***Synopsis for the Graphical abstract**

Phenylthiourea alters the toxicity of mercury compounds in zebrafish larvae, studied by X-ray fluorescence imaging, X-ray absorption spectroscopy and density functional theory.

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.

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 μ M PTU was used [10]. However, concentrations of 200 μ M 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 hours 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 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 were obtained from Alfa Aesar (Ward Hill, MA).

2.2 Zebrafish

All procedures were approved by the University of Saskatchewan Ethics Board. Adult zebrafish (*Danio rerio*) were mated using the marble technique [15]. Embryos were collected and raised to 22 hours 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 HgCl₂ in the presence or absence of PTU, both 0.2 and 0.5 µM CH₃HgCl 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 $K\alpha$ 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 intensity and removal of background signal by averaging the intensity per element in the area outside of the tissue then subtracting this amount from each pixel in the entire image. Certified highly-uniform thin film standards mounted on 6.3 μm Mylar substrates for zinc, gold, and thallium were obtained from Micromatter Co. (Vancouver, BC). To quantify the amount of zinc per pixel, the zinc standard was imaged, normalized and background subtracted using a blank foil. Because mercury amalgam standards

slowly decrease in mercury content over time, mercury was quantified using gold and thallium standards, as they are adjacent to mercury on the periodic table. These standards were imaged, normalized and background subtracted. Linear interpolation was then used to estimate a calibration for the mercury intensity for quantification of the mercury in the zebrafish sections. This method was verified by measurement of a new mercury-silver amalgam standard foil (Micromatter Co., Vancouver, BC). This worked only with a fresh mercury-amalgam standard as the mercury reading was found to decrease on storage, presumably due to loss of mercury as vapor.

Due to time constraints associated with limited allocations of beamtime and long acquisition time (an average of 3.5 h per sample), only a limited number of samples can be run. Therefore 3 representative zebrafish were selected for each treatment group for synchrotron imaging. Following the quantification of mercury in each sample, significant differences were evaluated with a Student's *t*-test on the amount of mercury (in $\mu\text{g}/\text{cm}^2$) in the livers of each treatment group.

2.7 X-ray Absorption Spectroscopy

Samples for X-ray absorption spectroscopy (XAS) were prepared at 10:1 mM PTU:Hg stoichiometries in aqueous solution (using CH_3HgOH or HgCl_2). Solution mixtures were incubated for 5 minutes at room temperature then were loaded into 2 mm $\times$ 3 mm $\times$ 22 mm acrylic cuvettes and were flash frozen in a slurry of liquid nitrogen-cooled isopentane.

Mercury L_{III} -edge XAS spectra was measured on the structural molecular biology beamline 7-3 at the Stanford Synchrotron Radiation Lightsource (SSRL) with the SPEAR storage ring operating at 3.0-GeV with a current of 500 mA. The beamline was equipped with a Si(220) double-crystal monochromator with harmonic rejection achieved by setting the cut-off

energy of an upstream Rh-coated mirror to 15 keV. Incident X-ray intensities were monitored using a nitrogen-filled gas ionization chamber and X-ray absorption of samples was measured as the X-ray fluorescence excitation spectrum using a 30-element germanium detector array [20]. Soller slits and Ga₂O₃ X-ray filters were used to attenuate unwanted scattered radiation and to preserve detector linearity. Samples were placed in a liquid helium cryostat to maintain an approximate temperature of 10K during data collection. Simultaneous absorption of a downstream standard Hg-Sn amalgam metal foil was measured by transmittance. Incident X-ray energy calibration was accomplished using the lowest energy Hg L_{III} inflection point of the standard foil, assumed to be 12,285.0 eV. Each sample was subjected to eight sweeps approximately 22 minutes long.

Extended X-ray absorption fine structure (EXAFS) oscillations $\chi(k)$ were analyzed using the EXAFSPAK program suite [21], as previously described [22] and assuming an Hg L_{III} threshold energy (E_0) of 12,305.0 eV. Phase-correction of Fourier transforms employed Hg—S backscattering. FEFF version 7 was utilized to compute theoretical phase and amplitude functions.

2.8 Density functional theory (DFT)

DFT calculations used the program Dmol³ and Biovia Accelrys Materials Studio V7.0 for geometry optimization [23,24]. Geometry optimization used the Perdew-Burke-Ernzerhof functionals [25] for both the potential during the self-consistent field procedure and the energy, and employing an all-electron relativistic core treatment. Solvent effects were simulated by using the Conductor-like Screening Model (COSMO) with a dielectric value representing water ($\epsilon = 78.39$) [26].

