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Investigating the application of a nitroreductase-expressing transgenic zebrafish line for high-throughput toxicity testing

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ABSTRACT

Nitroreductase enzymes are responsible for the reduction of nitro functional groups to amino functional groups, and are found in a range of animal models, zebrafish (*Danio rerio*) excluded. Transgenic zebrafish models have been developed for tissue-specific cell ablation, which use nitroreductase to ablate specific tissues or cell types following exposure to the non-toxic pro-drug metronidazole (MTZ). When metabolized by nitroreductase, MTZ produces a potent cytotoxin, which specifically ablates the tissue in which metabolism occurs. Uses, beyond tissue-specific cell ablation, are possible for the hepatocyte-specific *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish line, including investigations of the role of nitroreductase in the toxicity of nitrated compounds. The hepatic ablation characteristics of this transgenic line were explored, in order to expand its potential uses. Embryos were exposed at 48, 72, or 96 h post fertilization (hpf) to a range of MTZ concentrations, and the ablation profiles were compared. Ablation occurred at a 10-fold lower concentration than previously reported. Embryos were exposed to a selection of other compounds, with and without MTZ, in order to investigate alternative uses for this transgenic line. Test compounds were selected based on: their ability to undergo nitroreduction, known importance of hepatic metabolism to toxicity, and known pharmaceutical hepatotoxins. Selected compounds included nitrated polycyclic aromatic hydrocarbons (nitro-PAHs), the PAHs retene and benzo[a]pyrene, and the pharmaceuticals acetaminophen and flutamide. The results suggest a range of potential roles of the liver in the toxicity of these compounds, and highlight the additional uses of this transgenic model in toxicity testing.

1. Introduction

The zebrafish (*Danio rerio*) is unique among vertebrate model organism systems in that it is amendable to high-throughput developmental toxicity testing [1–3]. Zebrafish are easy to cultivate in a laboratory setting, have a high fecundity, develop externally, and the embryos are transparent during development. Zebrafish are also metabolically competent, in particular following development of the liver between 48 and 72 h post fertilization (hpf) [4,5]. Zebrafish have high genetic homology to humans, with approximately 70% of human genes and about 82% of potential human disease-related genes having at least one zebrafish orthologue [6]. This model is also highly amenable to genetic manipulation, with the addition or removal of genes of interest being relatively easy to achieve, including those involved in metabolism [2]. However, for some chemicals and chemical classes, there may be discordance in toxicity response between other model systems.

One difference in the metabolic capability of zebrafish compared to other model organisms is the enzyme nitroreductase. Nitroreductases are responsible for the reduction of nitro functional groups to amino functional groups. This has been implicated as an important component for the mechanism of toxicity for some compounds, such as nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) [7]. Zebrafish are not believed to have nitroreductase activity. This is supported by the distinctly different toxicological profiles for the corresponding pairs of amino- and nitro- compounds in zebrafish [8]. Some evidence suggests zebrafish may have nitroreductase activity in the yolk [9], and it is possible that zebrafish, like humans and other model organisms, may have nitroreductase activity in the intestine due to the presence of microbiota [10,11].

The lack of nitroreductase activity in zebrafish has been used to develop transgenic lines for tissue-specific cell ablation. Tissue-specific cell ablation has previously been used in other model systems [12,13], with more recent developments in zebrafish as well. One technique

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showing success in the zebrafish model uses the expression of nitroreductase genes controlled by tissue-specific promoters, to create the desired tissue specificity [14–17]. Animals are treated with a non-toxic pro-drug containing a nitro functional group, commonly metronidazole (MTZ), which becomes cytotoxic when reduced by nitroreductase [18]. Models have been developed where nitroreductase is expressed in a range of tissues, including hepatocytes, cardiomyocytes, pancreatic β-islet cells, oocytes and testis [14,15,19–21]. Specificity of the nitroreductase promoter is essential, as well as containment of the cytotoxin, to prevent ablation of other tissues and off-target effects, and the lack of endogenous nitroreductase expression in zebrafish makes them well-suited to this model for tissue ablation.

The use of transgenic zebrafish lines previously developed for nitroreductase-based tissue-specific cell ablation would be ideal for further investigating the role of nitroreductase in toxicity and metabolism. Of the previously developed nitroreductase-expressing transgenic zebrafish lines, those which express *Escherichia coli* nitroreductase using a hepatocyte-specific promoter most closely resembles nitroreductase expression in humans, with nitroreductase expressed in the liver, as humans do. This allows for a whole-animal system with a metabolic capability more similar to humans and mammalian model systems than the standard zebrafish lines.

Aside from the use in tissue-specific cell ablation, a zebrafish line with nitroreductase ability in the liver, and a more human-like metabolic capability, can have other uses as well. The nitroreductase capability makes this transgenic zebrafish a valuable resource in gaining insight on the metabolism of nitro-containing compounds, as well as identifying potential hepatotoxins. One potential use is the toxicity screening of nitrated environmental contaminants, such as nitrated polycyclic aromatic hydrocarbons (nitro-PAHs), for which nitroreduction has been implicated as a component of toxicity [8,22,23]. Previous data in the zebrafish model has indicated that amino-PAHs elicit a greater toxicity response than the corresponding nitro-PAH, with greater incidences of developmental malformations as well as mortality occurring following exposure to 1-aminopyrene and 9-aminophenanthrene, compared to embryos exposed to 1-nitropyrene and 9-nitrophenanthrene, respectively [8]. Zebrafish with this more human-like metabolic capability could also be useful in the investigation of pharmaceuticals and hepatotoxins, as well as investigating the role of the liver and hepatic metabolism in toxicity. Other PAHs, such as benzo[a]pyrene and retene, are known to undergo hepatic metabolism [24–26], and exposure results in developmental malformations, including edemas and craniofacial malformations, in the developing zebrafish [27–29]. Certain pharmaceuticals, including acetaminophen and flutamide, are known to result in human hepatotoxicity [30–32], and can result in developmental toxicity in the zebrafish model [33].

