Research article

Journal of Applied Toxicology

Received: 21 April 2015, Revised: 15 December 2015, Accepted: 15 December 2015 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jat.3288

Phenotypic and biomarker evaluation of zebrafish larvae as an alternative model to predict mammalian hepatotoxicity

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ABSTRACT: Zebrafish phenotypic assays have shown promise to assess human hepatotoxicity, though scoring of liver morphology remains subjective and difficult to standardize. Liver toxicity in zebrafish larvae at 5 days was assessed using gene expression as the biomarker approach, complementary to phenotypic analysis and analytical data on compound uptake. This approach aimed to contribute to improved hepatotoxicity prediction, with the goal of identifying biomarker(s) as a step towards the development of transgenic models for prioritization. Morphological effects of hepatotoxic compounds (acetaminophen, amiodarone, coumarin, methapyrilene and myclobutanil) and saccharin as the negative control were assessed after exposure in zebrafish larvae. The hepatotoxic compounds induced the expected zebrafish liver degeneration or changes in size, whereas saccharin did not have any phenotypic adverse effect. Analytical methods based on liquid chromatography–mass spectrometry were optimized to measure stability of selected compounds in exposure medium and internal concentration in larvae. All compounds were stable, except amiodarone for which precipitation was observed. There was a wide variation between the levels of compound in the zebrafish larvae with a higher uptake of amiodarone, methapyrilene and myclobutanil. Detection of hepatocyte markers (*CP*, *CYP3A65*, *GC* and *TF*) was accomplished by *in situ* hybridization of larvae to coumarin and myclobutanil and confirmed by real-time reverse transcription–quantitative polymerase chain reaction. Experiments showed decreased expression of all markers. Next, other liver-specific biomarkers (i.e. *FABP10a* and *NR1H4*) and apoptosis (i.e. *CASP-3 A* and *TP53*) or cytochrome P450-related (*CYP2K19*) and oxidoreductase activity-related (*ZGC163022*) genes, were screened. Links between basic mechanisms of liver injury and results of biomarker responses are described. Copyright © 2016 John Wiley & Sons, Ltd.

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Keywords: Non-animal alternative assay; hepatotoxicity; zebrafish larva; gene expression markers; compound uptake

Introduction

The liver is recognized as a critical target organ for xenobiotic chemicals and drugs. Owing to its functional position between the gastrointestinal tract and the systemic circulation, high concentrations of xenobiotics may end up in the liver after intestinal absorption. Seven basic mechanisms for xenobiotic-induced liver injury are known, i.e.: (1) disruption of calcium homeostasis and cell membrane injury; (2) canalicular and cholestatic injury; (3) metabolic bioactivation by cytochrome P450 enzymes; (4) mitochondrial injury, leading to interruption of lipid oxidation and steatosis; (5) stimulation of autoimmunity; (6) stimulation of apoptosis; and (7) (in)-direct activation of neutrophils and Kupffer cells. Many of these mechanisms can be triggered at the same time in an affected liver (Jaeschke *et al.*, 2002; Lee, 2003).

Hepatotoxicity evaluation is of high importance, but no single *in vitro*/*in vivo* assay or battery of screens currently being performed is able to predict reliably the biological effects of potentially toxic compounds (Hill *et al.*, 2012; Mennecozzi *et al.*, 2012; O'Brien *et al.*, 2006; Xu *et al.*, 2008). *In vivo* assessment of liver toxicity frequently involves histopathology requiring a large number of rodents (Ekor *et al.*, 2013). Rodent experiments are very laborious, costly and time-consuming, with often poorly consistent results, while the EU-animal directive 2010/63/EU recommends the reduction of scientific experiments on animals. Several alternative *in vitro* methods to evaluate liver injury have been developed, including rat liver slices (Boess *et al.*, 2003; Elferink *et al.*, 2008), rat or human primary hepatocytes (Boess *et al.*, 2003; Kienhuis *et al.*, 2009), and hepatic cell lines, e.g., HepG2 and HepaRG (Guillouzo *et al.*, 2007; Lecluyse *et al.*, 2012), each having their limitations related to simplification of a cellular system compared to the *in vivo* situation. Therefore, non-mammalian whole-organism approaches are currently explored for their suitability to screen for prediction of human toxicity and anticipate from the forementioned limitations.

Zebrafish embryos/larvae are often considered as alternative systems, as these developmental stages are likely to experience less or no pain, suffering, distress or lasting harm; therefore,

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developing zebrafish larvae are regarded as protected animals only from 120 h postfertilization (hpf) onwards (EU, 2012). Furthermore, fish embryos provide the complexity and interaction of an intact organism, enabling the evaluation of compound-induced effects on multiple target organs. The transparent nature of the embryo/larva, ability to screen the whole organism in a microwell plate format with small amounts of compounds, potential to utilize medium to high-throughput screening platforms (Pardo-Martin *et al.*, 2010), high degree of genetic conservation (Howe *et al.*, 2013) and their similar morphological and molecular basis of tissue and organ development (e.g., growth factor and gene expression during liver budding, processing of lipids, vitamins, proteins and carbohydrates in the tri-lobed liver) shared with other vertebrates, including humans, makes zebrafish an excellent model organism for studying complex biological processes (Chu & Sadler, 2009; Howe *et al.*, 2013; Vliegenthart *et al.*, 2014).

Zebrafish larvae have been used as a convenient model for assessing hepatotoxicity (Driessen *et al.*, 2013; He *et al.*, 2013; Hill *et al.*, 2012), as they complete primary liver morphogenesis by 48 hpf and the liver is fully formed and functioning by 72 hpf (Alderton *et al.*, 2010; Isogai *et al.*, 2001; Pack *et al.*, 1996). In addition, zebrafish homologs of mammalian lipid metabolizing enzymes are present in the zebrafish liver (Chng *et al.*, 2012; Goldstone *et al.*, 2010; Wiegand *et al.*, 2000). A transcriptomic-based comparison of hepatotoxicity for three compounds (amiodarone [AMI], acetaminophen [ACE] and cyclosporine A) between the zebrafish embryo and traditional models (i.e. *in vivo* mouse and rat liver, *in vitro* mouse and rat hepatocytes, and primary human hepatocytes) showed concordance at the pathway level (metabolism) and confirmed its potential as a model to screen for the hepatotoxic potential of compounds (Driessen *et al.*, 2015).

The objective of our research project was to explore zebrafish larvae as an alternative whole-organism approach to evaluate the potential of compounds to induce hepatotoxicity and compare with rodent studies and human health effects. Liver toxicity in zebrafish larvae was assessed using gene expression (*in situ* hybridization [ISH] and real-time reverse transcription–quantitative polymerase chain reaction [RT-qPCR]) as the biomarker approach, complementary to phenotypic analysis. A measure of compound uptake via bioanalysis (i.e. a liquid chromatography [LC]/mass spectrometry [MS] based approach) is rather unique and important to interpret appropriately the relevance of any observed toxicity outcome of the bioassay in relation to stability and bioavailability of the test compound, and allows explanation of the eventual false negative results. For that reason, analytical methods were optimized to analyze the stability, as well as the medium and internal concentration of selected hepatotoxic compounds. This multifaceted approach is of added value to existing studies with zebrafish larvae, which mainly study either gene expression (Driessen *et al.*, 2014, 2015), bioactivation of test compounds and metabolite profiling (Chng *et al.*, 2012), morphology and histopathology (He *et al.*, 2013; Hill *et al.*, 2012), or a combination of these approaches (Driessen *et al.*, 2013; Mesens *et al.*, 2015), but they all lack the extensive analytical study on the fate of the test compounds.