3. Results and Discussion

3.1. Toxicological Profiles

Prior to examining the effects of mercury, range-finding studies were conducted to determine the PTU dose that combined the best pigment inhibition with the lowest rate of adverse effects. Treatment groups were 0 (control), 25, 50, 75, 100, 200 and 300 μM PTU, each with 75 larvae at 22 hours post fertilization (hpf). Consistent with normal practice, this time was chosen as zebrafish pigment formation begins at ~ 24 hpf, with rapid progression thereafter [10] and to inhibit the pigment formation larvae must be exposed to PTU before to this stage [10]. Survival, hatch, deformity and transparency rates were assessed from 1 to 5 days post fertilization (dpf). Preliminary trials found that a dose of 100 μM PTU resulted in lower death rates and fewer deformities than the more commonly used higher dose of 200 μM PTU, but with comparable suppression of pigment formation. Therefore, 100 μM PTU was used in all subsequent work. Two different categories of mercury compound were tested (mercuric chloride and methylmercury chloride), based on the differential toxicology previously reported [7]. In these previous studies, the highest mercury concentrations in zebrafish exposed to mercuric chloride were found in the olfactory epithelial cells [7]. In contrast, methylmercury chloride preferentially targeted the developing lens [5-8]. While zebrafish exposed to mercuric chloride accumulated mercury in the ventricular region of the brain, zebrafish exposed to methylmercury chloride accumulated mercury in the muscle tissue [7]. As one would expect, mercury accumulated in the detoxification organs, the pronephros and liver, following exposure to both forms of mercury [7].

Zebrafish were raised to 3 dpf in either system water or in 100 μM PTU solution. Treatment solutions were replaced with fresh solution every 24 hours. Fish were examined for

deformities and deaths after 24, 48 and 72 hours following initial exposure, with deformities being defined as fin malformations, edema, and moribund behaviour. The results of toxicological screening with mercuric chloride and methylmercury chloride are shown in Figs. 2 and 3, respectively.

At 3 dpf, zebrafish larvae were exposed to 0 (control), 2 and 4 μM HgCl_2 in system water in the presence and absence of 100 μM PTU ($n = 100$ larvae per treatment). Two-way analysis of variance (ANOVA) was utilized to determine whether statistically significant differences existed for cumulative mortality between HgCl_2 and PTU + HgCl_2 treatment groups at 24, 48, and 72 hours post-exposure. The PTU + HgCl_2 treatment group showed very significant decreases in mortalities compared to HgCl_2 alone (Fig. 2), with all P values being $<10^{-3}$. Although at first sight the data seem to suggest that there may be more deformities in the fish exposed to both HgCl_2 and PTU when compared to HgCl_2 alone, this is actually due to the fact that there are significantly more dead larvae in the latter case (Fig. 2). Hence, the data indicate that PTU reduces the toxicity of HgCl_2 .

Methylmercury species can be considerably more toxic than inorganic mercury compounds [7] and can also have a very steep dose-response curve. To account for this, correspondingly lower levels of methylmercury chloride (CH_3HgCl) were used: 0.05, 0.1 and 0.5 μM CH_3HgCl in system water, in the presence and absence of 100 μM PTU. In contrast to HgCl_2 , which had decreased toxicity in the presence of PTU, CH_3HgCl showed enhanced toxicity in the presence of PTU (Fig. 3). While the trends were not as striking as for mercuric chloride, the increase in deaths of fish exposed to 100 μM PTU + 0.5 μM CH_3HgCl , was statistically significant at 48 and 72 hours post-exposure compared to fish exposed to 0.5 μM CH_3HgCl in absence of PTU, with $P < 0.0001$ for both (see supplementary material). The small

differences between control and 100 μ M PTU in Figs. 2 and 3 are primarily a reflection of the fact that the tests for Figs. 2 and 3 were done at different times. Such variations are not uncommon and are not considered statistically significant.

3.2 X-ray fluorescence imaging (XFI)

XFI [19] was used to map Hg accumulation in representative fish from each treatment group and to quantify Hg in regions of interest. The results of which are shown in Figs. 4-7, together with the corresponding histological images. Zinc, which is mapped simultaneously with mercury, is also shown for all sections; it is common practice to use the Zn image to ensure that the correct region is scanned [7]. Zinc is normally abundant in essentially all tissues but is present at notably high levels in pigment cells of zebrafish. Because PTU blocks pigment formation in the zebrafish, pigment levels in PTU treated fish were low and as expected, zinc levels were often correspondingly lower.

