

Zebrafish as model organisms for studying drug-induced liver injury

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Drug-induced liver injury (DILI) is a major challenge in clinical medicine and drug development. New models are needed for predicting which potential therapeutic compounds will cause DILI in humans, and new markers and mediators of DILI still need to be identified. This review highlights the strengths and weaknesses of using zebrafish as a high-throughput *in vivo* model for studying DILI. Although the zebrafish liver architecture is different from that of the mammalian liver, the main physiological processes remain similar. Zebrafish metabolize drugs using similar pathways to those in humans; they possess a wide range of cytochrome P450 enzymes that enable metabolic reactions including hydroxylation, conjugation, oxidation, demethylation and de-ethylation. Following exposure to a range of hepatotoxic drugs, the zebrafish liver develops histological patterns of injury comparable to those of mammalian liver, and biomarkers for liver injury can be quantified in the zebrafish circulation. The zebrafish immune system is similar to that of mammals, but the zebrafish inflammatory response to DILI is not yet defined. In order to quantify DILI in zebrafish, a wide variety of methods can be used, including visual assessment, quantification of serum enzymes and experimental serum biomarkers and scoring of histopathology. With further development, the zebrafish may be a model that complements rodents and may have value for the discovery of new disease pathways and translational biomarkers.

Introduction

Drug-induced liver injury (DILI) is a major problem in clinical medicine and drug development. The most common drug causing DILI in the UK and the USA is paracetamol (acetaminophen), a commonly used analgesic and antipyretic that is safe when used at therapeutic doses. However, when an accidental or deliberate overdose occurs, a metabolite of the drug is produced in excess, and this can lead to potentially fatal hepatocellular necrosis and acute liver failure. Each year, paracetamol overdose directly results in over 300 deaths in the USA [1] and around 150 in the UK [2]. The antidote, *N*-acetylcysteine, replenishes cellular glutathione [3] and is highly effective at preventing DILI if administered soon after overdose, but its efficacy declines substantially with delayed treatment [4].

Besides challenges with treatment of DILI in clinical medicine, in drug development DILI is a major safety concern and remains one of the main reasons for denial of drug approval, withdrawal of drugs from the market or 'black box' warnings by the US Food and Drug Administration (FDA) [5]. Drug-induced liver injury due to paracetamol overdose is dose dependent and, to an extent, predictable from the dose ingested and a timed measurement of blood drug concentration. In contrast, idiosyncratic liver toxicity is usually identified in late stages of drug development or after a new drug has already been released to the marketplace, occurring in less than one per 10 000–100 000 subjects who take the medication in therapeutic doses [6]. Partly because of its rarity, the pathogenesis of idiosyncratic DILI is incompletely understood, which makes it hard to predict at earlier stages of

drug development [7]. Therefore, high throughput and improved models are needed for predicting human DILI with potential therapeutic compounds and to identify new markers and mediators of DILI secondary to established hepatotoxic drugs, such as paracetamol.

The zebrafish (*Danio rerio*) is a promising animal for assessing drug-induced toxicity in a variety of organ systems [8]. Well-established zebrafish assays have frequently been used for measurement of cardiac and gastrointestinal function and for assessment of the central nervous system and developmental toxicity [9, 10]. The zebrafish liver can also be used to study drug toxicity; however, in comparison to other zebrafish organs, the zebrafish model of liver toxicity has been used less frequently.

This review highlights the strengths and weaknesses of zebrafish for studying DILI. The use of this model has the potential to identify new drug targets for the treatment of DILI and play a role in preclinical drug development. Also, the use of zebrafish is in line with the ‘3Rs’ (reduce, refine and replace) approach of animal use for scientific purposes by replacing higher-order animals with lower-order zebrafish (particularly, zebrafish embryos).

