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Phenylthiourea alters toxicity of mercury compounds in zebrafish larvae

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ABSTRACT

In recent years larval stage zebrafish have been emerging as a standard vertebrate model in a number of fields, ranging from developmental biology to pharmacology and toxicology. The tyrosinase inhibitor 1-phenyl-2-thiourea (PTU) is used very widely with larval zebrafish to generate essentially transparent organisms through inhibition of melanogenesis, which has enabled many elegant studies in areas ranging from neurological development to cancer research. Here we show that PTU can have dramatic synergistic and antagonistic effects on the chemical toxicology of different mercury compounds. Our results indicate that extreme caution should be used when employing PTU in toxicological studies, particularly when studying toxic metal ions.

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1. Introduction

Zebrafish (*Danio rerio*) are fresh-water fish that have been extensively and increasingly used as an animal model in a variety of research areas over the past 30 years [1]. Key characteristics that make the zebrafish an excellent model vertebrate system include nearly transparent embryos, rapid development outside the mother, large egg clutch, and a fully sequenced genome [2]. As a model vertebrate system for studying the development of the embryo, researchers have used zebrafish to explore metal toxicity [2–8] as well as various human diseases [1,9].

In developmental biology research, zebrafish embryos and larvae offer a particular advantage over other model systems in that they are nearly transparent. Transparency and development outside the mother have allowed researchers to create a well-characterized staging series from fertilized embryo to hatched larva [2]. Many techniques involve viewing fluorescent stains, probes or proteins using optical microscopy [10] which can be hindered by natural pigmentation in the fish as they age. Zebrafish develop black melanophores, yellow xanthophores and reflective iridophores [11]. One commonly used technique for inhibiting natural pigmentation is the inhibition of tyrosinase activity [12] by exposing embryos to 1-phenyl-2-thiourea (PTU) (Fig. 1) which inhibits melanogenesis in the melanophores [10,13]. Additionally, normal pigmentation is restored in zebrafish following PTU treatment after a two week recovery period in water with no exogenous agents such as PTU [10].

When the PTU pigment blocking process was initially developed, no significant effects on hatching or survival were noted when a dose of 75 μM PTU was used [10]. However, concentrations of 200 μM PTU are frequently used [14] with such concentrations considered standard protocols [15]. Recently, evidence has been emerging that commonly used doses of PTU may not be as benign as previously had been assumed [16–18]. Elsalini and Rohr [16] found that PTU can interfere with thyroid hormone production and thus may alter normal development after 60 h post fertilization. PTU also has been reported to affect extraocular muscle development and neural crest development [17]. Its use has also been seen to result in reduced body size and specifically in smaller eye lenses and more tightly packed eye cells [18]. To date, however, and despite the widespread use of zebrafish, the effects of PTU on the toxicity of exogenous chemical entities have not been studied. In the course of conventional microscopy studies of the effects of mercury compounds on the mechanosensory apparatus of the zebrafish, we uncovered dramatic changes in toxicology when PTU was used to generate

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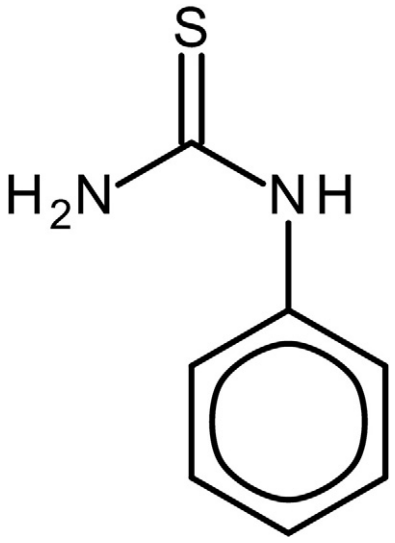

Fig. 1. Schematic structure of the tyrosinase inhibitor 1-phenyl-2-thiourea (PTU).

transparent fish. We present herein a study of the effects of PTU in combination with different mercury compounds on larval stage zebrafish and show that PTU substantially perturbs these systems in ways that are totally different for inorganic mercury (Hg^{2+}) species and methylmercury species.

2. Materials and methods

2.1. Chemicals

Phenylthiourea (PTU) and mercuric chloride were purchased from Sigma Aldrich (Oakville, ON) and methylmercury hydroxide from Strem Chemicals Inc. (Newburyport, MA). A 1000 ppm (3.98 mM) methylmercury chloride solution was obtained from Alfa Aesar (Ward Hill, MA).