Figs. 4-5 show sections from zebrafish treated with HgCl_2 solutions in the absence and presence of PTU. The olfactory pits have previously been shown to be a target organ for HgCl_2 [7]; Fig. 4 shows XFI of sections of this region. The comparative absence of mercury in the PTU-exposed larva is striking and is consistent with the protective effect of PTU observed in the toxicological profile. Fig. 5 shows XFI of sections through the liver, gut, muscle, and pronephros. Here, the pigment spots in the absence of PTU treatment are observed to be larger and to have higher zinc levels (Fig. 5, A & C) than the pigmented areas in the presence of PTU treatment (Fig. 5, B & D). As with olfactory pits in Fig. 4, the levels of mercury in the trunk region are substantially lower in PTU exposed fish, relative to fish exposed to HgCl_2 alone. Mercury concentrations in the livers of three separate fish from the 48-hour 4 μ M exposure group were quantified and averaged. A Student's two-tailed *t*-test determined the mean mercury

concentrations to be significantly greater in the HgCl_2 treatment compared to the PTU + HgCl_2 treatment with $P < 0.001$ for all. The mercury concentrations in the fish treated with solutions of PTU + HgCl_2 were 60-fold lower than in fish exposed to HgCl_2 solutions alone.

Fig. 6 shows zebrafish head sections from larvae exposed to $0.2 \mu\text{M}$ CH_3HgCl in absence and presence of PTU. Cells in the lens and retina are known to be among the primary targets of methylmercury compounds [5-8]. The differences in pigment levels in the retinal pigmented epithelia in the optical micrographs can be clearly seen as a loss of black coloration in Fig. 6B compared with Fig. 6A, and this is paralleled by the decrease in Zn levels in those regions (Fig. 6D compared with Fig. 6C). Fig. 7 shows XFI of the trunk region for the CH_3HgCl exposures; here also lower Zn levels in discrete regions that are normally pigmented are seen in the PTU-treated fish. In marked contrast to the HgCl_2 data, both head and trunk sections of the larvae treated with CH_3HgCl and PTU are visually higher in Hg than those treated with CH_3HgCl alone. This again is consistent with the increase in CH_3HgCl toxicity observed in PTU treated fish. Similar to the HgCl_2 treatment, a two-tailed Student's *t*-test with equal variance was conducted on average mercury levels in each liver of three fish per treatment group and showed $P < 0.05$. Zebrafish exposed to CH_3HgCl and PTU had approximately two fold higher mercury levels than those exposed to CH_3HgCl only.

The XFI results together with the observations of deformity and mortality show that PTU has opposite effects on the toxicology of the two different chemical forms of mercury, being antagonistic for inorganic mercury and synergistic with methylmercury. This suggests not only that PTU is interacting chemically with the mercury species, but also that it interacts differently with the two types of mercury species. We therefore investigated the possible solution chemistry

of PTU and the mercury species using X-ray absorption spectroscopy and density functional theory (DFT).

3.3 Extended X-ray Absorption Fine Structure (EXAFS) and Density Functional Theory (DFT)

We examined the EXAFS portion of the X-ray absorption spectrum, which can be used with quantitative analysis to obtain a radial structure [27]. EXAFS spectra of methylmercury and inorganic mercury in the presence of PTU, together with the associated Fourier transforms, are shown in Fig. 8. The results of EXAFS curve-fitting analysis are also shown in Fig. 8, with the derived structural parameters summarized in Table 1. Because EXAFS currently lacks the sensitivity to the micromolar levels used in the zebrafish exposures, we employed higher concentrations of both Hg and PTU, but maintaining an excess of PTU as in the fish exposures. In the case of methylmercury we used methylmercury hydroxide (CH_3HgOH) rather than the chloride because methylmercury chloride was insufficiently soluble; this species will in any case be the dominant form in dilute solution when the chloride is used [7]. Mercury typically forms compounds with either two-coordinate linear digonal coordination, three-coordinate trigonal planar coordination, or four-coordinate pseudo tetrahedral coordination [28]. The bond-lengths to Hg change systematically with coordination type; for example for Hg—S coordination environments, two, three and four coordinate species have bond lengths of 2.35, 2.46 and 2.54 Å, respectively [29].