The purpose of this study was to explore the importance of nitroreductase and hepatic metabolism in the developmental toxicity observed in zebrafish. Prior to adapting this line for use in high-throughput assays, further characterization of the *Tg(l-fabp:CFP-NTR)^{s891}* zebrafish line was necessary. Following more thorough line characterization, the role of nitroreductase and hepatic metabolism was investigated for a subset of selected chemicals, for which nitroreduction or hepatic metabolism had been previously implicated as an important component of toxicity.

2. Materials and methods

2.1. Fish care and husbandry

Adult zebrafish were maintained with a water temperature of 28° ± 1 °C on a recirculating system with a 14 h light 10 h dark photoperiod at the Sinnhuber Aquatic Research Laboratory (SARL). All experiments were conducted with wild-type 5D strain or *Tg(l-fabp:CFP-NTR)^{s891}* (background strain TL) [15]. Adult care and reproductive techniques were conducted according to the Institutional Animal Care and Use Committee protocols at Oregon State University (OSU). All 5D embryos used in exposures were collected following group spawning of adult zebrafish as described previously. Embryos from the *Tg(l-fabp:CFP-NTR)^{s891}* transgenic strain were collected following incross or outcross small group spawns [34].

2.2. Chemicals

Analytical-grade metronidazole (MTZ, CAS #443-48-1), acetaminophen (CAS #103-90-2), benzo[a]pyrene (CAS #50-32-8), 1-nitropyrene (CAS #5522-43-0), 1-aminopyrene (CAS #1606-67-3), 9-aminophenanthrene (CAS #947-73-9), and dimethyl sulfoxide (DMSO, CAS #67-68-5) were obtained from Sigma-Aldrich (St. Louis, MO). Analytical-grade 9-nitrophenanthrene (CAS #954-46-1) was obtained from AccuStandard (New Haven, CT). Analytical-grade retene (CAS #483-65-8) was purchased from Santa Cruz Biotechnology (Dallas, TX). Flutamide (CAS #13311-84-7) was provided by the NIEHS National Toxicology Project (NTP). The ROS-ID® Hypoxia/Oxidative Stress Detection Kit was purchased from Enzo Life Sciences (Farmingdale, NY). For each experiment, a fresh solution of MTZ was made in DMSO immediately prior to exposure, and protected from light prior to and during the course of the exposure to prevent photodegradation.

2.3. Basic *Tg(l-fabp:CFP-NTR)^{s891}* embryo exposure

Unless otherwise noted, embryos were exposed in 20 mL amber glass vials in groups of 10–12 animals per treatment, in 10 mL total volume of exposure solution (7 mL for flutamide exposure, with 7–8 embryos). Embryos were added to the vial prior to addition of appropriate chemical treatments. For experiments where exposures started at 6 hpf, embryos were distributed into vials prior to exposure. For experiments where exposures started at 48 hpf or later, embryos were kept in clean petri dishes of embryo media until prior to exposure, at which time embryos displaying normal development were placed into amber glass vials for treatment. During exposure, the amber glass vials containing embryos were rocked at 28 °C. Following exposure, embryos were evaluated for liver presence/ablation and imaged using a Keyence BZ-X700 fluorescence microscope (Keyence North America, Itasca, IL) with a green fluorescent protein (GFP) filter.

2.3.1. Initial characterization

Tg(l-fabp:CFP-NTR)^{s891} embryos (incross and outcross) were distributed into amber glass vials and exposed to 10 mM MTZ at 48, 72, or 96 hpf. Embryos were observed daily following exposure, until 120 hpf.

2.3.2. MTZ dilutions

Embryos were continuously exposed from 96 to 120 hpf to 1 mM to 10 mM MTZ on a 10-fold dilution scale. Based on the results from this experiment, a second group of 96 hpf embryos were exposed to a refined dilution series from 100 to 1000 μM MTZ, and evaluated at 120 hpf.

2.3.3. Liver ablation time course

96 hpf embryos were exposed to 10 mM MTZ, and evaluated at 30 min, every hour from 1 to 8, 12, and 24 h after MTZ exposure.

2.3.4. Ablation recovery time course

96 hpf embryos were exposed to 10 mM MTZ until 120 hpf, at which point the MTZ solution was removed, and the embryos rinsed three times and placed in clean embryo media (EM). Embryos were evaluated for regeneration of the liver 0 min, 30 min, and every hour from 1 to 12, 24, 36, 48, 60, and 72 h after MTZ removal.

2.4. The ROS-ID® Hypoxia/Oxidative Stress Detection Kit

Exposures were conducted based on the manufacturer's suggested

protocols for cells in suspension, with evaluation using fluorescent microscopy. Either wild-type or *Tg(l-fabp:CFP-NTR)*^{s891} embryos were distributed into individual wells of a 96-well plate containing 100 µL EM. At 48, 72, or 96 hpf, 56 µL of the EM was removed, and replaced with 56 µL of either EM, EM containing the hypoxia detection reagent at the suggested concentration (0.5 µM), or EM containing the detection reagent and the provided hypoxia inducer (deferoxamine, DFO) at the suggested concentration (200 µM). The plates were wrapped in foil, and embryos were exposed for 1, 3, 5, or 24 h in the 28 °C incubator. Following exposure, embryos were rinsed three times to remove excess reagent, and imaged using fluorescence microscopy.