Potential advantages of zebrafish as a model for studying DILI

Histopathology and clinical chemistry have been used traditionally to report liver toxicity in established animal models. In order to decrease the cost and time of toxicity studies, alternative test systems have been developed; these include liver slices [11], cultured primary hepatocytes [12] and immortal hepatic cell lines, such as the human hepatoma-derived HepG2 line [13] and the recently derived human hepatocyte HepaRG line [14]. The advantage of these *ex vivo* and *in vitro* approaches is that they can be used efficiently for high-throughput screening. However, the usefulness of these approaches for toxicological testing of compounds can be questioned based on differences in gene expression between the different systems [15] and the low sensitivity of the cytotoxicity assays, which can be <25% for the detection of hepatotoxic agents [16].

In order to perform liver toxicity testing with a higher degree of sensitivity, *in vivo* assessment is necessary. This allows study of the dose-dependent toxicity of a drug within the complex physiology of a whole organism. Higher vertebrate organisms (e.g. rodents and pigs) are physiologically similar to humans and have been used for this approach. However, smaller, lower-order vertebrates, such as the zebrafish, have similar molecular and cellular processes that can accurately model human physiology [8]. In addition, the zebrafish offers significant advantages in comparison to rodents (Table 1) and other larger animals. The zebrafish embryo is optically transparent and

Table 1

Comparative advantages of using zebrafish and mice to model drug-induced liver injury

Advantages of zebrafish	Advantages of mice
Optically large and transparent embryos	Characterized inbred strains, including knock-out and knock-in strains
<i>Ex utero</i> development	Complement of all mammalian organs and physiological similarity to humans
Similar cellular and subcellular processes to humans	Easier to draw blood from mice than fish
Rapid development of liver ~72–96 hours postfertilization	Feasible to perform pharmacokinetic/toxicokinetic studies
High fecundity (~200 eggs per female per week)	Genome duplication of fish results in multiple copies of genes
Large numbers of fish can be maintained easily	
Embryonic fish can survive up to 7 days without a cardiovascular system	
Low overall cost	
Easy drug delivery by dissolving in the tank water, with possibility of drug delivery by microinjection	
Feasibility of high-throughput screens	
High <i>n</i> numbers available per study, allowing improved statistical analysis	
Lower-order mammal (in line with ‘3Rs’ principles)	

grows outside the uterus. This makes it possible to detect and monitor developmental changes easily from the single-cell stage. For example, the zebrafish embryo has allowed researchers to study embryonic lethal phenotypes; something that was not possible with mammalian models [17]. Additionally, an early zebrafish embryo, at 3 days postfertilization, is ~3.5 mm long. This allows zebrafish embryos to be grown at high stocking densities in multiwell plates. The high fecundity of the zebrafish (each female can lay ~200 eggs per week) can generate hundreds of embryos for screening, each of which has very rapid development. This reduces the cost of zebrafish husbandry significantly, when compared with larger laboratory animals. Furthermore, the Wellcome Trust’s Sanger Institute has sequenced the genome of the zebrafish, and many of these sequences have been annotated (http://vega.sanger.ac.uk/Danio_rerio/Info/Index) [18]. Further advantages of the zebrafish have been described elsewhere in the literature [10, 19–21].

Zebrafish liver anatomy is different from that of rodents and humans

Studies examining the organs of zebrafish, specifically the liver, have revealed multiple similarities with higher verte-

brates [22]. When liver budding starts at 28 h post-fertilization, growth factor and gene expression similar to those of humans and rodents has been reported in zebrafish [23]. When hepatic organogenesis is completed at 72 h postfertilization, the liver is perfused with blood and is functional [24]. At 120 h postfertilization, the zebrafish is actively seeking food and the yolk sac reserves have become exhausted. By this time, the larval fish already has a fully functional liver. In comparison, in the embryonic mouse the primary liver bud starts to grow around embryonic day 8.5–9 and the liver is mature at embryonic day 18.5, just before birth [25]. The tri-lobed liver of the zebrafish is similar to the liver of mammals with regard to biological function; this includes processing of lipids, vitamins, proteins and carbohydrates and the synthesis of serum proteins [22]. The main difference between the mammalian and zebrafish liver is the structural organization of the liver tissue. Instead of having the large bile ducts, portal veins and hepatic arteries organized in portal tracts, these are randomly allocated throughout the liver parenchyma in the zebrafish. Hepatocytes in the mammal liver are arranged in plates, whereas in the zebrafish liver they are arranged in tubules. In zebrafish, the bile canaliculi radiate centripetally between hepatocytes to anastomose with a single ductular cell, forming a ductule at the centre of the tubule. These ductules form a network that transports the bile secreted by hepatocytes. Downstream, these ductules merge into intrahepatic bile ducts, converging at the cystic duct, which exits the liver at the hilum to connect with the gallbladder. Subsequently, the gallbladder empties into the intestine through the common bile duct (Figure 1) [23, 26]. The above-mentioned lack of lobular arrangement impairs morphological differentiation between venules from the portal and hepatic veins, because these vessels are histologically identical.

