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

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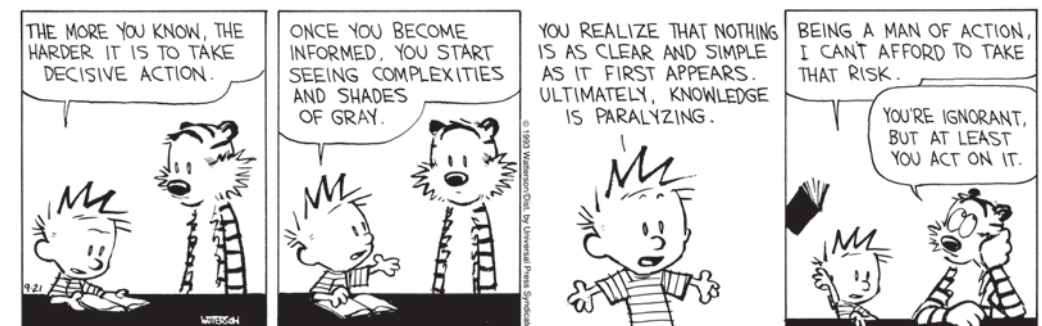
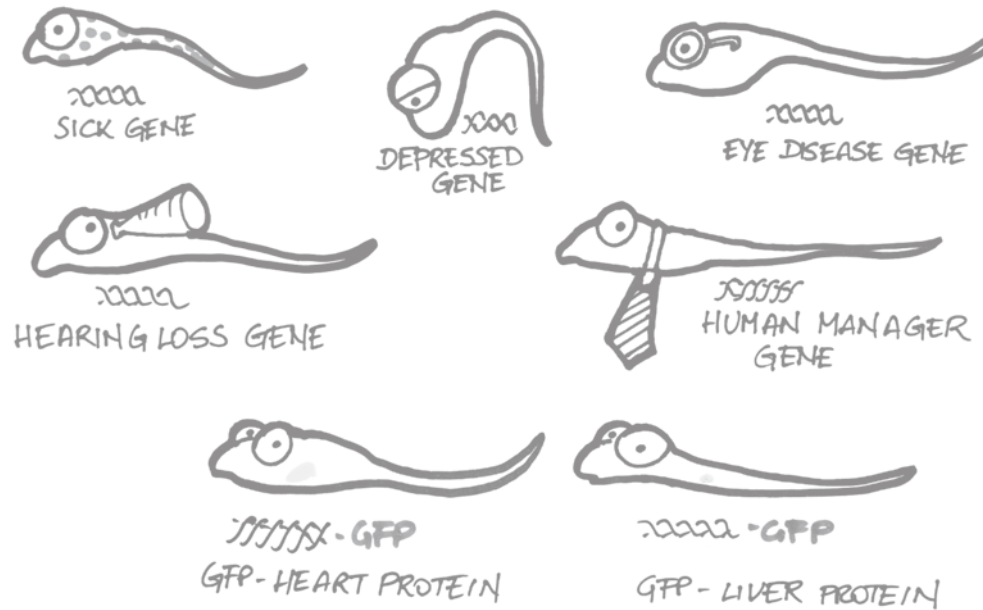
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General Discussion



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Hepatotoxicity-related pathologies as induced by drugs and industrial chemicals are traditionally measured in *in vivo* rat studies by histopathology and clinical chemistry. A disadvantage of *in vivo* studies is that they require enormous numbers of animals, which according to regulatory study protocols need to be exposed to high doses of compounds during extended periods of time. Apart from ethical and economical considerations, there is a scientific drawback because of the discordance between the hepatotoxic effects in rodents and humans, limiting the relevance of the outcome of *in vivo* studies regarding the prediction of human health risks. For these reasons, in recent years much effort was dedicated to the development of alternative test methods, and the zebrafish embryo is such a promising novel alternative test system. The general hypothesis of the work presented in this thesis is that the zebrafish embryo provides a simple and effective alternative animal model for hepatotoxicity testing, reproducing the complexity and functionality of the mammal liver *in vivo*. The main purpose of our studies was to develop and evaluate the zebrafish embryo model as an alternative system in a mechanism based way, using toxicogenomics approaches as a tool.