2.5. Exposure using 96-well plates

For windows of exposure experiments using the wild-type or *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish line, exposures were performed as previously described [8,35]. Briefly, embryos (dechorionated if wild-type, chorions on for the *Tg(l-fabp:CFP-NTR)*^{s891} line) were added to individual wells of a polystyrene 96-well plate already containing 100 µL of embryo media. Wild-type embryos were dechorionated and loaded in the plates using automation [36], while *Tg(l-fabp:CFP-NTR)*^{s891} embryos were not dechorionated due to low embryo production, and to minimize stress. Chemicals were dispensed into the plates using an HP D300 Digital Dispenser, utilizing previously optimized protocols, at 6, 24, 48, 72, or 96 hpf [35]. Following chemical addition, plates were moved to a temperature-controlled room (28 °C), and were placed on a custom-modified rotating shaker table from 6 to 24 hpf, and in a stationary incubator thereafter [35]. Embryos were evaluated at 24 and 120 hpf for mortality and a suite of developmental malformations [8,37,38].

2.6. Chemical co-exposures

At 48 hpf, *Tg(l-fabp:CFP-NTR)*^{s891} embryos were distributed into amber glass vials containing EM, and were exposed to either 10 mM MTZ in DMSO, or an equal volume of DMSO. At 72 hpf, embryos were exposed to retene (10, 12, 14, 16, 18 µM), benzo[a]pyrene (50, 65, 100 µM), acetaminophen (0.125, 2.5, 5, 10, 25 mM), or flutamide (5, 7, 10, 12 µM), or an equivalent volume of DMSO. Wild-type 5D embryos were also exposed to 5, 7, 10, and 12 µM flutamide in the presence and absence of MTZ. The final volume of DMSO in each vial was 1% for retene and flutamide exposures, and 1.5% for acetaminophen and benzo[a]pyrene exposures. Chemical concentrations for exposures were selected based on preliminary exposure data in the wild-type 5D embryos, for an approximate EC_{50} (data not shown). Vials were then placed horizontally on the rocking table in the incubator, until evaluation via fluorescent imaging at 120 hpf.

3. Results

3.1. Initial characterization

Homozygous adult *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish were either incrossed or outcrossed to 5D zebrafish to produce a uniform population of either homozygous or heterozygous eggs, respectively. Exposures to MTZ at 48, 72, and 96 hpf with either the homozygous or heterozygous embryos yielded the same result in terms of visual ablation of the liver at 120 hpf (Fig. 1). Fluorescence in the liver was substantially reduced or eliminated in embryos exposed to MTZ, regardless of the time of exposure or the genotype of the embryos. For subsequent experiments, heterozygous embryos were used. Additional characterization of the nitroreductase capability of this line was done using the ROS-ID® Hypoxia/Oxidative Stress Detection Kit (see SI 1), which was developed for use in cell culture and had not been previously adapted to be used in zebrafish. This assay showed nitroreductase activity in the neuromasts and head, but not in the liver as was expected (SI 1).

3.2. Liver ablation occurs at lower concentrations of MTZ

Embryos at 96 hpf were exposed to a range of MTZ concentrations, from 1 µM to 10 mM (10,000 µM), and evaluated after 24 h of exposure, at 120 hpf. Embryos exposed to 1, 10, or 100 µM MTZ had little to no tissue ablation, whereas the embryos exposed to 1000 or 10,000 µm MTZ had complete ablation, as indicated by the absence of a fluorescent signal in the liver (Fig. 2a). Low levels of developmental toxicity, primarily jaw malformations and slight truncation of the body axis, were observed in the 10,000 µM exposure animals. Further investigation of MTZ exposure concentrations from 100 to 1000 µM showed partial ablation from 700 to 900 µM, indicated by the decreasing fluorescence in the liver, with complete ablation of fluorescence occurring at 1000 µM (Fig. 2b).

3.3. Visual progression of liver ablation and recovery

Exposures to 10 mM MTZ on 96 hpf embryos were conducted as previously published with a freshly made MTZ solution [14,15], and the embryos evaluated following continuous MTZ exposure. Complete ablation was observed in some fish as early as 5 h post-exposure (hpe), with consistent ablation occurring by 12 h post exposure (Fig. 3a). Recovery of hepatic tissue, indicated by return of the fluorescent signal, in embryos exposed to 10 mM MTZ from 96 to 120 hpf was monitored from 120 to 192 hpf (0–72 hpe). Following removal of the MTZ solution and transfer to fresh embryo media, limited liver regeneration was observed by 10–12 hpe, as indicated by a faint fluorescent signal. Significant recovery of the liver had occurred by 24 hpe, and by 48 hpe the liver appeared to be nearly completely recovered (Fig. 3b).

3.4. Windows of exposure

The 120 hpf toxicity profiles elicited by 1-nitropyrene at 6, 24, 48, 72, and 96 hpf in the wild-type zebrafish embryos were nearly identical (Fig. 4a and SI 2). The primary endpoint observed, circulatory defects marked by pooling of blood in the torso, was observed in a dose-dependent manner for all exposure time points. The malformation profile for 1-aminopyrene (Fig. 4b) in the wild-type embryos was distinct from 1-nitropyrene, and changed with the different exposure windows. *Tg(l-fabp:CFP-NTR)*^{s891} embryos were exposed to 1-nitropyrene at the same concentration range and time points and evaluated at 120 hpf. The observed toxicity profile was similar to that of the wild-type embryos, indicating that metabolism of 1-nitropyrene was not occurring. To confirm that this line's toxicity profiles can be compared to the wild-type animals, *Tg(l-fabp:CFP-NTR)*^{s891} embryos were also exposed to 1-aminopyrene from 6 to 120 hpf. A similar profile to the wild-type embryo response was observed. Likewise, wild-type and *Tg(l-fabp:CFP-NTR)*^{s891} embryos shared similar developmental toxicity profiles in response to 9-nitrophenanthrene, which was distinct from the toxicity observed for 9-aminophenanthrene in the wild-type (Fig. 4c).