In this study, morphometric evaluation of the zebrafish liver, complemented with gene expression studies and analytical results are presented for one non-hepatotoxic compound, saccharin (SAC) and five hepatotoxic compounds, i.e. ACE, AMI, coumarin (COU), methapyrilene (MET) and myclobutanil (MYC). The expected positive and negative compounds were selected from mammalian studies, representing different mechanisms (cfr. Table 1 and discussion) and were evaluated in the zebrafish model with the purpose of assessing the predictive value of zebrafish in relation to the basic mechanisms for xenobiotic-induced liver injury. In summary, the novelty at the level of experimental approach is combining liver morphology, biomarker assessment (ISH and RT-qPCR) and fate of compound. The selection of appropriate biomarker(s) offers new perspectives for the development of a transgenic model as a screening tool to prioritize unknown substances for hepatotoxicity.

Materials and methods

Zebrafish

Adult, wild-type zebrafish (*Danio rerio*) were obtained from a commercial dealer, and were kept in the laboratory as breeding fish for this study and reared under standard conditions (temperature range 25–27°C) at a 14: 10 light/dark cycle. The fish were acclimated to laboratory conditions, and kept in quarantine for at least 2 weeks before use in experiments. Next, healthy male and female fish were used to generate zebrafish embryos by group breeding in breeding tanks. Eggs were rinsed in 0.0002% methylene blue (CAS no. 122965–43-9; Sigma-Aldrich, Diegem, Belgium), diluted in fish water (reverse osmosis water supplemented with $CaCl_2.2H_2O$ [0.294 g l^{-1}], $MgSO_4.7H_2O$ [0.123 g l^{-1}], KCl [0.006 g l^{-1}], and $NaHCO_3$ [0.065 g l^{-1}]; pH 7.0–8.5; conductivity 600–700 μS cm^{-1}; hardness 10–300 mg l^{-1} $CaCO_3$; oxygen level $>$ 80% [OECD TG 203, 1992]) and placed into large Petri dishes filled with fish water. At 6 hpf, unfertilized eggs were removed and the embryos were transferred to six-well plates (five embryos per well per 4 ml fish water) and placed in an incubator at a 14: 10 light/dark cycle at 28.5 ± 0.5°C until 72 hpf. The procedures described in this study were approved by the local ethics committee.

Chemical treatment

A hepatotoxic compound database containing 89 compounds was created based on mammalian data from peer-review articles (e.g., PubMed) and other databases (e.g., TOXNET-HSDB). For the selection of target hepatotoxic compounds, toxicological and analytical selection criteria were used, such as "different chemical classes, different mode-of-action, different physico-chemical characteristics, availability of compound in pure form." Five hepatotoxic compounds (i.e. ACE, AMI, COU, MET and MYC) and one non-hepatotoxic compound (SAC) were finally selected for assessing hepatotoxicity in larval zebrafish (see Table 1). All the compounds were purchased from Sigma-Aldrich. Stock solutions were prepared in either 100% dimethyl sulfoxide (DMSO, CAS no. 67–68-5; Lab-Scan, Gliwice, Poland) (AMI, COU, MET and MYC) or fish water (ACE and SAC) and dilutions were made in fish water with 0.1% DMSO or fish water.

An optimal experimental set-up was derived from our own preliminary experiments. At 72 hpf, the six-well plates with larval zebrafish were checked for normal development and the minority of abnormal larvae (e.g., non-hatched, edema $<$10%) was replaced before the start exposure to test compounds. For all experiments, 2× six-well plates with five zebrafish larvae each were statically exposed to the negative control (fresh fish water), if a solvent was used, to the solvent control (0.1% DMSO in fish water), or to three sublethal concentrations of test compounds for a treatment period of 48 h (from 72 to 120 hpf) in an incubator on a 14: 10 light/dark cycle at 28.5 ± 0.5°C. For real-time RT-qPCR, three technical repeats, each with 2× six-well plates containing five larvae, were set up for the same conditions as described above. The

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Table 1. Information on test compounds used for assessing zebrafish hepatotoxicity

Test compounds	Use	Potential mechanism of liver injury	CAS no.	Purity	Test concentrations (μM)	Log K_{ow} (Epi Suite; KOWWIN v1.68)
Acetaminophen (ACE), paracetamol	Analgesic and antipyretic drug, safe at therapeutic doses (Blieden *et al.*, 2014)	Metabolic bioactivation by cytochrome P450 enzymes; mitochondrial injury; stimulation of apoptosis	103–90-2	≥ 99%	2000–4000–5000	0.27
Amiodarone hydrochloride (AMI)	Lipophilic antiarrhythmic drug	Mitochondrial injury (steatosis)	19774–82-4	≥ 98%	0.2–1–5	7.29
Coumarin (COU)	Natural flavoring product used as fragrance ingredient and as therapeutic agent for the treatment of various cancers	Metabolic bioactivation by cytochrome P450 enzymes; mitochondrial injury	91–64-5	≥ 99%	250–500–1000	1.51
Methapyrilene hydrochloride (MET)	Antihistaminic compound	Disruption of calcium homeostasis and cell membrane injury; mitochondrial injury; metabolic bioactivation by cytochrome P450 enzymes; canalicular and cholestatic injury; stimulation of apoptosis	135–23-9	100%	2.5–5–10	2.74
Myclobutanil (MYC)	Triazole fungicide	Metabolic bioactivation by cytochrome P450 enzymes	88671–89-0	100%	12.5–25–50	3.50
Saccharin (SAC)	Artificial sweetener	–	81–07-2	≥ 99%	3400–6800–13 700	0.45

sublethal concentrations of the test compounds ranged from no or small effect on the liver to clear the toxic effect on the liver, while causing no larvae mortality, as these were derived in range-finding experiments using the optimal experimental set-up (Table 1).

Phenotypic evaluation of hepatotoxicity

After 48 h treatment, zebrafish larvae at 120 hpf were subject to visual observation of liver morphology under a stereomicroscope (Olympus IX2-UCB, Berchem, Belgium) at magnification 100×. Larvae were first immobilized in 2% methylcellulose (CAS no. 9004–67-5; Sigma-Aldrich). When viewed dorsolaterally, the liver is situated posterior to the pericardium and predominantly anterior to the gut. The liver is globular in structure, has a clearly recognizable periphery against the neighboring tissues and is perfused with circulating blood cells. Normal zebrafish liver is clear, whereas after treatment with hepatotoxic compounds, it became darker with gray coloration and the texture of liver tissue became amorphous, indicating degeneration and/or necrosis. Two specific phenotypic endpoints, respectively changes in liver size and liver degeneration, were visually assessed for hepatotoxicity. Other non-liver specific characteristics were noted, e.g., edema, non-inflated swim bladder, protruding mouth (not shown). At least two to five independent biological experiments using different batches of zebrafish larvae and freshly made test solutions were performed. For each experiment and each test concentration, the number of fish showing effect for each of the two endpoints was scored, as well as those showing a normal liver, and expressed in Table 3 as the percentage effect. In this way, percentage values in Table 3 show for each condition the range of effects across experiments, being more informative than the calculation of a mean value for the percentage effect, masking the variability between experiments.