Zebrafish drug metabolism is similar to that of rodents and humans

One of the key physiological functions of the liver is oxidative catalytic transformation, which leads to activation or inactivation of many endogenous and exogenous compounds. This metabolism is mainly performed by the cytochrome P450 (CYP) enzymes, which are predominantly localized in the liver. The metabolic reactions performed by the CYP enzymes include oxidation, reduction and hydrolysis. The CYP enzymes can be divided into two major groups. The first group of enzymes, with generally narrow substrate specificity, are predominantly involved in synthesis, activation or inactivation of endogenous regulatory molecules. The second group predominantly metabolize xenobiotics, but may also metabolize endogenous compounds [27, 28].

These reactions are divided in two phases. In phase I, the metabolized compound is oxidized, reduced or hydrolysed. These phase I reactions are predominantly mediated by CYP enzymes. In phase II, conjugation takes place (not CYP enzyme mediated). The rate of these reactions is controlled by expression levels and activity of the specific enzymes [29].

When selecting an animal model for toxicity testing, characterization of the metabolic properties of the selected species is very important. These properties influence DILI, for example, by creating reactive metabolites, and this will determine whether a compound is toxic [30]. Therefore, the application of zebrafish as a model of human (hepatic) endogenous and exogenous compound metabolism requires the full range of CYP genes; these have been identified in zebrafish and annotated with regard to their phylogenetic relationships to human CYP enzymes. This essential study was reported by Goldstone and colleagues, who characterised a total of 94 CYP genes in the zebrafish genome [27]. Based on homologous amino-acid sequences, they reported that these genes fitted into 18 CYP gene families that are also present in humans and other mammals. The CYP enzyme families 1–4, which predominantly metabolize exogenous compounds, are more diverse in zebrafish than in humans. However, analysis of shared synteny demonstrates an evolutionary relationship between human and zebrafish CYP genes. In the CYP families 5–51, zebrafish have single genes like humans, and there is a high degree of conservation between human and zebrafish sequences [27].

Metabolic experiments demonstrate that drugs are metabolized when exposed to zebrafish embryos by similar reactions to those in humans. An overview of reported metabolic experiments is presented in Table 2. The metabolic degradation of the widely used nonsteroidal anti-inflammatory drug ibuprofen is well studied in different mammals [31, 32]. The compound is metabolized by different reactions, including oxidation of the parent compound to hydroxyl-ibuprofen and carboxy-ibuprofen, as well as glucuronic acid conjugation of both parent and metabolite compounds [33]. In humans, the oxidation of ibuprofen is catalysed by the CYP2C8/9 isoforms [34]. When ibuprofen is exposed to zebrafish embryos, hydroxylated ibuprofen can be detected in the zebrafish extracts and water samples, suggesting that zebrafish have an analogous metabolic system to the human CYP2C8/9 [35].

