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The developmental stage is a critical parameter for accurate assessment of the drug-induced liver injury (DILI) potentials of drugs with the zebrafish larval liver model

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Abstract

Prediction of drug-induced liver injury (DILI) potential of drugs is one of the most challenging issues of drug development. Zebrafish larvae provide an *in vivo* and robust test platform. Due to the ease of handling developing larvae between 2 - 5 days post fertilization (dpf) has been extensively used as a DILI test model. However, the liver is not fully functional at this stage. Here, the importance of larval liver maturation was tested by applying selected known DILI-rank drugs to liver reporter zebrafish between 2-5 dpf and 5-7 dpf. Acetaminophen (most-DILI) treatment caused a significant dose-dependent reduction in liver size only at the early stage. Isoniazid (most-DILI) administration after liver maturation induced hepatomegaly, while it induced liver size reduction between 2-5 dpf. Chlorambucil (less-DILI) treatment induced opposing effects on liver size, in the two stages tested. A non-DILI agent chloramphenicol did not induce any liver size change in either larval stage. Clinical observations were better reproduced when isoniazid and chlorambucil were administered after liver maturation. Our findings show that often-overlooked liver maturity status is a critical parameter for the evaluation of DILI.

Introduction

Drug-induced liver injury (DILI) is a term describing liver damage observed after administration of various drugs, herbal products, and dietary supplements (Garcia-Cortes et al., 2020). Currently, the diagnosis of DILI is challenging due to the lack of specific biomarkers and therefore, DILI is a diagnosis of exclusion (Iruzubieta et al., 2015). Serum transaminase levels, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total serum bilirubin levels (TSB) are assessed to detect DILI using some calculations and score tables such as Roussel Uclaf Causality Assessment Method-RUCAM (Andrade & Robles-Díaz, 2020). Even though these methods are in clinical guidelines for the diagnosis of DILI, reliability and sensitivity are not high enough for early detection and diagnosis (Robles-Díaz et al., 2016). Drug-induced hepatotoxicity is not always dose-dependent and predictable, and the severity of DILI may depend on individual factors. DILI remains one of the most challenging issues in the discovery and pre/post-marketing stages of drug development (Andrade et al., 2019). Zebrafish larva due to it being time and cost-effective, has attracted interest as an *in vivo* model for prediction of a drug candidate's hepatotoxicity potential (Jagtap et al., 2022). The rapid maturation of the liver within 5 days post fertilization (dpf), transparency of the larvae, easy application of test compounds, and the possibility for medium-throughput live imaging are among attractive features of zebrafish (Chu & Sadler,

2009; Katoch & Patial, 2021; Vliegenthart et al., 2014). Different features of DILI can be screened with the help of numerous transgenic reporter zebrafish lines. Among these, the most widely used method is the liver size measurement in transgenic larva that expresses fluorescent protein in hepatocytes (Choi et al., 2014; Lin et al., 2019; Nguyen et al., 2017; Park et al., 2022; X. Zhang et al., 2014). Several studies confirmed that drug detoxification enzymes are conserved in zebrafish, and drugs are processed in zebrafish liver (Sato et al., 2023). Early zebrafish between 2-5 dpf are highly preferred for hepatotoxicity studies, this is a stage during which liver specification and maturation take place (Cassar et al., 2019; Chu & Sadler, 2009). While important drug-metabolizing enzymes such as Cytochrome p450 enzymes (CYPs) are expressed early on in extrahepatic tissues, the maturation of the larval liver is typically completed by the end of 5 dpf (Goldstone et al., 2010; Nawaji et al., 2020). Therefore, we hypothesized that the drug-induced liver toxicity observed before 5 dpf is partly due to interference with developmental processes, and the use of larvae with fully functional liver may provide a more direct hepatotoxicity assessment.

This study aims to investigate the effect of larval liver maturation status on liver damage. To this end, FDA-approved drugs with different DILI concern classifications were applied to zebrafish between 2-5 dpf and 5-7 dpf, and DILI potentials were evaluated with liver size changes.

Materials and Methods

Chemicals and reagents

Acetaminophen (APAP) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), chloramphenicol (CHLP) (Bioshop Canada Inc., Burlington, Ontario, Canada), isoniazid (INH) (Goldbio Technology, St. Louis, MO, USA), chlorambucil (CHB) (Sigma-Aldrich, St. Louis, MO, USA), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and ethanol (Sigma-Aldrich, St. Louis, MO, USA) were of analytical grade. Low melting point agarose and tricaine methanosulfate (MS-222) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

Preparation of drug solutions

APAP and INH were dissolved in deionized water. CHB and CHLP were dissolved in DMSO and 95% ethanol, respectively. Working solutions were prepared freshly by diluting in E3 medium (15 mM NaCl, 0.5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM $NaHCO_3$, 0.5% methylene blue, pH 7.5). The final doses applied were 1.25 mM - 10 mM for APAP, 1.25 mM - 10 mM for INH, 25 µM - 100 µM for CHB, and 0.25 mM - 2 mM for CHLP. Control groups were treated with E3 for APAP and INH, with 1% DMSO for CHB, and 0.5% Ethanol for CHLP. DMSO did not induce any systemic toxicity according to the previous study and did not cause any liver size change in our setup (Cornet et al., 2017).

Zebrafish treatments

All adult zebrafish were maintained under standard conditions. Embryos were obtained by crossing *fabp10a:mCherry* transgenics with AB+/+ wild-type fish and incubated in E3 at 28 °C at Izmir Biomedicine and Genome Center (IBG) zebrafish facility. All experiments were conducted according to the national regulations. The protocols were approved by IBG Animal Experimentation Local Ethics Committee by protocol no 2021-005. 10 larvae per well were maintained in 1 mL of medium, in 24-well plates for treatments. Medium was refreshed and larvae were monitored daily. The experiment was repeated two times with similar findings. One set of experiment results were presented in the figures.

Liver size measurements and statistical analysis

Larvae were anesthetized and embedded in low melting point agarose for imaging (Lecaudey et al., 2008). Liver images were captured with a fluorescent stereomicroscope (Olympus SZX16, XC50 camera). The liver area was measured with Fiji software. For each group 10 individual samples were measured, the average value was plotted, and standard deviation was used as the error bar. Statistical significance was calculated with one-way ANOVA, and significance was annotated: **P*<0.05, ***P*<0.01, ****P*<0.001.

