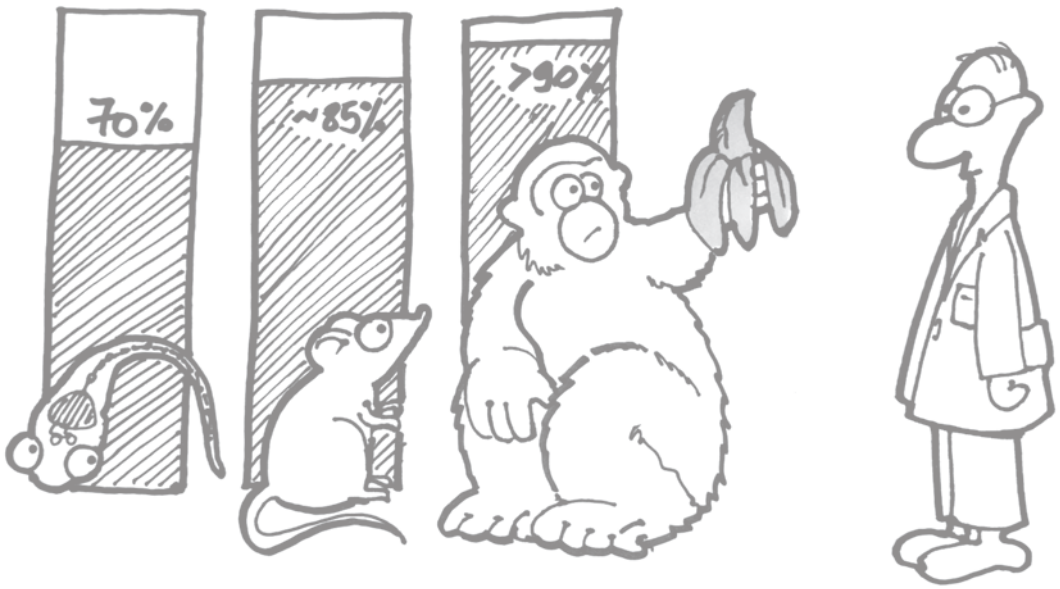
Evaluation of the zebrafish embryo as an alternative model for hepatotoxicity testing

Driessen, M.



5

A Transcriptomics-based Hepatotoxicity Comparison between the Zebrafish Embryo and established Human and Rodent *in vitro* and *in vivo* models using Cyclosporine A, Amiodarone and Acetaminophen

Marja Driessen[#], Alexa P. Vitins[#], Jeroen L.A. Pennings, Anne S. Kienhuis, Bob van de Water, Leo T.M. van der Ven

[#] Contributed equally

Abstract

The zebrafish embryo (ZFE) is a promising alternative, non-rodent model in toxicology, which has an advantage over the traditionally used models as it contains complete biological complexity, and provides a medium to high-throughput setting. Here, we assess how the ZFE compares to the traditionally used models for liver toxicity testing, i.e. *in vivo* mouse and rat liver, *in vitro* mouse and rat hepatocytes, and primary human hepatocytes. For this comparison we analyzed gene expression changes induced by three model compounds for cholestasis, steatosis, and necrosis. The three compounds, cyclosporine A, amiodarone, and acetaminophen, were chosen because of their relevance to human toxicity and these compounds displayed hepatotoxic-specific changes in the mouse *in vivo* data. Compound induced expression changes in the ZFE model shared similarity with both *in vivo* and *in vitro*. Comparison on single gene level revealed the presence of model specific changes and no clear concordance across models. However, concordance was identified on the pathway level. Specifically the pathway "Regulation of metabolism – bile acids regulation of glucose and lipid metabolism via FXR" was affected across all models and compounds. In conclusion, our study with three hepatotoxic model compounds shows that the ZFE model is at least as comparable to traditional models in identifying hepatotoxic activity, and has the potential for use as a pre-screen to determine hepatotoxic potential of compounds.

Introduction

A challenge in toxicology is to predict the hepatotoxic potential of compounds to which humans are exposed. In mammals and particularly for xenobiotics that rely on oral uptake, many compounds provoke first signs of toxicity in the liver. This is mainly due to the location of the liver (first pass effect) and liver cell activation. Therefore, hepatotoxicity is an important target to study, particularly in the context of adverse xenobiotic effects. Hepatotoxicity is the result of various xenobiotic-induced underlying molecular mechanisms, which produce different liver toxic phenotypes, of which cholestasis, steatosis and necrosis are most frequently observed. Cholestasis is a chronic condition and is phenotypically characterized by bile accumulation as a result of changes in intra- or extracellular bile flow or bile composition[34]. Steatosis may occur chronically as well and 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[19]. Necrosis is an acute condition and is characterized by cell death due to oxidative stress[4,33].

