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Identification of proteome markers for drug-induced liver injury in zebrafish embryos

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ABSTRACT

The zebrafish embryo (ZFE) is a promising alternative non-rodent model in toxicology, and initial studies suggested its applicability in detecting hepatic responses related to drug-induced liver injury (DILI). Here, we hypothesize that detailed analysis of underlying mechanisms of hepatotoxicity in ZFE contributes to the improved identification of hepatotoxic properties of compounds and to the reduction of rodents used for hepatotoxicity assessment. ZFEs were exposed to nine reference hepatotoxicants, targeted at induction of steatosis, cholestasis, and necrosis, and effects compared with negative controls. Protein profiles of the individual compounds were generated using LC-MS/MS. We identified differentially expressed proteins and pathways, but as these showed considerable overlap, phenotype-specific responses could not be distinguished. This led us to identify a set of common hepatotoxicity marker proteins. At the pathway level, these were mainly associated with cellular adaptive stress-responses, whereas single proteins could be linked to common hepatotoxicity-associated processes. Applying several stringency criteria to our proteomics data as well as information from other data sources resulted in a set of potential robust protein markers, notably Igf2bp1, Cox5ba, Ahnak, Itih3b.2, Psma6b, Srsf3a, Ces2b, Ces2a, Tdo2b, and Anxa1c, for the detection of adverse responses.

1. Introduction

The liver has a central role in the metabolism of xenobiotic substances in animals, which includes biotransformation targeted at inactivation of toxic compounds. However, this biotransformation may also produce toxic reactive metabolites, leading to xenobiotic-induced liver injury (Jaeschke et al., 2002; Lee, 2003). Various underlying mechanisms can result in a wide range of xenobiotic-induced toxic hepatic or hepatocellular phenotypes, including steatosis, cholestatic phenotypes, necrosis, and inflammation, and mixed forms (Kleiner, 2014). When focusing on drugs as xenobiotics, steatosis is the most prevalent phenotype and the leading diagnosis in drug-induced liver injury (DILI), associated with requirement for liver transplantation and with fatal outcomes (Kleiner, 2014). Steatosis is characterized as an increase in cellular lipid content due to an increase in *de novo* synthesis of fatty acids or reduced lipid secretion or oxidation (Anderson and Borlak, 2008). Cholestasis is a chronic condition and is phenotypically characterized by bile accumulation as a result of impaired intra- or extracellular bile flow or bile composition (Wagner et al., 2009). Necrosis is an acute condition and is characterized by cell death typically due to oxidative stress (Jaeschke et al., 2002, 2012).

A challenge in toxicology is to predict the hepatotoxic potential of compounds to which humans are exposed, preferably through new approach methodologies (NAMs), since the traditional in vivo rodent studies are associated with ethical (large numbers of animals potentially experiencing discomfort after exposure), economical (high costs due to high doses and long experimental periods), and scientific issues (the obtained results might not be fully predictive for the effects in humans). As such, the zebrafish embryo (ZFE) is an alternative test model, acting alone as a replacement model, or as part of a battery of NAMs, logically

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combined to inform toxicological events along a structured pathway, e.g. known as Adverse Outcome Pathways (AOPs; (Vinken, 2013)). Several AOPs for liver-specific adverse outcomes have been described (AbdulHameed et al., 2019; Bell et al., 2016; Gijbels et al., 2020; Gijbels and Vinken, 2017; Lichtenstein et al., 2020; Luckert et al., 2018; Mellor et al., 2016; Negi et al., 2021; Tsakovska et al., 2014; Vinken, 2015; Vinken et al., 2013), and some are being curated for inclusion in the OECD AOP wiki (https://aopwiki.org). Although the ZFE is not generally included as a test model in these AOPs so far, it has specific advantages because it combines the benefits of an in vivo model, namely an intact organism with complete biological complexity including interactions between tissues and cells (Jones et al., 2009; Peterson and Macrae, 2012; Sukardi et al., 2011), with the advantages of an in vitro model, that is, the ability for medium to high throughput testing. In addition, in view of its early developmental condition, the ZFE until 120 h post fertilization (hpf) are considered as non-protected stages under European legislation on laboratory animals (European Union, 2010).

The structure and the function of the liver in the adult zebrafish is similar to the mammalian liver (Hill et al., 2012), and a functional liver is present in the zebrafish embryo at 72 hpf, which is therefore a suitable time point to start hepatotoxicity testing in this model. Furthermore, there is high genetic conservation between humans and zebrafish, i.e. approximately 70 % of human genes have at least one obvious zebrafish orthologue (Howe et al., 2013). Although genetic conservation itself does not imply functional similarity per se, further analyses showed that conserved genes include those associated with hepatotoxicity and biotransformation (Goldstone et al., 2010; Hill et al., 2012). Next, mechanistic understanding of drug metabolism in humans compared to test species is essential for translation of effects between species (van Wijk et al., 2016). Zebrafish embryos express several cytochrome P450 enzymes, CYPs, which are grouped into the same families when compared to humans. In the zebrafish, the CYP subfamilies 1–4 are important for metabolizing xenobiotics, and these families are similar between the two species. The most important CYP in humans responsible for catalyzing the majority of known drug-metabolizing reactions, CYP3A4, has an ortholog in the zebrafish, namely, *cyp3a65* (Goldstone et al., 2010; Hill et al., 2012). The similarities between humans and zebrafish on the level of genetic make-up and metabolism support the human relevance of the zebrafish model in toxicology, however without ignoring the greater phylogenetic distance between these two species as compared to the phylogenetic distance between humans and rodents.

In view of immaturity of the developing liver, the level of metabolic activity in the ZFE has been a matter of dispute, but there is good support for active metabolism even in 72 hpf embryos (van der Ven et al., 2020). In previous studies, we showed that the ZFE can be used to identify specific hepatotoxic responses using histopathology and hepatotoxic-associated transcripts by next-generation sequencing (NGS) (Driessen et al., 2013), and possible biomarkers for general hepatotoxicity by gene expression analysis could be identified (Driessen et al., 2014). Thus, gene expression analysis resulted in a potential list of biomarkers to predict hepatotoxicity in a high-throughput manner, however, the measured mRNA levels are not per se predictive for levels of the translated protein product, let alone for protein activity (Baginsky et al., 2010). Proteomics analysis is an additional tool, which is already routinely applied on patient serum for diagnosis, and this can also be used to generate better understanding of the underlying molecular mechanisms of hepatotoxicity, while it provides quantitative information of molecular events at a more functional level (Kralj et al., 2021). Proteomics has been applied in the zebrafish embryo, albeit mainly targeted at unraveling developmental processes (Pina et al., 2018; Purushothaman et al., 2019). The objective of this study was to identify proteomic signatures which mark hepatotoxic events in the zebrafish embryo.