The EXAFS of PTU with inorganic Hg^{2+} indicated a 4-coordinate complex, with 2 Hg—S donors at 2.49 Å and 2 Hg—N or Hg—O at 2.43 Å (Fig. 8, Table 1). In support of this examination of the Cambridge Structural Database (CSD) [29] shows a marked predominance of four-coordinate Hg^{2+} compounds when coordination with thione ($\text{C}=\text{S}$) sulfur donors is present, with 190 database hits for 4-coordinate species, 5 hits for 3-coordinate species, and only 3 hits

for 2-coordinate species. Although EXAFS cannot normally distinguish between Hg—O and Hg—N due the similarity in ligand atomic number, the bond-lengths of four-coordinate Hg²⁺ species in CSD strongly support the assignment of an Hg—N coordination. Thus, while the average bond-length for four-coordinate species containing Hg—O bonds is 2.61 Å, that for four-coordinate species containing Hg—N bonds is 2.42 Å, which is in good agreement with our EXAFS-derived bond-length of 2.43 Å. Similarly, while there are no examples in the CSD of Hg²⁺ bound by two thione ligands with two additional nitrogen donors, the average Hg—S bond-length for thione-coordinate species is 2.52 Å [29], also in good agreement with our EXAFS derived Hg—S bond-length of 2.49 Å.

The thiourea moiety in PTU contains amide and thione groups, both of which potentially can coordinate mercury. DFT calculations indicate that monomercury four-coordinate species with two PTU bound to one Hg²⁺ each as a bi-functional chelator through both —NH and =S (Fig. 9A) will be unstable, because the bite angle of the (N—C=S) moiety is too acute to accommodate the Hg and DFT geometry optimizations yield two-coordinate species with Hg coordinated only through the thione sulfurs of the two PTU ligands. Coordination of a single Hg²⁺ by four PTU ligands is possible, with two PTUs bound through their thione sulfurs, and two PTUs contributing bound through their amide nitrogens. However, DFT indicates that such 4-coordinate S₂N₂ mononuclear entities are energetically unfavorable relative to the S₄ complex in which four thione sulfurs are bound, by some 40 kJ/Mol. Thus, in order to explain the four-coordinate model with mixed N and S donors to Hg²⁺, we need to postulate the formation of a polymeric entity in solution. While there are no crystal structures of polymeric or oligomeric thiourea or phenylthiourea Hg²⁺ structures, examples do exist of polymeric Cd²⁺ structures containing bridging thione ligands derived from phenylthiourea [30]. Furthermore, while the

samples of Fig. 8 were all solutions made by slow addition with stirring of Hg^{2+} to PTU solutions, it was observed that simple mixing of PTU with Hg^{2+} in solution created an amorphous precipitate, strongly suggesting the formation of some kind of polymer. A schematic fragment of one example of the very large number of possible structures for such oligomeric or polymeric species is shown in Fig. 9B. With our postulated N_2S_2 coordination, the nitrogen donors can be either protonated or deprotonated, corresponding to cationic or neutral oligomers, respectively. DFT calculations indicate expected Hg—N bond-lengths of protonated nitrogen donors ($\text{Hg} \leftarrow \text{:NH}_2\text{—C}$) to be 2.47 Å, some 0.25 Å longer than the bond-length of a deprotonated nitrogen donor (Hg—NH—C) and a good match for the EXAFS derived bond-length of 2.43 Å, arguing for protonated nitrogen donors and thus a cationic oligomer. We consider the nitrogen coordinated by the phenyl group a less likely metal ligand than the amino nitrogen due to crowding by the phenyl ring. The energy minimized geometry optimized structure of one possible oligomeric fragment is shown in Fig. 10A. Both Hg—S and Hg—N bond lengths of 2.55 and 2.47 Å, respectively, agree well with the EXAFS-derived values of 2.53 and 2.43 Å. A number of different coordination environments might be considered plausible, including S_4 , N_1S_3 , N_2S_2 , N_3S_1 and possibly even N_4 , which might combine to give an overall average coordination of N_2S_2 . However, each of the above coordination environments will have significantly different and characteristic Hg—N and Hg—S bond-lengths, so that a distribution in coordination environments would inevitably result in a spread of Hg—N and Hg—S bond-lengths. This in turn would substantially increase the Debye-Waller factor of the EXAFS [27]. This is quantified by σ^2 , the mean square deviation from the average bond-length R (Table 1). The σ^2 value has both static and vibrational components such that $\sigma^2 = \sigma_{\text{vib}}^2 + \sigma_{\text{stat}}^2$ where σ_{vib}^2 is the vibrational component and σ_{stat}^2 is the static component resulting from heterogeneity of the