Traditionally, these hepatotoxic properties of xenobiotics are determined using different *in vivo* and *in vitro* models. Detection of hepatotoxicity in *in vivo* studies is based on histopathology and clinical chemistry. However, these studies require high numbers of animals, interfere with animal welfare, and are associated with high costs and are not always predictive for the human situation. *In vitro* systems have limitations related to their reductionistic nature and to their loss of functionality compared to the liver *in vivo*[101]. In the effort to improve screening for hepatotoxicity regarding predictivity for humans and throughput and in the same time reduce animal experimentation, the development of alternative models has gained attention. As such, the zebrafish embryo is an alternative screening model with the potential to reduce animal experimentation. It combines the benefits of an *in vivo* model, namely complete biological complexity including interaction between tissues and cells[49,54,95], with the advantages of an *in vitro* model, that is reduced animal discomfort and the ability for median to high throughput testing. The structure and function of the adult zebrafish liver is similar to the mammalian liver[46,55], and fully functional liver tissue is present in the zebrafish embryo at 72 hours post fertilization (hpf), which is therefore a suitable time point to start hepatotoxicity testing. There is also high genetic conservation between humans and zebrafish, including genes functioning in biotransformation[46,55]. Previous studies showed that the zebrafish embryo could be used to identify human hepatotoxic responses using histopathology and gene expression analysis[149]. These studies have demonstrated that hepatotoxicity-associated gene expression remains detectable in the noise of other tissues and are distinct from developmental toxicity processes[149].

Although the above mentioned studies have shown promise for the zebrafish embryo as an alternative model, it still does not identify how comparable the obtained results are to the traditionally used models. Our anticipation is that the zebrafish embryo model shares commonalities with *in vivo* and *in vitro* models. To this end, we compared the ZFE to the *in vivo* mouse and rat liver, *in vitro* mouse and rat hepatocytes, and primary human hepatocytes using gene expression changes from existing experiments using a model compound for each hepatotoxic phenotype.

Three compounds, cyclosporine A (CsA), amiodarone (AMD), and acetaminophen (APAP), were chosen for the comparison because of their relevance to human toxicity and the availability of reference data in all of the selected models. The model compound CsA induces cholestasis. CsA competitively inhibits the bile salt export protein, which is the primary transporter involved in the biliary efflux of conjugated bile acids across the canalicular membrane[134,135]. AMD, an antiarrhythmic agent, induces steatosis in humans[137] by altering lipid homeostasis in the liver. Finally, necrosis is induced by APAP, an analgesic and antipyretic, which is considered safe at therapeutic doses. In this setting, APAP is inactivated by sulfation and glucuronidation. However, when the therapeutic doses are exceeded, APAP causes hepatic damage and may eventually lead to liver failure[1,138].

Although the zebrafish embryo model cannot distinguish between human hepatotoxicity phenotypes using transcriptomics or proteomics, mechanisms of general hepatotoxicity can be identified and these might overlap with those induced in the traditional models for which data is available (Fig. 1). Our current data suggest overlap in the activation of pathways of toxicity between ZFE and other models. Therefore, the zebrafish embryo may well serve as a preferred quick and accurate first round testing of new compounds.

Materials and methods

This study utilized data from multiple sources. In house (*in vivo* ZFE and mouse) and externally available data (*in vivo* rat and *in vitro* mouse, rat and human) were combined to assess the predictivity of the zebrafish embryo as a model for hepatotoxicity testing. In total, 161 treatment groups or conditions including a total of 161 Affymetrix CEL files with up to four replicates per conditions, were included in the analyses and an overview of each model is shown in Table 1 (Supplementary Table 1).

Zebrafish embryo model (ZFE). The studies on zebrafish embryos were conducted by the National Institute for Public Health and the Environment (RIVM) as described earlier[133]. Briefly, 72 hours old hatched embryos were randomly distributed over 48-well plates in a density of 5 embryos per well in 1 ml test or control medium. Following treatment, using previously described procedures[110,133,149], total RNA was isolated from the zebrafish embryo (1 sample consisted of 15 ZFE embryos) and used for gene expression analyses on

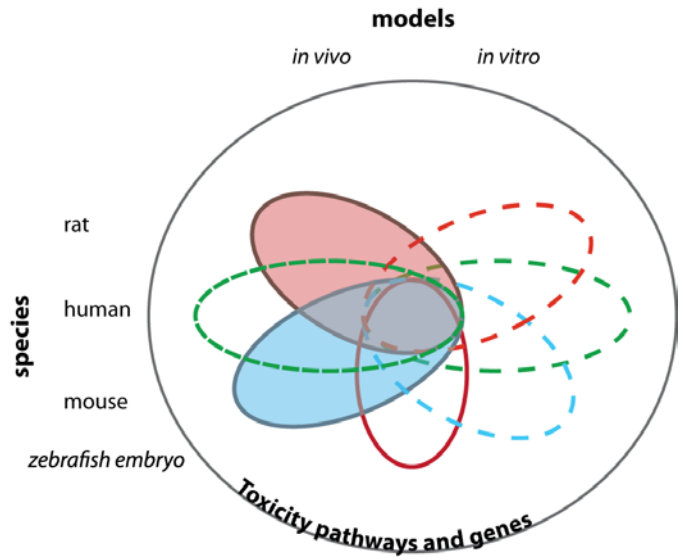


Figure 1 Concordance model for toxicogenomics-based extrapolation.
Central overlap represents species- and model-independent robust toxicity pathways or genes that are predictive for pathways/genes related to hepatotoxicity in humans. Solid red and blue lines, toxicity pathways/genes measured in *in vivo* models; large spaced dotted red, green, and blue lines, toxicity pathways/genes measured in *in vitro* models; small spaced dotted green line, the human *in vivo* toxicity pathways/genes to be predicted; red line, the zebrafish embryo toxicity pathways/gene contribution sharing similarity with *in vitro* as well as *in vivo* (adapted from Kienhuis *et al.*[5]).