Results

DILI-concern categories of selected drugs were determined according to the DILIrank database published by the Food and Drug Administration (FDA) (Chen et al., 2011). Most-DILI agents APAP and INH, less-DILI agent CHB, and non-DILI agent CHLP were tested. DILI was evaluated based on liver size change in *fabp10a:mcherry* liver reporter zebrafish transgenic larvae. The drugs were administered to zebrafish larvae between 2-5 or 5-7 dpf.

Acetaminophen-induced liver size reduction only at 2-5 dpf stage

APAP is a commonly used drug known for its analgesic and antipyretic effects and accepted as the model drug for dose-dependent, direct DILI studies (Klotz, 2012; Vliegenthart et al., 2014). While several studies employed zebrafish embryos and early larvae younger than 5 dpf showed that APAP induces hepatotoxicity in the zebrafish model, none of the previous studies tested larvae with fully functional liver (Guo et al., 2015; North et al., 2010; X. Zhang et al., 2014).

Here, previously reported hepatoxic doses (2 mM - 10 mM) of APAP were tested (North et al., 2010). 1.25 mM APAP did not cause any visible morphological changes or liver size change when applied between 2-5

dpf or 5-7 dpf. 10 mM APAP caused lethality when applied between 2-5 dpf. 2.5 mM APAP caused changes in melanocyte pattern, and 5 mM APAP caused smaller eyes and defects in pigmentation, which are previously reported phenotypes (Figure 1A) (Sato et al., 2023). However, the same concentrations of APAP did not induce any morphological or pigmentation defects when applied between 5-7 dpf (Figure 1B). 2.5 mM and 5 mM APAP induced a dose-dependent and significant liver size reduction when applied between 2-5 dpf. On the other hand, when APAP was applied after maturation of liver, between 5-7 dpf, no change in liver size was detected (Figure 1C and 1D).

Isoniazid-induced liver enlargement only at 5-7 dpf stage

INH is a widely used first-line drug for the treatment of tuberculosis. Unlike APAP, INH is known to cause idiosyncratic DILI (iDILI), which is more difficult to detect in preclinical test models (Chan & Benet, 2017).

It was demonstrated that the administration of 10 mM INH between 3-4 dpf resulted in liver size decrease (Jagtap et al., 2022). Another study reported induction of apoptosis upon exposure to 6 mM INH between 4-5 dpf (Higuchi et al., 2021). Here, 1.25 mM - 10 mM INH were applied between 2-5 dpf and 5-7 dpf. When applied between 2-5 dpf, 1.25 mM and 5 mM INH did not cause any morphological defects or apparent phenotypes, whereas 10 mM INH caused a significant reduction of larval body size (Figure 2A). When applied between 5-7 dpf, 1.25 mM INH did not cause any defect, while 10 mM was lethal. In this group, 2.5 mM INH caused the darkening of the liver, and 5 mM INH caused the darkening of the head and liver as well as the bending of the larval body (Figure 2B). The effect of INH treatment on liver size was different in tested two stages. While lower doses did not induce any change of liver size, 10 mM INH caused a significant decrease in liver size when applied before 5 dpf (Figure 2C, D). In contrast, INH caused a significant increase in liver size when applied between 5-7 dpf, at doses of 2.5 mM or 5 mM (Figure 2C, D).

Chlorambucil-induced liver enlargement only at 2-5 dpf stage

CHB is one of the best-tolerated oral alkylating agents prescribed for the treatment of chronic leukaemia and lyphomas as well as ovarian and breast cancers (Sienkiewicz et al., 2005). CHB is classified as a less-DILI drug which can still lead to moderate-to-serious clinical DILI manifestations (Patel, 2000; Patel et al., 2000). Clinical cases reported mild hepatomegaly or hepatosplenomegaly after CHB administration (Kyle et al., 2000; Skoutelis, 2000). CHB hepatotoxicity on zebrafish liver has not been studied previously. Microinjection of CHB (6 mM, 40 nL) into the yolk sac of 2 dpf zebrafish larvae resulted in 60% lethality, and body deformation was observed in survivors (Akdogan et al., 2022), while blastula treated with 10 µM - 50 µM of CHB exhibited delayed epiboly (Nakayama et al., 2021).

Here, CHB was applied at 25 µM, 50 µM, and 100 µM concentrations. When 25 µM - 50 µM CHB was applied between 2-5 dpf, no morphological change was observed in larvae. 100 µM CHB caused a systemic effect resulting in a reduction in the larval body length, smaller craniofacial region, and eye size (Figure 3A). When applied between 5-7 dpf; 25 µM caused mild darkening of the liver, 50 µM caused darkening of the liver and bending of the larval body, while 100 µM CHB was lethal (Figure 3B). When administered between 2-5 dpf 50 µM CHB caused liver enlargement. While 100 µM CHB-treated larvae were much smaller than controls, average liver size was similar to untreated healthy controls, which indicated liver size increase when compared to body size (Figure 3A, B, D). In contrast, when applied between 5-7 dpf, 50 µM CHB caused a small reduction in liver size, while 100 µM drug caused lethality (Figure 3).

Chloramphenicol did not cause liver size change

Chloramphenicol (CHLP) is an effective broad-spectrum antibiotic against meningeal pathogens, but its use is limited due to safety concerns such as hematological problems, neurotoxicity, and hypersensitivity reactions (Feder Jr, 1986; Singhal et al., 2020). CHLP was used here as a negative control since FDA-DILI rank dataset categorizes it as a non-DILI agent. Ali et al. (2012) reported LC_{50} values of 1.62 mM or 23.36 mM when applied to zebrafish between 1-5 dpf for different durations. Sublethal concentrations were verified in our preliminary studies and the drug was applied within a range of 0.25 mM - 2 mM. CHLP was well tolerated by zebrafish between 2-5 dpf, and no morphological changes were observed (Figure 4A). Similarly, larvae between 5-7 dpf tolerated CHLP well. While a minor darkening of the liver was observed at higher doses, the larvae looked healthy and normal (Figure 4B). Average liver sizes remained unchanged in all groups, and livers looked normal under stereomicroscope (Figure 4C, D).

