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Zebrafish Cardiotoxicity: The Effects of CYP1A Inhibition and AHR2 Knockdown Following Exposure to Weak Aryl Hydrocarbon Receptor Agonists

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Abstract

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates many of the toxic effects of dioxin-like compounds (DLCs) and some polycyclic aromatic hydrocarbons (PAHs). Strong AHR agonists, such as certain polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), cause severe cardiac teratogenesis in fish embryos. Moderately strong AHR agonists, such as benzo[a]pyrene and β -naphthoflavone, have been shown to cause similar cardiotoxic effects when coupled with a cytochrome P450 1A (CYP1A) inhibitor, such as fluoranthene (FL). We sought to determine if weak AHR agonists, when combined with a CYP1A inhibitor (FL) or CYP1A morpholino gene knockdown, are capable of causing cardiac deformities similar to moderately strong AHR agonists (Wassenberg and Di Giulio 2004; Wassenberg and Di Giulio 2004; Billiard, Timme-Laragy et al. 2006; Van Tiem and Di Giulio 2011). The weak AHR agonists included the following: carbaryl, phenanthrene, 2-methylindole, 3-methylindole, indigo, and indirubin. The results showed a complex pattern of cardiotoxic response to weak agonist inhibitor exposure and morpholino-knockdown. *Danio rerio* (zebrafish) embryos were first exposed to weak AHR agonists at equimolar concentrations. The agonists were assessed for their relative potency as inducers of CYP1 enzyme activity, measured by the ethoxyresorufin-*o*-deethylase (EROD) assay, and cardiac deformities. Carbaryl, 2-methylindole, and 3-methylindole induced the highest CYP1A activity in zebrafish. Experiments were then conducted

to determine the individual cardiotoxicity of each compound. Next, zebrafish were co-exposed to each agonist (at concentrations below those determined to be cardiotoxic) and FL in combination to assess if CYP1A inhibition could induce cardiac deformities. Carbaryl, 2-methylindole, 3-methylindole, and phenanthrene significantly increased pericardial edema relative to controls when combined with FL. To further evaluate the interaction of the weak AHR agonists and CYP1A inhibition, a morpholino was used to knockdown CYP1A expression, and embryos were then exposed to each agonist individually. In embryos exposed to 2-MI, CYP1A knockdown caused a similar level of pericardial edema to that caused by exposure to 2-MI and FL. However, CYP1A knockdown in phenanthrene and 3-methylindole only moderately increased pericardial edema relative to co-exposure to FL. AHR2 expression was also knocked down using a morpholino to determine its role in mediating the observed cardiac teratogenesis. Knockdown of AHR2 did not rescue the pericardial edema as previously observed with strong AHR agonists. While some of the cardiotoxicity observed may be attributed to the combination of weak AHR agonism and CYP1A inhibition, other weak AHR agonists appear to be causing cardiotoxicity through an AHR2-independent mechanism. The data show that CYP1A is protective of the cardiac toxicity associated with weak AHR agonists and that knockdown can generate pericardial edema, but these findings are also suggestive of differing mechanisms of cardiac toxicity among known AHR agonists.

Keywords

Aryl hydrocarbon receptor agonist; Cardiotoxicity; Zebrafish; Teratogenesis; Cytochrome P450; Morpholino

1. Introduction

The best-characterized high affinity ligands for the aryl hydrocarbon receptor (AHR) include a variety of anthropogenic and naturally occurring toxic and hydrophobic chemicals, such as certain polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls, and many polycyclic aromatic hydrocarbons (PAHs) (Poland and Knutson 1982; Safe 1990; Gillner, Bergman et al. 1993; Kafafi, Afeefy et al. 1993; Waller and McKinney 1995). However, a large number of chemicals can bind to the AHR and/or induce AHR-dependent gene expression (Denison and Heath-Pagliuso 1998; Denison and Nagy 2003; Nguyen and Bradfield 2008). Several weaker AHR agonists have been identified and some are environmental contaminants (Denison and Nagy 2003). The goal of this study is to determine whether CYP1A is protective against the toxicity of weak AHR agonists.