Affymetrix Zebrafish Gene ST Array. At least 4 biological replicates were conducted. The expression data set is publicly available at the Gene Expression Omnibus (GEO) database, (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE55618 (full dataset).

Mouse in vivo (MIV). The *in vivo* mouse liver studies were conducted by the RIVM as described previously[119,141,167]. Briefly, male C57BL/6Jlco mice, aged 10 weeks, were treated with a low, medium, and high dose of CsA and AMD for 1, 4, and 11 days, and for APAP, mice were given a single treatment at a low, medium, and high dose and assayed 1 and 2 days later. RNA from liver tissue was isolated and used for gene expression analysis by hybridizing to the Affymetrix HT Mouse Genome 430 PM Array. The expression data set is publicly available at the Gene Expression Omnibus (GEO) database, (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE31540 (CsA), GSE48126 (AMD) and GSE51969 (APAP). At least 4 biological replicates were conducted.

Table 1 Model Description.

		Model						
Compound	**Dose**	MIV (mg/kg) 1, 2, 4, 11 days (RIVM, Kienhuis et al.)	MPH (µM) 24, 48 hours (UM, van Summeren et al.)	ZFE (µM) 48 hours (RIVM, Driessen et al)	HPH (µM) 2, 8, 24 hours (TG-Gates)	RIVS (mg/kg) 3, 6, 9, 24 hours (TG-Gates)	RIVR (mg/kg) 4, 8, 15, 29 days (TG-Gates)	RPH (µM) 2, 8, 24 hours (TG-Gates)
AMD	High	60	25	10	7	2000	200	7
	Medium	20	1	3.3	1.4	600	60	1.4
	Low	6.7	0	1.1	0.28	200	20	0.28
APAP	High	300	10000	660	5000	1000	1000	10000
	Medium	225	1000	220	1000	600	600	3000
	Low	168.75	0	73.3	200	300	300	1000
CsA	High	26.7	50	6	6	300	100	6
	Medium	8.9	10	3	1.2	100	30	1.2
	Low	3	0	1	0.24	30	10	0.24

Primary mouse hepatocytes (MPH). Samples were from a previous transcriptomics study from Maastricht University (UM) on mouse primary hepatocytes (MPH) treated with AMD, APAP and CsA[168]. In short, primary hepatocytes were isolated from adult male C57Bl/6 mice and brought into culture in a collagen-collagen sandwich formation. Following treatment, using previously published procedures[169], total RNA was isolated from cultured mouse hepatocytes and used for gene expression analyses on high-density oligonucleotide gene chips (Affymetrix Mouse Genome 430 2.0 GeneChip arrays). Three independent biological replicates were conducted.

Rat in vivo and in vitro rat and human. Experimental study designs for AMD, CsA and APAP for rat primary hepatocytes (RPH), rat *in vivo* repeated dosing (RIVR), rat *in vivo* single dosing (RIVS) and human primary hepatocytes (HPH) performed in the Japanese Toxicogenomics Project and Toxicogenomics Informatics Project (TGP) (Open TG-GATEs; http://toxico.nibio.go.jp)[170,171]. These studies can be obtained from ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under accession numbers E-MTAB-797 (rat primary hepatocytes), E-MTAB-798 (human primary hepatocytes), E-MTAB-799 (*in vivo* rat single dose) and E-MTAB-800 (*in vivo* rat repeated dose).

Data preprocessing and quality control. For all systems, the data was annotated with a MBNI custom CDF specifically designed for the chips (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/CDF/)[118] and RMA normalized using the Robust Multichip Average algorithm[76]. RMA normalized data was Log_2 transformed and the quality of the micro array images was inspected visually (http://arrayanalysis.org, BiGCaT Maastricht University)[172].

Homolog conversion. The unique Entrez GeneIDs for each species was converted to human homologs by downloading the full HomoloGene data file from NCBI (ftp://ftp.ncbi.nlm.nih.gov/pub/HomoloGene/current) and matching the species GeneIDs to the human homologs using an Entrez ID conversion script in R in order to compare the model systems. Only the homologs present in all model species were used for further analysis which resulted in 7828, 7825, 7623 and 8855 Entrez ID for mouse, rat, human and zebrafish, respectively.

Gene expression analysis.

Single gene expression. To ensure that an equal number of genes were compared between the different treatment/condition groups, the top-500 ranked genes based on Log_2 fold-change (Log_2FC) were used. This analysis was performed because of differences in the power between the experimental designs.

Single gene overlap. Per model, the unique genes found above in the "Single gene expression" were compiled. Similarity between these lists was visualized by means of a heatmap as well as Principal Component Analysis (PCA).

Pathway analysis. Each of the lists compiled in the section "single gene overlap" were submitted to MetaCore from Thomsom Reuters and pathways having a p-value of < 0.05 were identified.

Biplot. Biplot representation of the pathway was done to display information on both the samples and the variables of the data matrix. In a biplot the samples are displayed as points, whereas the variables of the datamatrix are displayed as vectors. The biplot was generated using the "ade4" package in R (http://pbil.univ-lyon1.fr/ade4/home.php?lang=eng)[173,174] with an in-house R script based on the *ggplot2* R-package[175] function, with minor changes that helped improve graphical representation.

