In vivo assessment of drug-induced hepatotoxicity using *Xenopus* embryos

Katy Saide and Grant N. Wheeler

School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK

Correspondence: email grant.wheeler@uea.ac.uk

ABSTRACT

Failure to predict drug-induced toxicity reactions is a major problem contributing to a high attrition rate and tremendous cost in drug development. Drug screening in *X. laevis* embryos is high-throughput relative to screening in rodents, potentially making them an ideal model organism for this use. *Xenopus* embryos have been used as a toxicity model in the frog teratogenesis assay-*Xenopus* (FETAX assay) for the early stages of drug safety evaluation. We have previously developed compound-screening methods using *Xenopus* embryos and believe they could be used for *in vitro* drug-induced toxicity safety assessment prior to expensive preclinical trials in mammals. Specifically, *Xenopus* embryos could help predict drug-induced hepatotoxicity and consequently aid lead candidate prioritization. Here we present methods, which we have modified for use on *Xenopus* embryos, to help measure the potential for a drug to induce liver toxicity. One such method is the release of the liver-specific micro-RNA (miRNA), miR-122, from the liver into the vasculature as a result of hepatocellular damage, which could be due to drug-induced acute liver injury. Paracetamol, a known hepatotoxin at high doses, was a positive control. We have previously shown that some of the phenotypes of mammalian paracetamol overdose are reflected in *Xenopus* embryos. Consequently, we have also included a method that measures the concentration of free glutathione (GSH), which is an indicator of paracetamol-induced liver injury. These methods can be used as part of a panel of protocols to help predict the hepatoxicity of a drug at an early stage in drug development.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Agarose gel (2% [w/v]; Sigma-Aldrich

Bradford assay kit (BIO-RAD)

GSH (0.1 mM; Sigma)

GSH assay reagent <R>

GSH buffer <R>

GSH reductase (13 U/mL in GSH buffer; Sigma Aldrich)

HCl (10 mM)

Liquid nitrogen (optional; see Step 6)

MEM salts (10X; pH 7.4) <R>

MEMFA fixative for *Xenopus* <R>

miRCURY^{TM} LNA PCR Primer mix for *Xenopus* miR-122 (Exiqon)

miRCURY^{TM} LNA PCR Primer mix for *Xenopus* miR-103 (Exiqon)

MMR (10X; pH 7.5) <R>

Paracetamol and/or drug to be texted (2X final desired concentration)

PBS (P) <R>

PBS; 0.1 % tween-20 (PBST)

RNA isolation and cDNA synthesis kit

RNase free water

5-sulfosalycylic acid hydrate (SSA; 6.5 % (w/v))

SYBR® Green PCR Master Mix (Applied Biosystems)

Tricaine (0.5 mg/mL in 0.1X MMR)

X. laevis embryos (Nieuwkoop and Faber (NF) stage 1; Nieuwkoop and Faber, 1994)

Equipment

Applied Biosystems 7500 Fast Real-Time PCR system

Breathe-Eazy^{R} Sealing membranes (Sigma, UK)

Culture incubator (set to temperature indicated below)

Culture plate (96 or 24-well; non-cell culture grade; Fisher Scientific)

Dumont #5 forceps (stainless steel; Sigma-Aldrich)

Ultrafine for careful manipulation of embryos

Eppendorf pestle

Freezer (-20 ^{o}C)

Glass vials with screw caps (3.5 ml; SGL)

Light microscope with charge coupled-device (CCD) digital camera for whole-mount imaging of embryos

Long-handled scalpel (10A blades)

MicroAmp optical 96 well plate (Applied Biosystems)

Microcentrifuge

Microcentrifuge tubes

Parafilm M^{TM} wrapping film (Fisher Scientific)

Pasteur pipette

To use plastic Pasteur pipettes, cut the end off with scissors. For glass pipettes, mark the end with a diamond pen, break off cleanly and fire the end briefly to melt any sharp edges.