individual bond-lengths R which is not directly resolved in the EXAFS [27]. Because the values obtained for σ^2 (Table 1) are close to the expected σ_{vib}^2 values [27] the aforementioned heterogeneity cannot be very pronounced, and we conclude that the bulk of the Hg is actually bound with N₂S₂ coordination. The observed lack of distinct mercury-mercury interactions in the EXAFS (Fig. 8) means that well-defined relatively rigid bridged structures, such as rhombs with two Hg separated by bridging sulfurs and/or nitrogens, cannot be uniformly present. Investigation of this possibility with DFT indicates that such structures tend to be sterically crowded in any case because of the phenyl groups on the PTU ligands.

The EXAFS data of methylmercury plus excess PTU clearly indicate the formation of a 2-coordinate species, with Hg—C and Hg—S bond lengths of 2.06 and 2.37 Å, respectively (Table 1). The CSD [29] contains a number of structures in which a thione group (C=S) is coordinated to an aliphatic organo-Hg entity in a 2-coordinate complex, with mean Hg—C and Hg—S bond-lengths of 2.08 and 2.39 Å, respectively, which agree very well with the EXAFS derived bond-lengths. The presence of a two-coordinate compound with PTU is in accord both with the known stability of Hg—S complexes, and with the known predilection of CH₃—Hg species to form two-coordinate complexes; the CSD contains 178, 23 and 17 entries with two-, three- and four-coordinate CH₃Hg, respectively [29]. The energy-minimized geometry-optimized density functional theory (DFT) model of a solution structure is shown in Fig. 10B, for which the computed bond-lengths of 2.05 and 2.39 Å are in excellent agreement with the EXAFS derived values (Table 1).

Our results clearly show that oft-assumed innocuous additive PTU significantly modifies the toxicology of both inorganic mercuric and methylmercury species. In the case of inorganic mercury the toxicity is remarkably decreased, whereas with methylmercury it is substantially

increased. XFI reveals that this can be attributed to differences in mercury uptake by the zebrafish larvae, with PTU treatment causing substantially decreased Hg levels for inorganic mercury and causing increased Hg levels for methylmercury. We hypothesize that inorganic Hg^{2+} forms oligomeric or polymeric species with PTU which, due to their relatively large size, would not readily cross cellular membranes and would thus be unable to enter the zebrafish. In the case of methylmercury, XAS and DFT indicate that a molecular entity $[\text{CH}_3\text{Hg}(\text{PTU})]^+$ is generated in solution through formation of an Hg—S bond with the PTU thione sulfur. Since such a complex would bear a single positive charge, one possible explanation for its increased toxicity may lie in the surface chemistry of cells. It is well known that glycoproteins and polysaccharide chains on cell membranes contribute a negative charge to the cell surface [31]. Electrostatic attraction between positively charged CH_3HgPTU and the cell surface might increase the local availability of $[\text{CH}_3\text{Hg}(\text{PTU})]^+$, which might then enter the cell and exert its lethal effects.

At the time of writing there is widespread use of PTU for generating transparent zebrafish larvae. However, other methods of obtaining essentially transparent zebrafish larvae are available as alternatives to using PTU and wild-type fish. The *roy orbison* mutant zebrafish lacks iridophores, has few melanocytes and thus has translucent skin and uniformly pigmented eyes [32], while Lister et al. [33] have reported a mutant named *nacre* that lacks melanophores but has increased numbers of iridophores. White et al. [32] subsequently created the *casper* strain that is doubly mutant for *roy orbison* and *nacre*, thus lacking both melanocytes and iridophores from embryogenesis to adulthood [32].

In conclusion, while PTU is used very widely in studies of larval phase zebrafish, our results indicate that it can interfere remarkably with the toxicology of mercury compounds. Our

study was initiated following control experiments when we sought to use PTU together with conventional microscopy to study aspects of mercury toxicology, but our findings have broad implications that extend outside mercury toxicology. Even though the coordination chemistry of PTU itself has only been studied in few cases, a search of the CSD [29] indicates that PTU forms structurally characterized complexes with Cu, Zn, Ag and Cd. PTU would be expected to coordinate a wide variety of metals, and a CSD search for thiourea-related ligands to metals returns some 1,636 hits. Transition metal complexes account for 1,504 of these, including pharmacologically-relevant platinum and ruthenium species, with the remainder including complexes with toxic metals such as indium, tin, antimony, lead and bismuth. The fact that many of these metals have been studied using the larval stage zebrafish model expands the implications of our work, and we suggest that extreme caution should be exercised when employing PTU in toxicological studies of metal ions using zebrafish larvae.