Biomarker evaluation of hepatotoxicity

Potential gene markers for liver injury were studied by whole-mount ISH and real-time RT-qPCR to complement the phenotypic hepatotoxicity assessment.

Biomarker selection. Six liver-specific biomarkers with a clear orthologous gene in zebrafish and four non-liver specific biomarkers were selected from mammalian and zebrafish studies (occurrence in liver mammals, evidence for changes due to hepatotoxicity in general) and are shown in Table 2.

Whole-mount in situ hybridization. Whole-mount ISH for the markers *CP*, *CYP3A65*, *GC* and *TF* was performed following an adapted protocol (Mavropoulos *et al.*, 2005). At 24 hpf, pigmentation was blocked by adding 0.0003% phenylthiocarbamide (CAS no. 103–85-5; Sigma-Aldrich) to improve visual observation. ISH experiments were performed using fixed larvae at 120 hpf after 48 h exposure to COU (250 and 500 μM), MYC (12.5 and 25 μM) or the solvent control (0.1% DMSO in fish water). Details on the protocol can be found in Supplementary Information.

Real-time reverse transcription–quantitative polymerase chain reaction. Biomarkers (*CP*, *CYP3A65*, *GC* and *TF*) with a clear difference in expression in treated larvae compared to control larvae in the ISH experiments were quantitatively confirmed using real-time RT-qPCR. In addition, other liver-specific biomarkers (i.e. *FABP10a* and *NR1H4*), apoptosis as well as additional metabolism/oxidoreductase-related markers i.e. *CASP3A*, *TP53*, *CYP2K19* and *ZGC163022* were selected to verify whether these genes were influenced by exposure to hepatotoxicants. After 48 h exposure, the two wells with five larvae each for each condition were pooled in a single tube ($n = 10$ larvae), snap-frozen in liquid nitrogen and homogenized in TRIzol (TRI reagent; Ambion, ThermoFisher Scientific, Gent, Belgium) using the SilentCrusher S (Heidolph Instruments, Schwabach, Germany). Three technical repeats were performed for each exposure condition within the same experiment, and for each compound at least three independent biological experiments were set up. Details on the protocol and statistical analysis can be found in Supplementary Information.

Chemical analysis

For chemical analysis, two replicate wells of five zebrafish larvae were statically exposed to the negative control, eventually to the solvent control, and to three sublethal concentrations of test compounds (Table 1). The identification and quantitation of the selected compounds were executed using a LC (1200 series; Agilent Technologies, Diegem, Belgium), equipped with a binary pump and coupled to a triple quadrupole MS (Agilent Technologies 6410) equipped with an electrospray ionization source. The LC parameters for each chemical are given in Supplementary Information Table S2. For SAC, an Agilent Zorbax SB-C18 column (2.1 × 50 mm, 3.5 μm) was used, while all other compounds were analyzed using a Phenomenex Kinetex C18 column (2.1 × 100 mm, 2.6 μm). The mobile phase was composed from eluent Ag (MilliQ water with 0.1% formic acid; Merck, Overijse, Belgium) and eluent B (methanol or acetonitrile, Merck with 0.1% formic acid). Supplementary Information Table S3 gives an overview of the MS parameters for each compound and its internal standard, the latter being chosen from the same chemical class as the analyte.

The stability of each chemical was determined during the exposure period of 48 h at 28.5 ± 0.5°C in a plastic well plate at 14: 10 light/dark cycle, storage at 4°C in a glass vial, and transport at room temperature in a glass vial. The highest exposure concentration was used to determine the stability at 28.5°C in a plastic well plate, except for MET which was twice as high (Supplementary Information Table S4, 21.3 μM).

Test compound concentration measurement in medium. At the start of exposure (72 hpf; $n = 1$ technical repeat/condition) and at the end of exposure after 48 h treatment (120 hpf; $n = 2$ technical repeat/condition), the exposure medium was collected and the concentration of test compound was measured. The media of blank wells were also collected to demonstrate compound availability in the absence of larvae (i.e. how much compound may have degraded or adsorbed to the plastic during the assay). The average of measured concentrations of two independent experiments and standard deviation were calculated. The medium samples were diluted in fish water and the calibration for each compound was made in fish water or in 0.1% DMSO in fish water. Supplementary Information Table S4 gives for each compound the limit of detection, limit of quantification, range of linearity range, accuracy at low and high reference level and precision at low and high reference level in fish water.

Determination of compound concentration in zebrafish larvae. After exposure (120 hpf), two replicates of five larvae each ($n = 2$/condition) were washed three times in 1% DMSO, if 0.1% DMSO as solvent was used for exposure, or fish water to eliminate compound adsorbing to the outside of the larvae and collected to measure actual systemic compound uptake. Compound

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Table 2. Overview biomarkers. The relation of the liver markers to the basic mechanisms of xenobiotic-induced liver injury is mentioned between brackets in the column "relevance in mammals:" (1) disruption of calcium homeostasis and cell membrane injury; (2) canalicular and cholestatic injury; (3) metabolic bioactivation by cytochrome P450 enzymes; (4) mitochondrial injury; (5) stimulation of autoimmunity; (6) stimulation of apoptosis; (7) (in)direct activation of neutrophils and Kupffer cells

Gene name	Gene symbol	Relevance in mammals	Occurrence in zebrafish
Albumin-like	*GC*	Liver marker (1) (Lee *et al.*, 1987)	(Noel *et al.*, 2010)
Ceruloplasmin	*CP*	Liver marker (2, 7) (Koruk *et al.*, 2003)	(Noel *et al.*, 2010)
Cytochrome P450, family 3, subfamily A, polypeptide 65	*CYP3A65*	Synteny with human *CYP3A-se1*, *−se2*; 54% identical to human *CYP3A4* (3)	Liver and gut marker (Tseng *et al.*, 2005)
Liver fatty acid binding protein	*FABP10a*	Liver marker (*), reflective of liver health and homeostasis (Chu and Sadler, 2009; Monbaliu *et al.*, 2005)	(Noel *et al.*, 2010)
Nuclear receptor subfamily 1 group H member 4	*NR1H4*	Liver marker (2,3,4) (Heni *et al.*, 2013)	Personal communication Hill A. (2010)
Transferrin	*TF*	Liver marker (*) (Amacher, 2002)	(Mudumana *et al.*, 2004; Noel *et al.*, 2010)
Caspase 3a	*CASP3A*	Non-liver specific apoptosis marker (Porter and Jänicke, 1999)	(Shi *et al.*, 2011)
Cytochrome P450, family 2, subfamily K, polypeptide 19	*CYP2K19*	Shares synteny with human *CYP2W1*, a tumor-specific CYP that oxidizes indole and chlorzoxazone, but not fatty acids (Goldstone *et al.*, 2010)	Cytochrome P450 metabolism-related marker (Driessen *et al.*, 2014)
Tumor suppressor protein p53	*TP53*	Non-liver specific apoptosis marker (Amaral *et al.*, 2010)	Lam *et al.*, 2013
Ferric chelate reductase 1	*ZGC163022*	–	Oxidoreductase metabolism (Driessen *et al.*, 2013)