Results

Model-dependent gene expression changes. Changes in gene expression may be informative regarding how different models react to xenobiotic treatment. Therefore, to ensure equal numbers of differentially expressed genes (DEGs) for all 161 treatment conditions, we decided to use the top-500 ranked genes to determine the overlap between the conditions on single gene level and identify similarities between groups (Fig. 2, Supplementary Figure 1). Based on this stringent selection model dependent changes were observed; as expected the greatest amount of overlap was found within a model. Additionally, within a model, the different dose and time points of a given compound tend to overlap. Yet different compounds tend to overlap less within a model. Furthermore, when comparing a model to the other models, there is no difference in the overlap. Therefore, to identify whether common regulated genes were found across all models a gene overlap comparison was performed.

Gene overlap across models. Our goal was to determine the feasibility of the ZFE to pick relevant transcriptomics changes that mimic other more standard models. Therefore we determined the overlap at the single gene level between ZFE and all other models. Per model, we used the unique genes across the top-500 of each treatment/condition group; since per compound (AMD, APAP and CSA) we had several concentration/doses the total DEGs for the analysis typically 500 genes per model. Because conceptually the ZFE shares likely similarities with the *in vivo* models as well as with the *in vitro* models, a separation was made between *in vivo* and *in vitro*. More single DEGs were found to be in common across all the different *in vivo* models per compound (22 AMD, 14 APAP, 19 CsA) than in the *in vitro* model comparison (5 AMD, 11 APAP, 5 CsA) (Fig. 3A) (Supplementary Table 2). For both the *in vivo* and *in vitro* comparisons of AMD and CsA, ZFE shared most genes with the mouse model. Yet for APAP, there was not much difference in overlap between the pair-wise comparisons. Furthermore, to identify whether there were common genes altered across hepatotoxic states, the common genes identified from the above overlap were compared across compounds. Almost all compound specific changes across species were unique. Only one gene, CYP8B1, was found in common *in vivo* across AMD, APAP, and CsA, but no genes were found in common from the *in vitro* studies (Figure 3B).

Concordance identified at the pathway level. Next overrepresented pathways were determined using the unique top-500 ranked gene list for each model species to identify whether there were common pathways induced after reference compound exposure (Supplementary Table 3). Also here a separation between *in vivo* and *in vitro* models was made (see Fig. 4). Highest concordance for *in vivo* pathway activation was observed between rat and mouse; only several pathways overlapped between ZFE and rat and mouse, with mouse given the highest concordance with ZFE for CsA. Overlap for all the *in vivo* models was observed for only several pathways: for AMD one pathway that was shared between all models, which was the "Regulation of metabolism – Bile acids regulation of glucose and lipid metabolism via FXR". This pathway was also observed after exposure to APAP and CsA. Another pathway that was regulated after APAP exposure was the "Regulation of CFTR activity". For CsA, the pathways "Transcription – Transcription regulation of amino acid metabolism" and the pathway "Signal transduction – cAMP signaling" were enriched (Fig. 4).

For the *in vitro* comparison, at the pathway level ZFE correlated overall best with the mouse primary hepatocytes with respect to AMD and CsA. Overlapping pathways included the regulation of lipid metabolism (Supplementary Table 4). Interestingly, also *in vitro* APAP and CsA exposure resulted in the enrichment of the "Regulation of metabolism – bile acids regulation of glucose and lipid metabolism via FXR" pathway. After exposure

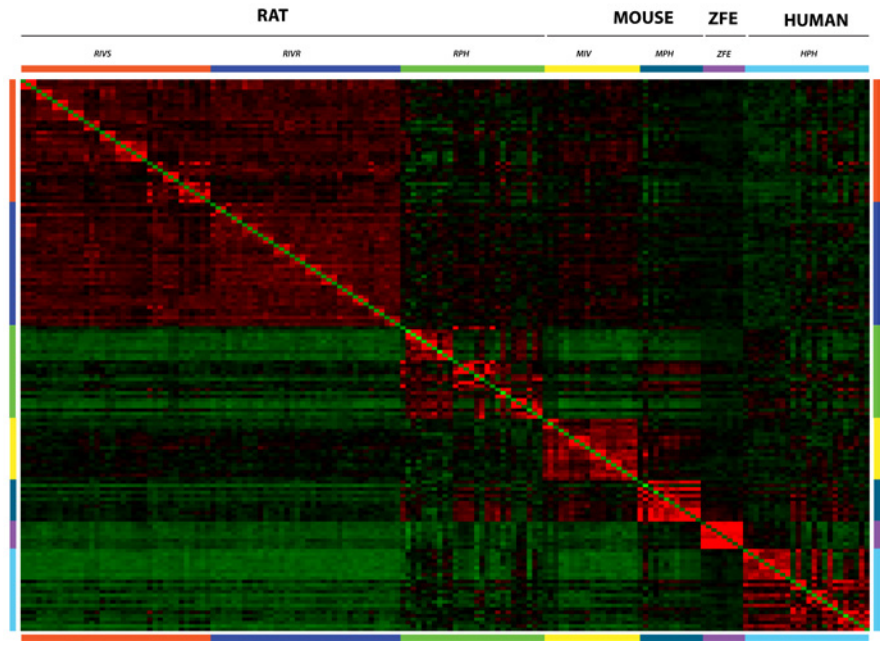


Figure 2 Single gene overlap between all conditions.
The top-500 ranked genes, for all 161 treatment conditions, were used to determine the overlap between the conditions on single gene level and identify similarities between groups. All conditions included in this study are color coded. RIVS is rat *in vivo* single dosing, RIVR is rat *in vivo* repeated dosing, RPH is rat primary hepatocytes, MIV is Mouse *in vivo* liver, MPH is mouse primary hepatocytes, ZFE is zebrafish embryos and PHH is human primary hepatocytes. Red color represents more overlap whereas green color represents less overlap.