Petri dish (3 cm^{2} and 10 cm^{2}; Fisher Scientific)

Razor

Spectrophotometer such as GloMax^{R} Explorer System (Promega)

Stereomicroscope with two-armed fiber optic illuminator to allow easy adjustment of the angle of illumination

Vortex

METHODS

Before examining hepatoxicity, the overall toxicity of a small molecule or compound needs to be determined, including the maximum dose that embryos can be exposed to before they all consistently die (Saide et al., 2019). This will determine a range of concentrations to use to test for hepatoxicity below the maximum dose.

Assays for liver toxicity

These protocols were developed using paracetamol, which is associated with acute liver injury in humans after overdose administration, as a model drug. This drug can be used as a positive control in these methods and for comparisons with drugs/compounds being tested.

1. Harvest NF stage 1 *X. laevis* embryos as in Al-Yousuf et al., 2017 and incubate in 0.1X MMR in petri dishes between 13–23^{o}C until stage 38. Regularly observe the embryos (at least twice daily or more at early stages) to remove any dead embryos and ensure the correct NF stage has been reached. It should take roughly 4 days to reach stage 38.
2. In a 96-well plate, add 125 μL of a 2X final concentration of paracetamol or the drug to be tested dissolved in 0.1X MMR. For the negative control add 125 μL 0.1X MMR to the well. Add 1 stage 38 embryo (Nieuwkoop and Faber; 1994) in 125 μL 0.1X MMR to each well using a cutoff pipette tip. The final volume of each well is 250 μL.

   When testing paracetamol, a range of 0 – 5 mM generated a paracetamol-induced liver injury phenotype that was similar to that observed in other animal models and humans (Saide et al., 2019; Verstraelen et al., 2016; Vliegenthart et al., 2014).

3. For 1 biological replicate (n=1), use up to 10 embryos per drug concentration. Consequently 5 biological replicates (n=5) use a total of 50 embryos.

1 biological replicate is defined as the embryos from one female.

4. Seal plates with Breathe-Eazy^{R} sealing membranes to prevent evaporation, but allow exchange of gases.

5. Incubate embryos from stage 38 to stage 45 at 23^{o}C. This takes approximately 2-3 days.

Stage 38 corresponds to late organogenesis when the heart, vascular system, and other organs such as the liver are well developed. Between stages 38-45 the mouth opens and the gills and gut start to function.

6. Proceed to Step 7 to measure differences in gene expression due to drug treatment, or Step 14 to measure changes in free glutathione due to drug treatment.

Expression of miR-122 in different tissues of embryos

7. Following drug treatment until stage 45, place the embryos into a clean petri dish of tricaine (0.5 mg/mL in 0.1X MMR) and incubate at 23°C for 1 h. Transfer to a petri dish coated with 2% agarose and dissect the embryos into tail and gut tissue using a simple razor as shown in Fig 1.

Following this step, samples can be stored at -80^{o}C. Place the samples in a 1.5-mL microfuge tube, remove all fluid, and snap freeze in liquid nitrogen for at least 2 min. We usually freeze 10 embryos per 1.5mL microfuge tube.

The gut section contains the liver. The tail section does not contain any part of the liver and so liver markers observed are assumed to be in the extensive vasculature of the tail.

8. Pool the guts and tails separately for 10 embryos. Using a commercial kit, isolate the RNA and synthesize cDNA. We routinely use around 10 ng of RNA for cDNA synthesis.

qRT-PCR

9. Dilute the cDNA 1:80 in RNAse-free water.

We empirically determined that this was the optimum dilution factor.

10. Prepare reactions in a MicroAmp optical 96 well plate. Add 5 µL of the diluted cDNA, 0.5 µL of miRCURY^{TM} LNA PCR Primer mix for *Xenopus* miR-122 (Exiqon), 7.5 µL of 2x SYBR® Green PCR Master Mix (Applied Biosystems) and 2 µL of RNase free water for a final volume of 15 µL.

11. Carry out PCR using an Applied Biosystems 7500 Fast Real-Time PCR system under the following conditions: 95°C for 10 min, 40 cycles at 95°C for 10 s and 60°C for 1 min.