Discussion

Drug metabolism in the liver converts the drug molecules into more polar and soluble forms that can be excreted and this can occur in three phases: modification (phase I), conjugation (phase II), and transported mediated elimination (phase III) (Almazroo et. al, 2017). The zebrafish liver is accepted to gain function as of 4 dpf but levels of most drug metabolising enzymes increase at day five or later (Chu & Sadler, 2009; Cakan-Akdogan et. al, 2023). Although zebrafish has been increasingly used as a hepatoxicity screening tool, majority of the studies use early larvae (younger than 5 dpf), a stage in which the liver is still developing (Vliegenthart et. al, 2014). Here, the importance of stage for hepatotoxicity assessment was investigated by comparing affects of selected drugs in early developing larvae and 5 dpf larvae with a fully functional liver.

Interestingly, hepatotoxicity potentials and the liver size phenotypes varied according to the stage and drug applied.

The first selected drug was APAP, a most-DILI agent, which has been extensively studied in zebrafish in early larvae and adult stages. Liver size reduction caused by 2.5 mM - 5 mM APAP before 5 dpf reported here is in line with previous findings (North et al., 2010). However, to our knowledge, exposure of larvae to APAP after full development of the liver is performed for the first time in this study. Interestingly, 5 mM APAP did not induce liver size change in larvae older than 5 dpf. These findings suggest that the enhanced tolerance of zebrafish towards APAP after completing liver development may be associated with the rapid increase in glutathione levels or more effective functioning of alternative detoxification pathways, such as glucuronidation and sulfation. Supporting this hypothesis, the glutathione-*S*-transferase levels of zebrafish larvae increase gradually between 3-5 dpf, followed by a dramatic increase after 5 dpf (Tierbach et al., 2018).

The second tested drug, INH (most-DILI) was chosen as a model drug for iDILI in this study. Previous studies reported that INH induces apoptosis and a liver size reduction in early larvae (Higuchi et al., 2021; Y. Zhang et al., 2019). Interestingly, we showed that the liver size reduction is only observed at a dose when the overall larval size is also reduced, hence it is likely not liver-specific. On the other hand, larvae treated with INH after the liver is functional (5-7 dpf) had hepatomegaly without any apparent toxicity in the rest of the larval body. Hepatomegaly is a known clinical manifestation of liver injury induced by INH administration (Nanton et al., 2004; Shah et al., 2016; Wolf & Lavine, 2000). To our knowledge, this is the first study demonstrating INH induced hepatomegaly in the zebrafish model. Our findings indicate that the utilization of zebrafish larvae older than 5 dpf may represent a preferable approach for modeling INH-induced DILI effects in zebrafish. The hepatotoxicity of INH is suggested to be either due to the bioactivation of the acetyl hydrazine metabolite generated during the metabolism of INH or due to the protein adducts formed by INH, which in turn trigger an immune response (Metushi et al., 2016). Hepatomegaly, liver enlargement, is known to involve five underlying mechanisms: inflammation, inappropriate storage, infiltration, vascular congestion, and biliary obstruction (Wolf & Lavine, 2000). Since all these functions are active only after 5 dpf in zebrafish, the observation of liver enlargement is expected only at stages older than 5 dpf in agreement with our results.

The third test drug CHB is a less-DILI agent, which was not previously studied in the zebrafish liver toxicity model. A consistent trend of hepatomegaly was observed at all tested concentrations of CHB during the 2-5 dpf period. In contrast, when applied after 5 dpf, CHB exposure resulted in liver damage and a reduction in liver size. Notably, hepatomegaly is rarely reported in patients. Therefore, examination of CHB effects in both stages reflected possible clinical manifestations in the fish larval model. The 5-7 dpf larval stage proved to be better for studying CHB-induced hepatotoxicity. Hepatotoxicity caused by CHB is not very common, and the mechanisms of the toxicity are not well described. The toxicity detected in the zebrafish DILI model reported here supports the sensitivity of the test model.

The fourth test drug CHLP is a non-DILI agent that was selected as a negative control. As expected, CHLP did not affect the liver size in larvae of 2-5 dpf or 5-7 dpf. Our findings further demonstrate the suitability of CHLP as a negative control in the liver size-based toxicity screening studies conducted on the zebrafish larvae.

In the present study, we revealed that DILI potentials of drugs significantly change depending on the stage of treatment in zebrafish (Figure 5). The toxic effects of APAP were found to be more prevalent in the developing liver, whereas the functional liver was more tolerant to APAP. On the other hand, the functional larval liver (between 5-7 dpf) was affected more by CHB treatment. Interestingly, iDILI agent INH-induced hepatomegaly was reproduced only in the larvae older than 5 dpf. On the other hand, non-DILI CHLP did not induce liver size change in either stage.

Conclusion

We found that the developmental stage of the zebrafish is critical for reproducing the clinical hepatotoxicity effects of drugs. Although zebrafish up to 5 dpf has been almost exclusively used for DILI testing, this study is the first to show that maturity status of larval liver is a critical factor affecting the test outcome. Researchers are advised not to overlook the importance of the zebrafish stage in drug-induced liver injury studies. This difference is likely to be due to difference in expression levels of drug detoxification enzymes in tested stages. Testing of a wider collection of drugs in both developmental stages, together with metabolite and gene expression profiling may provide a better understanding of the underlying mechanisms.

Ethical Statement

Experimental procedure was approved by IBG Local Ethics Committe with 2021-005 protocol number.

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Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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References

Akdogan, Y., Sozer, S. C., Akyol, C., Basol, M., Karakoyun, C., & Cakan-Akdogan, G. (2022). Synthesis of albumin nanoparticles in a water-miscible ionic liquid system, and their applications for chlorambucil delivery to cancer cells. Journal of Molecular Liquids, 367, 120575. https://doi.org/10.1016/-j.molliq.2022.120575

Ali, S., Champagne, D. L., & Richardson, M. K. (2012). Behavioral profiling of zebrafish embryos exposed to a panel of 60 water-soluble compounds. Behavioural Brain Research, 228(2), 272–283. https://doi.org//0.1016//j.bbr.2011.11.020

Almazroo, O. A., Miah, M. K., & Venkataramanan, R. (2017). Drug metabolism in the liver. *Clinics in liver disease*, *21*(1), 1-20. https://doi.org/10.1016/j.cld.2016.08.001

Andrade, R. J., Chalasani, N., Björnsson, E. S., Suzuki, A., Kullak-Ublick, G. A., Watkins, P. B., Devarbhavi, H., Merz, M., Lucena, M. I., & Kaplowitz, N. (2019). Drug-induced liver injury. Nature Reviews Disease Primers, 5(1), 58. https://doi.org/10.1038/s41572-019-0105-0