The distinct phenotypical classes of interest including steatosis, cholestasis, and necrosis could be observed in the liver of adult zebrafish, however variant phenotypes were observed in the zebrafish embryos (Driessen et al., 2013). Also, differential gene expression was more related to hepatotoxicity in general than to the specific nominal phenotypes (Driessen et al., 2013, 2014). Therefore, we here hypothesize whether hepatotoxicity in general can be discerned by a specifically altered proteome profile. Finally, we assessed whether specific hepatotoxicity-associated proteomics markers could be derived from such a proteome, to complement the changes as observed with gene expression after exposure to reference hepatotoxicants. For this purpose, nine reference hepatotoxicants were defined, with confirmed hepatotoxic phenotypes in humans and in rodent studies, namely steatosis (amiodarone, tetracycline and valproic acid) (Leitner et al., 2010; Santangeli et al., 2012; Silva et al., 2008), cholestasis (cyclosporine A, chlorpromazine and 17alpha-ethinylestradiol) (Ansede et al., 2010; Bohan and Boyer, 2002; Yamamoto et al., 2006; Zimmerman, 1999), and necrosis (acetaminophen, paraquat and thioacetamide) (Jaeschke et al., 2002; Zimmerman, 1999). Zebrafish embryos were treated with each of these compounds for 48 h starting from 72hpf, with a single dose which, in a dose response analysis, caused discernable effects on the level of phenotype and gene expression (Driessen et al., 2013, 2014). Whole body extracts of these exposed embryos were then used for proteomics profiling by liquid chromatography coupled with tandem mass spectrometry.

2. Materials and methods

2.1. Chemicals

All tested chemicals (purity > 95 %), were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands), and included acetaminophen (N-acetyl-para-aminophenol; paracetamol; APAP, CAS no.103-90-2), paraquat (1,1′-Dimethyl-4,4′-bipyridinium dichloride; PQ, CAS no.1910-42-5), thioacetamide (CH3-C(S)NH2); TA, CAS no.62–55–5), amiodarone hydrochloride (2-butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]−3,5-diiodophenyl ketone hydrochloride; AM, CAS no.19774-82-4), valproic acid (2-propylpentanoic acid sodium; VPA, CAS no.1069-66-5), tetracycline (TET, CAS no.64-75-5), cyclosporine A (CsA, CAS no.59865-13-3),17α-ethynylestradiol (17α-Ethynyl-1,3,5(10)-estratriene-3,17β-diol; EE2, CAS no.57-63-6), chlorpromazine (2-Chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride; CPZ, CAS no.69–09–0), D-Mannitol (Mannite; DM, CAS no. 69-65-8). Dutch Standard Water or 0.2 % Dimethylsulfoxide (DMSO, CAS no. 67-68-5, Fisher-Scientific) served as vehicle controls.

2.2. Fish treatment

Wild-type zebrafish (*Danio rerio*) were originally obtained as commercially bred Singapore import (Ruinemans Aquarium BV, Montfoort, The Netherlands), which was maintained and bred in our facilities for more than 5 generations. Egg production was optimized by separation of the male and female fish before spawning, and female zebrafish were fed only thawed *Artemia naupli* prior to spawning. Egg predation was prevented by using a breeding tank with a perforated bottom, in which male and female zebrafish were paired in a 2:2 ratio before spawning. Spawning was triggered by morning light and was usually completed within 30 min. After spawning, the eggs were collected using a glass siphon and debris was removed to rinse the fertilized batches of eggs at least three times in Dutch Standard Water (DSW; demineralized water supplemented with $NaHCO_3$ (100 mg/l)), $KHCO_3$ (20 mg/l), $CaCl_2{\cdot}2H_2O$ (200 mg/l), and $MgSO_4{\cdot}7H_2O$ (180 mg/l) which was then. aerated for 24 h at 27 °C). After rinsing, fertilized eggs from different batches were pooled and placed in a petri dish in an incubator at 26.5 ± 1 °C with a light cycle of 14 h light/10 h dark. After 72 h, hatched embryos were randomly distributed over 48-well plates in a density of 5 embryos per well in 1 ml test or control medium. Per test compound, three statistical replicates, each consisting of N = 10 embryos (2 wells), were used. In total, 12 conditions were tested including

9 human hepatotoxicants, two vehicle controls and one negative (non-hepatotoxic) control compound (D-mannitol) (Table 1). The selected concentrations were the highest applied in a previous gene expression study (Driessen et al., 2014), and induced no mortality nor observed morphological or teratological effects. At 120 hpf, the embryos were evaluated under a Leica Labovert FS microscope for deviations from normal development (e.g. delay in development, teratogenic effects) and indications of toxicity (e.g. abnormal swimming behaviour) and snap frozen in liquid nitrogen to be used for proteomic analysis. Compound concentration during the 48 h of exposure period (72–120 hpf) was not analysed, however, all compounds were indicated to be relatively stable according to NLM Hazardous Substances Database (https://pubchem.ncbi.nlm.nih.gov/source/11933).

2.3. Protein extraction and digestion

Protein extraction and digestion was adapted from a previously reported method (van der Plas-Duivesteijn et al., 2014). In short, at 120 hpf, 10 embryos from each control and treated groups were homogenized using 0.5 mm zirconium oxide beads and a Bullet Blender (Next Advance, New York, United States), and proteomes were extracted using a lysis buffer containing 8 M urea, 75 mM NaCl, 50 mM Tris-HCl pH 8.2, 50 U/ml benzonase (E1014–5KU, Sigma-Aldrich), 2 mM $MgCl_2$, and protease inhibitors (Complete ULTRA Tablets, mini, EDTA-free, Roche). The lysate was kept at 4 °C for 30 min and the supernatant was collected after centrifugation at 16,000 ×*g* for another 30 min at 4 °C. Total protein concentration was estimated by the use of a BCA assay (Product #23235, Bio-Rad) based on bovine serum albumin as standard. In solution digestion of the proteins was performed by adding 60 mM dithiothreitol (DTT) to the protein extract and incubation for 45 min at 56 °C for cystines reduction. For alkylation and protection of the cysteines, 100 mM iodoacetamide was added to the sample followed by incubation for one hour at room temperature in the dark. Then, the protein mixture was diluted by adding 50 mM ammonium bicarbonate. Next, the volume of the samples was reduced to 30 µl by centrifuging at room temperature for 30 min at 14,000 ×*g* using the Millipore Amicon Centrifugal Filter ultra 0.5 (3000 Da MWCO). The samples were collected by reversing the column in a new tube and centrifuging at 1000*g* for 2 min. After this, sequencing-grade trypsin, which specifically cleaves at the carboxylic side of lysine and arginine residues, was added to each sample to initiate digestion, and after overnight incubation, 10 % trifluoroacetic acid (TFA) was added to quench the digestion producing peptides that are readily identified by mass spectrometry. Samples were centrifuged at 2500×*g* for 10 min at room temperature, whereafter supernatant was transferred to a new tube. Peptide digests were stored at − 80 °C until further analysis.