Strong agonists of the AHR (e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-TCDD and the polychlorinated biphenyl, PCB-126), cause cardiac teratogenesis, namely pericardial edema and stringy heart in embryonic fish (Wassenberg and Di Giulio 2004; Antkiewicz, Burns et al. 2005). Complex hydrocarbon mixtures are also capable of causing cardiac defects (Meyer and Di Giulio 2002; Meyer, Nacci et al. 2002; Incardona, Collier et al. 2004; Wassenberg and Di Giulio 2004; Incardona, Carls et al. 2009). Incardona *et al.* (2004, 2009) demonstrated that low levels of weathered crude oil exposure causes cardiac dysfunction and pericardial edema in developing zebrafish (*Danio rerio*) and herring (*Culpea harengus*). Polycyclic aromatic hydrocarbon (PAH)-contaminated sediments from the Elizabeth River

in Portsmouth, Virginia, cause cardiac toxicity, including the stringy heart phenotype and cardiac effusion, in the Atlantic killifish (*Fundulus heteroclitus*) (Meyer and Di Giulio 2002; Wassenberg and Di Giulio 2004).

Current risk assessment models for PAH exposures assume that toxicity is additive (Barron, Carls et al. 2004); however, research has demonstrated that mixtures can result in synergistic embryotoxicity, specifically pericardial edema and stringy heart. In previous studies, we have shown that co-exposure to a PAH that is a moderate AHR ligand such as benzo[a]pyrene (BaP), benzo[k]fluoranthene (BkF), or the model AHR agonist β -naphthoflavone (BNF) and one that is a cytochrome P450 1A (CYP1A) inhibitor such as fluoranthene (FL) or the model PAH α -naphthoflavone (ANF) also cause cardiac effects through the AHR in embryos of zebrafish (*Danio rerio*) (Billiard, Timme-Laragy et al. 2006; Van Tiem and Di Giulio 2011) and Atlantic killifish (*Fundulus heteroclitus*) (Wassenberg and Di Giulio 2004). Effects of these binary exposures are significantly greater-than-additive (Wassenberg and Di Giulio 2004; Wassenberg, Nerlinger et al. 2005). Billiard et al. (2006) demonstrated that CYP1A knockdown exacerbated AHR agonist toxicity in zebrafish. This synergy is in marked contrast to responses with combined exposures to TCDD and CYP1A inhibitors, where enzyme inhibition or protein knockdown either reduces (Cantrell, Lutz et al. 1996; Dong, Teraoka et al. 2002) or does not alter toxic response to TCDD during zebrafish development (Carney, Peterson et al. 2004). Clark et al. (2010) demonstrated that the AHR2 is a primary mediator of cardiac teratogenesis caused by some PAHs in the Atlantic killifish. The synergy we have observed raises compelling questions concerning mechanisms underlying these greater-than-additive toxicities and the role of the AHR and CYP1A activity in mediating cardiotoxicity.

Studies have shown that the AHR is a promiscuous receptor and several weak agonists including potential endogenous ligands have been identified (Denison and Heath-Pagliuso 1998; Denison and Nagy 2003; Nguyen and Bradfield 2008). There are two key issues that have emerged from studying the cardiotoxic impacts of AHR agonists (1) Whether all AHR ligands (strong or weak) cause cardiotoxicity through the AHR (like dioxin/PCBs) and (2) Whether there are metabolic interactions between AHR ligands CYP1A metabolism that are driving toxicity. The effects of exposure to weak AHR agonists in conjunction with CYP1A inhibition have not been well studied. Therefore, the goals of this study were to determine if CYP1A is protective of cardiac toxicity or if it instead, contributes to toxicity by producing toxic metabolites. Weak AHR agonists were chosen based on literature from a variety of *in vitro* and cell based publications that have reported novel AHR-ligand interactions (Chen, Riby et al. 1995; Wei, Helleberg et al. 1998; Adachi, Mori et al. 2001; Adachi, Mori et al. 2004; Incardona, Carls et al. 2005; Bohonowych and Denison 2007; Nguyen and Bradfield 2008). Some of the compounds used in this study are potential endogenous ligands for the AHR (indigo and indirubin) (Chen, Riby et al. 1995; Wei, Helleberg et al. 1998; Adachi, Mori et al. 2001; Adachi, Mori et al. 2004) and others are anthropogenic environmental contaminants (carbaryl, phenanthrene, and fluoranthene) (Denison and Heath-Pagliuso 1998; Phelan, Winter et al. 1998; Incardona, Carls et al. 2005; Denison, Soshilov et al. 2011). To this end, zebrafish embryos were exposed to the weak agonists carbaryl (Carb), phenanthrene (Phe), 2-methylindole (2MI), 3-methylindole (3MI), indigo, and indirubin