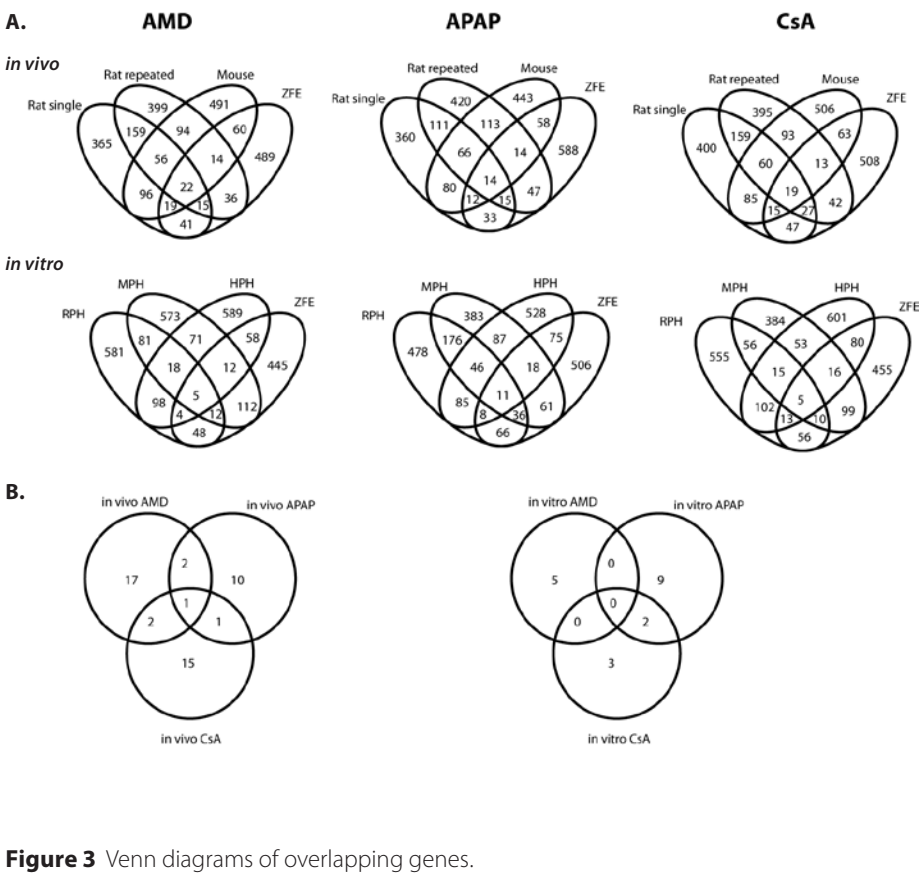


Figure 3 Venn diagrams of overlapping genes.
Unique top-500 genes for each model are compared between all species and overlap is presented in Venn diagrams. **A.** Gene overlap of compound dependent *in vivo* and *in vitro* models. **B.** Gene overlap of compound independent *in vivo* and *in vitro* models.

to AMD, this pathway was only observed in MPH, RPH and ZFE and not in HPH. However, HPH shared an enriched pathway with MPH and RPH, which was "Reproduction – Progesterone – mediated oocyte maturation" (Fig. 4) (Supplementary Table 3).

Species/model differences for genes within the enriched pathway. Overlap on single gene level resulted in one gene overlapping between the compounds in the *in vivo* models. This gene is part of the "Regulation of lipid metabolism_FXR-dependent negative-feedback regulation of bile acids concentration" pathway. For the *in vitro* models no overlap was found. However, pathway analysis resulted in one pathway enriched across most compounds and models being the "Regulation of metabolism – Bile acids regulation of glucose and lipid metabolism via FXR" pathway. To gain further insight into this specific