Through the mechanisms identified in our studies we provide evidence that the zebrafish embryo is a representative model to predict human hepatotoxicity, hence this discussion will focus on the critical factors related to hepatotoxicity testing in this model. First, the toxicokinetic properties of the zebrafish embryo will be discussed and how these differ from the human situation. Next, the histopathological changes as observed in the adult zebrafish liver and zebrafish embryo will be reviewed. Further, the additional value of toxicogenomic techniques presented in this thesis in identifying the underlying molecular mechanisms of hepatotoxicity will be discussed. Finally, further perspectives and applicability of the model will be considered.

Toxicokinetics in the zebrafish embryo

For a valid prediction of toxicity from fish to the mammal situation it is essential to take toxicokinetics in the zebrafish embryo toxicokinetics into account. Generally, toxicokinetics captures information on the administration and absorption, distribution, metabolism or biotransformation and excretion (ADME) of a toxic compound in an organism¹⁸¹.

First, absorption of xenobiotic compounds greatly differs between mammals and zebrafish embryos. The difficulty to translate toxicity data from zebrafish embryos to humans starts with the xenobiotic characteristics, whereby water soluble compounds such as paracetamol may have completely different uptake and distribution patterns compared to more lipophilic compounds such as cyclosporine A. Lipophilicity may partly explain differences in absorption efficiency between fish and mammals, in view of the different surfaces through which absorption takes place. In fish, a major route of absorption of a compound is through the gills and skin, in addition to oral uptake^{182,183}, whereas in

humans most xenobiotic compounds are mainly orally ingested. Therefore, the zebrafish gills can be considered as an important first-pass organ in addition to the liver, which is the single first pass organ in mammals/humans. The importance of gills as a first pass organ is underlined by the presence of CYP450¹⁸⁴. In addition, an important issue related to absorption difference between lipophilic and hydrophilic compounds in exposure studies in zebrafish embryos is the barrier that is formed by the chorion (egg shell). However, here we circumvented this problem by exposing after hatching of the embryo, which is a relevant timing for hepatotoxicity studies in view of the presence of functional liver tissue in the embryo.

After the administration and uptake of a compound, the distribution of a compound is responsible for inducing the toxicity in the target organs, and distribution modeling can therefore help to understand details of observed effects. In the zebrafish embryo, whole organism concentration studies are only beginning to emerge¹⁸⁵, while information regarding distribution is presently extremely limited, excluding direct comparison between relevant concentration in the fish versus plasma concentration in humans. Here, xenobiotic compounds dissolved in DMSO were added to the exposure medium, and were taken up by the zebrafish embryo presumably by passive diffusion, mainly through gills and skin¹⁸². The biotransformation capacity of the gills might influence the distribution of parent compound concentration and its metabolites at the site of the liver, and is a factor that needs to be taken into account when comparing liver toxicity profiles between ZFE and mammals. Furthermore, the distribution through the body might be influenced by the presence of the yolk sac, which is the internal food supply in the zebrafish embryo essential for initial development. However, the yolk is a lipid rich compartment allowing hydrophobic compounds to accumulated here. This accumulation could then lead to an overestimation for the effective concentrations required to induce organ toxicity¹⁸².

Regarding the biotransformation of xenobiotic compounds, expression of most metabolizing enzymes is already present in early developmental stages^{55,182}. Furthermore, enzymes that are part of the metabolizing process are highly conserved compared to mammals¹⁸² and many orthologs are found in humans^{55,182}. However, development of the ZFE is associated with stage dependent changes of expression levels of metabolizing enzymes, which might result in a different sensitivity to the xenobiotic compounds compared to mammals. This difference in sensitivity is further anticipated by the large evolutionary distance between humans and zebrafish, which is around 445 million years¹⁸⁶.