Andrade, R. J., & Robles-Díaz, M. (2020). Diagnostic and prognostic assessment of suspected drug-induced liver injury in clinical practice. Liver International, 40(1), 6–17. https://doi.org/10.1111/liv.14271

Cassar, S., Adatto, I., Freeman, J. L., Gamse, J. T., Iturria, I., Lawrence, C., Muriana, A., Peterson, R. T., Van Cruchten, S., & Zon, L. I. (2019). Use of zebrafish in drug discovery toxicology. Chemical Research in Toxicology, 33(1), 95–118. https://doi.org/10.1021/acs.chemrestox.9b00335

Chan, R., & Benet, L. Z. (2017). Evaluation of DILI predictive hypotheses in early drug development. Chemical Research in Toxicology, 30(4), 1017–1029. https://doi.org/10.1021/acs.chemrestox.7b00025

Chen, M., Vijay, V., Shi, Q., Liu, Z., Fang, H., & Tong, W. (2011). FDA-approved drug labeling for the study of drug-induced liver injury. Drug Discovery Today, 16(15–16), 697–703. https://doi.org/10.1016/j.drudis.2011.05.007

Choi, T., Ninov, N., Stainier, D. Y. R., & Shin, D. (2014). Extensive conversion of hepatic biliary epithelial cells to hepatocytes after near total loss of hepatocytes in zebrafish. Gastroenterology, 146(3), 776–788. https://doi.org/10.1053/j.gastro.2013.10.019

Chu, J., & Sadler, K. C. (2009). New school in liver development: lessons from zebrafish. Hepatology, 50(5), 1656–1663. https://doi.org/10.1002/hep.23157

Cornet, C., Calzolari, S., Miñana-Prieto, R., Dyballa, S., Van Doornmalen, E., Rutjes, H., Savy, T., D'Amico, D., & Terriente, J. (2017). ZeGlobalTox: an innovative approach to address organ drug toxicity using zebrafish. International Journal of Molecular Sciences, 18(4), 864. https://doi.org/10.3390/ijms18040864

Feder Jr, H. M. (1986). Chloramphenicol: what we have learned in the last decade. Southern Medical Journal, 79(9), 1129–1134.

Garcia-Cortes, M., Robles-Diaz, M., Stephens, C., Ortega-Alonso, A., Lucena, M. I., & Andrade, R. J. (2020). Drug induced liver injury: an update. Archives of Toxicology, 94, 3381–3407. https://doi.org/10.1007/s00204-020-02885-1

Goldstone, J. V, McArthur, A. G., Kubota, A., Zanette, J., Parente, T., Jönsson, M. E., Nelson, D. R., & Stegeman, J. J. (2010). Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC Genomics, 11(1), 1–21. https://doi.org/10.1186/1471-2164-11-643

Guo, Q., Yang, W., Xiao, B., Zhang, H., Lei, X., Ou, H., Qin, R., & Jin, R. (2015). Study on early biomarkers of zebrafish liver injury induced by acetaminophen. Toxin Reviews, 34(1), 28–36. https://doi.org/10.3109/15569543.2014.986282

Higuchi, A., Wakai, E., Tada, T., Koiwa, J., Adachi, Y., Shiromizu, T., Goto, H., Tanaka, T., & Nishimura, Y. (2021). Generation of a Transgenic Zebrafish Line for In Vivo Assessment of Hepatic Apoptosis. Pharmaceuticals, 14(11), 1117. https://doi.org/10.3390/ph14111117

Iruzubieta, P., Arias-Loste, M. T., Barbier-Torres, L., Martinez-Chantar, M. L., & Crespo, J. (2015). The need for biomarkers in diagnosis and prognosis of drug-induced liver disease: does metabolomics have any role? BioMed Research International, 2015. https://doi.org/10.1155/2015/386186

Jagtap, U., Basu, S., Lokhande, L., Bharti, N., & Sachidanandan, C. (2022). BML-257, a Small Molecule that Protects against Drug-Induced Liver Injury in Zebrafish. Chemical Research in Toxicology, 35(8), 1393–1399. https://doi.org/10.1021/acs.chemrestox.2c00100

Katoch, S., & Patial, V. (2021). Zebrafish: An emerging model system to study liver diseases and related drug discovery. Journal of Applied Toxicology, 41(1), 33–51. https://doi.org/10.1002/jat.4031

Klotz, U. (2012). Paracetamol (acetaminophen)–a popular and widely used nonopioid analgesic. Arzneimittelforschung, 62(08), 355–359. https://doi.org/10.1055/s-0032-1321785

Kyle, R. A., Greipp, P. R., Gertz, M. A., Witzig, T. E., Lust, J. A., Lacy, M. Q., & Therneau, T. M. (2000). Waldenström's macroglobulinaemia: a prospective study comparing daily with intermittent oral chlorambucil. British Journal of Haematology, 108(4), 737–742. https://doi.org/10.1046/j.1365-2141.2000.01918.x

Lecaudey, V., Cakan-Akdogan, G., Norton, W. H. J., & Gilmour, D. (2008). Dynamic Fgf signaling couples morphogenesis and migration in the zebrafish lateral line primordium. https://doi.org/10.1242/dev.025981

Lin, H.-S., Huang, Y.-L., Wang, Y.-R. S., Hsiao, E., Hsu, T.-A., Shiao, H.-Y., Jiaang, W.-T., Sampurna, B. P., Lin, K.-H., &

Wu, M.-S. (2019). Identification of novel anti-liver cancer small molecules with better therapeutic index than sorafenib via zebrafish drug screening platform. Cancers, 11(6). https://doi.org/739. 10.3390/cancers11060739

Metushi, I., Uetrecht, J., & Phillips, E. (2016). Mechanism of isoniazid-induced hepatotoxicity: then and now. British Journal of Clinical Pharmacology, 81(6), 1030–1036. https://doi.org/10.1111/bcp.12885

Nakayama, J., Tan, L., Li, Y., Goh, B. C., Wang, S., Makinoshima, H., & Gong, Z. (2021). A zebrafish embryo screen utilizing gastrulation identifies the HTR2C inhibitor pizotifen as a suppressor of EMT-mediated metastasis. Elife, 10, e70151. https://doi.org/10.7554/eLife.70151

Nanton, S. E., Bu-Ghanim, M., Ponnambalam, A., Nathan, R., & Fisher, S. E. (2004). Isoniazid induced fulminant hepatic failure in a teenager: 971. Official Journal of the American College of Gastroenterology| ACG, 99, S319.

