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) to which it is converted by cytochrome P450 (CYP) enzymes. Thioacetamide (TAA) is also metabolised by CYP enzymes to a reactive metabolite which increases the ROS production. Mechanism of paraquat (PQ) is not completely understood, but it probably also induces an increase in ROS production. One important damaging action of ROS/RNS is the decrease of mitochondrial permeability transition (MPT) leading to loss of ATP, which contributes to cell death. Kupffer cells secrete cytokines which may aggravate ROS/RNS production leading to cell death (Adapted from Hinson *et al.* (2010) and Jaeschke et al. (2012) [32,33])

The *in vitro* cell system of primary hepatocytes is one of the most prominent models for studying hepatotoxicity and allows for easy interspecies comparison[7,11,37]. Generally, these cells maintain expression of Phase I and Phase II metabolic enzyme activities for longer periods of time, however, they are not fully representative of the *in vivo* liver due to a loss of original cell shape and a deviating gene expression response to xenobiotics[7,11,37].
Alternatively, several permanent, immortalized cell models are available for toxicity screening. Of these, the frequently used human hepatocarcinoma derived HepG2 cell line has a long history in xenobiotics testing. The advantage of this cell line is that it provides an infinite reservoir of identical cells[7,11,37]. HepG2 cells show activity of several Phase I and Phase II metabolic enzymes, however, not all metabolizing enzymes are present and otherwise the expression level is generally much lower than in the *in vivo* situation or primary human hepatocyes, leading to a different response after compound exposure[7,11,37]. However, recent advances allowed to improve the expression of Phase I enzymes in the HepG2 cells constructing Adv-HepG2 cell line[38].

Table 1 Reference compounds and phenotypes.

Phenotype	Compound	Proposed mechanism of action	References
Cholestasis	17α-ethinylestradiol	Inhibition of bile acid transporters	Wagner *et al.,*[12,34]
	Cyclosporine A	Inhibition of BSEP	Antherieu *et al.,*[12]
	Chlorpromazine	Decreases bile acid dependent bile flow	Yamamoto *et al,.*[16]
Steatosis	Amiodarone	Inhibition of mitochondrial β-oxidation and CPTI dependent transport	Anderson and Borlak[19]
	Tetracycline	Down-regulation of PPARs which play a role in lipid desposition	Yin *et al.,*[21,22]
	Valproic acid	Inhibition of mitochondrial β-oxidation	Silva *et al.,*[23]
Necrosis	Acetaminophen	CYP2E1 metabolism releasing NAPQI (toxic metabolite) inducing mitochondrial dysfunction	McGill *et al.*[35]
	Paraquat	Taken up by redox cycle inducing release of active oxygen radicals	Burk *et al.,* and Bus and Gordon[30,36]
	Thioacetamide	Metabolism via CYP2E1 resulting in reactive intermediate	Hunter *et al.,*[31]

An overview of the phenotypes of interest in this thesis and the associated reference compounds

Other hepatocyte cell lines are also used, and HepaRG is an example of a recently isolated and cultured hepatoma cell line. Several studies have shown the presence of xenobiotic metabolizing enzymes, much similar to the primary hepatocytes, enabling them to be suitable in assessing the xenobiotic compounds hepatotoxicity potential[39–41]. Another recent advancement is use of 3D cell culture systems. HepG2 cells cultured in 3D stopped proliferating, self-organize and differentiate to form multiple polarized spheroids acquiring hepatotocyte functions including glycogen storage, transportation of bile salts and the formation of structures resembling bile canaliculi[42].

The isolated perfused liver and liver slices are considered to be the system closest related to the *in vivo* condition, because these systems maintain 3D-architecture including bile flow and cell-cell interactions. Furthermore, these systems contain the non-parenchymal cells which have an important role in mediating the toxicity[7,43]. A downside of these systems is the relative short period of viability, limiting these models to test only compounds which are expected to have an effect within a few hours[7,43].