The final component in ADME is the excretion of the compound. However, excretion is not seen as a very important aspect in the zebrafish embryo model while the exposure is mainly static^{182,187}.

In future studies, it is imperative to improve characterization of the kinetic properties in fish to gain a better understanding of their influence on the development of hepatotoxicity. In this thesis, we have not measured the internal concentration of our compounds or their

metabolites in the ZFE, but relied on the observed compound-induced phenotypic changes in the liver, which were suggestive for internal ZFE drug exposure.

Identification of hepatotoxicity using histopathology

Histopathology can be used to differentiate the three hepatotoxic phenotypes cholestasis, steatosis and necrosis. 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 composition³⁴. 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 oxidation¹⁹. Necrosis is an acute condition and is characterized by cell death due to oxidative stress and/or mitochondrial damage leading to loss of cellular ATP levels^{4,33}. The model compounds CsA, EE2 and CPZ induce cholestasis. AMD, TET and VPA induce steatosis in humans¹³⁷. Finally, necrosis is induced by APAP, PQ and TAA¹⁴.

In **chapter 2**, we showed that the zebrafish embryo demonstrates clear histopathological changes after xenobiotic exposure, but failed to display the classical cholestatic and necrotic phenotypes as observed in humans. To verify whether this difference was life stage dependent or represents a response difference between fish and mammals, the adult zebrafish was also exposed to the same reference hepatotoxicants. The adult zebrafish liver was found particularly sensitive to the development of cholestasis and displayed a phenotype similar to humans. Cholestasis could not be identified in the zebrafish embryo probably due to the underdevelopment of bile production mechanisms, in line with the observation that genes involved in the formation of bile ducts are first expressed at 48-hpf¹²⁶ and that onset of fully operational bile production in the embryo is only from 5-dpf onwards⁴⁴. The observed steatosis in both zebrafish life stages was consistent with the nominal phenotype as observed in mammals. The necrotic phenotype was represented in both life stages as hepatocellular vacuoles, but no apparent apoptosis or necrosis was induced.

The onset of necrosis typically involves lipid peroxidation, which is especially the case for acetaminophen. Lipid peroxidation is generally coupled to the formation of reactive oxygen species (ROS) and is a result of oxidative stress in cells. The Nrf2 transcription factor plays a very important role in the defense against oxidative stress¹⁸⁸. In the zebrafish embryos, the Nrf2 pathway was not identified as a critical reporter for most of our hepatotoxicants. The Nrf2 pathway is conserved between humans and zebrafish and is even present in the zebrafish embryos. However, it has been shown that the *nrf2* mutant zebrafish embryo is not sensitive to all ROS productions as is the case within the mammalian systems¹⁸⁹, suggesting that alternative defense mechanisms are active. This explains why this pathway has not been found as critical in the zebrafish embryo. Overall, there are apparently species and life stage dependent apical phenotypes, suggesting that toxicity pathways diverge between life stages and species. This is in line with the conclusion that our toxicogenomic methods could not distinguish the nominal pathological classes represented in our set of model compounds in the zebrafish embryo.

Identification of hepatotoxicity using gene expression profiling

Histopathology is the classical method to verify toxic events, but it does not provide information about the underlying molecular mechanisms leading to hepatotoxicity, and thus may limit full comparison of effects between testing models. Our working hypothesis was that the underlying molecular mechanisms leading to a toxic phenotype might be conserved between humans and zebrafish embryos, and therefore be more informative than histopathology regarding the translation of effects. In case of zebrafish, toxicogenomics techniques, including transcriptomics and proteomics, were already applied mostly in the area of developmental and ecotoxicological toxicity in the zebrafish embryo^{53,66–70,187}. However, the implementation of transcriptomics or proteomics in the zebrafish embryo for predicting hepatotoxicity had not been applied, and our initial results described in **chapter 1** indeed supported that the application of toxicogenomics in hepatotoxicity appeared to be of great added value regarding better understanding of the underlying molecular mechanisms of toxicant induced effects.