3.5. Co-exposure experiments

Structures of all chemicals (retene, benzo[a]pyrene, acetaminophen, and flutamide) selected for co-exposure experiments are shown in Fig. 5a. Exposure to DMSO alone did not result in toxicity to the zebrafish embryos, and the fluorescence in the liver was not impacted following exposure to 1% or 1.5% DMSO (Fig. 5b). The concentration of DMSO selected was based on compound solubility. Embryos exposed to MTZ at 48 hpf were phenotypically normal, except for a slight (not statistically significant) increase in jaw malformations and body truncation in some animals. Complete ablation of the liver was observed in all MTZ-treated animals. For compounds where hepatic

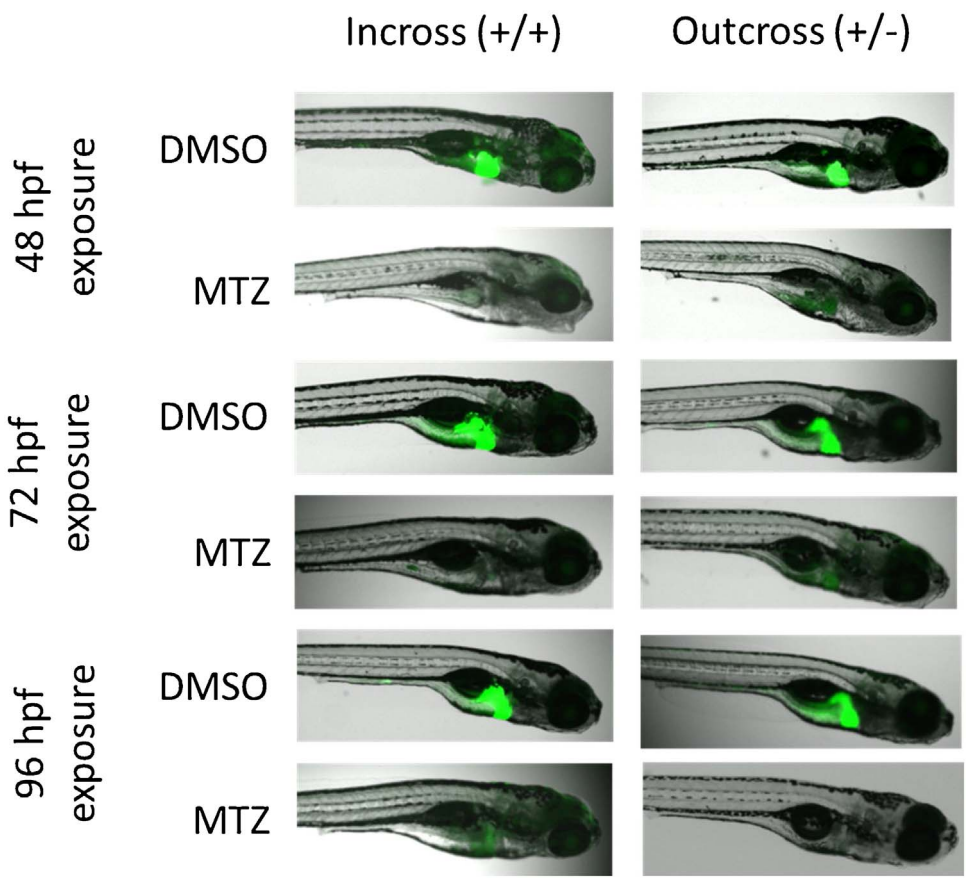

Fig. 1. Comparison of incrossed (homozygous positive) and outcrossed (heterozygous) *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish. Embryos were exposed to either DMSO or 10 mM metronidazole (MTZ) at 48, 72, or 96 hpf until imaging at 120 hpf. Presence of hepatocytes is indicted by green fluorescence.

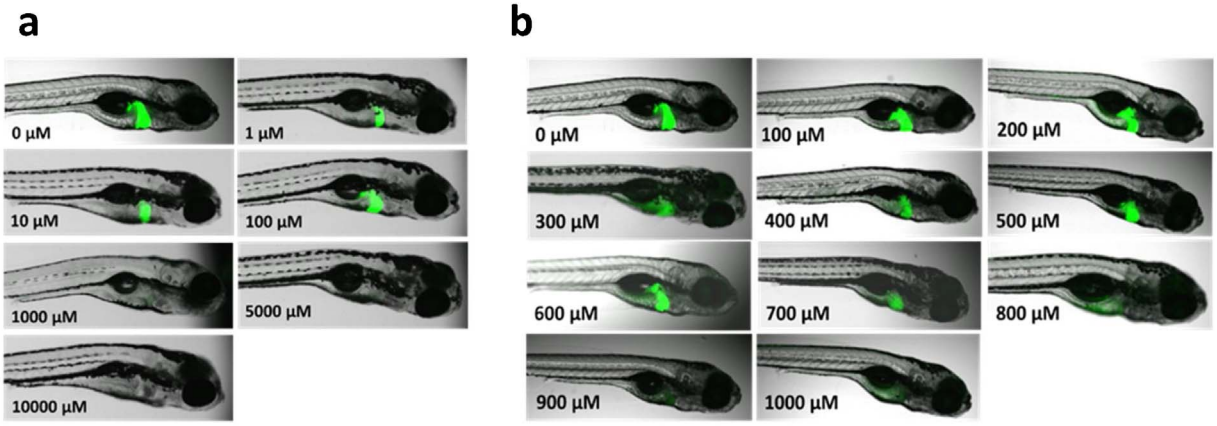

Fig. 2. Dose-response of *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish embryos exposed to metronidazole (MTZ) at 96 hpf, and imaged at 120 hpf, at a broad (a) and refined (b) range of MTZ concentrations. Ablation of hepatocytes was assessed based on visible green fluorescent signal, where a decreasing fluorescent signal indicates hepatocyte ablation.

metabolism is a detoxifying pathway, chemical exposure in the presence of MTZ would result in an increase in observed toxicity. Where hepatic metabolism is a toxicologically activating pathway, co-exposure with MTZ would result in a decrease in observed toxicity.