Following exposure to high-dose paracetamol, in humans, rats and mice, the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) is formed by phase I metabolism of paracetamol, predominately by CYP3A4 [36–38]. Recently, Chng and colleagues used a glutathione trapping assay for NAPQI to determine that zebrafish generate the same reactive metabolite as humans. The same authors reported that the zebrafish CYP3A65, orthologue

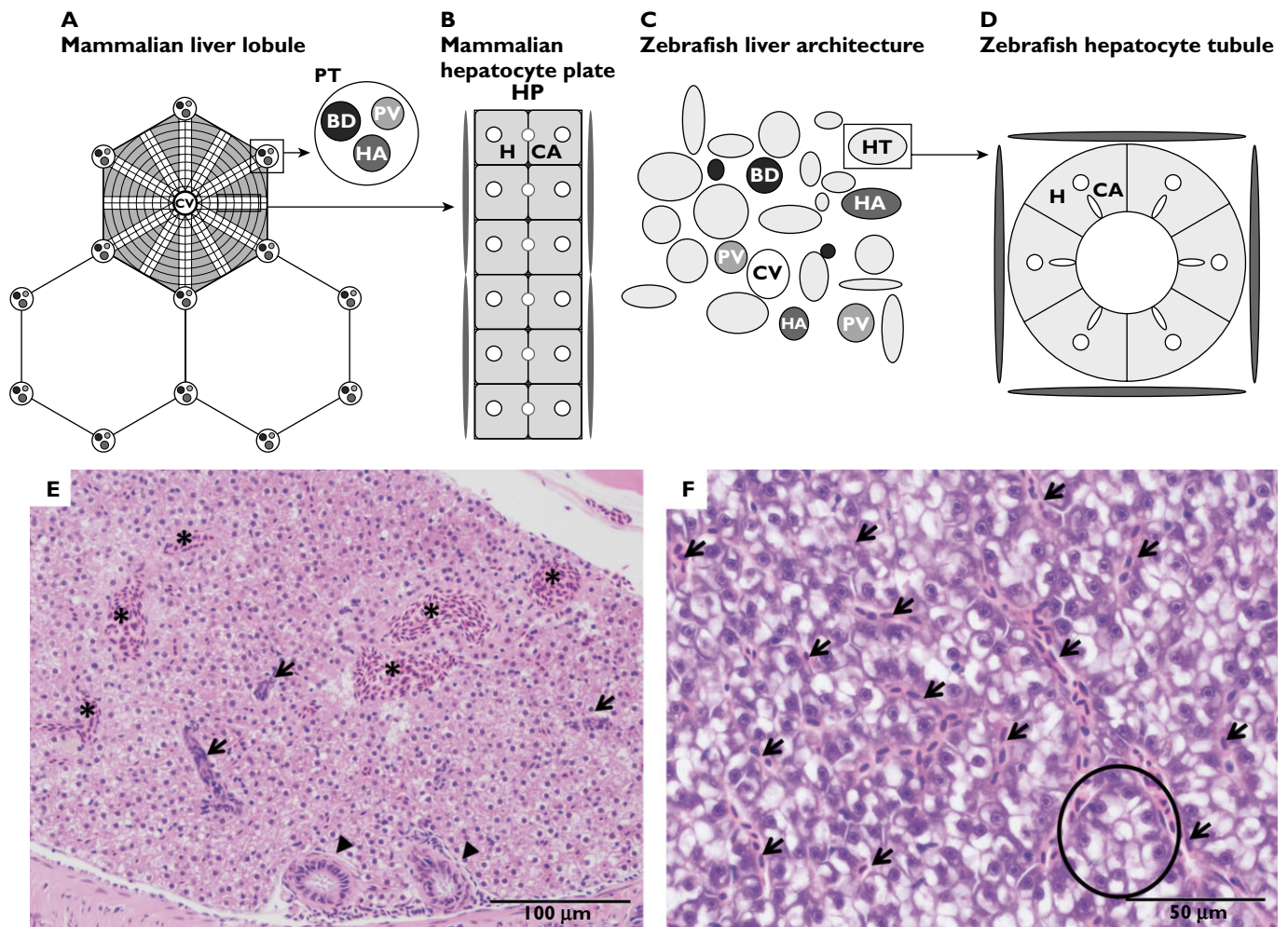


Figure 1

Schematic transverse representations of mammalian and zebrafish liver architecture. (A) The mammalian liver lobule, arranged with plates of hepatocytes radiating outward from a central vein (CV). At the corners of each lobule are portal tracts (PT), containing a portal vein (PV), a hepatic artery (HA) and a bile duct (BD). (B) Mammalian bilayered hepatocyte plate. Bicellular canaliculi (CA) are located adjacent to the hepatocytes (H) in the hepatocyte plate (HP). A basal hepatocyte membrane allows transport of oxygen, proteins and different macromolecules to the hepatocytes. Blood enters the liver through the portal vein and hepatic artery, after which it enters the central vein through sinusoid vessels, located between the plates. (C) The zebrafish liver architecture. The portal vein (PV), hepatic artery (HA), bile duct (BD), hepatocyte tubule (HT) and the central vein (CV) are scattered throughout the parenchyma. (D) Zebrafish hepatocytes (H) are arranged in tubules around small bile ducts, which receive bile from the hepatocyte canaliculi (CA). Sinusoids are located at the periphery of these tubules. (E) Histological image of male zebrafish liver (haematoxylin and eosin staining at $\times 200$ magnification). Note the presence of several biliary ducts (arrows), bile ductules (arrowheads) and blood vessels (*), with lack of lobular arrangement. (F) Histological image of female zebrafish liver (haematoxylin and eosin staining at $\times 400$ magnification). This high-power image displays sinusoidal spaces between hepatocytes (arrows) and an instance of the tubular arrangement of hepatocytes (encircled), which is frequently not visible histologically. Note the difference in staining of male and female zebrafish liver