Nawaji, T., Yamashita, N., Umeda, H., Zhang, S., Mizoguchi, N., Seki, M., Kitazawa, T., & Teraoka, H. (2020). Cytochrome P450 expression and chemical metabolic activity before full liver development in zebrafish. Pharmaceuticals, 13(12), 456. https://doi.org/10.3390/ph13120456

Nguyen, X.-B., Kislyuk, S., Pham, D.-H., Kecskés, A., Maes, J., Cabooter, D., Annaert, P., De Witte, P., & Ny, A. (2017). Cell imaging counting as a novel ex vivo approach for investigating drug-induced hepatotoxicity in zebrafish larvae. International Journal of Molecular Sciences, 18(2), 356. https://doi.org/10.3390/ijms18020356

North, T. E., Babu, I. R., Vedder, L. M., Lord, A. M., Wishnok, J. S., Tannenbaum, S. R., Zon, L. I., & Goessling, W. (2010). PGE2-regulated wnt signaling and N-acetylcysteine are synergistically hepatoprotective in zebrafish acetaminophen injury. Proceedings of the National Academy of Sciences, 107(40), 17315–17320. https://doi.org/10.1073/pnas.1008209107

Park, Y. M., Dahlem, C., Meyer, M. R., Kiemer, A. K., Müller, R., & Herrmann, J. (2022). Induction of Liver Size Reduction in Zebrafish Larvae by the Emerging Synthetic Cannabinoid 4F-MDMB-BINACA and Its Impact on Drug Metabolism. Molecules, 27(4), 1290. https://doi.org/10.3390/molecules27041290

Patel, S. P. (2000). First report of liver failure: case report. Reactions, 818, 9. https://doi.org/10.2165/00128415-200008180-00016

Patel, S. P., Nast, C. C., & Adler, S. G. (2000). Chlorambucil-induced acute hepatic failure in a patient with membranous nephropathy. American Journal of Kidney Diseases, 36(2), 401–404. https://doi.org/10.1053/ajkd.2000.8995

Robles-Díaz, M., Medina-Caliz, I., Stephens, C., Andrade, R. J., & Lucena, M. I. (2016). Biomarkers in DILI: one more step forward. Frontiers in Pharmacology, 7, 267. https://doi.org/10.3389/fphar.2016.00267

Sato, Y., Dong, W., Nakamura, T., Mizoguchi, N., Nawaji, T., Nishikawa, M., Onaga, T., Ikushiro, S., Kobayashi, M., & Teraoka, H. (2023). Transgenic Zebrafish Expressing Rat Cytochrome P450 2E1 (CYP2E1): Augmentation of Acetaminophen-Induced Toxicity in the Liver and Retina. International Journal of Molecular Sciences, 24(4), 4013. https://doi.org/10.3390/ijms24044013

Shah, R., Ankale, P., Sinha, K., Iyer, A., & Jayalakshmi, T. K. (2016). Isoniazid induced lupus presenting as oral mucosal ulcers with pancytopenia. Journal of Clinical and Diagnostic Research: JCDR, 10(10), OD03. https://doi.org/10.7860/JCDR/2016/22543.8629

Sienkiewicz, P., Bielawski, K., Bielawska, A., & Pałka, J. (2005). Inhibition of collagen and DNA biosynthesis by a novel amidine analogue of chlorambucil is accompanied by deregulation of β1-integrin and IGF-I receptor signaling in MDA-MB 231 cells. Environmental Toxicology and Pharmacology, 20(1), 118–124. https://doi.org/10.1016/j.etap.2004.11.001

Singhal, K. K., Mukim, M. D., Dubey, C. K., & Nagar, J. C. (2020). An Updated Review on Pharmacology and Toxicities Related to Chloramphenicol. Asian Journal of Pharmaceutical Research and Development, 8(4), 104–109. https://doi.org/10.22270/ajprd.v8i4.671

Skoutelis, A. (2000). Chlorambucil/methylprednisolone. Reactions, 811, 22. https://doi.org/10.2165/00128415-200008110-00011

Tierbach, A., Groh, K. J., Schönenberger, R., Schirmer, K., & Suter, M. J.-F. (2018). Glutathione S-transferase protein expression in different life stages of zebrafish (Danio rerio). Toxicological Sciences, 162(2), 702–712. https://doi.org/10.1093/toxsci/kfx293

Vliegenthart, A. D. B., Tucker, C. S., Del Pozo, J., & Dear, J. W. (2014). Zebrafish as model organisms for studying drug-induced liver injury. British Journal of Clinical Pharmacology, 78(6), 1217–1227. https://doi.org/10.1111/bcp.12408

Wolf, A. D., & Lavine, J. E. (2000). Hepatomegaly in neonates and children. Pediatrics in Review, 21(9), 303–310. https://doi.org/10.1542/pir.21-9-303

Zhang, X., Li, C., & Gong, Z. (2014). Development of a convenient in vivo hepatotoxin assay using a transgenic zebrafish line with liver-specific DsRed expression. PloS One, 9(3), e91874. https://doi.org/10.1371/journal.pone.0091874

Zhang, Y., Cen, J., Jia, Z., Hsiao, C.-D., Xia, Q., Wang, X., Chen, X., Wang, R., Jiang, Z., & Zhang, L. (2019). Hepatotoxicity induced by isoniazid-lipopolysaccharide through endoplasmic reticulum stress, autophagy, and apoptosis pathways in zebrafish. Antimicrobial Agents and Chemotherapy, 63(5), e01639-18. https://doi.org/10.1128/aac.01639-18