Toxicological science is in need of a model that combines these advantages and overcomes the drawbacks of these systems. In this respect, the zebrafish embryo could be considered as a complementary alternative model to screen for potential hepatotoxic compounds and to investigate the mechanisms by which chemicals induce liver injury[44–49].

The zebrafish embryo

The zebrafish (*Danio rerio*) is a diploid minnow of the teleost family Cyprinidae and was introduced several decades ago to study development and neurobiology. Thereof, the zebrafish emerged as a powerful model to study human disease and the effects of chemical exposure[45,50–53]. Several features make the zebrafish embryo attractive as an experimental model, among which are the potential for use in a high-throughput screening setting and the small size of both the adult zebrafish and zebrafish embryo. Furthermore, maintenance in large stocks is easy due to their high fecundity. In the embryo, direct observation and experimental manipulation of tissue and organs is relatively simple due to the transparency and the rapid development. As a result of the rapid development, most organs become fully functional between three and five days post fertilization[50]. In addition, during the earliest developmental stages (until independent, free-feeding) the zebrafish embryo is not a protected animal under present European legislation (Directive 2010/63/EU) and can therefore be considered as an alternative to animal experimentation[54]. A fully functional liver is present in the zebrafish embryo by three days post fertilization, at which time it is perfused with blood. In terms of xenobiotic biotransformation capacity, zebrafish embryos express 94 cyps, which occur in the same 18 gene families as in humans, and most of them are human cyp orthologs. The cyp1-4 families are mainly involved in metabolizing xenobiotics, similar to humans. Most important is the presence of the human CYP3A4 ortholog, *cyp3a65*, because this cyp catalyzes the majority of known drug-metabolizing reactions[46,55,56].

The zebrafish embryo and hepatotoxicity testing. While most of the important cytochromes are present in the zebrafish embryo, this model might be suitable to detect hepatotoxicity. Some studies showed that zebrafish embryos are suitable to detect hepatotoxicants. In a study by Jones *et al*., the whole zebrafish embryo model was evaluated through morphological endpoints after exposure to a set of compounds, including falsely classified DILI drugs by HepG2 cells. In this study, the whole zebrafish embryo model successfully detected hepatotoxicants with higher specificity than the HepG2 cells[49]. In another study, Amali *et al*., carried out histopathological, molecular and biochemical analysis in zebrafish embryos exposed to a single dose of thioacetamide and showed that the whole zebrafish embryo model is suitable to detect steatohepatitis[57]. These studies indicate the potential of the zebrafish embryo as a model for hepatotoxicity testing at the level of the phenotype, but they provide limited insights into the underlying mechanisms of toxicity[58]. Toxicogenomics is hypothesized to contribute understanding of the underlying mechanisms of hepatotoxicity and discrimination between various hepatotoxic phenotypes, such as cholestasis, steatosis and necrosis.

Toxicogenomics

For drug development, understanding the mechanism of toxicity is imperative, not only to compare between reference toxicants and test compounds, but also to differentiate between toxic and targeted effects, and to develop relevant markers of toxicity. The method to analyze and describe mechanisms of toxicity comprises a set of tools, which generally involves toxicogenomics[61].

Traditionally, hepatotoxic properties of chemicals and compounds are determined using rodent studies which rely on different toxicity endpoints including body and organ weight, death rate, serum toxicity biomarkers and histopathological changes[11]. Although these phenotypical endpoints give an indication of toxicity, they do, however, not provide insight into the underlying molecular mechanisms of hepatotoxicity. By applying molecular techniques, such as transcriptomics, proteomics and next generation sequencing, in the toxicology setting will provide an insight into the underlying molecular mechanisms[61–63]. The assumption is that changes in for example toxicity markers and histopathology are preceded by changes on gene and/or protein level after exposure to compounds. The changes in genes and/or proteins will provide us with a more detailed view on the onset of the toxic events.

Transcriptomics analysis can be implemented in different ways including the use of micro arrays and the more recent technique next generation sequencing. Next generation sequencing provides multiple advantages over the traditionally used mRNA microarrays. In contrast to microarrays, NGS does not rely on the probe design and probe selection, thus enabling detection of non-predefined transcripts, including diverse splicing variants of a single gene.