We used miR-103 as a quantitative control for miR-122 expression of treated embryos using 0.5 µL of miRCURY^{TM} LNA PCR Primer mix for *Xenopus* miR-103 (Exiqon)

Samples derived from embryos produced from different mothers are biological replicates. We test biological replicates were three times (technical replicates).

qRT-PCR statistical analysis

12. Analyze gene expression using the Livak method (Livak and Schmittgen, 2001). Take an average Ct value from the technical replicates and normalize that value to the Ct value for miR-103 expression and also to the untreated samples (embryos that received no drug treatment).

13. Determine the fold change in gene expression using the formula: $2^{-\Delta\Delta CT}$ and convert this number into a logarithmic ($\log_{10}$). Perform Mann-Whitney tests

between gut and tail tissues from embryos treated with the same concentration of drug to determine statistical significance.

Measuring free glutathione (GSH)

Many compounds are metabolized in the liver to facilitate their excretion. In many cases including paracetamol this involves the metabolic addition of glutathione to a molecule. Here we provide a method to measure the depletion of free GSH in response to a drug.

14. At the end of the incubation period described in Step 5, transfer 10 embryos that were treated with the same conditions into 1 microcentrifuge tube. Place the tube on ice.
15. Remove as much of the incubation solution as possible. Add 125 μL of 10 mM HCl.
16. Homogenize embryos using an Eppendorf pestle and vortexing.
17. Centrifuge at 14000*g* for 5 min at 4^{o}C.
18. Transfer 25 μL of the supernatant to another tube and store at -80^{o}C for protein quantification using a Bradford assay. Replace the 25 μL with 25 μL of 6.5 % (w/v) 5-sulfosalycylic acid hydrate (SSA).
19. Remove all of the supernatant including the SSA and flash freeze in liquid Nitrogen. Store at -80^{o}C until ready to perform the GSH assay.

GSH Assay

20. Prepare the following standard GSH concentrations: 0, 1, 2, 5, 10, 20, 30 and 40 nmol/mL using GSH buffer and 0.1 mM GSH. Keep on ice.
21. Mix 20 μL of the standard or sample with 200 μL GSH assay reagent in 1 well of a 96-well plate and incubate for 5 min at room temperature.

22. To each well add 50 μL of 13 U/mL GSH reductase and gently shake before placing on the plate reader.

23. Immediately measure the absorption of the plate at 405 nm using a spectrophotometer.

24. Take 11 readings at 15s intervals.

25. Use the reading with the best standard curve to calculate the sample results (nmol/mL).

Bradford assay

26. Prepare standard concentrations of BSA dissolved in 10 mM HCl: 0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2 and 4 mg/mL.

27. Perform the assay according to the manufacturer's instructions as follows.

28. Add 5 µL of each sample from Step 18 to 1 well of a 96-well plate with 25 µL of reagent A + S (1 mL reagent A + 20 µL reagent S) and 200 µL of reagent B.

29. Incubate for 15 min at room temperature.

30. Read the plate absorption at 595 nm absorption using a spectrophotometer.

31. Divide the GSH results (nmol/mL) by the Bradford result (mg/mL) for each sample to give the final result (nmol/mg).

DISCUSSION

Predicting drug-induced toxicity is a big problem for Pharma in the 21st century. *X. laevis* embryos could be an ideal model organism to be used for screening new drugs and compounds as screening in *Xenopus* is comparatively high-throughput relative to screening in rodents (Tomlinson et al., 2009). We have previously developed methods to both screen compounds for novel phenotypes and test toxicity (Al-Yousuf et al., 2017; Tomlinson et al., 2012; Tomlinson et al., 2009; Webster et al., 2016; Wheeler

and Liu, 2012, Saide et al., 2019). In addition, *Xenopus* embryos have been used as a toxicity model for drugs in their early stages of drug safety evaluation in the frog teratogenesis assay-*Xenopus* (or FETAX assay) (Leconte and Mouche, 2013).