for the human CYP3A4, contributed to the formation of NAPQI, as well as the phase I hydroxylation of testosterone [39].

Alderton and colleagues [40] confirmed that zebrafish embryos are able to perform the metabolic phase I reactions, oxidation, *N*-demethylation, *O*-demethylation and *N*-dealkylation, as well as the metabolic phase II metabolic reactions, sulfation and glucuronidation. The metabolites of three compounds, namely cisapride, verapamil and

chlorpromazine, were profiled. With cisapride, the mammalian phase I reactions (piperidine *N*-dealkylation and fluorophenyl ring oxidation) and phase II reactions (glucuronidation, resulting in glucuronide conjugates) were not observed in zebrafish [41]. However, following exposure of zebrafish to verapamil, a number of metabolites were formed by *N*-dealkylation and hydroxylation; these reactions are also present in mammals [42]. Three major metabolites of chlorpromazine, which are excreted

Table 2

Specific metabolic drug reactions reported in zebrafish compared with humans

Compound	Drug metabolism in zebrafish Reaction observed in zebrafish	Similar to human	Human P450 isotype	Ref.
Ibuprofen	Hydroxylation	Yes	CYP2C8/9	[33]
Paracetamol	Hydroxylation	Yes	CYP3A4	[37]
Testosterone	Hydroxylation	Yes	CYP3A4	[37]
Cisapride	Sulfate conjugation	No	CYP3A4	[38]
Verapamil	<i>N</i> -Dealkylation and hydroxylation	Yes	CYP3A4, CYP2C8/9, CYP1A2	[38]
Chlorpromazine	Hydroxylation, oxidation, <i>N</i> -demethylation, glucuronidation and sulfation	Yes	CYP1A2, CYP2D6	[38]
Phenacetin	De-ethylation	Yes	CYP1A2	[38]
Dextromethorphan	Demethylation	Yes	CYP2D6	[38]
Bupropion	Hydroxylation	Yes	CYP2B6	[38]

in human urine, were also excreted by zebrafish; these metabolites were formed by hydroxylation, oxidation, *N*-demethylation, glucuronidation and sulfation. Alderton and colleagues also reported that zebrafish embryos were able to de-ethylate phenacetin, demethylate dextromethorphan and hydroxylate bupropion [40].