Another advantage of the implementation of molecular techniques in toxicology research is that it allows the researcher to extrapolate the findings to other organisms. In the area of

Table 2 Testing models for hepatotoxicity.

Model	Strength	Weakness
Liver slices	Fairly high throughput Retain liver structure; contain all cell types Functional bile canaliculi Good *in vitro/in vivo* correlation of xenobiotic metabolism Maintain zone-specific CYP activity; maintain toxicity mechanisms Stability of phase II enzymes, albumin production, gluconeogenesisis for 20-96 hours	Cellular necrosis after 48-72 hours CYP levels quickly decrease (6-72 hours) Poor concordance with liver for intrinsic clearance rates and *Km* values Diffusion-limited gradient of the exposure to a compound across the slice
Immortalized hepatic cell lines	Throughput depends on application Unlimited amount of cells available Some cell lines retain expression of many liver-specific functions	Lacking most phenotypic and functional characteristics of the liver tissue
Primary hepatocyte suspensions	Fairly high throughput Better estimate of internal clearance than monolayer cultures Retain high level of enzyme functionality (close to *in vivo*)	Loss of cell-cell interactions Loss of cell-matrix interactions Limited viability allows short-term use only (<4 hours) Loss of cellular polarity No bile canaliculi
Primary hepatocyte cultures	Throughput depends on the application Cells can re-establish cell-cell interactions and polarity Cells retain some morphology and liver specific functionality in short-term cultures (2-4 days) Induction/inhibition of the metabolizing enzymes can be studied	Inability to maintain *in vivo* liver-specific functionality for long-term culture Quick reduction in functionality and phenotype (24-48 hours) May not develop functional bile canaliculi
Primary hepatocyte cultures Sandwich	Throughput depends on the application Restores *in vivo* polygonal morphology Better maintains liver-specific functionality Prevents loss of viability Functional bile canaliculi	Loss of liver-specific functionality, morphology and phenotype in long-term cultures Decline in metabolic enzyme activity in long-term culture
HepaRG cell line	Functional activities remain stable for long time period Enzyme activity can be modulated allowing to more closely mimic inter-individual variations in xenobiotics metabolizing enzymes	Loss of cell-cell interactions Loss of cell-matrix interactions No bile canaliculi
Zebrafish embryo	*Ex vivo* development and optical clarity of the embryo Embryogenesis is completed at 72 hpf, liver is fully functional Biological complexity of *in vivo* models Easy to handle and cheap Genome of the zebrafish is sequenced. Molecular biology tools are available for genetic manipulation High-throughput screening	Developmental stage of the hepatocytes Extrapolation to human First pass effect Teleost fish possess two copies of many mammalian genes due to gene duplication events

Adapted from Delvecchio *et al.* (2011), Guillouzo (1998) and Soldatow *et al.* (2013)[37,59,60]

hepatotoxicity, several studies have shown that toxicogenomics can be applied to investigate the underlying molecular mechanisms leading to toxicity[61,64]. Mostly, these toxicogenomics techniques are applied in traditionally used models for hepatotoxicity including mice, rats and *in vitro* models[64,65]. With regards to the ZFE, several recent studies show that toxicogenomics can be applied to investigate the underlying mechanisms of toxicity. For example, the study of Yang *et al.*, showed that the ZFE allows for the robust identification of genes to distinguish between toxicants[53]. More specifically, several transcriptomics studies identified the underlying mechanisms of teratogenicity[66–70] of compounds showing the suitability of applying toxicogenomics in the ZFE. Few studies have been performed with toxicogenomics in ZFE with regard to hepatotoxicity[46,49,57]. Nevertheless, these transcriptomics studies show that the zebrafish embryo model is suitable for detecting differences between chemical classes illustrating the possibilities of this model. Several proteomics studies have been performed in other models, such as *in vitro* and *in vivo* systems, to gain further insight into the differences in proteomes after exposure to hepatotoxicants[17,71,72]. These studies show the potential of this technique to assess the molecular mechanisms of hepatotoxicity.