Retene is known to undergo hepatic metabolism, and therefore elimination of the liver would reduce this metabolic capacity. The toxicity profile of retene would be expected to reflect if hepatic metabolism is primarily a toxifying or detoxifying pathway. Embryos exposed to 10 µM retene were phenotypically normal in the presence or absence of MTZ. Fifty percent of embryos exposed to 12 µM retene in the absence of MTZ were malformed, with the common malformations being axial deformities, edema, and craniofacial malformation, and the remaining embryos survived with no malformations. In the presence of MTZ, 80% of embryos died, with the remaining embryos malformed. Embryos exposed to 14 µM retene without MTZ had almost complete incidence of malformations, with about 20% mortality. Embryos co-exposed with MTZ and 14 µM retene had 100% mortality (Fig. 5c). Exposure to 16 or 18 µM retene, in the presence or absence of MTZ, resulted in complete mortality.

Thirty-five percent of embryos exposed to 50 or 65 µM B[a]P in the absence of MTZ were phenotypically normal, whereas co-exposure with MTZ increased the incidence of edemas and facial malformations, in particular at 65 µM benzo[a]pyrene (B[a]P), to 60% (Fig. 5d). Embryos exposed to 100 µM B[a]P in the absence or presence of MTZ showed a

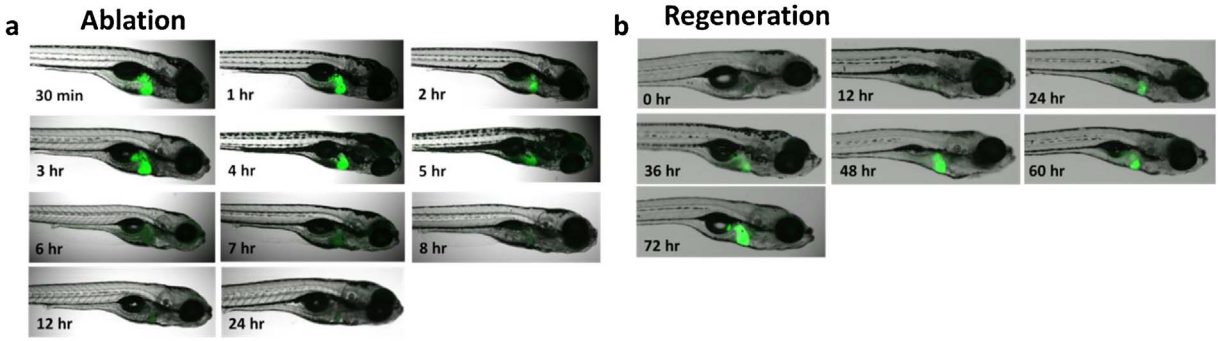

Fig. 3. Time course of tissue ablation (a) and regeneration (b) using 10 mM metronidazole (MTZ) in the hepatocytes of *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish. For the ablation study, embryos were dosed at 96 hpf with 10 mM MTZ, and ablation was evaluated by visible fluorescence, following continuous exposure until the indicated time point. For the regeneration study, embryos were exposed to 10 mM MTZ from 96 to 120 hpf, then rinsed and moved to clean media, with imaging at the indicated time points after MTZ removal. The presence of hepatocytes is indicated by green fluorescence.

90% incidence of edemas and other malformations, although with greater severity in the presence of MTZ. Significant fluorescence from B[a]P particles in the media and in the yolk sac was also visible, although clearly distinguishable from liver fluorescence.

None of the embryos exposed to 0.125 mM acetaminophen showed any morphological toxicity, either in the presence or absence of MTZ (Fig. 5e). For increasing concentrations of acetaminophen, a positive correlative relationship was observed with increasing malformation incidence, in the presence and absence of MTZ. Embryos exposed to 2.5 mM acetaminophen without MTZ displayed a 40% incidence of malformations (primarily pericardial edema and craniofacial deformities) and partial hepatic ablation. In the presence of MTZ, the malformation incidence was approximately 65%. Exposure to 5 mM acetaminophen in the absence of MTZ resulted in 70% malformation incidence, as well as partial hepatic ablation. MTZ co-exposure with 5 mM acetaminophen resulted in 100% incidence of malformations. Exposure to 10 mM acetaminophen, with or without MTZ co-exposure, resulted in approximately 80% malformation incidence, with hepatic ablation also observed in the non-MTZ treated embryos. Treatment with 25 mM acetaminophen, in the presence or absence of MTZ, resulted in complete mortality.

Embryos exposed to 5 or 7 μM flutamide were phenotypically normal, with most showing some degree of hepatocyte ablation. (Fig. 5f). Embryos exposed to MTZ and 5 or 7 μM flutamide showed a similar pattern of malformations and edemas as non-MTZ exposed embryos. Embryos exposed to 10 μM flutamide had a toxicity profile similar to the lower flutamide exposure concentrations. However, embryos exposed to MTZ and 10 μM flutamide experienced 100% mortality, as did embryos exposed to 12 μM flutamide, with or without MTZ co-exposure. Wild-type embryos exposed to 5 μM flutamide were phenotypically normal in the absence of MTZ, with a 50% incidence of edemas and craniofacial malformations in the presence of MTZ (Fig. 5g). Exposure to 7 μM flutamide also resulted in approximately 50% incidence of malformations, and co-treatment with MTZ resulted in complete mortality. Exposure to 10 or 12 μM flutamide, in the presence or absence of MTZ, resulted in 100% mortality.