The nuclear receptor, pregnane X receptor (PXR), is involved in the transcriptional regulation of cytochrome P4503A (CYP3A) and the multidrug resistance 1 transporter (MDR1) [43, 44]. Studies have confirmed that CYP enzymes can be induced and inhibited in zebrafish as reported in mammals. Bresolin and colleagues [45] studied the *in vivo* expression of *PXR*, *CYP3A* and *MDR1* genes in the liver of zebrafish treated with the synthetic steroid pregnenolone 16 α -carbonitrile, a potent PXR agonist [46]. The liver of the fish treated with pregnenolone 16 α -carbonitrile had a 1.9-fold increase in *PXR*, followed by a 1.8-fold increase of *CYP3A* and 1.6-fold increase in *MDR1*. This suggests that the regulation of *PXR*, *CYP3A* and *MDR1* is conserved in zebrafish and similar to mammals [45].

Tseng and colleagues [47] studied the effect of different drugs on CYP3A expression in zebrafish. They found that CYP3A65 expression was upregulated in the embryo (84 h postfertilization) intestine by rifampicin and dexamethasone. In addition to the PXR pathway, the aryl hydrocarbon receptor (AHR2) has a role in the pathway that regulates gene expression and is activated by endogenous and exogenous compounds, such as drugs and xenobiotics [48]. The AHR2 is present in different mammals, such as humans, mice and rats, and regulates expression levels of enzymes involved in phase I metabolism, including CYP1A2, CYP1B1 and aldehyde dehydrogenase 3A1 (ALDH3A1), as well as phase II metabolism, including NAD(P)H dehydrogenase quinone 1 (NQO1), UDP glucuronosyltransferase 1A2 (UGT1A2) and glutathione S-transferase α 1 (STA1) [49]. Expression of CYP3A65 was increased by exposing fish to 2,3,7,8-tetrachloro-dibenzo-

p-dioxin, an AHR2 ligand [50], during early embryonic stages, and inhibition of AHR2 translation by antisense morpholino oligonucleotides inhibited both normal and 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin-stimulated CYP3A65 transcription in embryonic intestine. These data suggest that AHR2 regulates CYP3A65 expression in zebrafish [47].

In summary, the zebrafish liver contains enzymes that metabolize a variety of endogenous and exogenous compounds in a similar manner to human liver. Additionally, these enzymes are subject to similar regulation mechanisms to those reported in humans. These findings support the potential of the zebrafish as an animal model for DILI.

The zebrafish immune system is similar to that of rodents and humans

Hepatic inflammation is commonly reported in various liver diseases, including DILI. Hepatotoxic drugs can have a direct effect on liver cells to release damage-associated molecular patterns (DAMPs) that stimulate immune cell secretion of chemokines and cytokines. Various immune cells, such as lymphocytes, neutrophils and macrophages, can subsequently infiltrate the liver. This complex immune response has been widely described by several authors [51–53]. Additionally, specific genetic backgrounds can be a risk factor for idiosyncratic DILI in humans [54, 55]. For example, a variety of leukocyte antigen (HLA) haplotypes are associated with immunological drug hypersensitivity (e.g. amoxicillin/clavulanate and abacavir) [56–59].

Many similarities exist between the zebrafish and the mammalian immune systems. Different studies of haematopoiesis in zebrafish have demonstrated that most, if not all, cell types of the human immune system

have zebrafish counterparts, although the sites of origin differ [60]. There is a variation in the repertoire of chemokine receptors in different species, regardless of the specific evolutionary position. Despite this, the expression and function of orthologous chemokine receptors in lower and higher vertebrates are highly similar [61]. While the zebrafish metabolizes drugs using similar pathways to humans, whether a similar immune response takes place with DILI in zebrafish is yet to be confirmed.

A range of drugs induce liver toxicity in zebrafish

Different methods have been used to assess liver toxicity in zebrafish, for example, visual assessment of gross and microscopic morphological changes, serum enzyme and biomarker tests, hepatic excretory tests and assessment of alterations in chemical constituents of the liver.

Gross/subgross visual phenotypic assessment

The ability to perform assays for liver toxicity with visually assessable phenotypic end-points enables the transparent larval zebrafish to be used in high-throughput screening.