* Drug-induced liver injury marker, not specified.

concentration in larvae was calculated according to Berghmans *et al.* (2008 where the volume of a larva was equated to 1.67 µl. The concentration in larvae was calculated (µM) and relative uptake of compound into larvae was presented as percentage of the start concentration in the medium measured at the beginning of the experiment, except for AMI where nominal concentration was used. The average compound concentration in larvae of two independent experiments was calculated with the corresponding standard deviation. For the analysis of exposed larvae, we have used matrix-matched calibration using non-exposed larvae to account for the matrix effect during the analysis. The calibration was first made in 100% methanol, the standards were then evaporated and reconstituted in 100 µl of blank larvae in methanol/water (1: 1). A volume of methanol/water 1: 1 was added to the larvae samples, the larvae were ultrasonicated until they were dissolved (typically 10 min), then vortexed for 1 min. The entire sample was centrifuged for 1 min at 10 000 rpm and the supernatant was injected into the LC–MS/MS.

The quality control reference samples were prepared by spiking the analytes to non-exposed larvae. The solvent ratio in the non-exposed larvae was methanol/water (1: 1). More details are given in Supplementary Information Table S4.

Results

Phenotypic evaluation of hepatotoxicity

Zebrafish treated with the negative (fish water) and solvent control solution (0.1% DMSO in fish water) exhibited clear liver tissue (Fig. 1a). After treatment with certain tested concentrations of a hepatotoxic compound, e.g., MET, zebrafish liver lost transparency and became dark (Fig. 1c,d) or changes in liver size were observed (Fig. 1d), while the lowest concentration of MET (2.5 µM) did not show phenotypic effects on the liver (Fig. 1b) compared to the control. The non-hepatotoxic compound SAC did not exhibit any of these phenotypic effects on larval zebrafish liver for all tested concentrations. The effect percentages of evaluated phenotypic endpoints after 48 h exposure of zebrafish larvae to hepatotoxic compounds and a non-hepatotoxic compound SAC, compared

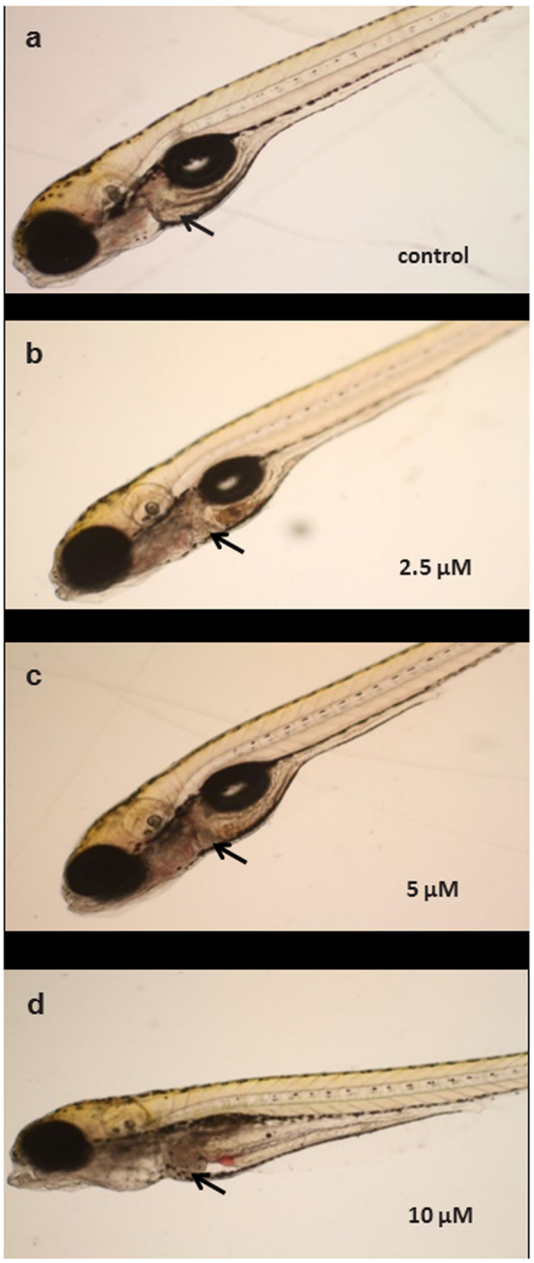

Figure 1. Phenotypic identification of hepatotoxicity at 120 hpf treated for 48 h, the liver region is indicated by an arrow (magnification × 100). Control larva (a) and larva treated with 2.5 µM methapyrilene (b) exhibit a clear, healthy liver. Larvae treated with higher concentrations of the hepatotoxicant methapyrilene exhibit tissue degeneration (c,d) and changes in liver size (d), with more toxic responses as was observed by pericard edema, protruding mouth and an uninflated swim bladder at the highest concentration (d).

to the corresponding negative/solvent control are shown in Table 3. For each of the experiments, percentage effect values are presented that point to rather a high variability at the highest concentrations, e.g., for liver degeneration, 1000 µM COU resulted in a percentage effect range of 11–100% across five experiments.

Biomarker evaluation of hepatotoxicity

Whole-mount in situ hybridization. In Fig. 2, ISH results are shown for the marker *TF* as example. An overview of all ISH results is presented in Supplementary Information Table S5. Exposure to both concentrations, COU affects all hepatocyte markers and a strongly decreased expression in the liver was observed compared to the solvent-treated larvae. MYC causes a slightly reduced expression of hepatocyte markers for both concentrations compared to the solvent control. Only exposure to 12.5 µM MYC showed no change in expression for the marker *GC* compared to the solvent control.

Real-time reverse transcription–quantitative polymerase chain reaction. Results are shown in Fig. 3(a–c). Below, we describe the exposure conditions that induced a significant change in expression ($P < 0.05$) relative to solvent control by mixed-effect model analyses followed by Tukey's *post-hoc* testing in R and exceeding the threshold of effect size (abs($\log_2$(FC)) above $\log_2$(1.5)). The exposure conditions not inducing significant changes and/or for which the threshold of effect size was not met, are not described in detail in the text but can be derived from the figures. Significance of pairwise comparisons between different treatments (concentration) is given by Supplementary Information Table S7 and enables information to be derived on concentration–response relationships.

Hepatotoxicity-associated gene expression in 120 hpf zebrafish larvae exposed for 48 h (Fig. 3a). The ISH results (see "Whole-mount in situ hybridization") were confirmed for COU. Exposure to COU reduced the expression of *CP*, *CYP3A65*, *GC*, *FABP10a* and *TF*. Next, the hepatotoxicity-associated markers were also screened for ACE, AMI, MET and SAC. Strong effects on gene expression were induced by ACE and AMI. ACE induced downregulation of *TF*, *CP*, *FABP10a* and *GC*. A similar response could be observed after exposure to AMI. The highest concentration of AMI also upregulated the expression of *CYP3A65*. MET and MYC were the least potent chemicals based on gene expression changes of the selected markers. Overall, SAC showed no change in expression, except for the highest concentration that induced little effect on *CP* and *GC*. The marker *NR1H4* is expressed in zebrafish, but no change in expression above the threshold of effect was observed for this marker for any compound.