4. Discussion

Zebrafish are a powerful model for vertebrate development, that can be made more versatile through the use of transgenic lines, in particular when the metabolic capability of zebrafish is made to more closely resemble humans or other model systems. Transgenic lines developed for one purpose can also be useful for others. Tissue ablation has been used to study zebrafish tissue regeneration in a range of organs [15,17,21,39,40]. Using the nitroreductase system for tissue ablation suggests there could be additional uses for the added nitroreductase metabolic capacity. Other potential uses of these types of transgenic lines include toxicity testing of nitrated environmental contaminants, such as nitro-PAHs, since the addition of nitroreductive capability in the liver specifically increases the similarities between the zebrafish and human metabolic pathways. The ability to completely ablate the

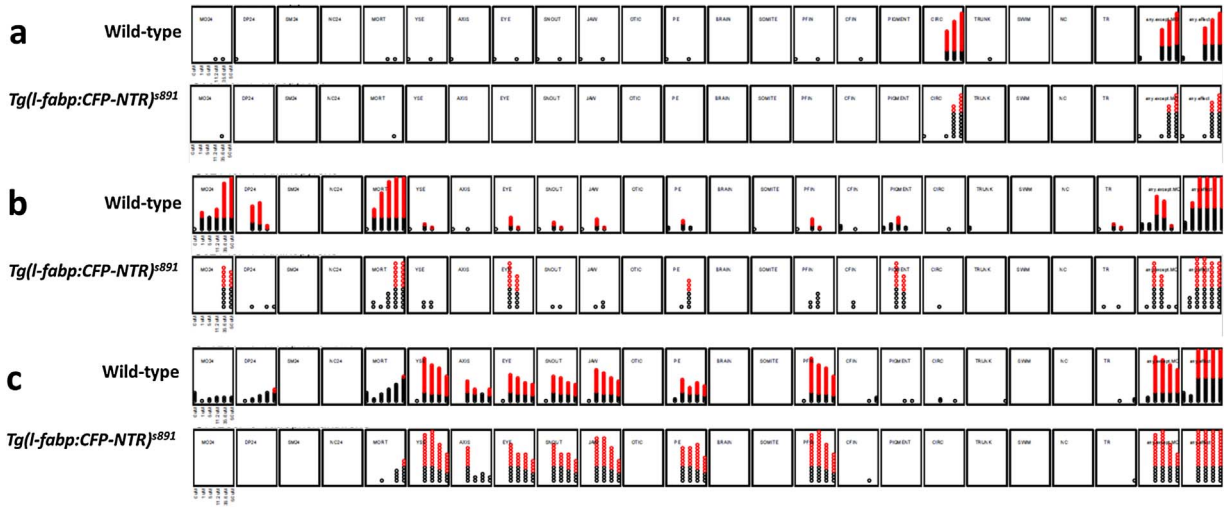

Fig. 4. Windows of exposure in wild-type and *Tg(l-fabp:CFP-NTR)*^{s891} embryos exposed to 1-nitropyrene (a), 1-aminopyrene (b), and 9-nitrophenanthrene (c). Embryos were exposed at 6 hpf with evaluations at 120 hpf. Bar height indicates incidence of each individual endpoint, where red dots indicate statistical significance. Toxicity profiles for exposures at other time points are shown in SI 2.