2.2. Zebrafish

All procedures were approved by the University of Saskatchewan Ethics Board. Adult zebrafish (*D. rerio*) were mated using the marble technique [15]. Embryos were collected and raised to 22 h post fertilization (hpf) in system water in a 28 °C incubator with a 14:10 hour light: dark cycle.

2.3. Statistical analysis

For comparisons involving deformity and death rates, significance was determined using ANOVA with a Tukey's post-hoc pairwise test to compare treatments within each day. A Student's *t*-test was used to compare Hg values in the livers of fish imaged using X-ray fluorescence imaging (XFI). For all statistical analyses a P-value < 0.05 was considered to be significant.

2.4. XFI sample preparation

Half of the zebrafish were raised in 100 μM PTU until 3 days post fertilization (dpf) while the second half were raised to 3 dpf in system water. All zebrafish were then randomly divided into the following treatment groups: control, 100 μM PTU, both 2 and 4 μM $HgCl_2$ in the presence or absence of PTU, both 0.2 and 0.5 μM CH_3HgCl in the presence or absence of PTU. These doses were selected to represent a high mercury exposure; however, the number of deaths in the 0.5 μM CH_3HgCl group prohibited collection of any samples. The dose 0.2 μM CH_3HgCl was selected because fish in the 0.1 μM CH_3HgCl were found to have few deformities and no deaths. After a 48 hour exposure all fish were rinsed 3 times in fresh system water to remove excess mercury on the surface of the fish. Zebrafish were fixed, embedded and sectioned as previously described [7].

2.5. X-ray fluorescence imaging (XFI)

XFI data were collected at the Advanced Photon Source (APS) in Argonne, IL, USA on beamline 20-ID-B. The storage ring was operating in continuous top-up mode at 102 mA and 7.0 GeV. An incident X-ray energy of 13.45 keV was selected to avoid the Br K edge while being able to monitor the Hg $L\alpha_{1,2}$ and Zn Kα fluorescence lines. A Si(111) double crystal monochromator and Rh-coated mirrors were used for focusing and harmonic rejection. Samples were mounted at 45° to the incident X-ray beam and raster scanned, with a silicon-drift Vortex detector at 90° to the incident X-ray beam [19]. Kirkpatrick–Baez Rh-coated focusing mirrors were used to generate a micro-focused beam of 5 μm diameter. Samples were raster scanned using a step size of 5 μm with a beam exposure time of 0.6 s per point.

2.6. XFI data analysis

Data collected from X-ray fluorescence imaging were processed as previously described [7], with normalization to the incoming beam

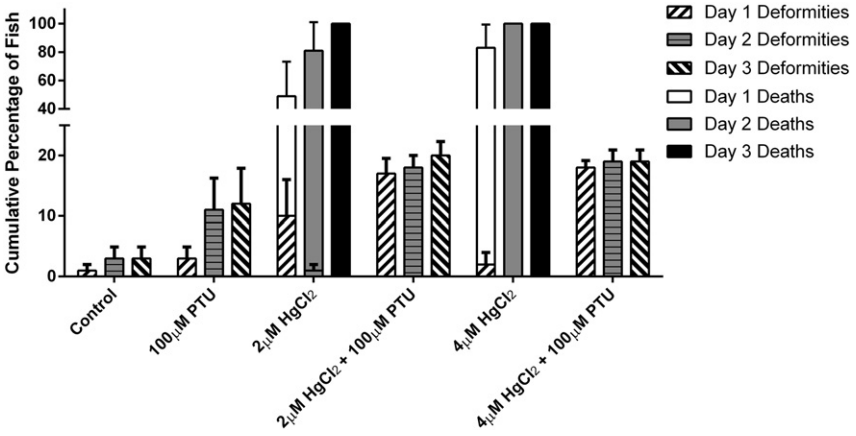

Fig. 2. Cumulative mortality (%) stacked on top of cumulative deformity (%) (+SE) of zebrafish larvae over 3 days of exposure to various $HgCl_2$ treatments, with and without PTU. Note the absence of deaths following PTU addition, showing that PTU *decreases* the toxicity of $HgCl_2$. The control was system water from the fish facility.

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