2.4. Liquid chromatography - tandem mass spectrometry

As previously described (van der Plas-Duivesteijn et al., 2014), 2 µl of each sample was loaded and desalted on a C18 PepMap 300 µm, 5 mm-i.d., 300 Å precolumn (Thermo Scientific) and separated by reversed-phase liquid chromatography using two identical 150 mm 0.3 mm–i.d. ChromXP C18CL, 120 Å columns (Eksigent, Dublin, CA, USA) coupled parallel and connected to a split less NanoLC-Ultra 2D plus system (Eksigent) with a linear 90-min gradient from 4 % to 35 % acetonitrile in 0.05 % formic acid and a constant (4 µl/min) flow rate. The LC system was coupled to an amaZon speed ETD ion trap (Bruker Daltonics, Bremen, Germany) equipped with an Apollo II ESI source. After each MS scan, up to 10 abundant multiply charged species in *m/z* 300–1300 were selected for MS/MS and actively excluded for one min after having been selected twice. The LC system was controlled by HyStar 3.2 and the ion trap by trapControl 7.1.

2.5. Data analysis

2.5.1. Data processing

Raw LC-MS/MS data were converted to line spectra mzXML files (Pedrioli et al., 2004) with the Bruker compassXport tool version 3.05. All further data processing was done with the Trans-Proteomic Pipeline 4.6 rev 3 (Deutsch et al., 2010; Keller et al., 2002). Database search was performed with X!Tandem (2009.10.01.1) (Craig and Beavis, 2004; Keller et al., 2002) using the following parameters: precursor mass tolerance window between − 2.0 and 4.0 Da, with fragment tolerance of 0.4 Da; modifications for carbamidomethylation of cysteine and oxidation of methionine, k-scoring enabled, maximum missed cleavages set to 2, and scoring done for *b* and *y* ions. The X!Tandem output files were converted to pepXML file format with tandem2xml without applying any cut-offs. X!Tandem pepXML files were merged into PeptideProphet and analyzed with the decoy option enabled (Keller et al., 2002). The resulting posterior probabilities for the peptide spectrum matches were further refined by iProphet (Shteynberg et al., 2011). Finally, ProteinProphet was used to compute a probability that each protein was present in the sample and to estimate a global false discovery rate (FDR). ProteinProphet output was filtered using the probability threshold that corresponded to an FDR of 1 % and proteins with an individual probability of zero were discarded (Craig and Beavis, 2004; Keller et al., 2002). Protein abundance was estimated by spectral counting (label-free quantitation). Spectral counts of each protein or protein group, normalized by the total number of spectral counts and log2-transformed, were used a proxy for protein expression (mass concentration) without further adjustment or modification. The spectral counts by protein (group) were used as reported by ProteinProphet in the Trans-Proteomic-Pipeline with default settings, i.e. Peptide-Spectrum Matches (PSMs) with posterior error probability < 0.2 are not counted.

2.5.2. Protein expression analysis

Following our hypothesis, the basis for protein expression analysis was the association of changes in protein expression with histopathological appearance, which was either defined as the phenotype as observed in humans or rodents after compound exposure (nominal phenotype) or as the actual observed histopathology in ZFE (observed

Table 1
Compound exposures in ZFE experiments.

Compound	Abbreviation	CAS	Exposure concentration	Vehicle	N replicates	Nominal phenotype
Amiodarone	AM	19774-82-4	10	DMSO	3	steatosis
Tetracycline	TET	64-75-5	200	DMSO	3	steatosis
Valproic Acid	VPA	1069-66-5	600	DSW	3	steatosis
Chlorpromazine	CPZ	69-09-0	3	DMSO	3	cholestasis
Cyclosporine A	CsA	59865-13-3	6	DMSO	3	cholestasis
17alpha-ethynylestradiol	EE2	57-63-6	3.5	DMSO	3	cholestasis
Acetaminophen	APAP	103-90-2	660	DMSO	1	necrosis
Paraquat	PQ	1910-42-5	3000	DSW	3	necrosis
Thioacetamide	TAA	62-55-5	10,000	DSW	3	necrosis
D-Mannitol	DM	69-65-8	3000	DSW	2	negative control
Dutch Standard Water	DSW				4	vehicle
Dimethylsulfoxide	DMSO	67-68-5	0.20 %		4	vehicle

phenotype, see (Driessen et al., 2013, 2014)). The nominal phenotypes included three classes, steatosis (AM, TET and VPA), cholestasis (CPZ, CsA and EE2), and necrosis (APAP, PQ and TAA). The observed phenotypes consisted of four classes: lipid vacuolization (AM, CPZ and VPA), chromophobic vacuolization (CsA, EE2, PQ and TAA), eosinophilic vacuolization (CPZ, CsA, TET and APAP), and chromatin condensation (CPZ, EE2, and PQ). To determine whether the phenotypes, nominal or observed, corresponded to pathways at the protein level, a phenotype-directed analysis was performed. For each specific phenotype, a set of discriminating proteins was identified and this set was used for the determination of enriched processes. Discriminating proteins were identified using a two group-comparison (phenotype group against no-phenotype controls) with a $p < 0.05$ in QluCore Omics Explorer version 3.0 (Qlucore AB, Lund, Sweden), and overrepresented pathways per phenotype were determined using STITCH version 5.0 (Szklarczyk et al., 2016) with a 5 % FDR.

2.5.3. Marker protein selection

To detect commonly induced proteins among hepatotoxic compounds, the protein expression values of each compound were compared to those of their respective vehicle controls (see Table 1 for details). Proteins with an absolute FC > 1.5 were considered to be differentially expressed, and proteins showing common up- or down-regulation by at least six compounds were then analysed for overrepresented pathways by STITCH (Szklarczyk et al., 2016). Additionally, STITCH was used to identify protein interactions among the set of common up- and down-regulated proteins, respectively. Interactions with high confidence (score > 0.7) were downloaded and further visualized as a network using Cytoscape (Shannon et al., 2003) version 3.7.2, where network hubs were related to processes through expert view. Proteins that were either up- or down-regulated by 8 or 9 compounds were considered as candidate markers for general hepatotoxicity. These were mapped to biological functions as given in the Zebrafish Information Network (ZFIN) database (https://zfin.org), and visualized as a heatmap with clustering (Euclidean distance, Ward linkage) using R (version 4.1.0). Links to liver toxicity were confirmed using the online databases: human protein atlas (HPA; https://www.proteinatlas.org) and the gene annotation portal BioGPS (http://biogps.org), and a literature search in PubMed, combining protein names with key terms related to liver functions and liver toxicology.

2.5.4. Comparing protein to gene expression

Gene expression was obtained from a previous study (Driessen et al., 2014) and matched with the observed proteins in this study. Protein names from UniProt were matched against the microarray transcripts gene IDs (Palmblad et al., 2013). This resulted in a set of 373 proteins/genes. Next, for each compound the direction of expression was compared between these gene-protein pairs for the subset of proteins that had an absolute FC > 1.5 for that compound. Corresponding direction of gene and protein expression was implicated as a complementary criterion for protein marker definition.