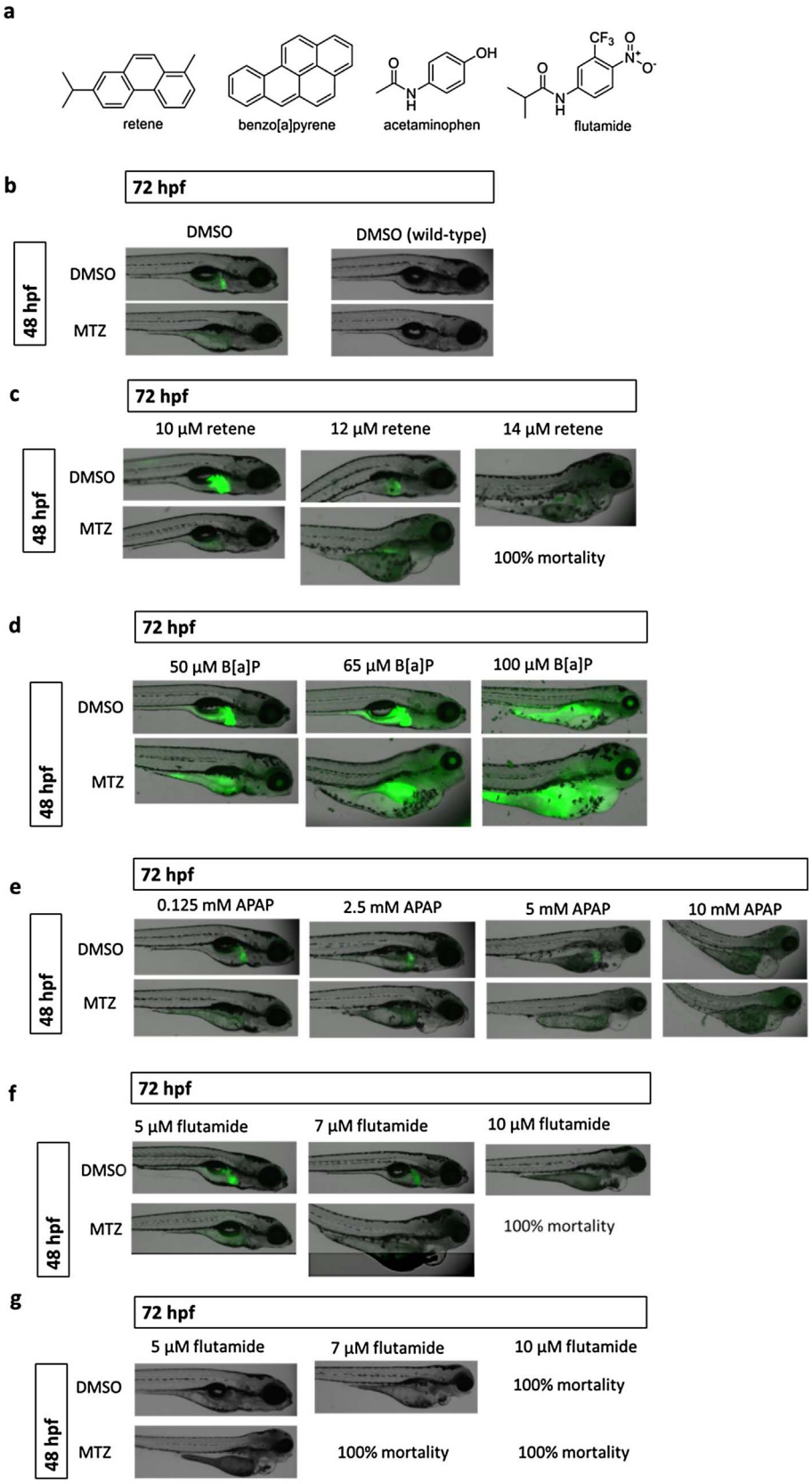

Fig. 5. Co-exposures with 10 mM metronidazole (MTZ) in *Tg(l-fabp:CFP-NTR)*^{s891} and wild-type zebrafish embryos. Structures of all compounds tested are shown in (a). Embryos were exposed to MTZ at 48 hpf, and to 1% DMSO (1.5% DMSO-exposed animals were phenotypically indistinguishable) (b), retene (c), benzo[a]pyrene (B[a]P) (d), acetaminophen (APAP) (e), or flutamide (f) at 72 hpf. Additionally, wild-type embryos were exposed to flutamide (g). Imaging and evaluations were done for all animals at 120 hpf. Concentrations tested but not shown resulted in 100% mortality both in the presence and absence of MTZ.

liver, and all associated metabolism, would be a powerful tool to interrogate compounds with toxicity mechanisms dependent on their hepatic metabolism or toxicity. These compounds include pharmaceuticals and environmental contaminants, in particular those containing a nitro functional group. This model could be useful for a relatively high-throughput screen investigating the role of nitroreductase or hepatic metabolism in toxicity.

In the initial published methods using the *Tg(l-fabp:CFP-NTR)*^{s891} transgenic line [14], heterozygous adult animals were incrossed to yield a mixture of genotypes within the offspring. Embryos were visually screened for fluorescence at 96 hpf, immediately prior to exposure with MTZ. Our work confirms that homozygous positive (embryos with two copies of the nitroreductase gene) and heterozygous embryos have the same visual tissue ablation patterns in response to MTZ exposure. We also demonstrated that exposure to MTZ during development of the liver, and before 96 hpf, will result in complete ablation of the liver. This allows for expanded use of the hepatic ablation capabilities of this line during development, such as developmental toxicity testing at earlier stages of development.

Use of a lower MTZ concentration was also effective in causing tissue ablation, which is advantageous as it both reduces the amount of chemical used, and reduces the off-target effects that result from exposure to MTZ. MTZ is generally considered to be non-toxic, with no evidence for developmental toxicity [41], although genotoxicity and neurotoxicity have been reported [42,43]. The ability to expose embryos to lower concentrations of MTZ, and at earlier stages of development, expands the possibilities of this model for use in other developmental biology and toxicology studies, in particular at early developmental time points, as well as during normal hepatic development.

The time course of liver ablation following MTZ exposure also showed that visual ablation occurred within 8–12 h, in contrast to the 24 h in the previous tissue ablation protocol [14,15]. Visual regeneration of the liver occurred starting at 10–12 h and was more substantial by 24 h. This is consistent with other studies on liver regeneration after damage, where the hepatocytes were the first cell population to recover following hepatectomy [44], although complete recovery of hepatic tissue took several days [40]. This expands the potential use of this transgenic line for the investigation of liver recovery and the impacts on recovery which could result from exposure to chemicals or other stressors.

We also attempted to use the ROS-ID® Hypoxia/Oxidative Stress Detection Kit to further characterize the nitroreductase activity in the transgenic and wild-type zebrafish. The kit failed to detect nitroreductase activity in the liver of the transgenic embryos (SI 1). This kit was developed for use in cell culture, and had not been previously published for use in zebrafish or any similar whole-animal systems. The results from this kit was not consistent with the other assays, suggesting that this kit, as provided, may not be suitable for use in zebrafish.

Use of the wild-type 5D line to characterize the toxicity of the nitrocompounds of interest prior to use in the *Tg(l-fabp:CFP-NTR)*^{s891} line provided a background toxicity against which to measure later exposures using the *Tg(l-fabp:CFP-NTR)*^{s891} zebrafish line. The nitro-PAHs studied here had a developmental toxicity profile which was consistent across the range of time points tested [8], allowing for exposures later in development with the *Tg(l-fabp:CFP-NTR)*^{s891} line. While not substantially impacting the overall toxicity profile, the presence of the chorion for the transgenic line exposures could explain the slightly lower incidence of toxicity in the *Tg(l-fabp:CFP-NTR)*^{s891} embryos compared to the dechorionated wild-type exposure [45].

Had the *Tg(l-fabp:CFP-NTR)*^{s891} fish significantly reduced the nitro-PAHs to the amino-PAHs (or a partially-reduced intermediate), the toxicity profile would have been expected to shift, to be more similar to the amino-PAH. The lack of observable changes in toxicity could be explained by a lack of nitroreduction, potentially due to a low affinity of the nitroreductase enzyme for the nitro-PAHs. It is also possible that other metabolic pathways, such as metabolism by the cytochrome P450s, compete with the nitroreduction pathway, resulting in less substrate available for nitroreduction [46,47]. Nitro-PAHs are known to undergo nitroreduction in bacterial systems, and development of a transgenic line similar to *Tg(l-fabp:CFP-NTR)*^{s891} line, but with a different nitroreductase enzyme, would allow for further investigations into the metabolism of nitro-PAHs.