Statistical evidence of concentration–response relationships can be derived from Supplementary Information Table S7 in which pairwise differences between chemical concentrations were analyzed. The results support that there is indication for concentration–response (systematic increase or decrease) for the following liver-specific markers: *CP* after exposure to AMI; *CYP3A65* after exposure to AMI and COU; *FABP10a* after exposure to ACE, AMI and COU; *GC* after exposure to ACE, AMI and COU; *TF* after exposure to ACE and AMI.

Apoptosis and/or metabolism-related/oxidoreductase activity in exposed 120 hpf zebrafish larvae. Two apoptosis-related markers (*CASP3A* and *TP53*; Fig. 3b) and metabolism/oxidoreductase-related markers (*CYP2K19* and *ZGC163022*; Fig. 3c) were tested to check if these genes are influenced by exposure to the test compounds. In larvae, exposed to ACE and AMI, transcription of *CASP3A* was stimulated for the two highest concentrations. The same was true for the marker *TP53* after ACE exposure. The marker *ZGC163022* was increased in expression by ACE (5000 µM) and COU exposure (all concentrations). On the other hand, for AMI, downregulation of *ZGC163022* was observed after exposure to 1 µM. *CYP2K19* was increased in expression for 5 µM AMI. On the contrary, for ACE and COU (all concentrations), *CYP2K19* was downregulated.

Potential gene markers to predict human hepatotoxicity. The compounds ACE, AMI and COU show a decreased expression of most

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Table 3. Effect percentages of evaluated phenotypic endpoints after 48 h exposure of zebrafish larvae to hepatotoxic compounds and a non-hepatotoxic compound SAC, compared to corresponding NC/SC. Effect percentages were calculated only for the living larvae, and are given for each of separate experiments, except for condition SC with for all experiments ($n = 18$) 100% clear and healthy liver

Test compounds	Nominal concentration (μM)	Effect percentages for phenotypic endpoints (%)		
		Clear, healthy liver	Changes liver size	Liver degeneration
ACE ($n = 2$)	2000	80–100	0–0	0–20
	4000	11–56	11–22	22–56
	5000	10–11	0–33	56–80
AMI ($n = 3$)	0.2	90–100–100	0–0–0	0–0–10
	1	60–100–100	0–0–10	0–0–30
	5	20–40–60	0–20–50	40–50–60
COU ($n = 4$–5)	250	50–70–80–100–100	0–0–0–10–50	0–0–0–10–20
	500	0–10–30–89	0–11–30–60	11–30–30–80
	1000	0–0–0–22	0–0–40–56	11–20–70–100
MET ($n = 5$)	2.5	100–100–100–100–100	0–0–0–0–0	0–0–0–0–0
	5	90–100–100–100–100	0–0–0–0–10	0–0–0–0–0
	10	0–20–60–60–70	10–10–25–40–70	0–20–30–70–75
MYC ($n = 3$–5)	12.5	90–90–100–100–100	0–0–0–0–10	0–0–0–0–10
	25	89–100–100	0–0–0	0–0–11
	50	10–20–30–40	0–0–20–40	30–40–60–80
SAC ($n = 2$)	3400	100–100	0–0	0–0
	6800	100–100	0–0	0–0
	13 700	100–100	0–0	0–0
NC ($n = 4$)	Fish water	100–100–100–100	0–0–0–0	0–0–0–0
SC ($n = 18$)	0.1% DMSO in fish water	100	0	0

[a]ACE, acetaminophen; AMI, amiodarone; COU, coumarin; DMSO, dimethyl sulfoxide; FC, fold change; MET, methapyrilene; MYC, myclobutanil; *n*, number of independent biological experiments; NC, negative control; SAC, saccharin; SC, solvent control.

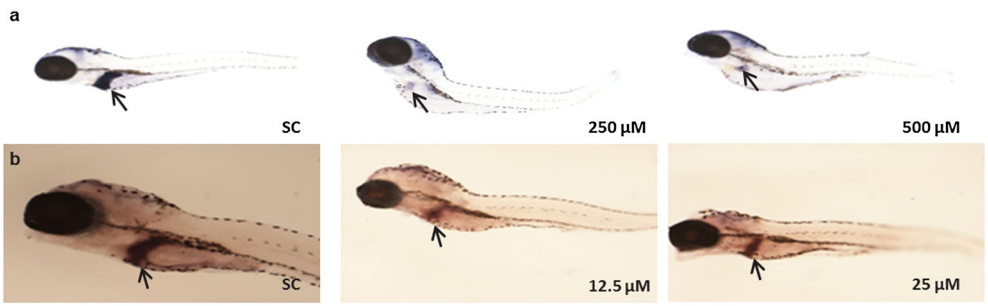

Figure 2. *In situ* hybridization images of 120 hpf zebrafish larvae after 48 h exposure to the SC (0.1% dimethyl sulfoxide in fish water), 250 and 500 μM coumarin (a), and 12.5 and 25 μM myclobutanil (b) for the marker *transferrin*. The liver region is indicated by an arrow. SC, solvent control.

hepatotoxic markers. On the other hand, MET and MYC show a minor response for differential gene expression of only a few markers indicating that these compounds are less potent in the zebrafish model compared to ACE, AMI and COU, despite phenotypic effects at the highest test concentration.

Chemical analysis

Test compound concentration measurement in medium. An overview of the compound concentration measurements in the medium as a function of the nominal concentration at the beginning and end of exposure, and in blank wells at the end is presented in Fig. 4(a–e). These results show a good agreement between the measured concentration of the test compounds in the medium compared to the nominal concentration and between the measured concentrations at the beginning of an experiment versus the concentration at the end for all compounds.

Determination of compound uptake by zebrafish larvae. The whole body concentration of compound uptake in zebrafish larvae as a function of the nominal concentration in the medium is shown in Fig. 5(a–f). For exposure to ACE, AMI, MET and MYC an increase in compound uptake in the larvae is shown with increasing concentration in the exposure medium. For AMI, MET and MYC (highest nominal concentration), the uptake concentration in the larvae was higher than the nominal concentration, indicating a bioconcentration of test compound in the larvae. Larvae exposed to 250 and 500 μM COU showed the same low