With regard to the ZFE, proteomics studies have been performed, but are mainly targeted at the differences between proteomes during different life-stages. Less is known about

the changes occurring after exposure to hepatotoxicants. The most practical way to study hepatic gene expression in ZFE is to use whole ZFE RNA extract for analysis, instead of liver extracts, but this may result in masking of signals or lowering the signal/noise ratio of the regulated genes. Although available, sophisticated methods such as micro-dissection to study hepatic gene expression in small organism hinder the throughput of the system[73].

Toxicogenomics data analysis. The amount of data generated by applying these molecular techniques in such a toxicology experiment is enormous, and it is therefore imperative that the proper methods are applied which might translate to biological conclusions. For all the "omics-techniques" analysis, the data needs to be corrected for the background and normalized after the samples are either hybridized to the array (transcriptome) or measured by mass spectrometry (proteome). Background corrections are applied to improve the signal of the measurements, and thus adjust for artifacts, including non-specific hybridizations of the samples on the arrays or peak distributions in proteins[74]. After background correction, the data is routinely log-transformed. This improves the characteristics of the data distribution and enables the use of classical parametric statistics[75,76]. In the next step, the gene expression data is normalized, which corrects for the systematic difference between the arrays. After the preprocessing phase, the analysis of the data is continued by identifying the genes/proteins which differ from the control situation. This is mostly achieved by a selection based on significance level using *p-values*, or false discovery rate (FDR) combined with a fold change (FC). Genes/proteins fitting the selection criteria can be visualized using different methods including biplots, PCA and hierarchical clustering methods. By using these available tools, it is possible to identify the mode-of-action of compounds. In this case, certain groups of compounds are used to establish a 'fingerprint' for certain classes of toxicity. A new compound can thus be identified as toxic based on the basis of overlap based on the fingerprint of the toxicity classes, thereby contributing to the predictive toxicology.
To compare the results from the experiments with the literature, several databases are available, for example Gene Ontology[77], UniProt[78,79], National Center for Biotechnology Information (NCBI)[80], comparative toxicology database[81], and specific for the zebrafish is the Zebrafish Model Organism Database from the Zebrafish Information Network (ZFIN)[82]. Identification of genes which differ from the control situation is not enough to improve the understanding of the molecular mechanism behind the toxic event. Therefore, the genes are tested for overrepresentation in certain biological processes (Gene Ontology[77]) or pathways (KEGG, Wikipathways, MetaCore, Tox-Profiler, DAVID)[83–89]. Also for proteomic expression several databases exist to compare the results to literature. The analysis for proteomics expression can be done using Trans Proteomic Pipeline (TPP) which is a completely open-source analysis method for MS/MS-based proteomics[90]. For example, STITCH, UNIPROT, and again DAVID. STITCH is a resource to explore known and predicted interactions of chemicals and proteins which is based on literature and experiments[91–94].

UniProt (Universal Protein database) provides a freely accessible resource of protein sequence and functional information[78,79].
The implementation of toxicogenomics in the area of hepatotoxicity testing has advanced the understanding of the underlying molecular mechanisms of the different hepatotoxic classes[65]. In addition, this technique allows to extrapolate the findings to the human situation.

Aim and outline of this thesis

Aim of this thesis. The hepatotoxic potential of compounds and chemicals are traditionally assessed using *in vivo* rodent studies. Since these *in vivo* rodents studies are associated with scientific issues (limited predictivity for effects in humans), economical (high costs due to high doses and long experimental periods), and ethical (large numbers of animals to be exposed), there is a need to develop and validate alternative approaches. As such, the zebrafish embryo is presented as an alternative test model that may replace or support the traditional *in vivo* rodent studies. This zebrafish embryo model combines the benefits of an *in vivo* model, namely biological complexity including interactions between tissues and cells[49,54,95], with the advantages of *in vitro* models, that is, the ability for medium to high throughput testing. Therefore, the objective of this thesis is to evaluate the whole zebrafish embryo as an alternative model system to screen for the hepatotoxic potential of compounds using different "-omics" technologies including next-generation sequencing, transcriptomics, and proteomics, thereby improving the prediction for effects in humans. To achieve this objective, a dedicated set of approaches was applied in the studies schematically represented in Fig. 4 and outlined below.