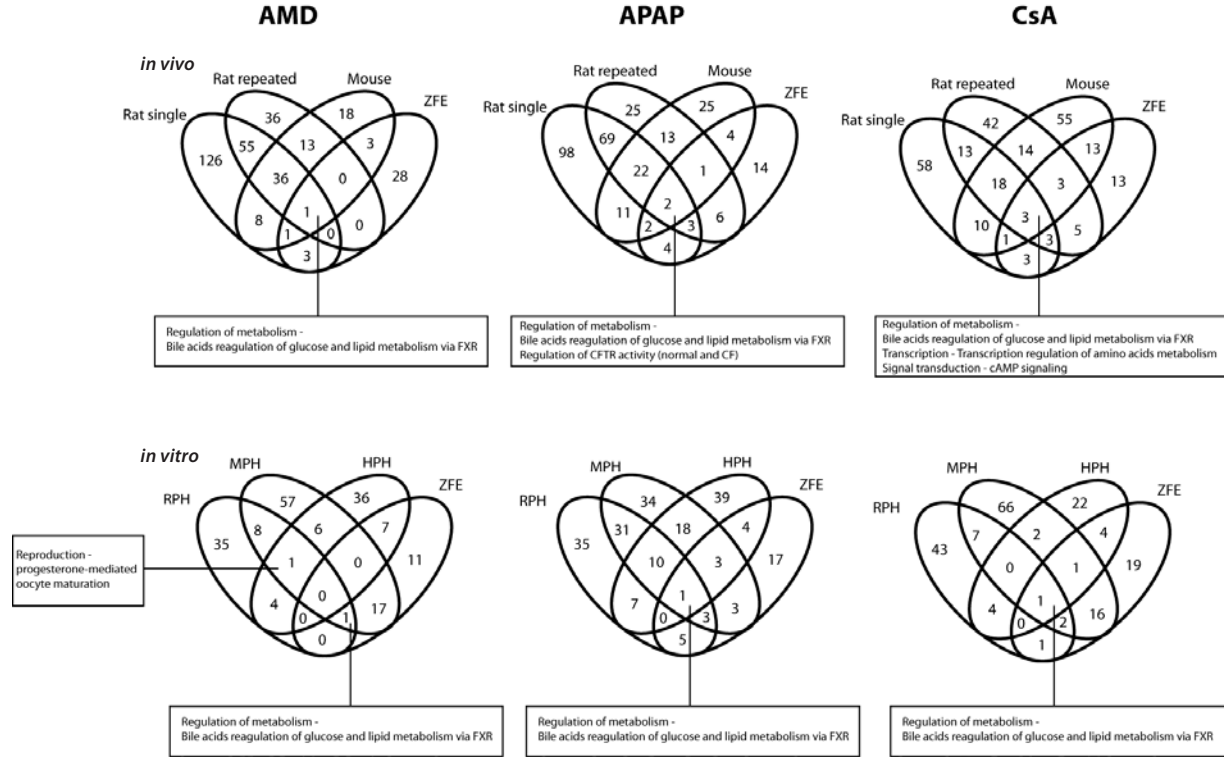


Figure 4 Venn diagrams of overlapping pathways.
Pathway overlap determined using venn diagrams. Pathways were based on the unique top-500 ranked genes per model and compound. Pathways were determined using MetaCore, a pathway was significant when $p < 0.05$.

pathway, a biplot was created to determine the effect of gene expression changes on each model (Fig. 5). It was observed that the genes APOE, LXR-α and HNF3-β had the lowest effect signified by their placement on the intersection of the axes. Genes were separated into the four different clusters: 1-topleft) SHP, PPAR-α, and ME1; 2-topright) MTP, APOB, HNF3-γ, F16P, HNF4-α, and FXR; 3-bottomleft) NIPK, SCD, ACACA, SREBP1 (nuclear), Insulin processed, GSK3-β, PLTP, HNF3-β, and FKHR; 4-bottomright) PPCKC and G6PT.
Models were also separated into the four different clusters: 1) RIVR_CsA, RIVR_AMD, HPH_APAP, HPH_AMD, RIVS_CsA, MPH_APAP and RIVS_APAP; 2) RIVS_AMD, RPH_AMD, ZFE_AMD, ZFE_APAP, ZFE_CsA, MIV_APAP; 3) HPH_CsA, RPH_CsA, MPH_AMD, MPH_CsA, RIVR_APAP, MIV_CsA and MIV_AMD; 4) RPH_APAP. Based on the gene expression changes, there was no separation between *in vivo* and *in vitro* models. The ZFE model treatments

Figure 5 Biplot of the "Regulation of metabolism – Bile acids regulation of glucose and lipid metabolism via FXR" pathway.

This pathway was found enriched across most compounds and models, and a biplot was created to determine the effect of gene expression changes on each model. Arrows represent the different conditions (model and compound). The arrow length indicates the strength of the effect.

were found in close proximity to each other on the biplot, and ZFE_AMD shared close similarity with AMD treated RPH *in vitro*, RIVS *in vivo*, and HPH *in vivo* models. These data indicate that although in all models an effect on the same pathway was observed, the components that were changed within the pathway were rather model dependent than compound dependent.

Discussion

Hepatotoxic potential of compounds to which humans are exposed are traditionally determined using rodent studies including mice and rats. However, these studies rely on long term exposure of the animals, and in light of the 3Rs, there is a need for new alternative testing models. The zebrafish embryo is a potential alternative testing model, which combines the benefits of an *in vivo* model, namely the complete biological complexity including the interactions between tissues and cells[49,54,95], with the advantages of an *in vitro* model, that is reduced animal discomfort and the ability for medium to high throughput testing. Here, we assessed the similarities of the zebrafish embryo model in relation to the traditionally used models including rat and mouse *in vivo* and *in vitro* to help in the identification of common hepatotoxic responses.

The changes on single gene level give information about how the different models respond to the xenobiotic treatment. Based on single gene overlap, the overlap within a species regardless of compound exposure was greater than between species. This might suggest that, within the limits of the data used for this analysis, every species produces a unique gene expression signature regardless of time, dose and compound. Also, comparing one model to the other models we found no difference in gene overlap suggesting that no two models are more similar to each other than the rest. When the DEGs were restricted to each individual model and compared across compounds, more DEGs were found in common for the *in vivo* comparison than for the *in vitro* comparison. This suggests that *in vivo* models may be more similar in the way they react to xenobiotic treatment than *in vitro* models. Alternatively the isolation procedure and culture procedures between mouse, rat and human hepatocytes affects the differentiation status of the cells preventing an equivocal comparison. Intriguingly, ZFE shared more genes with the mouse *in vivo* and *in vitro* models in the AMD and CsA comparison suggesting a strong similarity between the ZFE and mouse models.

