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

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1-Aminopyrene (PubChem CID: 15352)
9-Nitrophenanthrene (PubChem CID: 70382)
9-Aminophenanthrene (PubChem CID: 13695)
Retene (PubChem CID: 10222)
Benzo[a]pyrene (PubChem CID: 2336)
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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