Outline of this thesis. First, we evaluated whether the whole zebrafish embryo can be used for the determination of hepatotoxicity. In **Chapter 2**, we determined whether hepatotoxicity-associated gene expression was detectable in whole zebrafish embryos using next-generation sequencing.
To unravel the underlying molecular mechanisms, we investigated in **Chapter 3** whether gene expression analysis after hepatotoxicant exposure could produce a limited set of informative biomarkers for human hepatotoxicity. To characterize the model even further, we applied proteomics in **Chapter 4**, to strengthen the applicability of the model.
To assess the additonal value of the zebrafish embryo as a model for hepatotoxicity, we performed a meta-analysis based on gene-expression changes including different species in **Chapter 5**.
A general discussion and possible future perspectives are discussed in **Chapter 6** including discussion on the implementation of a better toxicokinetics characterization of the model, while a summary is provided in **Chapter 7**.

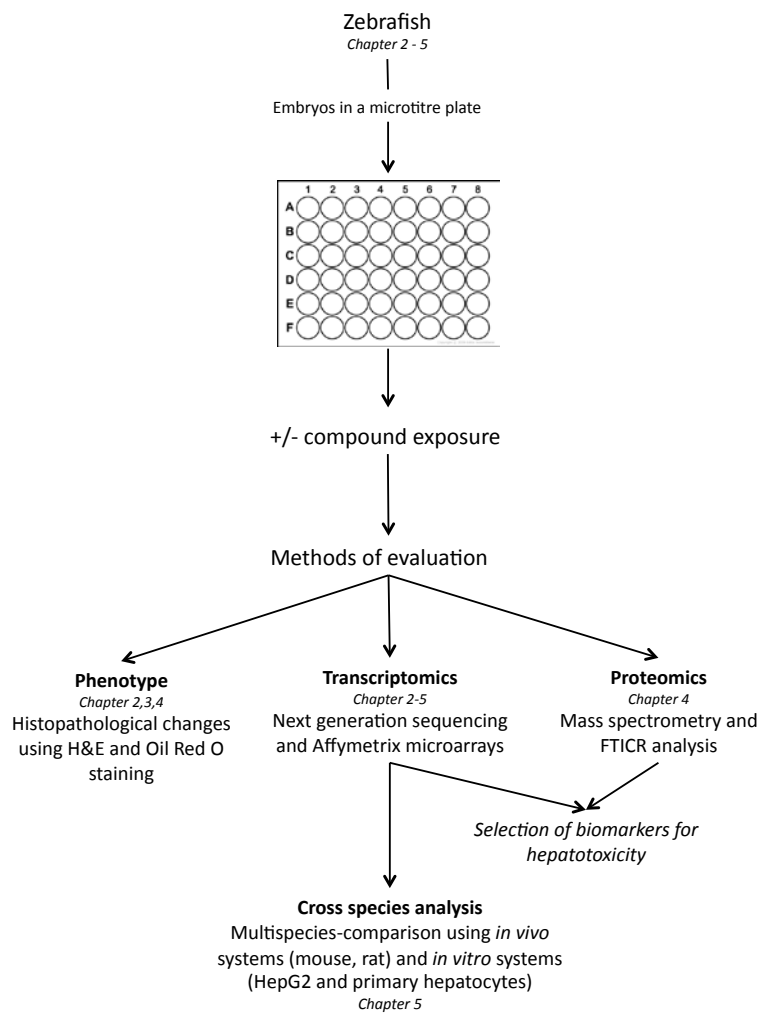

Figure 4 Schematic representation of experiments in this thesis.