The liver is an important component of metabolism and toxicity for many compounds, and the ability to study toxicity, both in the presence and absence of the liver, would be a useful tool for toxicity testing. The ability to expose a whole-animal system, in the presence and absence of functioning hepatic tissue, can provide powerful insight into the mechanism of toxicity for a variety of compounds. Exposures to the environmental contaminant retene had increased incidence and severity of malformation in the absence of a functioning liver, indicating that hepatic metabolism is a likely a detoxifying pathway for retene. Previous work has implicated hepatic metabolism, particularly by CYP 450s, as an important component of retene toxicity [24,48]. Exposure to concentrations of retene greater than 14 μM, in the presence or absence of hepatic tissue, resulted in 100% mortality. This suggests other mechanisms of toxicity as well, potentially including CYP 450 metabolism in extrahepatic tissues [49]. B[a]P also had increased developmental toxicity in the absence of the liver, indicating that hepatic metabolism is also a detoxification pathway with regard to the developmental toxicity of this compound. Hepatic metabolism of B[a]P by CYP 450 enzymes is known to generate powerful mutagenic and carcinogenic metabolites [25,26], but the pathway for developmental toxicity is less clear. AHR2 has been implicated as essential for B[a]P induced behavioral endpoints [50]. However, AHR2 is primarily located in tissues other than the liver [51], so it would not be expected to change significantly as a result of hepatic ablation. The increased toxicity in the absence of the liver indicates that hepatic metabolism is responsible for detoxification with regards to developmental toxicity, in contrast to the mutagenic activation pathways typically noted for B[a]P.

Flutamide, an anti-androgenic compound used for the treatment of prostate cancer, has been known to cause hepatic injury or necrosis for some human patients [30,31], and was developmentally toxic with and without hepatic ablation in zebrafish. Flutamide exposure alone caused hepatocyte ablation, indicating hepatotoxicity consistent with previous medical reports [31]. Flutamide caused effects other than hepatoxicity, including pericardial edema and craniofacial malformations, and is known to undergo metabolism by several subfamilies of CYP 450 enzymes in humans and other model organisms [52–55]. While nitroreduction of the nitro group is possible [56], the primary mechanism of metabolism is believed to be through CYP 450 oxidation. Nitroreduction does not appear to be a primary mechanism of hepatotoxicity, because the profile of malformations in the hepatocyte-ablated and hepatocyte-present embryos was similar. To further investigate the role of nitroreductase in developmental toxicity, wild-type embryos were exposed to MTZ and flutamide at the same concentrations as the *Tg(l-fabp:CFP-NTR)*^{s891} embryos. The greater observed toxicity at lower concentrations of flutamide and in the presence of MTZ also indicates that hepatic metabolism is again important in mediating the toxicity of flutamide. As wild-type embryos are not capable of reducing the nitro group, the nitroreduction pathway does not appear to be a driver for toxicity, and instead may play a protective and detoxifying role in the metabolic pathways of flutamide.

In contrast to flutamide, the structurally similar acetaminophen, a known hepatotoxin [32], required exposure at concentrations orders of magnitude higher than the other compounds tested to cause toxicity [57–59]. Acetaminophen exposure results in hepatocellular necrosis, caused by metabolites of acetaminophen [60]. Acetaminophen hepatotoxicity has been previously established to be dependent on oxidative metabolism, by hepatic CYP 450 enzymes, into the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). Mice lacking these CYP 450

isoforms were protected against APAP-induced hepatotoxicity [61]. Multiple conjugate metabolites are formed as well, although these are relatively non-toxic and readily excreted [62]. Liver ablation was observed following exposure to acetaminophen without MTZ, demonstrating the hepatotoxicity of acetaminophen. Toxicity following exposure to acetaminophen in the absence of a liver indicates that acetaminophen can be toxic to other tissues, and that hepatic metabolism is not required for toxicity to occur.

For all four compounds tested, exposure following ablation of the liver increased the observed toxicity. This demonstrates the importance of the liver in metabolic and detoxifying pathways in zebrafish. The ability to selectively ablate hepatic tissue allows for investigation of the role of hepatic metabolism in the toxicity of a compound of interest. The presence of nitroreductase in this zebrafish line increases the relevance of hepatic metabolism to human health and metabolism.

5. Conclusions

As intended and previously described, treatment with MTZ results in ablation of the hepatic tissue of *Tg(l-fabp:CFP-NTR)^{s891}* zebrafish. We demonstrated that the published methods could be expanded upon, allowing for additional uses of the *Tg(l-fabp:CFP-NTR)^{s891}*, and similar transgenic zebrafish lines, in chemical screening assays. This transgenic line does not appear to be useful in the investigation of nitroreduction as a mechanism for nitro-PAH toxicity, potentially due to the binding affinity of the nitroreductase enzyme used in the development of this line. Development of a similar transgenic line, where the nitroreductase has a higher binding affinity for nitro-PAHs, would be a useful model system in the investigation of the toxicity and metabolism of nitro-PAHs and other nitroaromatic compounds, as well as expanded use in pharmaceutical development and testing. We also demonstrated novel uses for this transgenic zebrafish line, including toxicity testing in the absence of hepatic metabolism which could be used in a high-throughput manner. The ability to determine the role of hepatic metabolism in compound toxicity is a powerful tool for further mechanistic investigations, in particular for determining the role of the liver in toxicity.

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Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2017.04.005.

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