CYP8B1 was identified in the *in vivo* models as a common gene altered in all hepatotoxic states. CYP8B1 is a cytochrome P450 family member that catalyzes numerous drug and lipid metabolizing reactions, and it is found in the pathway "Regulation of lipid metabolism_ FXR-dependent negative-feedback regulation of bile acids concentration". The expression of CYP8B1 is directly controlled by a combination of bile acids and the FXR receptor and indirectly by the PPAR-α receptor[176], supporting an important role of this gene in hepatotoxicity.

Inter- and intra-species comparison based on single genes is complicated because biological meaning is based on lists of differentially expressed genes. Instead, pathway analysis offers functional interpretation of these genes to a common, and therefore a more easy to compare endpoint[83]. Most importantly, in both *in vitro* (APAP and CsA) and *in vivo* (AMD, APAP and CsA) models, one pathway "Regulation of metabolism – Bile acids regulation of glucose and lipid metabolism via FXR" was found to be commonly regulated.

Bile acids are produced in the liver and aid in the adsorption of dietary lipids. Additionally, bile acids can activate various downstream signaling pathways including those of nuclear receptors, like the farnesoid X receptor (FXR), regulating bile acid and drug metabolism among others[34,177]. This pathway has been linked before to cholestatic liver diseases[164,178], and it has been regulated after exposure to AMD and APAP. After *in vivo* exposure of APAP, the pathway "Regulation of CFTR activity" was found in common. The cystic fibrosis transmembrane conductance regulator (CFTR), which is a member of the ATP-binding cassette (ABC) transporter family, is found in epithelial cells and acts as a glutathione channel among other functions. Furthermore, CFTR maintains water-salt homeostasis on the cell surface and normal function of epithelia lining airways, intestinal tract, and liver[80]. CFTR is significantly upregulated after APAP exposure in the mouse liver[179]. Since Kuppfer cells are suggested to play an important role in the upregulation of CFTR, this explains why this pathway is not observed in the *in vitro* models after APAP exposure because of their lack of Kupffer cells[180].

After *in vivo* exposure of CsA, the pathways "Transcription – Transcription regulation of amino acid metabolism" and "Signal transduction – cAMP signaling" were enriched. Amino acid metabolism is altered in the *in vivo* mouse model from CsA treatment[119], and cAMP signaling is important in many biological processes including the regulation of glycogen, sugar and lipid metabolism, which occurs in the liver[80]. From the AMD *in vitro* studies, the pathway "Reproduction – Progesterone – mediated oocyte maturation" was only observed in MPH, RPH and HPH and not in ZFE. Within HPH, this pathway contains genes that are generally responsible for cell growth, like p90RSK1 and PLK1, suggesting this pathway is important in liver cell function during xenobiotic exposure.

To gain more insight into how each model alters this particular pathway, a so-called biplot (Fig. 5) was made to identify the behavior of the genes, which resulted in their separation into five clusters. Additionally, the connection between the genes in each cluster was analyzed by plotting their location within the pathway (Fig. 6). In general, separation of the genes can be identified by their placement within the pathway. The genes within a particular cluster tend to have an interaction between them in a linear fashion then being scattered randomly. SHP and ME1 are found in cluster 1 where ME1 is controlled upstream by SHP in the regulation of lipogenesis. From cluster 2, HNF4-α is upstream of F16P in the modulation of gluconeogenesis and also upstream of MTP and APOB in the regulation of lipid transport. From cluster 3, NIPK and insulin processed are upstream of FKHR and GSK3β in the regulation of glycogenolysis and gluconeogenesis. Additionally, SREBP1 is upstream of ACACA and SCD in the regulation of lipogenesis, but is also separate from the cluster 1 genes in this regulation. From cluster 4, G6PT and PPCKC both modulate gluconeogenesis. Lastly, the genes found at the intersection of the axes (cluster 5 in Fig. 6), APOE, LXR-alpha and HNF3-beta, identify that the changes in gene expression are fairly similar among all models leading to these genes having the least effect and being randomly distributed throughout the pathway.