A comparative toxic screen of 50 different compounds classified to be hepatotoxic by the US FDA, and non-toxic controls, was performed in zebrafish embryos. The compounds were screened in a researcher-blinded fashion for evaluation of three specific phenotypic end-points of liver toxicity, i.e. change in liver size, liver morphological abnormality and yolk-sac retention. A sensitivity for hepatotoxic drugs of 86% and specificity for nonhepatotoxic drugs of 77% was reported, which resulted in an overall correlation of 84% with mammalian *in vivo* data [62]. However, when four compounds were excluded from the analysis because of low uptake into the embryo from the tank water, an increased sensitivity, specificity and overall predictability of 97, 77 and 91%, respectively, was reported [63].

He and colleagues [64] exposed zebrafish embryos at 120 h postfertilization to six known mammalian hepatotoxic drugs (acetaminophen, aspirin, tetracycline hydrochloride, sodium valproate, cyclophosphamide and erythromycin) and two nontoxic compounds (sucrose and biotin), after which three phenotypic visual end-points of liver toxicity were assessed quantitatively. These end-points were liver degeneration score, changes in liver size and shape and yolk-sac retention. These end-points were easily measured under a light microscope without the need for dissection. All six hepatotoxic compounds induced liver degeneration, reduced liver size and led to yolk-sac retention, which suggested that this assay could be predictive for liver toxicity. Zhang and colleagues [65] have developed a transgenic zebrafish line (LiPan) that expresses a liver-specific fluorescent protein (DsRed) under the *fabp10a* promoter. They reported that the LiPan line could identify hepatotoxic drugs by detecting

changes in both liver red fluorescence and liver size in a dosage-dependent fashion. This was demonstrated by exposing the LiPan line to the hepatotoxic drugs paracetamol, aspirin, isoniazid and phenylbutazone.

Liver histopathology

Specific changes in zebrafish liver histology have been reported. North and colleagues reported hepatic necrosis after zebrafish were treated with paracetamol [66]. As described for other mammals, exposure of zebrafish to hexachlorocyclohexane results in specific histological changes, such as hepatic macrovesicular triglyceride droplets, glycogen depletion and the presence of club-shaped mitochondria [67]. Exposure of zebrafish to thioacetamide induces steatohepatitis, which is accompanied by the accumulation of fatty droplets and apoptosis [68]. Zebrafish exposed to ethanol display histological changes such as steatosis, as seen in alcoholic liver disease in humans [69]. In conclusion, both embryonic and adult zebrafish are amenable to study of the histological changes that accompany different liver pathologies, such as steatosis, apoptosis and necrosis.

Circulating biomarkers

Whilst zebrafish embryos offer a range of advantages that facilitate high-throughput screening, adult zebrafish are needed if circulating biomarkers are to be measured.

Murtha and colleagues determined multiple serum biochemical values in zebrafish (± 1 year old), such as total bilirubin concentration (mean \pm SD, 0.38 ± 0.1 mg dl⁻¹; range, 0.2–0.6 mg dl⁻¹) and serum alanine transaminase (ALT) activity (mean \pm SD, 376 ± 25.3 U l⁻¹; range, 343–410 U l⁻¹) [70]. However, in our laboratory we have reported lower ALT activity (range, 12–137 U l⁻¹) in serum from zebrafish (5–24 months old). In a paracetamol-induced liver toxicity model in adult zebrafish, North and colleagues demonstrated that ALT activity increased in zebrafish in a dose- and time-dependent fashion [66]. Injury was reduced by acetylcysteine treatment of paracetamol-exposed zebrafish, as is the case in humans [4]. We have observed similar effects of paracetamol on zebrafish in our laboratory. In the same model, we reported an increase in the circulating concentration of microRNA-122, a new experimental biomarker for liver toxicity in humans [71], in fish with liver injury [72]. Cox and colleagues reported that after paracetamol exposure, inhibition of the enzymic regulator S-nitrosoglutathione reductase (GSNOR) minimized liver toxicity in zebrafish. A GSNOR-specific inhibitor improved survival and histology and lowered ALT activity through the cytoprotective Nrf2 pathway. Paracetamol toxicity studies in GSNOR-deficient mice confirmed conservation of the hepatoprotective properties of S-nitrosothiol signalling across vertebrates [73]. This supports use of the zebrafish as a translational model of human paracetamol toxicity and biomarker research.