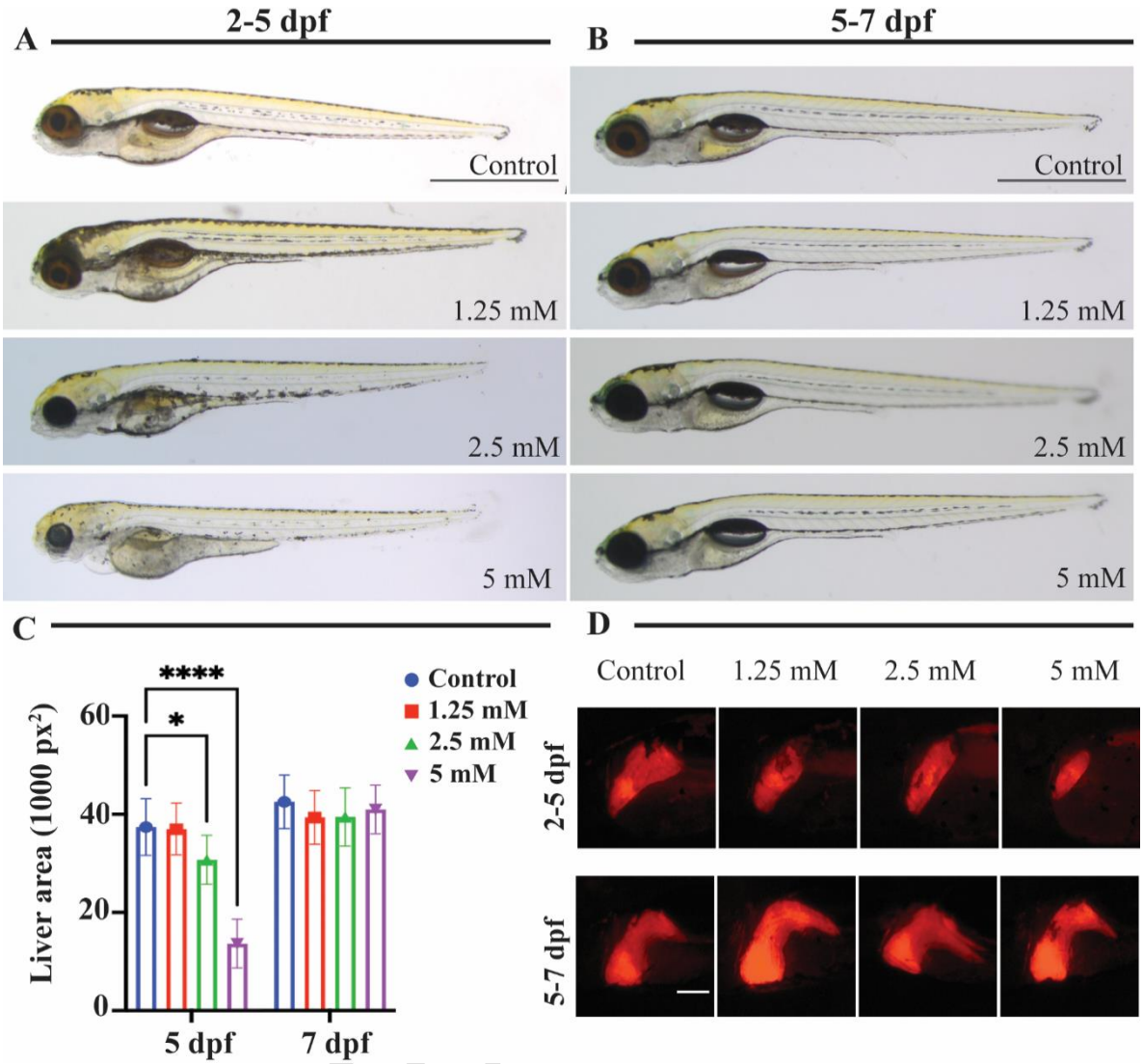

Figure 1. Morphology and liver size of acetaminophen (APAP) treated larvae. Whole body lateral images of controls and APAP treated larvae at the end of treatment applied between **A)** 2-5 dpf, **B)** 5-7 dpf. **C)** Average liver area ± std measured at the end of treatment, n=10 for each group. **D)** Representative liver images of *fabp10a:mCherry* transgenic fish. Scale bars **A, B)** 1 mm, **D)** 200 µm.