Defining liver-associated transcripts

In rodent experiments, the liver is dissected and subjected to RNA isolation for the determination of gene expression changes, but the zebrafish embryo is too small for liver dissection. Although sophisticated procedures such as micro-dissection can be applied to collect small amounts of liver material, this will hamper the throughput and robustness of the testing system. Therefore, we first determined whether liver-associated gene expression can be detected in the whole zebrafish embryo using whole body homogenates. In the zebrafish embryo, the liver accounts for only approximately 20% of the total body weight, and this relatively small proportion might limit the detectability of liver specific signals. Therefore, to ensure that we were able to determine liver-specific gene expression, whole embryo homogenates were compared to isolated adult zebrafish livers. This comparison was complicated by the necessity to use pools of 15 zebrafish embryos per sample meaning that the specific signal of the individual embryo is diluted, but on the other hand, the observed gene expression changes are more robust¹⁰⁶. In this comparison we used next-generation sequencing, which is a relatively new technique, which can be used for mapping as well as quantifying transcriptomes. The advantage over the use of the traditional RNA arrays is that this technique provides a more sensitive and precise measure of transcript levels resulting from the relative low background signal and the absence of an upper limit of detection⁶⁴. The results in **chapter 2** support the additional value of this still developing technique for toxicity testing. Furthermore, we confirmed through next generation sequencing that liver-associated gene expression is present in the whole zebrafish embryo and that hepatotoxicity-associated signals are detectable over the noise of other tissues, and that the whole zebrafish embryo can therefore be used as a proxy for the assessment of hepatotoxicity. Building on this premise, a detailed analysis of mechanisms of hepatotoxicity was conducted using RNA arrays as

shown in **chapter 3**. This resulted in a better description of hepatotoxic responses and to the development of expression markers predictive for hepatotoxicity in humans. To assess the liver-specificity of the found markers, we assessed their location using *in situ* hybridization (data not shown), and could thus confirm liver specificity of eight marker genes. This supports that the small proportion of the liver and the pooling of the samples did not influence the liver associated gene expression.

Analysis of gene expression resulted in markers for general hepatotoxicity, but specific phenotypes were not reflected, possibly due to suboptimal exposure concentrations and durations. Additional efforts to discriminate between nominal phenotypes using proteomics (**chapter 4**) produced a similar result, *i.e.* identification of general markers for hepatotoxicity but not for specific phenotypes. Furthermore, we observed that most of the expressed proteins were linked to a more general hepatotoxic stress response, possibly also related to the used concentrations and exposure durations. On the other hand, we did not observe interference of yolk sac related proteins, which is a general problem in proteomics studies with zebrafish embryos, supporting robustness of the liver specific protein signals. This suggests that de-yolking of a 5-dpf old whole zebrafish embryo is not necessary which improves the throughput of the system when applying proteomics.

Determination of hepatotoxic classes

The reference compounds used in this thesis were selected to represent three different histopathological endpoints (cholestasis, steatosis or necrosis), but as described in **chapter 1**, within each class, individual compounds have unique underlying molecular mechanisms for these histopathological changes. In **chapter 3 and 4**, we identified markers for general hepatotoxicity, while the applied zebrafish toxicogenomics strategy was unable to discriminate between the hepatotoxic classes of interest. This is either due to species dependent differences in liver physiology or to experimental conditions. With regard to the latter, the selected exposure concentrations were based on the maximum tolerable concentration inducing no teratological deviations or death. It might be that this single concentration of the compounds was not optimal for inducing the specific hepatotoxic phenotypes; moreover, the exposure period might be too short to induce the specific phenotype. Alternatively, zebrafish specific responsiveness to xenobiotics could be in line with deviating responses in other models, such as hepatoma-derived cell lines which strongly differ from the whole liver gene expression profile^{64,101}. It should be noted that we only tested a set of nine compounds, and a more comprehensive range of compounds will probably generate more robust results. A first attempt was made to validate our markers by using an additional set of seven hepatotoxic compounds (not shown). Zebrafish embryos were exposed to this new set of model hepatotoxicants and compared to three old reference hepatotoxicants. After exposure, the expression of the marker genes obtained in **chapter 2 and 3**, were determined using qPCR. Most of the additional hepatotoxicants had similar expression patterns as the original set of hepato-

toxicants, confirming the validity of our markers. However, this additional exercise was not designed to improve class distinction, and only a larger drug library screen in a more high-throughput setting including more hepatotoxicants and non-hepatotoxicants may provide a better estimate whether the zebrafish embryo is able to discriminate between classes.