3. Results

3.1. General proteomics, association with histopathology

We identified 287000 PSMs in total, corresponding to 3461 unique peptides and 1258 unique proteins, of which 1011 proteins were present in at least one sample with a probability of 99 % (i.e. an FDR of 1 %). These 1011 proteins were used for association analysis with histopathology, revealing 50, 37, and 84 discriminating, i.e. statistically significantly regulated, proteins for the nominal phenotypes steatosis, cholestasis and necrosis, respectively (Tables S1–3), and 47, 18, 43, and 47 for the observed phenotypes, lipid vacuolization, chromophobic vacualization, eosinophilic vacuolization and chromatin condensation, respectively (Tables S4-7). In turn, these discriminating proteins were the basis for a histopathology-associated pathway analysis, leading to 5, 11, and 13 enriched biological processes for the nominal phenotypes, steatosis, cholestasis, and necrosis, respectively (Table S8). For the observed phenotypes, lipid vacuolization, chromophobic vacualization, eosinophilic vacuolization and chromatin condensation this analysis resulted in 15, 9, 18, and 1 enriched biological process, respectively (Table S8). However, considerable overlap was present in the enriched biological processes (Fig. 1). For example, the biological process “translation” was enriched for each phenotype except eosinophilic vacuolization (Table S8). The phenotype steatosis completely overlapped with the phenotypes necrosis and cholestasis based on enriched processes, whereas there were three necrosis-specific processes and one for steatosis. For the observed phenotypes, eosinophilic vacuolization had 15 specific enriched processes and only shared 3 processes with lipid vacuolization phenotype (Table S8). The other three observed phenotypes shared enriched processes with the lipid vacuolization phenotype (Fig. 1, Tables S8).

3.2. Identification of common hepatotoxicity marker proteins

In view of the large overlap of enriched processes and the subsequent scarcity of phenotype-specific processes, we set out to determine marker proteins for general hepatotoxicity. For this purpose, all.

hepatotoxicants were pooled into and treated as a single group and we determined the number of compounds that led to up- and down-regulation of each protein. This resulted in 427 proteins having a FC > 1.5 and being modulated by 6 or more compounds, evenly representing the categories (phenotypes). Of these, 190 proteins appeared to be up-regulated and 237 down-regulated. Using these 427 proteins, we determined enriched processes, which resulted in 15 up-regulated processes and 10 down-regulated processes (Table 2). Processes involving the most proteins, both up- and downregulated, were broadly defined categories related to metabolism, such as “metabolic process”, “cellular metabolic process”, and “primary metabolic process”. However, the most significantly regulated processes included more defined categories, such as “translation”, “gene expression”, and “protein folding”.

Protein interaction network analysis using STITCH revealed that proteins grouped together into sub-networks of proteins having multiple mutual relations (such as interactions, co-expression or text-mining co-occurrence). These sub-networks generally corresponded to specific cellular processes. Among up-regulated proteins (Fig. 2A), we found major sub-networks for proteins involved in translation (left of center), muscle function (right) and glycolysis (top). For down-regulated proteins (Fig. 2B), we found major sub-networks for proteins involved in translation (bottom left), mRNA splicing (top left), tricarboxylic acid cycle (center), heat shock proteins (center), muscle function (top right) and skin (bottom right). These sub-networks partly overlapped with the processes identified above, and particularly the identification of liver-related sub-networks, such as glycolysis and tricarboxylic acid cycle is of interest. Furthermore, the appearance of a sub-network of mitochondrial proteins involved in the tricarboxylic acid cycle and mitochondrial heat shock proteins among down-regulated proteins and a sub-network of glycolytic enzymes among up-regulated proteins would support mitochondrial damage.

To identify candidate markers for hepatotoxicity, we focused on the proteins where eight or nine compounds gave either up- or down-regulation (in the same direction) as a first criterion, limiting the total to 75 proteins (Table S9). For the upregulated proteins, two proteins were induced by all nine compounds, i.e. Pcnxl3 and Si:ch211–212n6.8. There were 19 proteins induced by eight compounds and these proteins were Mylpfa, Zgc:163069, Rpsa, Psap, Psma8, Crygm2d19, Snrpa1, Epb41b, Msi2a, Glud1b, Nbeal2, Nasp, Agr1, Col1a1a, Eno1a, Znf143, Stil, Cirbp and Fdx1 (Table S9). For the downregulated proteins, 13 proteins were induced by nine compounds and 41 were induced by eight compounds. The 13 proteins induced by nine compounds included Murc, Ttnb, Srsf3a, Paics, Cfap65, Anxa1c, Hspe1, Cox5ba, Kat6b,

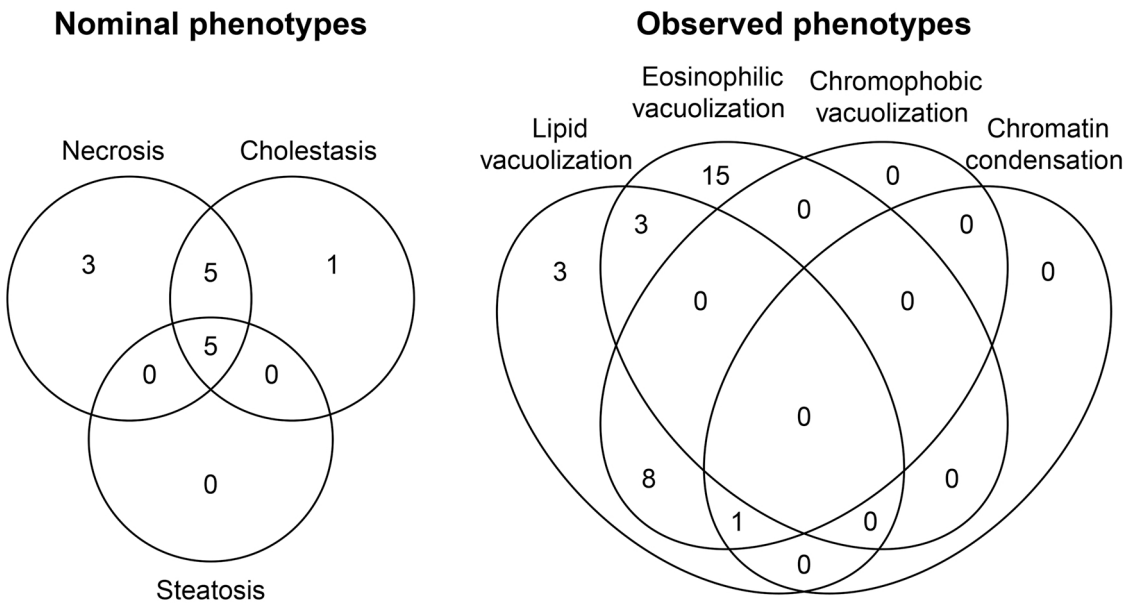

Fig. 1. Venn diagram for pathway enrichment. The Venn diagrams show overlap between pathways that were overrepresented in proteins associated with nominal (left) or observed (right) phenotype. The nominal phenotypes necrosis and cholestasis have three and one unique biological process respectively, while they have five processes in common. Five biological processes are shared among the three phenotypes. Of the observed phenotypes, lipid and eosinophilic vacuolization have three and 15 unique processes, respectively, while there are none for the other two phenotypes. In total 12 processes are shared among the phenotypes, although chromatin condensation has only one identified process. Processes are explained in Table S8.

Rps14, Tnw, Ldhba, and Psma6b (Table S9). Additional cluster analysis of protein fold change values of these 75 proteins separated the compounds into two groups, i.e. CsA and PQ in one group versus the remaining compounds (Fig. 3). Again, none of the subclusters (CSA and PQ; APAP and EE2; TAA and VPA) could be linked to either nominal or observed phenotypes.