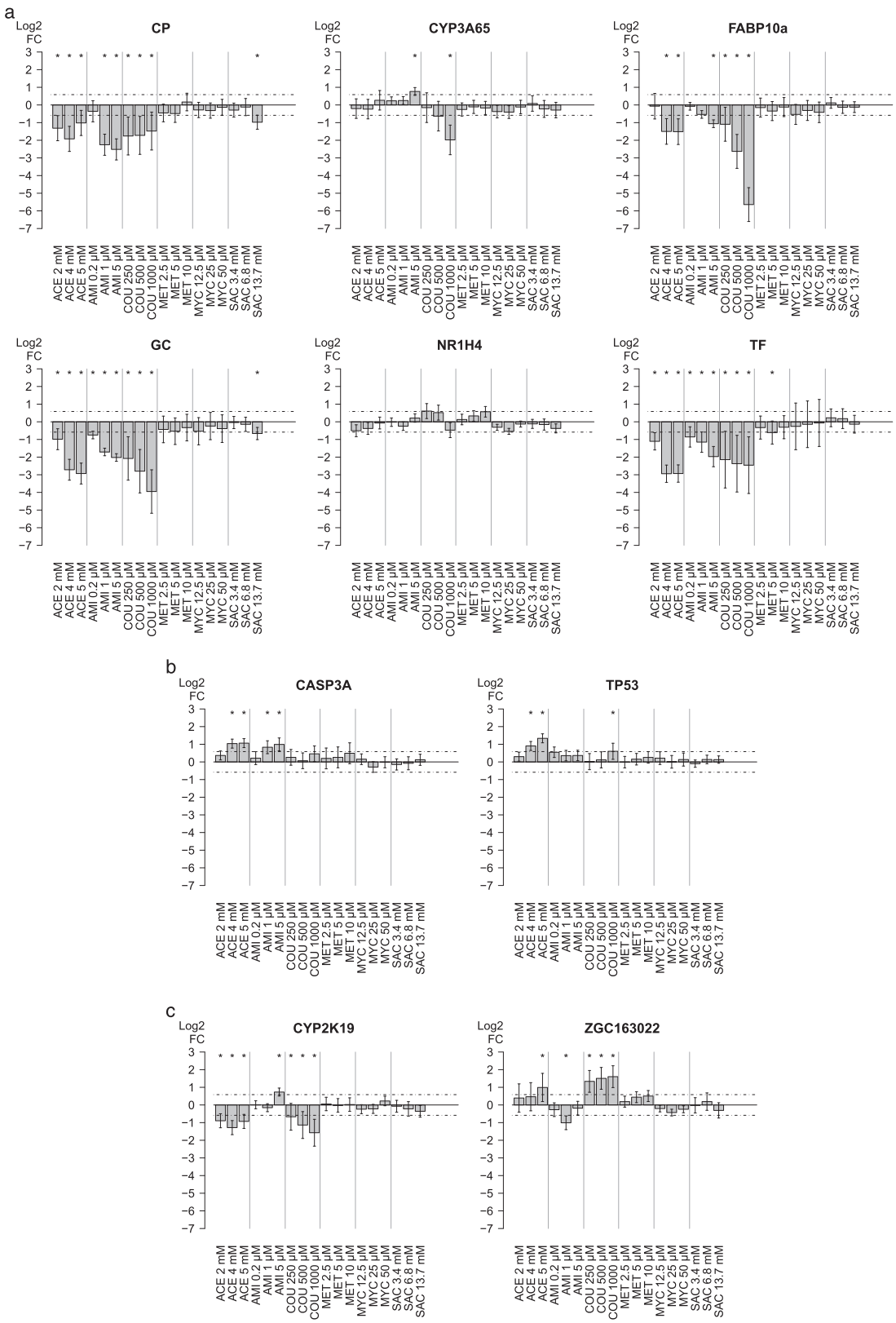

Figure 3. (a–c) Real-time reverse transcription–quantitative polymerase chain reaction results of (a) liver-specific markers (*CP*, *CYP3A65*, *FABP10a*, *GC*, *NR1H4*, *TF*), (b) apoptosis-related markers (*CASP3a* and *TP53*) and (c) metabolism−/oxidoreductase-related markers (*CYP2K19* and *ZGC163022*) after 48 h exposure of zebrafish larvae to three sublethal concentrations of hepatotoxic compounds (shown on *x*-axis) ACE, AMI, COU, MET and MYC and the non-hepatotoxic compound SAC. The average log2 FC and 95% confidence interval (error bars) of three independent biological experiments are shown. The threshold FC of log2 (1.5) is shown by a dotted line. Treatments for which the effect crosses the threshold of effect and for which the effect is statistically significant compared to the solvent control group ($P < 0.05$ Tukey's test) are presented by "*". ACE, acetaminophen; AMI, amiodarone; COU, coumarin; FC, fold change; MET, methapyrilene; MYC, myclobutanil; SAC, saccharin.

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level (~3 μM) of compound uptake, which doubled when exposed to the highest concentration (1000 μM). Low whole body concentrations compared to medium were also seen for exposure to SAC, but actual concentrations reached 80–120 μM, which is of same order of magnitude as for some hepatotoxic compounds (ACE, MYC, MET).

On the second *y*-axis of Fig. 5(a–f), the relative compound uptake is shown, which is the percentage of the measured concentration in the medium at start in function of the nominal concentration. Results on relative uptake can be expressed as high (> 100%), medium (5–100%) or low (< 5%) uptake relative to the test solution concentration as proposed by Gustafson *et al.* (2011). A low relative uptake compared to the nominal concentration was observed for the compounds COU and SAC. ACE exposure resulted in a medium uptake (from 5 to 12%) in zebrafish larvae. High relative compound uptake (range 100–1000%, or up to 10-fold bioconcentration for MET) in zebrafish larvae was observed after exposure to AMI (nominal concentration: 1 and 5 μM), MET and MYC (nominal concentration 50 μM).

Discussion

Phenotypic evaluation

In the current model, 72 hpf zebrafish was found to be an optimal developmental stage with a fully functional liver (Isogai *et al.*, 2001) and 48 h exposure was sufficient to see the compound effects under the microscope (Hill *et al.*, 2012). Visualizing zebrafish liver requires manual manipulation and proper orientation of the animals in 2% methylcellulose. Here, phenotypic assessment of gross morphological changes was used to screen for liver toxicity and to define sublethal concentrations for gene expression experiments. Despite some variation between the independent experiments (cfr. Table 3), we found that all the assessed mammalian hepatotoxic compounds induced the expected liver degeneration or changes in liver size for certain tested concentrations, whereas the non-hepatotoxic compound SAC did not have any phenotypic adverse effect on zebrafish liver. This method to assess liver toxicity in zebrafish larvae, supplemented with observations on decreased yolk sac retention upon hepatotoxicity was also successfully applied by others (He *et al.*, 2013; Hill *et al.*, 2012), though scoring of liver morphology remains subjective and difficult to standardize unless automated imaging systems are developed (Pardo-Martin *et al.*, 2010). Phenotypic evaluations in zebrafish larvae were extended with histopathological studies of the liver (Driessen *et al.*, 2013, 2014) for nine hepatotoxicants, categorized according to their known phenotypes in humans: cholestasis, steatosis and necrosis. Distinct histopathological changes (lipid vacuoles, chromatin condensation, eosinophilic vacuolization) were observed in some zebrafish larvae depending on the treatment. Frequency and type of effects did not allow to distinguish nominal mammalian phenotypes and the incomplete maturation of liver in the zebrafish larvae (Driessen *et al.*, 2013, 2014). Two of the nine compounds, ACE and AMI, were in common with our study with, respectively, a lower concentration range (up to 660 μM) and higher range (up to 10 μM) compared to our range-finding experiments (up to 5000 μM for ACE and 5 μM for AMI). The concentrations were differently defined in both studies. In the studies of Driessen *et al.*, the highest concentration had no observable general morphological or teratological effects and no mortality. In our study, the highest concentrations were sublethal and specifically defined as those having a clear toxic effect on the liver.