Interspecies extrapolation

As mentioned above, the zebrafish embryos were able to determine the hepatotoxic potential of xenobiotic compounds. It is important to extrapolate the observations to mammalian species and where possible even to the human situation, to underpin major added value of the zebrafish embryos. Therefore, in **chapter 2 and 5**, we compared the zebrafish embryo to the *in vivo* mouse and rat liver, *in vitro* mouse and rat hepatocytes, and primary human hepatocytes using gene expression changes from experiments using a model compound for each hepatotoxic phenotype to identify commonalities. Three compounds, CsA, AMD, and APAP, were chosen for the comparison because of their relevance to human toxicity and the availability of reference data in the selected models. This comparison revealed that the zebrafish embryo shares similarities at the pathway level after xenobiotic exposure with both *in vivo* and *in vitro* models, with the highest concordance to the mouse model. One pathway which was shared between all models was the pathway "Regulation of metabolism – bile acids regulation of glucose and lipid metabolism via FXR". While bile acids are important in the regulation of the normal liver, they also activate various signaling pathways including those of nuclear receptors^{34,177}. In this particular pathway, it was observed that each species targeted a specific part suggesting species differences. Still, the common regulation of this particular pathway between all included species indicates its robustness. This supports that the zebrafish embryo model has a similar response to the hepatotoxicants as the traditionally used models and therefore, the zebrafish embryo could be used as a prescreen for hepatotoxic compounds.

Optimization of the zebrafish embryo screening for high throughput

The studies presented in this thesis have shown the promise and applicability of the embryo model for the detection of hepatotoxic potential of compounds. In the current work, the model was not optimized for high throughput screening. Optimization of the model can be achieved in multiple ways. First, the experimental setup including handling of the zebrafish embryos and the distribution of the xenobiotic compound of interest can be improved. The handling of the embryos can be automated by using an embryo sorting machine, for example the COPAS XL^{59,190}. The distribution of the xenobiotic compound to the well plates can be improved by either using a multichannel pipette or automated by the use of a robotic liquid-handling machine⁵⁹. Furthermore, the whole genome sequencing or the microarray technologies that we applied here provide highly detailed

information, but are rather time consuming. Dedicated arrays or selected qPCR with marker genes for hepatotoxicity could improve the throughput of the screening. A drawback of these methods is that these still require time consuming RNA extraction, and have no information on the tissue distribution, *i.e.* liver expression, of marker genes. To overcome these drawbacks, another optimization could be to generate transgenic reporter zebrafish, which allows screening in high throughput manner. The technique of generation reporters is well developed for zebrafish, and could be applied for the markers from **chapter 2 and 3**. For this purpose, DNA constructs enabling the expression of a fluorescent protein after induction of the marker of hepatotoxicity should be designed and injected in wild type one cell stage zebrafish embryos. Selected embryos which show the intended presence of the fluorescent protein are further raised to adulthood and paired to produce fluorescent embryos which can subsequently be used for screening purposes¹⁹¹. With the use of transgenic zebrafish embryos for hepatotoxicity testing, large drug screens can be tested in a more high-throughput manner using COPAS or confocal microscopy which has been shown to be a valid approach for developmental toxicity screening^{192–194}. Preliminary testing with one such a reporter fish, *fabp10*^{195,196}, has been initiated.