An established relation to liver function of the proteins was used as a second criterion. For this purpose, we first retrieved the mapping of these most consistently up- and down-regulated marker proteins to cellular processes/pathways from the ZFIN database. Some of the proteins up-regulated by eight or nine compounds could thus be linked to enriched liver-associated pathways, such as carbohydrate metabolism, oxidoreductase activity and alcohol dehydrogenase activation, although also general processes/pathways were identified, such as zinc ion binding, calcium ion binding, heat shock proteins (Table S9). Proteins downregulated by eight or more compounds were linked to enriched processes/pathways, including calcium ion binding, glycolysis and cytoskeleton related processes (Table S9).

Further indications for a relation to liver function was retrieved from the HPA and BioGPS databases, and from a PubMed search, details of which are given in the Discussion. This led to a total of 23 proteins with a suggested relation to liver function (Table 3).

3.3. Comparing transcripts and proteins

This set of 23 proteins was further prioritized by comparing protein and gene expression, where the availability and similarity of the direction of gene and protein expression was set as a third criterion. Gene expression data were based on the same time point and concentration as published previously (Driessen et al., 2014). Analysis was based on the availability of matching direct protein-mRNA combinations ($n = 373$), where matching of direction can be grouped into four up-down combinations. This analysis revealed that a more or less equal distribution among the four sets was mostly observed (Fig. 4). Predominant protein down-regulation (light shaded bars in Fig. 4) was obvious with particularly PQ and CsA, in line with their separate position in the cluster analysis (heatmap, Fig. 3), and to a lesser extent also with EE2 and VPA. In contrast, predominant upregulation of proteins (dark-shaded bars in Fig. 4) was observed with APAP and CPZ. Notably, the compounds from the nominal phenotype Steatosis (TET, VPA, AM) had the overall lowest count of regulated gene-protein sets (N = 210–220) compared to the six other compounds (N = 223–311). Of the 23 robustly regulated proteins with suggested relation to liver function, only 17 had gene expression regulation in the same direction (Table 3), however of these, only 10 showed this concordance in more than half of the test compound set ($n > 4$) or in at least one of each of the three nominal phenotype classes (as was the case for all concordant sets observed with $n = 4$ compounds; Table 3).

4. Discussion

In this study, we analyzed the regulation of protein expression in ZFE after exposure to nine reference hepatotoxicants, to improve mechanistic understanding of processes underlying hepatotoxicity and derive predictive biomarkers hereof. Furthermore, we compared gene and protein expression to detect interrelations between these levels in the dynamics of liver toxicity.

The enrichment analysis with STITCH (Szklarczyk et al., 2016) to detect overrepresented pathways did not produce discriminating processes related to nominal or observed histopathological phenotypes. Only common pathways and/or processes related to hepatotoxicant exposure were identified, including “translation”, “cellular protein metabolic process”, “gene expression” and “metabolic process”, which are all known to be disrupted in global stress responses (Paschen et al., 2007; Petrak et al., 2008). As explained in the Introduction, the distinct phenotypical classes of interest, steatosis, cholestasis, and necrosis could be reproduced in the liver of adult zebrafish (Driessen et al., 2013), but not in zebrafish embryos, possibly related to inherently short exposure duration in an immature hepatic tissue. The short exposure duration and immaturity possibly also relate to detection of more acute processes, such as general stress response, instead of specific phenotype-related processes. While this difference clearly limits a one-to-one comparison with chronic effects in an adult human, the zebrafish embryo may still represent a valuable model for identification of DILI effects, although based on zebrafish embryo-specific markers.

Thus, the analysis was repeated based on common hepatotoxicity

Table 2
Enriched processes related to common hepatotoxicity.

GO_id	Term	Number of Proteins	p-value	p-value fdr
UP (190 proteins)				
GO:0006412	translation	21	5.18E-19	3.03E-15
GO:0010467	gene expression	32	4.41E-10	1.29E-06
GO:0044237	cellular metabolic process	46	6.04E-09	1.18E-05
GO:0044249	cellular biosynthetic process	28	1.66E-08	2.42E-05
GO:1901576	organic substance biosynthetic process	28	2.62E-08	3.06E-05
GO:0008152	metabolic process	52	5.24E-08	5.10E-05
GO:0009058	biosynthetic process	28	6.12E-08	5.10E-05
GO:0044267	cellular protein metabolic process	27	7.54E-08	5.51E-05
GO:0034645	cellular macromolecule biosynthetic process	23	1.29E-07	8.09E-05
GO:0009059	macromolecule biosynthetic process	23	1.39E-07	8.09E-05
GO:0042274	ribosomal small subunit biogenesis	4	1.32E-06	7.02E-04
GO:0071704	organic substance metabolic process	44	1.62E-06	7.87E-04
GO:0044238	primary metabolic process	43	2.47E-06	1.11E-03
GO:0019538	protein metabolic process	28	3.16E-06	1.32E-03
GO:0044260	cellular macromolecule metabolic process	33	7.36E-06	2.86E-03
DOWN (237 proteins)				
GO:0006412	translation	19	2.20E-12	1.28E-08
GO:0006457	protein folding	13	6.13E-11	1.79E-07
GO:0044267	cellular protein metabolic process	39	4.06E-09	7.91E-06
GO:0019538	protein metabolic process	44	1.40E-08	2.05E-05
GO:0044238	primary metabolic process	66	7.96E-08	9.30E-05
GO:0044237	cellular metabolic process	62	1.98E-07	1.73E-04
GO:0071704	organic substance metabolic process	66	2.07E-07	1.73E-04
GO:0008152	metabolic process	74	2.62E-07	1.91E-04
GO:0043170	macromolecule metabolic process	52	5.46E-06	3.54E-03
GO:0044260	cellular macromolecule metabolic process	47	6.68E-06	3.90E-03

Processes were produced by analysis in STITCH based on 427 proteins commonly regulated by 6 or more compounds. Upregulated processes are in the upper half of the table, downregulated processes in the lower half. Processes are ordered by p-value, prioritizing processes which may be more active in liver (see text for details).

markers, and this confirmed broad processes, including "protein folding", "translation", and "gene expression" as highly over-represented, i.e. most significantly regulated, processes. This can be understood because protein folding is a key function in protein processing in the endoplasmic reticulum (ER), and therefore particularly active in secretory cells such as hepatocytes (Rashid et al., 2017). Disruption of protein folding is associated with a stress response in the ER, and a prolonged stress response will eventually lead to apoptosis (Fribley et al., 2009). Protein folding in the liver is a vulnerable process and impairment associated with various liver diseases (Rashid et al., 2017), and the unfolded protein response has also been reported as important in the context of DILI (Fredriksson et al., 2014; Wijaya et al., 2021). Regulation of translation is a downstream effect of the unfolded protein response, targeted at resolving the protein folding impairment (Malhi and Kaufman, 2011) and this may explain the observed concomitantly, highly significantly regulation of both processes. Similarly, impairment of several other cellular processes, including gene expression, as emerging at the protein expression level after hepatotoxicant exposure in our ZFE, may thus be related with this ER stress response.