Challenges in using zebrafish as a new model for DILI

Although a substantial amount of research demonstrates the potential of zebrafish as a model of liver toxicity, there are a number of challenges.

Zebrafish are often exposed to a drug by dissolving the drug in the water, which enables easy and fast drug administration. This is an advantage of the model that allows for high-throughput phenotypic screening, especially if transgenic lines are used [74]. The problem with this method of drug administration is that, although the concentration of drug in water is known, the amount taken up by the fish is imprecise and variable, which limits the study of toxicokinetics. Berghmans and colleagues [75] studied the uptake of nine compounds in zebrafish embryos by dissolving the compounds in the water and found a large variability in the bioavailability of the different compounds. This was because the physiochemical properties of different compounds determine the absorption of the compounds into the fish through the gills and intestine, rather than simply their aqueous concentration. If required, drugs can be injected into the yolk sac of embryonic fish, and this method can therefore quantify the administered dose at the expense of being time consuming [76].

The relationship between the lipophilicity of the drug and the amount of compound penetrating the zebrafish has been determined [75]; however, no single physiochemical property can accurately predict the uptake of different types of compounds [77]. Therefore, bioanalysis should be performed to correlate the amount of drug in the fish (the real body burden) and the observed toxic effects [78]. For instance, sodium valproate, a potentially hepatotoxic drug in humans, did not cause toxic effects in zebrafish embryos, possibly due to poor uptake of this drug. In contrast, valproic acid did cause liver toxicity in zebrafish embryos with higher blood concentrations, indicating increased uptake [62]. To overcome the possible problem of absorption, the amount of drug taken up by the fish can be determined by using radio-labelled compound and liquid scintillation counting or radio high-performance liquid chromatography. Other methods that determine the uptake of drug into fish include extracting the embryos with acetonitrile and then using liquid chromatography–tandem mass spectrometry analysis [79], and determining the uptake of compounds by drawing blood from adult zebrafish and measuring the concentration of the compound in blood plasma. However, because of the low blood yields obtained from zebrafish, typically 3–20 μl of whole blood [80–84], 10–20 fish may have to be pooled. Zang and colleagues [84] described a recovery method that allows for serial blood sampling from adult zebrafish, but it takes 1–2 weeks for fish to recover normal haemoglobin values after taking a small blood

sample of 2 μl . This delayed recovery may limit the application to toxicity studies.

Goldstone and colleagues [27] demonstrated that 66 of the 88 studied CYP genes in zebrafish embryos have a differential level of expression during development between 3 and 48 h postfertilization. This stresses the importance of age on toxicity when zebrafish embryos are used. In liver toxicity studies, embryos must be >3 days postfertilization [27]. Circadian rhythms will influence an organism's susceptibility and responses to xenobiotic exposure. It is established that ATP binding cassette (ABC) transporters have a significant impact on bio-availability, metabolism and excretion of drugs. The gene expression of some transporters, including P-glycoprotein, could be under circadian transcriptional regulation in zebrafish, as reported in mice [85]. Therefore, age-related and circadian-related gene expression profiles will impact on the relative higher amount of toxic metabolite, which might influence susceptibility. It is for this reason that standardization of protocols when using embryonic stages is an important consideration in high-throughput screens.

Conclusion

Early identification of hepatotoxic compounds would accelerate the process of drug discovery and development and reduce the enormous costs. The zebrafish appears to be a model that may complement established models. However, before the model can be applied on wider scale more validation is needed to confirm the translatability of the model to humans. This may include testing established human hepatotoxic and nonhepatotoxic compounds, comparing dose responses between fish and humans and developing translational biomarkers that bridge between fish, rodents and humans. Furthermore, the immunological response observed with DILI in humans has to be studied in zebrafish to confirm mechanistic similarity. Ultimately, use of the zebrafish as a model for DILI is promising and may enable better decision making in the early stages of drug discovery, before a compound is tested in higher mammals.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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