Xenopus embryos could assist *in vitro* drug-induced toxicity safety assessment in the early phases of drug development before moving on to expensive preclinical trials in mammals (Fig 2). The procedures outlined here can provide an assessment of the potential for a novel compound to cause hepatotoxicity in the tadpole model. Previous work in *Xenopus* suggests this could be translatable to the incidence of toxicity in higher vertebrates such as mice and humans (Saide et al., 2019). Many drugs such as paracetamol are metabolized in the liver and it is the metabolites that are often toxic. Preliminary work has shown that using mass spectroscopy of tadpole tissue to identify drug metabolites, such as those generated from paracetamol, is feasible (G. N. Wheeler, unpublished observation).

The tail/vascular assay described here is also amenable to protein assays such as ELISAs. Known biomarkers that are released into the blood in hepatotoxic situations that could be tested in this way include alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, glutamate dehydrogenase, cytochrome C and keratin 18.

REFERENCES

Al-Yousuf, K., Webster, C.A., Wheeler, G.N., Bombelli, F.B., Sherwood, V., 2017. Combining Cytotoxicity Assessment and Xenopus laevis Phenotypic Abnormality Assay as a Predictor of Nanomaterial Safety. Curr Protoc Toxicol 73, 20 13 21-20 13 33.

Leconte, I., Mouche, I., 2013. Frog embryo teratogenesis assay on Xenopus and predictivity compared with in vivo mammalian studies. Methods Mol Biol 947, 403-421.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Nieuwkoop, P.D., Faber, J., 1994. Normal Table of Xenopus laevis (Daudin). Garland Publishing Inc, New-York.

Saide, K., Sherwood, V., Wheeler, G.N., 2019. Paracetamol-induced liver injury modelled in Xenopus laevis embryos. Toxicol Lett 302, 83-91.

Tomlinson, M.L., Hendry, A.E., Wheeler, G.N., 2012. Chemical genetics and drug discovery in Xenopus. Methods Mol Biol 917, 155-166.

Tomlinson, M.L., Rejzek, M., Fidock, M., Field, R.A., Wheeler, G.N., 2009. Chemical genomics identifies compounds affecting Xenopus laevis pigment cell development. Mol Biosyst 5, 376-384.

Verstraelen, S., Peers, B., Maho, W., Hollanders, K., Remy, S., Berckmans, P., Covaci, A., Witters, H., 2016. Phenotypic and biomarker evaluation of zebrafish larvae as an alternative model to predict mammalian hepatotoxicity. J Appl Toxicol 36, 1194-1206.

Vliegenthart, A.D., Tucker, C.S., Del Pozo, J., Dear, J.W., 2014. Zebrafish as model organisms for studying drug-induced liver injury. Br J Clin Pharmacol 78, 1217-1227.

Webster, C.A., Di Silvio, D., Devarajan, A., Bigini, P., Micotti, E., Giudice, C., Salmona, M., Wheeler, G.N., Sherwood, V., Bombelli, F.B., 2016. An early developmental vertebrate model for nanomaterial safety: bridging cell-based and mammalian toxicity assessment. Nanomedicine (Lond) 11, 643-656.

Wheeler, G.N., Liu, K.J., 2012. Xenopus: an ideal system for chemical genetics. Genesis 50, 207-218.

Figures:

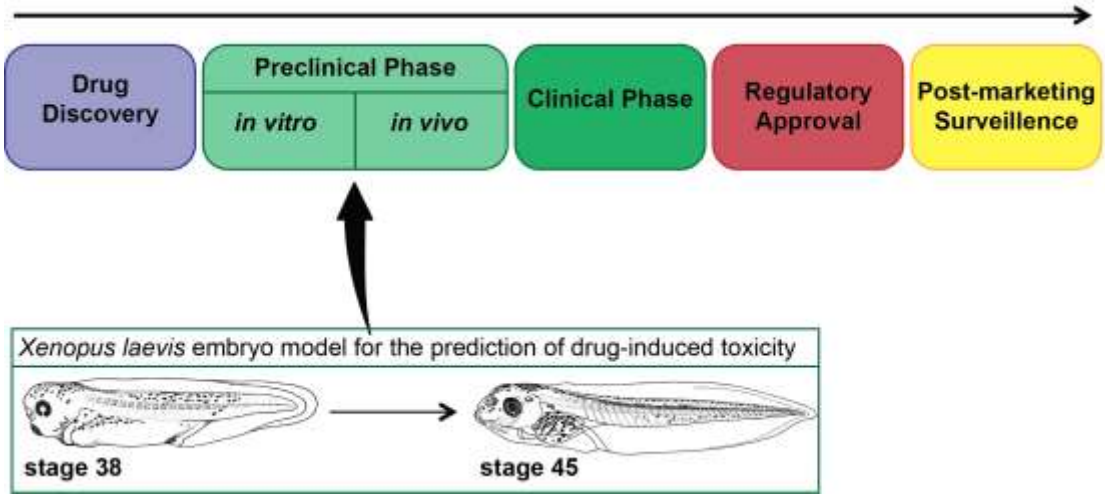

Figure 1: Drug development with Xenopus laevis

The proposed use of *Xenopus laevis* embryos in assessing hepatoxicity would be in the early preclinical phase for the prediction of drug-induced toxicity. *Xenopus* embryos could bridge the gap between *in vitro* and *in vivo* safety studies.

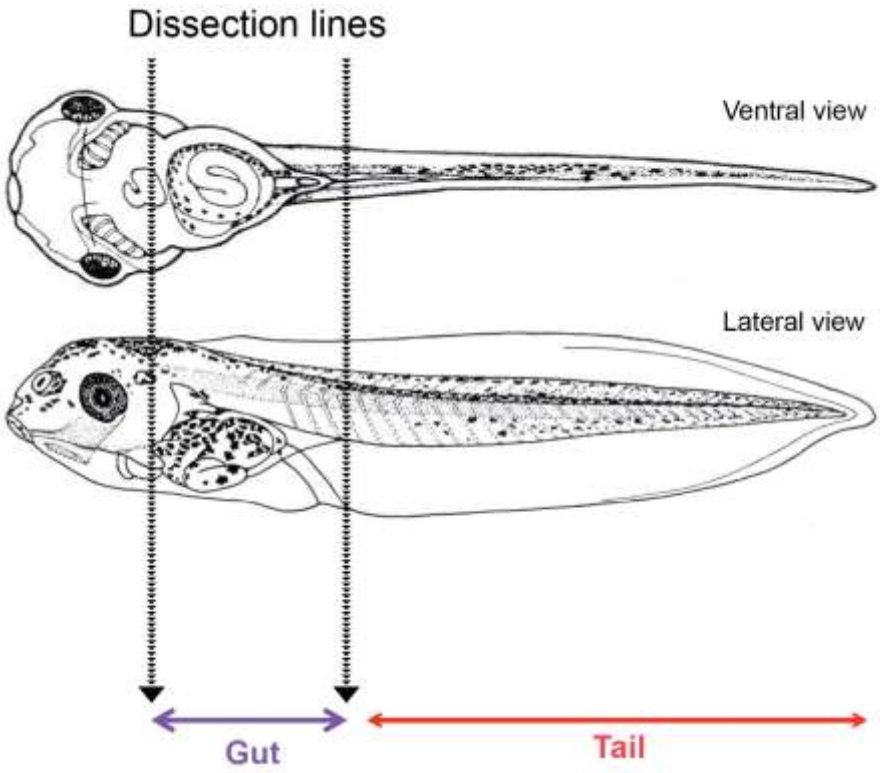

Figure 2: Dissection for qRT-PCR miR-122 detection
After drug exposure, stage 45 *Xenopus* embryos were anaesthetised and dissected along the lines indicated here (black arrows) to obtain gut (purple arrow) and tail (red arrow) regions. These regions are then processed to detect miR-122 expression by qRT-PCR. (Figure taken from Saide et al 2019)