While there was a large overlap among the phenotypes on the pathway and/or process level, we identified a set of commonly regulated key proteins, where regulation by eight or nine reference hepatoxicants was the first identifier for candidate protein markers (criterion 1). It should be kept in mind that the proteins were extracted from whole embryos, and consequently, contribution of regulated proteins in other tissues than liver to the analysis is likely. Although effects in other tissues may still be predictive for hepatotoxicity, a known function in the liver was considered as a support for robustness of markers (criterion 2). Such involvement could be established through mapping in ZFIN, HPA and BioGPS databases for some of the candidate markers (Table S9), while additional associations were found in literature, as outlined below.

From the top score protein markers in Table 3, Igf2bp1 appears to have a role in liver functioning in zebrafish, although established in relation to normal liver development (Wu et al., 2020), rather than hepatotoxicity. Proteome studies in zebrafish related to hepatotoxicity are scarce, supporting information was therefore retrieved from mammalian studies. As such, Igf2bp1 was shown to be related to liver tumorigenesis, rather than to DILI, in mice (Lin et al., 2021; Yang et al., 2021). Another high scoring protein, Ahnak, appeared to be a mediator in diet-induced fatty liver in mice (Kim et al., 2021). Srsf3 was decreased in hepatic steatosis, fibrosis and inflammation in humans and in mice, and in the mice model, prevention of Srsf3 degradation was protective against liver disease (Kumar et al., 2019). The Ces2b protein is a carboxyl esterase with multiple functions (https://zfin.org/ZDB-GENE-041014-96), and known to be involved in the hydrolysis of xenobiotics (Takemoto et al., 2021). Liver-specific Ces2b was the only significantly regulated protein overlapping with our results in an 21d exposure APAP study in zebrafish, however, the poor overlap may be due to model differences (proteomics analysis on excised liver of adult fish versus ZFE, 21d versus 2d exposure). Another protein, Anxa1c, is predicted to enable calcium ion binding activity, calcium-dependent phospholipid binding activity, and phospholipase A2 inhibitor activity, and involved in several processes, including organ development, regulation of T cell activation, and regulation of cell migration (https://zfin.org/ZDB-GENE-030131-5274). In mice, overexpression of annexins A1 and A2 inhibited APAP-induced expansion of liver injury (Dadhania et al., 2016). In mice and humans, expression of AnxA1 is associated with development of non-alcoholic steatohepatitis (NASH), and in a mouse model, macrophage-derived AnxA1 was shown to play a functional role in modulating hepatic inflammation and fibrogenesis during NASH progression (Locatelli et al., 2014).

Of the markers with lower scores (Table 3), upregulation of Col1a1 was linked with liver cirrhosis in several studies, e.g. as a result of exposure to TAA in rats (Zeweil et al., 2020). Sirt1 has well established (protecting) roles in alcoholic liver disease (Ren et al., 2020), non-alcoholic fatty liver disease (Colak et al., 2014), or DILI, e.g. in APAP-induced hepatotoxicity (Rada et al., 2018). Uqcrc2 is a mediator in the protection against alcohol-induced liver injury in mice (Lu et al., 2021), and similarly, Hsp90b1 showed increased expression in alcohol-associated liver inflammation in human and mice livers (Ratna et al., 2021). Crygm2d19 (https://zfin.org/ZDB-GENE-081105-90) is a gamma-crystallin, which, in addition to the eye lens, are also expressed in other organs (Fagerberg et al., 2014; Yue et al., 2014). Like Anxa1c, crystallins may play a role in calcium ion binding (Bhat, 2003), which could be relevant in hepatotoxic processes in view of the role of calcium

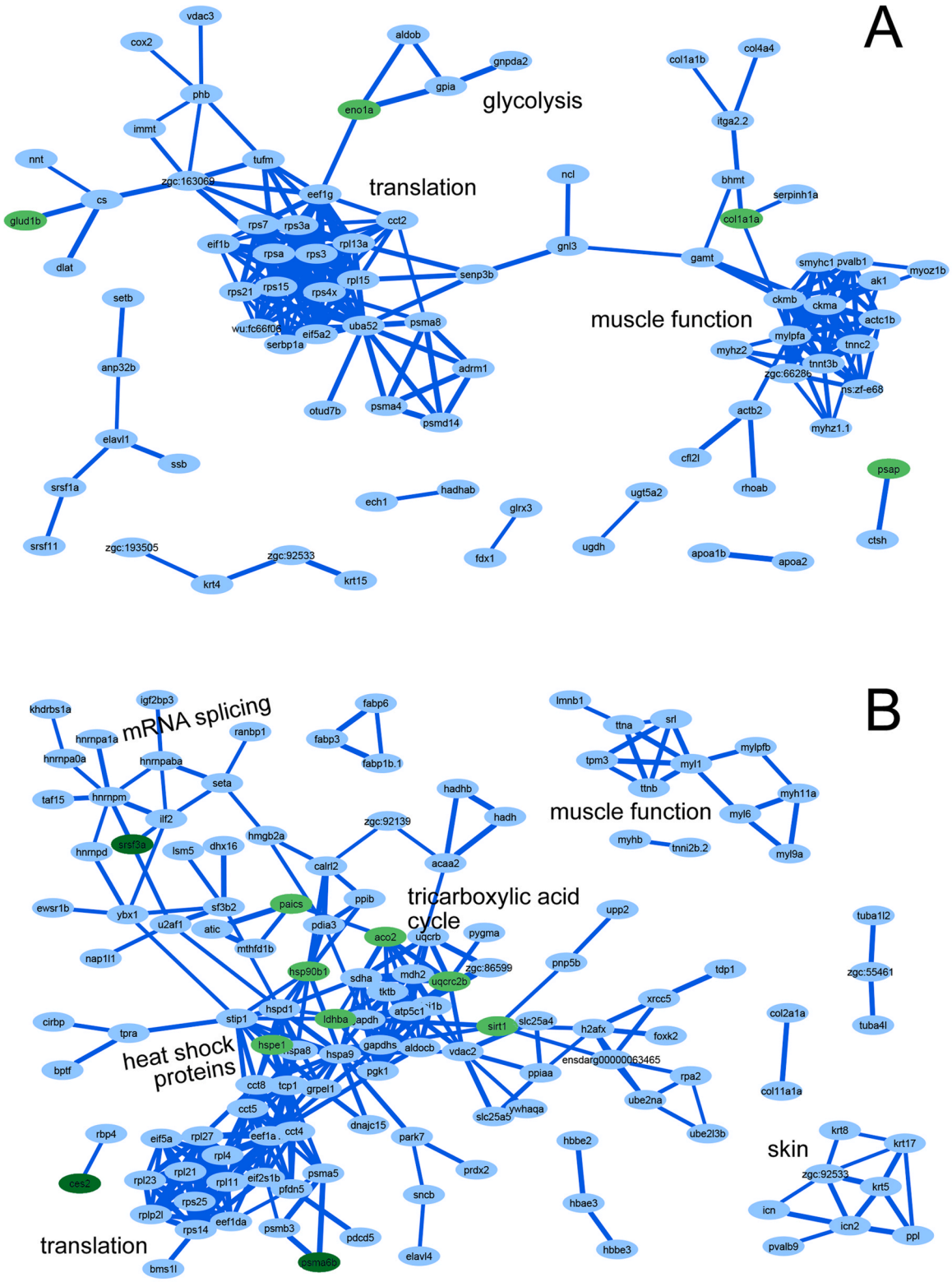

Fig. 2. *Network representation of the common up-regulated (A) or down-regulated proteins (B)*. Proteins identified as having a FC > 1.5 and being up- or down-regulated by 6 or more compounds (n = 427) were analyzed by STITCH for significant relations (such as interactions, co-expression, text-mining co-occurrence). Results were visualized using Cytoscape. Proteins without established interactions were not included. Subnetworks related to specific biological processes were identified by expert assessment. Blue lines indicate relations, line widths indicate the evidence scores. Candidate marker proteins for hepatotoxic effects (Table 3) are marked light green (all markers) or dark green (strict selection).