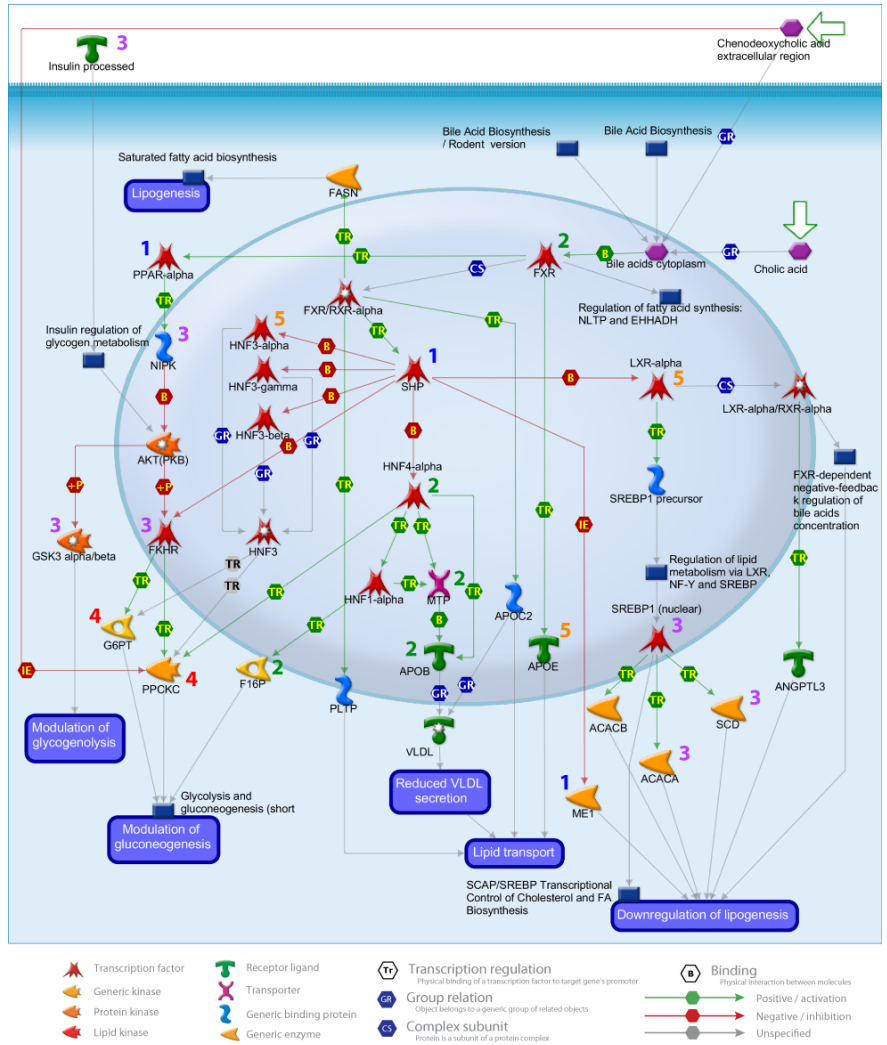


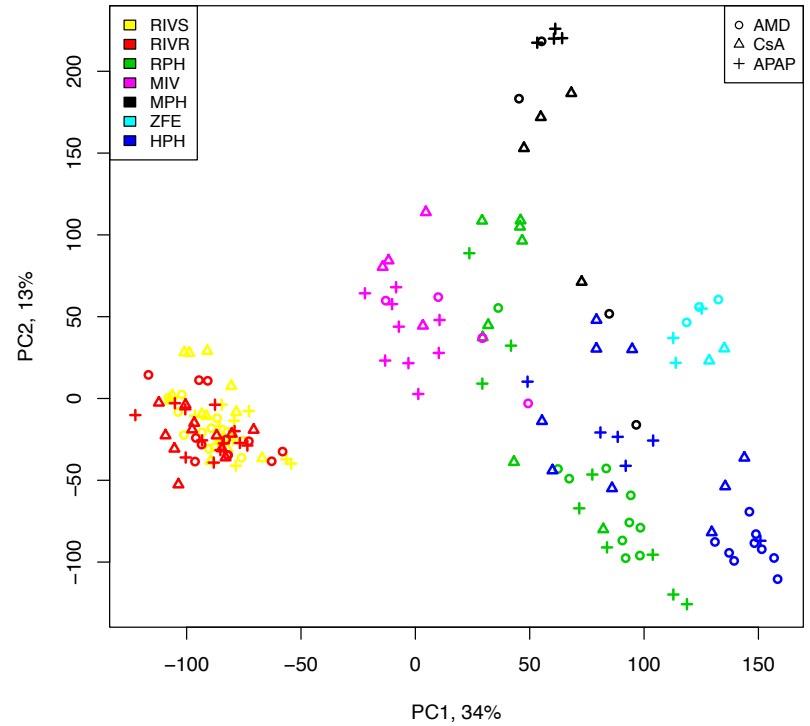
Figure 6 Diagram of the "Regulation of metabolism – Bile acids regulation of glucose and lipid metabolism via FXR" pathway from Metacore.

The relationship between the genes in each cluster from the biplot in Figure 5 was analyzed by plotting their location within the pathway (numbered genes on the figure correspond to the quadrants from Figure 5). In general, separation of the genes can be identified by their placement within the pathway.

In conclusion, the ZFE model shares similarity at the pathway level after xenobiotic exposure with both *in vivo* and *in vitro* models, using three reference compounds that cover a variety of hepatotoxic phenotypes. Concordance on the pathway level identified a single pathway to be altered across all hepatotoxic phenotypes, although with variations producing model and compound dependent clustering. Comparison at the single gene level showed that there were model specific changes and that outside of a model the overlap was fairly similar across models suggesting that on the gene level concordance cannot be readily identified. Therefore, the ZFE could be used as a prescreen for hepatotoxic compounds as it shares biological complexity with *in vivo* models and ease of testing found in *in vitro* models.

Acknowledgements

The authors wish to thank Paul Igor Costea PhD, of the European Molecular Biology Laboratory (EMBL), for helping to generate the biplot. In addition, we would like to thank Anke van Summeren and Edwin Mariman from Maastricht University (UM) for providing us with the mouse primary hepatocyte data. This work was supported by the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NWO) grant number: 050-060-510.



Supplementary Figure 1 PCA plot of the samples used within this study.
PCA plot of all the samples used within this study. PCA was based on a distance matrix using the overlap of the top-500 ranked genes between the samples. Samples are color-coded similar to as in Figure 2, and the different compounds are represented by the different shapes. Percentages on the x- and y-axis indicate the explained variances for the two first principal axes.