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Table 1. Extended X-ray absorption fine structure (EXAFS) parameters^a and curve-fitting results of phenylthiourea with mercury compounds at stoichiometry of 10:1.

aqueous complex	bond	<i>N</i>	<i>R</i>	σ^2	ΔE_0	<i>F</i>
PTU: MeHgOH	Hg-C	1	2.063(3)	0.0025(3)	-15.0(5)	0.3785
	Hg-S	1	2.374(2)	0.0038(2)		
PTU: HgCl ₂	Hg-N	2	2.430(2)	0.0026(3)	-10.3(3)	0.2497
	Hg-S	2	2.493(1)	0.0030(1)		

a. Coordination number *N*, interatomic distances *R* (Å), Debye-Waller factors σ^2 (Å²), and threshold energy shift ΔE_0 (eV). Values in parentheses represent the standard uncertainties between the curve fitting parameters and spectroscopy data. Goodness of fit function *F* is defined as $[\Sigma k^6(\chi(k)_{\text{calc}} - \chi(k)_{\text{expt}})^2 / \Sigma k^6 \chi(k)_{\text{expt}}^2]^{1/2}$, where $\chi(k)_{\text{calc}}$ and $\chi(k)_{\text{expt}}$ are the calculated and experimental EXAFS, respectively, with *k* being the photoelectron wavevector.

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

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.

Fig. 3. Cumulative mortality (%) stacked on top of cumulative deformity (%) (+SE) of zebrafish larvae over 3 days of exposure to various CH_3HgCl (MeHg) treatments with and without PTU. Note the increased deaths at 0.5 μM MeHg with added PTU, showing that PTU *increases* the toxicity of CH_3HgCl . The control was system water from the fish facility.

Fig. 4. Transverse sections through the olfactory region of zebrafish treated with 4 μM HgCl_2 in absence (A, C and E) or presence (B, D and F) of 100 μM PTU. In each column the section stained with methylene blue for the optical image (A or B) was serial (immediately adjacent) to the unstained section used for X-ray fluorescence imaging of zinc (C or D) and mercury (E or F). White scale bars represent 100 μm . The colour bars show the quantification of zinc (C, D) and mercury (E, F) in $\mu\text{g}/\text{cm}^2$. oe= olfactory epithelium.

Fig. 5. Transverse sections through the trunk region of zebrafish treated with 4 μM HgCl_2 in absence (A, C and E) or presence (B, D and F) of 100 μM PTU. White scale bars represent 100 μm . mo= medulla oblongata, sm= somitic muscle, pd= pronephric duct, gt= gut tube, lv= liver, yl= yolk. Additional details as for Fig. 4.

Fig. 6. Transverse sections through the eye region of zebrafish treated with 0.2 μM CH_3HgCl in absence (A, C and E) or presence (B, D and F) of 100 μM PTU. White scale bars represent 100 μm . br= brain, el= eye lens, rpe= retinal pigmented epithelium. Additional details as for Fig. 4.

Fig. 7. Transverse sections through the trunk region of zebrafish treated with 0.2 μM CH_3HgCl in absence (A, C and E) or presence (B, D and F) of 100 μM PTU. White scale bars represent 100 μm . mo= medulla oblongata, sm= somitic muscle, pd= pronephric duct, gt= gut tube, lv= liver, yl= yolk. Additional details as for Fig. 4.

Fig. 8. Mercury L_{III} -edge extended X-ray absorption fine structure (EXAFS) for phenylthiourea complexes with (a) HgCl_2 and (b) MeHgOH , each at a stoichiometry of 10:1. **A** shows the EXAFS oscillations and **B** the corresponding Fourier transforms phase-corrected for Hg—S backscattering. Experimental data are shown as solid lines, with results from curve-fitting analyses (Table 1) as broken lines.

Fig. 9. Schematic structures of four-coordinate complexes involving PTU and Hg^{2+} . (A) Hg^{2+} coordinated by two bidentate PTU ligands. As discussed in the text, density functional theory calculations indicate that A is unstable. (B) Fragment of one possible oligomeric species. See the discussion in the text for additional details.

Fig. 10. Density functional theory energy-minimized geometry-optimized structures of the products resulting from reacting phenylthiourea with (A) HgCl_2 or (B) CH_3HgOH in aqueous solution.

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10