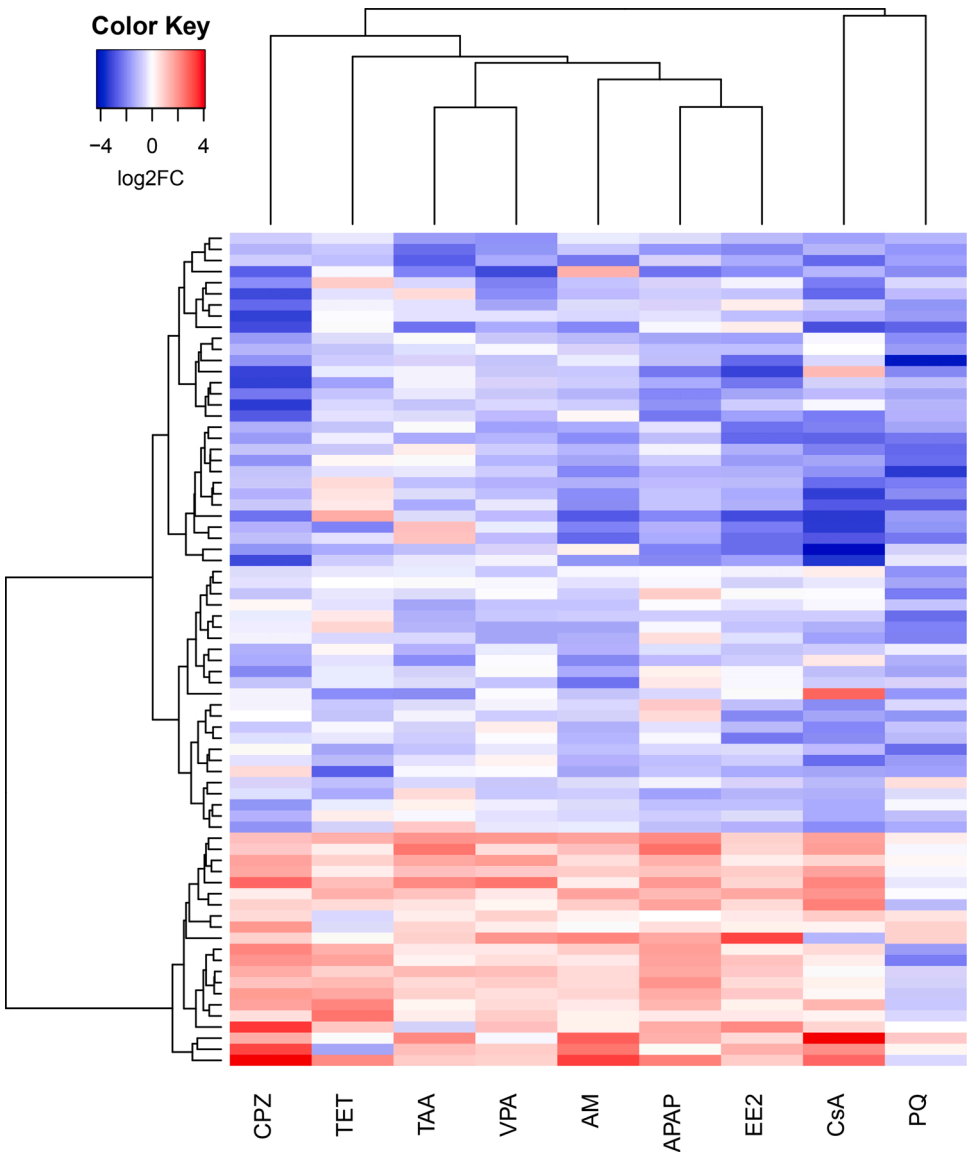

Fig. 3. *Cluster analysis of differentially expressed proteins.* Included proteins had an absolute FC > 1.5 and showed common up- or down-regulation by 8–9 compounds (n = 75). CsA and PQ separate from the other compounds. None of the subclusters appear to relate to either nominal or observed phenotype. Red and blue indicate up- and down-regulation, respectively; color intensity represents the level of regulation (absolute fold change compared to respective controls).

as an important second messenger in response to stress (Amaya and Nathanson, 2013). Crystallins were found to be differentially expressed after toxic stress in PFOS treated zebrafish larvae (Hanisch et al., 2010; Shi et al., 2009) and in teratogen-exposed zebrafish embryos (Hermsen et al., 2013). Crystallins are highly stable proteins, which may explain the discordance with the direction of gene expression, as observed in eight of the nine reference compounds. The mitochondrial 10 kDa heat-shock protein, Hspe1, was down-regulated in our ZFEs after exposure to the hepatotoxicants suggesting the occurrence of mitochondrial damage. This is in agreement with the observed down-regulation in mouse liver slices after CsA exposure (Szalowska et al., 2013). Heat-shock proteins are involved in folding/unfolding of proteins and protein translocation. The ER stress after CsA exposure negatively affects the function of heat-shock proteins (Szalowska et al., 2013). The protein Glud1b plays a key role in nitrogen and glutamate metabolism, and also in the energy homeostasis (https://zfin.org/ZDB-GENE-030828-1). The human ortholog GLUD1 is a serum marker for hepatotoxicity along with ALT and AST (Hu et al., 2014), and Glud1 was identified as a marker in TCDD-induced hepatotoxicity in rats (Watson et al., 2017). The Eno1a protein is involved in glycolysis and was previously reported to be differentially expressed in zebrafish embryo after exposure to CsA (Ponnudurai et al., 2012), supporting a role in hepatotoxicity. In summary, although the regulated processes and/or pathways point towards more general stress and oxidative stress responses, analysis of individual genes/proteins also reveals more hepatotoxicity-associated changes. This supports that protein expression changes can identify hepatotoxicity-specific responses in the ZFE.

An uncertainty in many biological studies is the concordance of direction for changes of transcripts and protein levels. Comparison of protein expression signatures with their corresponding gene expression (Driessen et al., 2014), revealed that generally, all four combinations of gene and protein up- and downregulation occurred in a similar ratio, although some compounds induced predominant protein up- or down-regulation. Difficulties in correlating mRNA and protein expression are generally acknowledged, and relate to post-transcriptional processing, different half-lives between mRNAs and proteins (particularly relevant in cases of downregulation (Metz et al., 2017)), and the noise in both mRNA and protein expression measurements, preventing an easy mechanistic interpretation (Greenbaum et al., 2003). Some of this lack of correlation between mRNA and protein expression in ZFEs was shown

Table 3
Candidate protein markers for hepatoxicity.