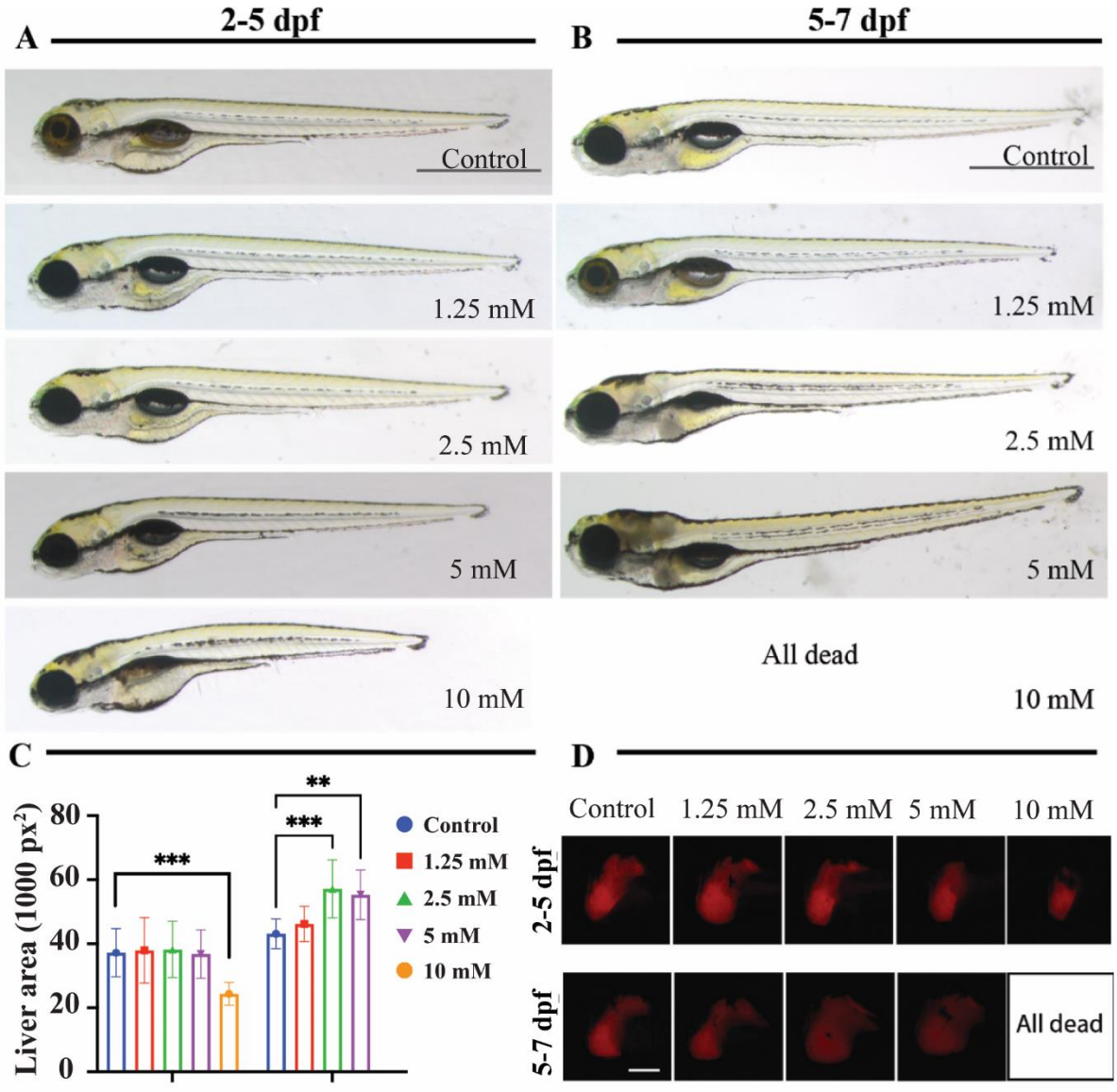

Figure 2. Morphology and liver size of isoniazid (INH) treated larvae. Whole body lateral images of controls and INH treated larvae at the end of treatment applied between **A)** 2-5 dpf, **B)** 5-7 dpf. **C)** Average liver area ± std measured at the end of treatment, n=10 for each group. **D)** Representative liver images of *fabp10a:mCherry* transgenic fish. Scale bars **A, B)** 1 mm, **D)** 200 µm.

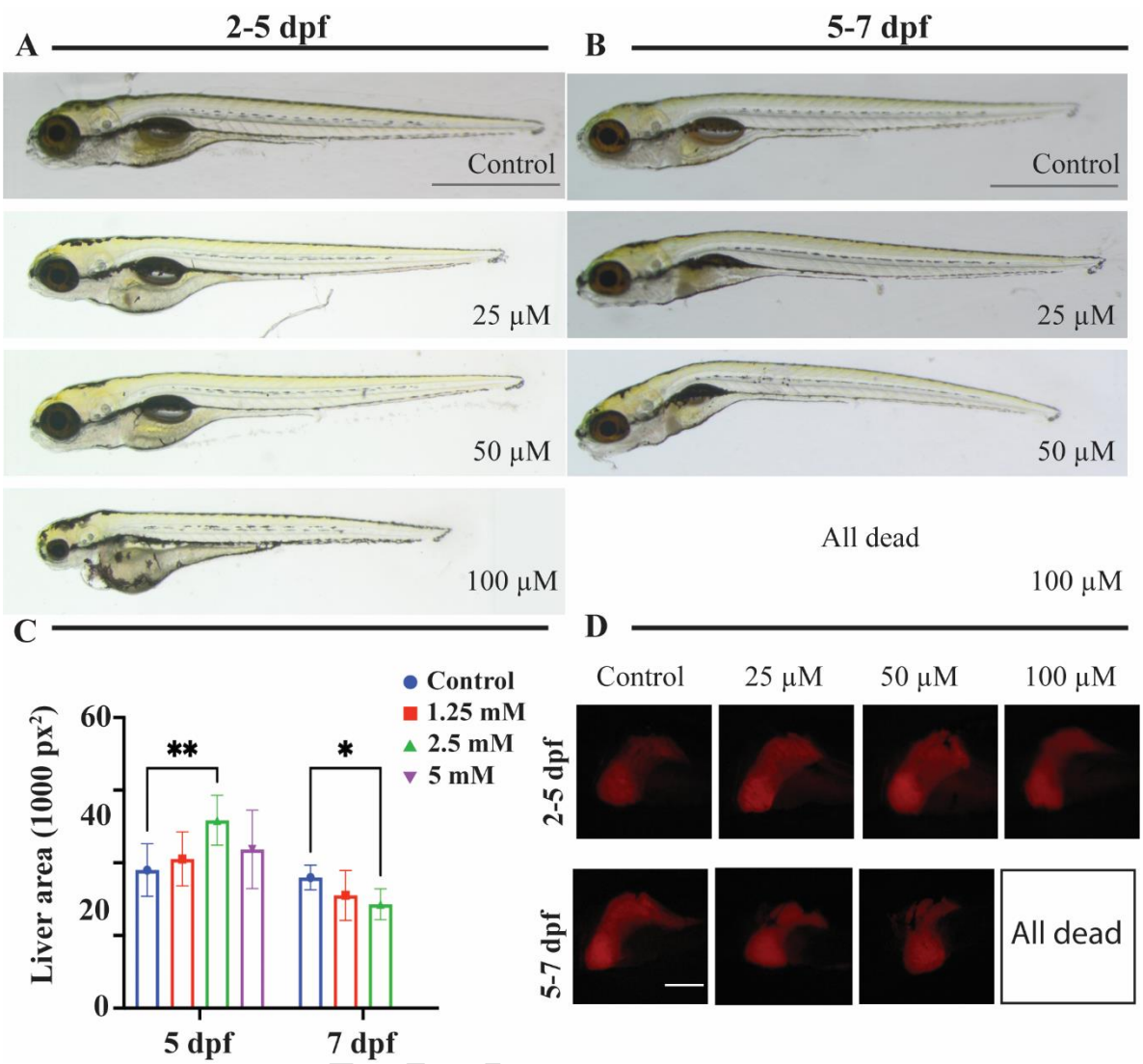

Figure 3. Morphology and liver size of chlorambucil (CHB) treated larvae. Whole body lateral images of controls and CHB treated larvae at the end of treatment applied between **A)** 2-5 dpf, **B)** 5-7 dpf. **C)** Average liver area ± std measured at the end of treatment, n=10 for each group. **D)** Representative liver images of *fabp10a:mCherry* transgenic fish. Scale bars **A, B)** 1 mm, **D)** 200 µm.