Biomarker evaluation

Driessen *et al.* (2013 extended the fore-mentioned histopathological evaluation with gene expression analysis in whole zebrafish embryos (120 hpf) compared to the liver of adult zebrafish for a set of reference hepatotoxic compounds, including ACE and AMI. Some hepatotoxicity-associated genes were only present in whole zebrafish embryo, e.g., *FABP10a*. Mesens *et al.* (2015 showed promising predictivity for the hepatotoxic effects in zebrafish larvae by studying the expression of *FABP10a* as an appropriate endpoint. This marker was also studied in our experimental set-up. To study hepatic gene expression, it was decided to use whole zebrafish larvae RNA extracts as the most practical way in this study. Hepatotoxicity-associated gene expression responses remain detectable in the noise of other tissues and were not hampered by the developmental stage of the whole zebrafish larvae.

The most common drug causing drug-induced liver injury is ACE. When an accidental or deliberate overdose occurs, the reactive metabolite *N*-acetyl-*p*-benzoquinone imine is produced by CYP450 enzymes (Nelson, 1990). Excess of this reactive metabolite causes oxidative stress in humans and mice by depleting glutathione and results in mitochondrial damage and necrotic cell death (McGill *et al.*, 2012). Compounds that damage mitochondrial structure, enzymes or DNA synthesis can disrupt β-oxidation of lipids and oxidative energy production within the hepatocytes. Prolonged interruption of β-oxidation leads to micro/macrovesicular steatosis (Cullen, 2005). In this zebrafish study, high concentrations (mM range) of ACE were used to observe any phenotypic effects on the liver, which is in analogy with other zebrafish reports (He *et al.*, 2013). CYP3A65 gene expression was not altered after ACE exposure, which is possible as not all CYPs are regulated at the mRNA level by their substrates. All other hepatotoxicity-associated genes, except *NR1H4*, were decreased in expression after ACE exposure. In patients with ACE overdose, total and free GC-globulin serum levels were decreased as a sign of hepatotoxicity (Amacher *et al.*, 2005; Lee *et al.*, 1987; Schiodt *et al.*, 2001). As a cellular defense response against oxidative stress induced by ACE (McGill *et al.*, 2012), genes involved in oxidoreductase metabolism were increased in the expression as also observed in the zebrafish model in this study for *ZGC163022* (5 mM ACE) and in the study of Driessen *et al.* (2014 it was 660 μM.

In this gene expression study of zebrafish, all hepatotoxic markers, except *NR1H4*, were decreased in expression for at least one tested concentration of AMI. The apoptotic marker *CASP3A* and metabolism-related gene *CYP2K19* were increased in expression. The other marker *ZGC163022* was decreased in expression, whereas in the study of Driessen *et al.* (2014 this marker was increased after exposure to 10 μM AMI, which is a twofold higher concentration than in our study.

COU exhibits marked species differences in both metabolism and hepatotoxicity. In zebrafish larvae, all liver-associated gene markers, except *NR1H4*, were decreased in expression, as also observed for the other known model hepatotoxic compounds ACE and AMI. As also observed in rat studies (Lake *et al.*, 2002; Uehara *et al.*, 2008), increased expression of *ZGC163022* points to increased oxido-reductase activity.

MET is a known hepatotoxin in rats. It was found that enterohepatic recirculation of metabolites was important for its hepatotoxicity (Ratra *et al.*, 2000). MET was also indicated as a severely hepatotoxic compound in humans (O'Brien *et al.*, 2006). In this experimental set-up, only *TF* was statistically

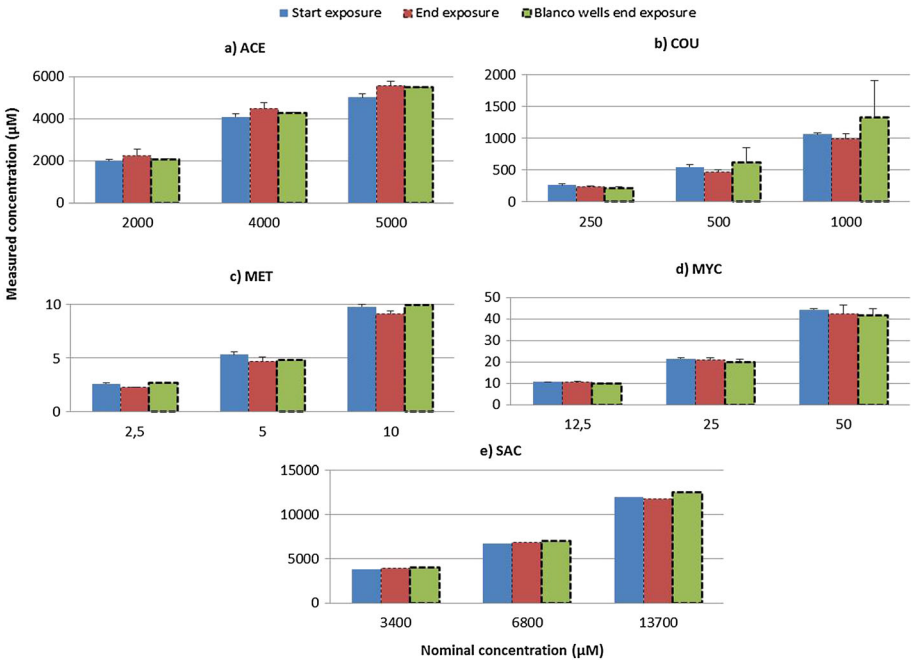

Figure 4. (a–e) Test compound concentration measurement in medium. The average measured concentration (µM, *y*-axis) with standard deviation of two independent experiments was plotted against the nominal concentration (µM, *x*-axis) for exposure to three sublethal concentrations of four hepatotoxicants. Only one experiment was performed for SAC. For MET, concentration in blank wells was measured for only one experiment. ACE, acetaminophen; COU, coumarin; MET, methapyrilene; MYC, myclobutanil; SAC, saccharin.