Proteins regulated by 8 or 9 compounds AND identified relation to liver functions	Number of compounds in which the protein has matching direction of expression	Criteria
Igf2bp1	6	1,2,3+
Cox5ba (Zgc:86599)	6	1,2,3+
Ahnak	5	1,2,3+
Itih3b.2 (Zgc:112265)	5	1,2,3+
Psma6b	5	1,2,3+
Srsf3a	5	1,2,3+
Ces2b (si:ch211–93f2.1)	4	1,2,3+
Ces2a (ces2)	4	1,2,3+
Tdo2b	4	1,2,3+
Anxa1c	4	1,2,3+
Col1a1a	3	1,2,3
Aco2	3	1,2,3
Psap	2	1,2,3
Krt18b (Zgc:77517)	2	1,2,3
Sirt1	2	1,2,3
Uqcrc2b	2	1,2,3
Crygm2d19 (Si:ch211–212n6.8)	1	1,2,3
Hspe1	no data	1,2
Ldhba	no data	1,2
Glud1b	no data	1,2
Eno1a	no data	1,2
Hsp90b1	no data	1,2
Paics	no data	1,2

Criteria for selection of protein markers for hepatotoxicity:
1. Proteins are regulated by nine or eight compounds.
2. Proteins have a known relation to liver functions.
3. Proteins are regulated in the same direction as their coding mRNAs.
3+. Criterion 3 is observed in at least half of the compounds (>4)/in at least one compound from each nominal phenotype (>1 +1 +1).
Italics, proteins showing interactions in STITCH network analysis (Fig. 2). Proteins without a known relation to liver functions (criterium 2) are not included in this list but can be found in Table S9.

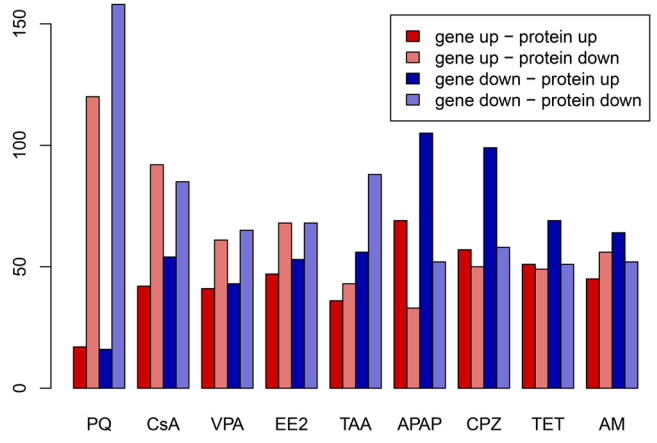

Fig. 4. *Concordance between gene and protein expression per compound.* Representation of the matched directionality of corresponding respective gene – protein expression combinations, of which 373 combinations were available. All four possible combinations are more or less equally represented, with protein downregulation dominating in the left-most compounds, and protein upregulation in APAP-CPZ.

to derive from three important categories: ribosomal proteins, histones and vitellogenins (Palmblad et al., 2013). The specific noise in our analyses relates to the selective counting of the number of genes and/or proteins being regulated; as a consequence, potentially relevant and usable gene-protein combinations may be lacking. Furthermore, because mRNA and protein data were derived from the same time point, the different expression kinetics between mRNAs and proteins will also reduce the degree of correlation or even result in negative correlation, particularly for transiently induced genes with slow protein turnover. Despite, or perhaps because of these limitations, mRNA-protein sets with matching expression directionality support the use of these entities as markers for hepatotoxicity (criterion 3, Table 3), as they suggest non-transiently up- or downregulated gene expression with a corresponding and rapid change in protein abundance. In consequence, the method and timing of analysis for these matching sets is less critical, which renders them more reliable and solid as biomarkers, particularly when applied in AOPs, where they should be used to inform robustness of changes in a chain of events. For enhanced robustness, criterion 3 should be observed in more than half of the compounds ($n > 4$), or in at least one representative of each of the nominal phenotypes (as is the case in all $n = 4$, which can thus be included). By applying criterion 3 in such a strict way to the selection of proteins regulated by eight or nine compounds (criterion 1) and with a known relation to liver function (criterion 2), ten candidate marker proteins remain, i.e. Igf2bp1, Cox5ba, Ahnak, Itih3b.2, Psma6b, Srsf3a, Ces2b, Ces2a, Tdo2b, and Anxa1c (Table 3), in that order of robustness.

It appears that different tools and databases can produce different results regarding identification of biological processes and/or their relevance for liver functioning. For instance, there was not much overlap between processes identified by the STITCH analysis on the full set of 427 commonly regulated proteins (Table 2) and processes associated to robustly regulated individual proteins in ZFIN (Table S9), and only few of the robustly regulated protein markers (three out of ten) occurred in the STITCH interaction network analysis, although this is true for 14 out of 23 proteins of the initial marker set (marked proteins in Fig. 2). Furthermore, there is apparent hierarchy in the informative value of the databases used to identify associations of proteins with liver functions (Table S9); associations obtained from a Pubmed search are obviously more detailed and informative regarding functionality than high expression of a protein in human liver (HPA), or high expression ($> 100 \times$ the median expression level across all tissues) in human or murine liver in BioGPS. The selection of tools and databases thus represents a source of bias, however, the flow of our analysis and definition of criteria may support the robustness of the identified protein markers for hepatotoxicant-induced effects.

In conclusion, our exposure study with a set of reference hepatotoxicants in ZFEs, each in a dose matching an effective dose in dose-response analysis of phenotype and differential gene expression, identified proteomics markers for enriched biological processes associated with general hepatotoxicity. The most prominent process was stress response, as indicated by translation, gene expression and metabolic synthetic processes. We identified ten potential robust protein markers, listed above, which are potential candidates for the prediction of human hepatotoxicants. Such markers could be further developed into high throughput screening using more dedicated methods such as Western blots or ELISA assays. Finally, as noted in the Introduction, this protein analysis in ZFE should be used to build evidence for hepatotoxic effects of test compounds in combination with other alternative testing models, preferably structured along an AOP for DILI, rather than as a stand-alone prediction model.

Authors contribution

Marja Driessen: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Suzanne van der Plas – Duivesteijn:** Methodology, Investigation, Writing – review & editing. **Anne S. Kienhuis**: Conceptualization, Writing – original draft, Writing – review & editing, Supervision. **Evert-Jan van den Brandhof**: Methodology, Writing – review & editing. **Marianne Roodbergen**: Investigation, Writing – review & editing. **Bob van de Water**: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Herman P. Spaink**: Methodology, Writing – review & editing. **Magnus Palmblad**: Methodology, Writing – review & editing. **Leo T.M. van der Ven**: Conceptualization,

Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Jeroen L.A. Pennings**: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2022.153262.

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