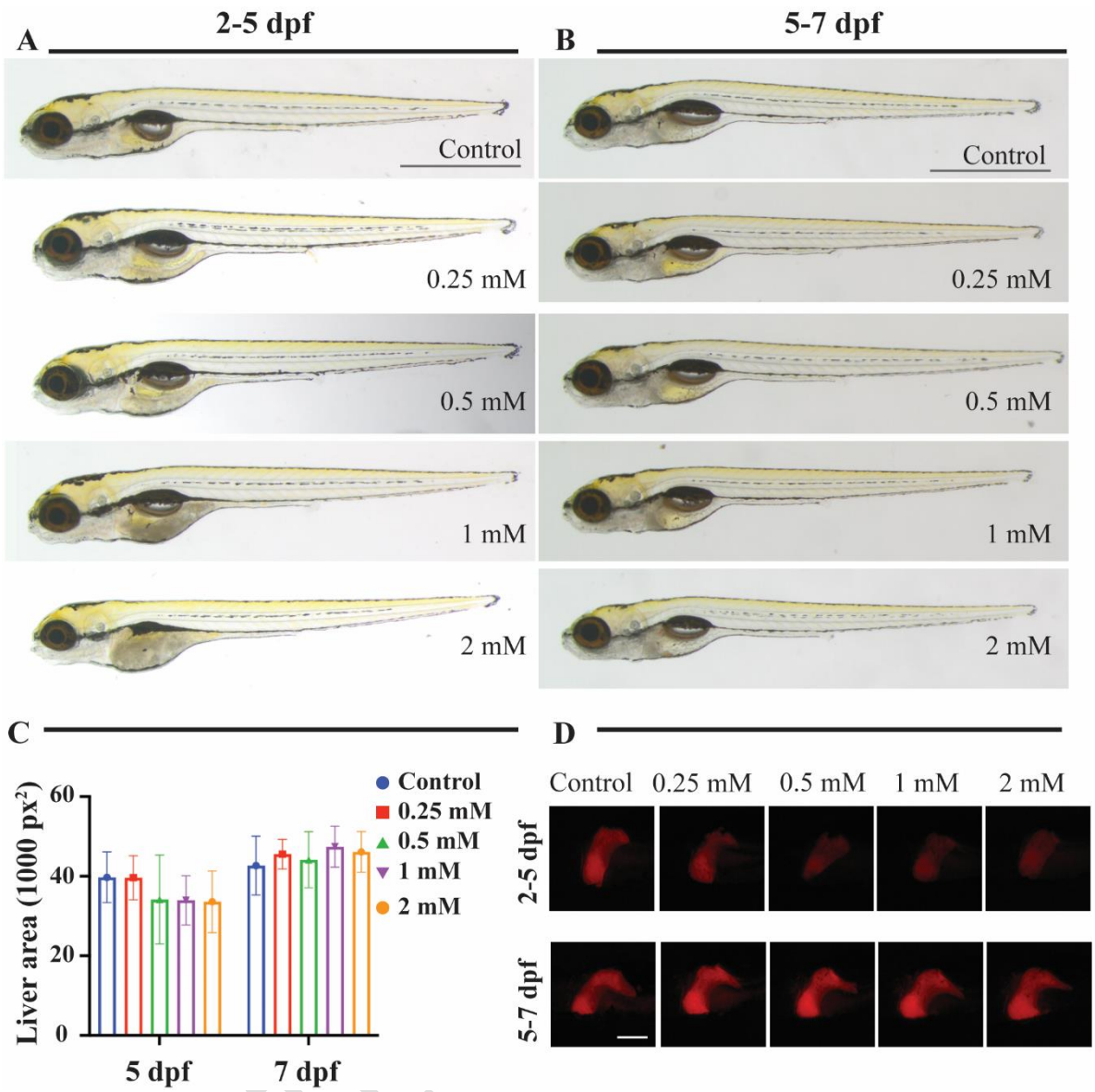

Figure 4. Morphology and liver size of chloramphenicol (CHLP) treated larvae. Whole body lateral images of controls and CHLP treated larvae at the end of treatment applied between **A)** 2-5 dpf, **B)** 5-7 dpf. **C)** Average liver area ± std measured at the end of treatment, n=10 for each group. **D)** Representative liver images of *fabp10a:mCherry* transgenic fish. Scale bars **A, B)** 1 mm, **D)** 200 µm.

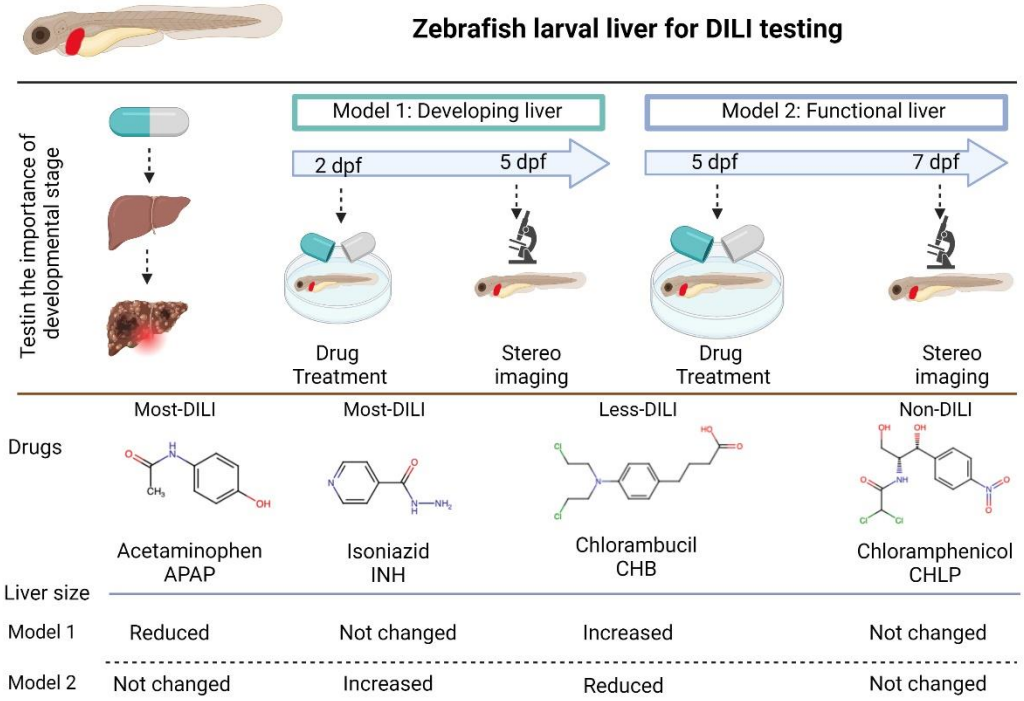

Figure 5. Graphical representation of study design and findings.