Implementation of the zebrafish embryo in drug discovery process

The zebrafish embryo testing model fits into multiple stages of the drug discovery pipeline, ranging from lead and target identification to lead optimization and ADME studies^{193,197}. Application of this model is attractive because it is relatively cheap, it contributes to reduction of animal experimentation, and relatively small amounts of the xenobiotic compounds are needed. The model can be incorporated easily into the existing tiered approach for the prediction of hepatotoxicity. Combining multiple *in vitro* screening models in a tiered approach could overcome the limitations of each individual *in vitro* model in the prediction of toxicity, and in a series of models, they can complement each other for information regarding toxicity^{164,197}. Presently, the earliest test models in such a tiered approach for hepatotoxicity are typically the *in vitro* hepatoma cell lines, for example HepG2 or HepaRG. Next, primary hepatocytes from either rat or human origin would be tested, to ensure incorporation of drug metabolism and transport capabilities as well as bioenergetics that are closer to the *in vivo* situation. Precision cut tissue slices, which provide an intact architecture similar to the organ of interest, provide an alternative method that can be used next in the line of *in vitro* models^{164,197}. Although all these models are suitable for the detection of hepatotoxic potential of compounds, the zebrafish embryo offers biological complexity similar to *in vivo* models with the ease of testing as is possible with *in vitro* models. Therefore, the zebrafish embryo can be used to prioritize the compounds, which should be tested in an *in vivo* set-up, thereby contributing to the reduction and refinement of animal experimentation.

Current and future application of the zebrafish embryo

The implementation of the zebrafish embryo as a model for hepatotoxicity testing requires a number of steps. Firstly, as mentioned above, the toxicokinetic parameters, which limit assessment of particular compounds in the zebrafish embryo model, should be characterized better. As discussed, a clear example in this respect is the yolk sac which might cause underestimation of the results obtained by the more lipophilic xenobiotics compounds.

Here, we tested only nine compounds which are known to induce hepatotoxicity in humans. To validate the model further and determine the predictability of the model, a large screen of many hepatotoxicants needs to be performed. For practical reasons, we had to limit our studies to a single sub-lethal concentration of each compound, but obviously confirmation of concentration-dependent responses would improve robustness of the identified markers. As indicated, improvement of the throughput of testing could be achieved through the development of transgenic zebrafish. In addition, humanized transgenic fish that would express relevant biotransformation enzymes could further strengthen the zebrafish embryo system.

The zebrafish embryo can be of added value to elucidate the underlying molecular mechanisms by using morpholino techniques to establish a knock down of genes of interest. Morpholinos allow the temporary knockdown of genes thus identifying their role in the involved toxic mechanism of hepatotoxicity. For example genes that were induced by any of our model compounds in the present study could be interrogated for involvement in the onset of the phenotypic hepatotoxic changes in the liver. In particular those genes that would then be functionally associated with the onset of hepatotoxicity and have translational relevance would be of the highest interest to serve as marker for hepatotoxicity liabilities of novel chemical entities.

Conclusion

In this thesis we showed the applicability of the zebrafish embryo as an alternative model for hepatotoxicity testing using analysis of mechanisms through toxicogenomics. By applying a variety of toxicogenomics techniques, we were able to characterize specific responses. NGS revealed that hepatotoxicity-associated gene expression remains detectable even in non-tissue specific analysis in whole body zebrafish embryo homogenates. Gene and protein expression profiling resulted in the identification of a set of marker genes that could be linked to pathways and processes, which are associated with a general hepatotoxic response. Application of such markers will increase the throughput of the system. Finally, we showed that the zebrafish embryo model shares similarities with *in vivo* and *in vitro* models for hepatotoxicity, where the model has more commonality with the mouse *in vivo* and *in vitro* models than with the other models.

Overall, the model has the advantages of the *in vitro* models with the biological complexity of an *in vivo* response, and we thus anticipate that the zebrafish embryo can contribute to the 3R strategy by reducing, refining and replacing animal studies. Our observations support that this model can have added value in a tiered approach as a pre-screen for the detection of hepatotoxic potential of compounds.