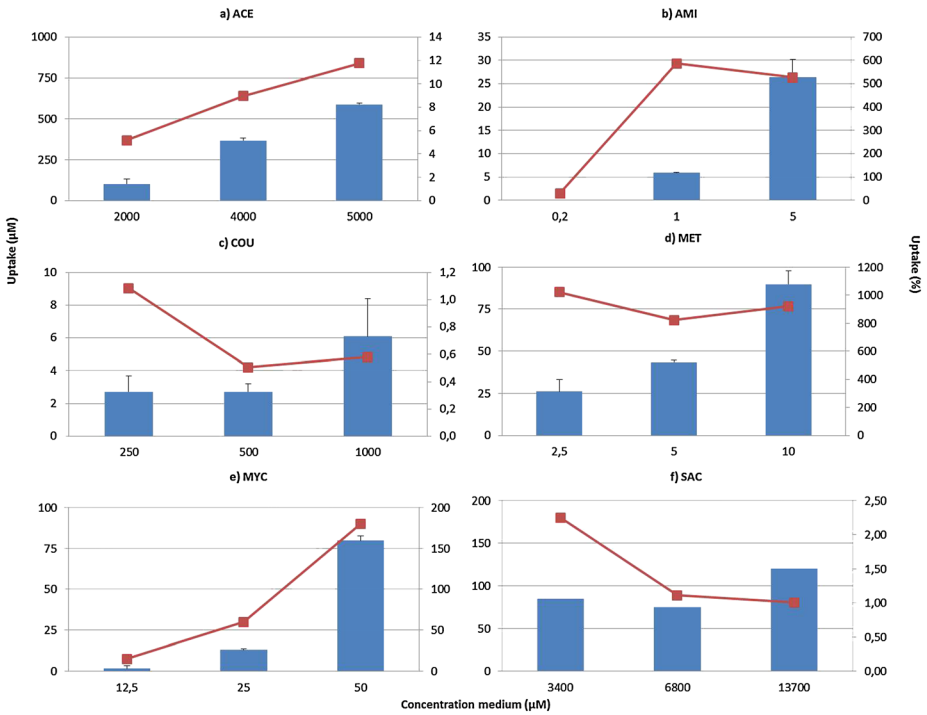

Figure 5. (a–f) Compound uptake by zebrafish larvae. The average and standard deviation of measured compound concentration in larvae (µM, left *y*-axis, column) and the relative uptake of the compound in larvae as a percentage of the nominal concentration in the medium at the start (%, right *y*-axis, line) is plotted against the nominal concentration in the medium for exposure to three sublethal concentrations of hepatotoxicants (µM, *x*-axis). Results of two independent experiments, except for SAC only one experiment was performed. ACE, acetaminophen; AMI, amiodarone; COU, coumarin; MET, methapyrilene; MYC, myclobutanil; SAC, saccharin.

significantly decreased in expression after 5 µM MET exposure, which is not really convincing for a good model for hepatotoxic compounds.

MYC induces hepatotoxicity in mouse and rat by CYP450 metabolic bioactivation leading to perturbation of fatty acid, steroid and xenobiotic metabolism pathways through an adrostane

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receptor and pregnane X receptor signaling pathways (Goetz and Dix, 2009; Goetz *et al.*, 2006). The hepatic gene markers and metabolism/oxidoreductase or apoptosis-related genes were not statistically significantly affected after MYC exposure in zebrafish.

Exposure to MET and MYC resulted in no statistically significant response of any hepatotoxic marker, except for 5 μM MET for the marker *TF*, and none of the apoptosis- or stress-related markers compared to the strong gene responses induced by ACE, AMI and COU exposure.

A good marker to predict hepatotoxicity should give a response after exposing zebrafish larvae to hepatotoxic compounds and not non-hepatotoxic compounds. Our gene expression studies demonstrated that four of six tested hepatotoxic markers, more specifically *CP*, *FABP10a*, *GC* and *TF* are promising markers as a step towards the development of transgenic models for screening (Fig. 3a), which is the final goal of this project. These four genes showed a decreased expression after ACE, AMI and COU exposure, which was often concentration dependent. This was clearly different from the negative compound SAC, which only induced a small effect at the highest concentration on *CP* and *GC*. At first, a *TF* transgenic zebrafish will be developed and with successful construction, its complementarity will be checked to the existing *FAPB10a* transgenic line (Mesens *et al.*, 2015). *TF* is highly expressed in the adult mammalian liver and is secreted by hepatocytes into the serum where it functions as an iron transport protein and growth factor for a variety of cells (de Jong *et al.*, 1990; Zakin, 1992). For that reason, transferrin enzyme among others is used in preclinical animal studies to predict adverse liver effects in humans (Amacher, 2002), and is seen in our zebrafish study as a marker for hepatotoxicity of three tested compounds. The decreased expression of TF gene, resulting into disruption of normal iron metabolism by affecting transferrin as a major protein involved in this process (Hertz *et al.*, 1996) might thus be one of the mechanisms inducing liver damage after xenobiotic exposure.

Chemical analysis

Zebrafish are often exposed by dissolving the compound in the water, which enables easy and fast administration. This is an advantage of the model that allows for high-throughput screening. As part of this study, the concentration of test compound in the surrounding aqueous medium and within zebrafish larvae were measured to assess uptake and to interpret appropriately the relevance of any observed toxicity outcome. The protocol described herein was successful at determining the amount of compound present in tissue samples as small as five pooled larvae (120 hpf stage). All compounds were stable within the setup of these experiments, except AMI for which precipitation was observed already after 24 h of exposure. A good agreement was observed between the measured concentrations in the medium and the nominal concentrations at the start of the experiments at 72 hpf and after 48 h exposure for all compounds. There was a wide variation between the actual concentrations of compound in the zebrafish larvae with a higher compound uptake of AMI, MET and MYC being concentrated in the larvae up to 527, 920 and 180% respectively of the concentration in the medium at the start in function of the nominal concentration (for the highest concentration) after a 48 h exposure period. Conversely, levels of COU and SAC were $<5\%$ of the external concentration at 120 hpf, indicating a low compound uptake, whereas ACE exposure resulted in a medium uptake (from 5 to 12%). Despite low relative uptake, it is clear that the actual body concentration of the non-hepatotoxic compound SAC is of the same order of magnitude as some hepatotoxic compounds (COU, AMI, MYC) while there is no phenotypic effect on liver (true negative). We also need to remark that only parent compounds were measured in the larvae, and levels of low to medium uptake might rather be the result of slow to fast biotransformation and clearance of the parent compounds, while metabolites could not be quantified in our analytical approach. There was an indication that compounds with low log K_{ow} (< 1.5) (or less lipophilic character) (Table 1) resulted in low relative uptake. However, more compounds should be evaluated and other physical–chemical properties should be included to confirm this hypothesis.

A relationship between log K_{ow} and compound uptake was shown in a study using zebrafish-based assays for the assessment of cardiac, visual and gut function (Berghmans *et al.*, 2008), while no correlation of compound uptake with log K_{ow} values was observed in another study using zebrafish as a screening tool for developmental toxicity (Van den Bulck *et al.*, 2011). On the other hand, an association could be made between the amount of compound uptake and the strength of gene expression responses, e.g., high uptake of AMI was observed in larvae and exposure resulted in a strong decreased expression of all hepatotoxicity markers. No conclusion can be made for the other compounds (ACE, COU), as they could have been metabolized, and no metabolites were measured in this study. Nonetheless, this could be the subject of further studies. Measurement of the internal concentration of a parent compound or corresponding metabolites, considering uptake and clearance should be further investigated for their value as a "threshold" for gene expression changes and hepatotoxic effects.

Conclusions

Our data support the use of zebrafish larvae as a predictive animal model for assessing compound-induced hepatotoxicity. This convenient, predictive animal model can serve as an intermediate step between cell-based evaluation and mammalian animal testing. Mammalian evidence is available to confirm the hepatotoxic nature of the five selected liver toxic compounds. Both ISH and real-time RT-qPCR support the phenotypic observations on liver toxicity in zebrafish. The results of the current study are part of the ongoing development and validation of a test strategy using a transgenic zebrafish line for a quick screening to prioritize unknown compounds. Our goal is to develop a *TF* transgenic zebrafish. With successful construction, the latter should first be tested for its complementarity to the existing *FABP10a* transgenic line for an extended panel of chemicals covering all the seven mechanisms of human hepatotoxicity.

Acknowledgments

The ZETOX project is sponsored by Federal Public Service, Health, Belgium (RF 10/6232). For technical assistance, the authors are grateful to Francis Boonen (VITO), Virginie Von Berg (ULg) and Marianne Voz (ULg).

Informed consent

The manuscript does not contain clinical studies or patient data.

Conflict of interest

The authors did not report any conflict of interest.

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