individually, and CYP1A activity and cardiac toxicity were measured. Zebrafish embryos were exposed to AHR agonists at equimolar concentrations to assess their relative potency as inducers of CYP1 enzyme activity, measured by the EROD assay, and cardiac deformities. Embryos were subsequently co-exposed to each agonist and FL in combination to assess if CYP1A inhibition could enhance cardiac deformities. A morpholino approach was also used to knockdown CYP1A, and embryos were then exposed to each agonist individually. AHR2 morpholino knockdown was used to determine if the cardiac teratogenesis caused by co-exposure of the weak agonists and FL could be rescued, as has been observed with more potent PAH-based agonists (Clark, Matson et al. 2010; Van Tiem and Di Giulio 2011). Agonists were used at concentrations that did not cause cardiac teratogenesis when administered individually (however, provisional experiments did show that some agonists were capable of producing cardiac teratogenesis at high concentrations by themselves, i.e. carbaryl). Some agonists did cause cardiac teratogenesis when coupled with CYP1A inhibition or knockdown; however, AHR2 morpholino injection did not rescue any of the observed cardiac deformities.

2. Materials and methods

2.1. Fish care

Adult Ekkwill zebrafish (*D. rerio*; Ekkwill Waterlife Resources, Ruskin, FL, USA) were maintained at 28 °C in a recirculating AHAB system (Aquatic Habitats, Apopka, FL, USA) under a 14:10 h light:dark cycle. Adults received a diet of brine shrimp and a mix of Ziegler's Adult Zebrafish Complete Diet (Aquatic Habitats, Apopka, FL, USA) and Cyclop-eeze (Argent Chemical Laboratories, Redmond, WA, USA). Embryos were collected after natural spawning of adult zebrafish and were maintained in 30% Danieau (Nasevicius and Ekker 2000) at 28 °C and the same photoperiod as adults. Adult care and reproductive techniques were non-invasive and approved by the Duke University Institutional Animal Care & Use Committee (A279-08-10).

2.2. Chemicals and exposure

Fluoranthene (FL) was purchased from AccuStandard (Hamden, CT, USA). Dimethyl sulfoxide (DMSO), 7-ethoxyresorufin (7-ER), carbaryl (Carb), phenanthrene (Phe), 2-methylindole (2MI), 3-methylindole (3MI), indigo, indirubin, and tricaine methanesulfonate (MS-222) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All standards were dissolved in DMSO and kept at -20 °C in a vial box for protection from light until use.

Embryos exhibiting normal development were exposed at 24 hours post fertilization (hpf) in 7.5 mL 30% Danieau in 20-mL glass scintillation vials, with five embryos per vial and three vials per treatment. For equimolar potency experiments examining CYP1 activity via the 7-ethoxyresorufin-O-deethylase (EROD) assay, embryos were exposed to 0.1, 1.0, and 10 µM concentrations of each weak agonist. The EROD substrate 7-ER was added at a final concentration of 21 µg/L at the time of exposures.

All weak agonists were assessed in a series of dose-response experiments to determine threshold concentrations (for subsequent co-exposure experiments) at which no increased pericardial edema was observed relative to DMSO controls (data not shown; concentrations

for co-exposures are listed below). In co-exposure experiments, embryos were exposed to DMSO, 500 µg/L FL, or a co-exposure of a weak agonist (5 µM Carb, 6 µM Phe, 75 µM 2MI, 50 µM 3MI, 50 µM Indigo, and 50 µM Indirubin) and 500 µg/L FL. The final DMSO concentration was 0.03% in all experiments. Embryos remained in the initial dosing solutions until the deformity assessment and EROD assay were performed at 96 hpf. Agonists were combined with 500 µg/L FL (which does not cause pericardial edema by itself) in co-exposure experiments to evaluate if the combination caused significant pericardial edema.

2.3. Morpholino gene knockdown

In separate experiments, translation-blocking morpholinos were used to knockdown either CYP1A or AHR2. The sequence of the AHR2-morpholino is 5'-TGTACCGATACCCGCCGACATGGTT-3' (Teraoka, Dong et al. 2003), and the sequence of the CYP 1A-morpholino is 5'TGGATACTTTCCAGTTCTCAGCTCT-3' (Carney, Peterson et al. 2004). Gene Tools' standard control morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a morpholino injection control. The morpholinos were fluorescein-tagged at the 3' end to monitor injection success and were diluted to 100 µM working stocks in 30% Danieau. The morpholinos (approximately 3 nL) were injected into the yolk of zebrafish embryos at the 1–4 cell stage using a microinjection system consisting of a Nikon SMZ-1500 zoom stereomicroscope (Nikon Instruments Inc., Lewisville, TX, USA) and an MDI PM 1000 Cell Microinjector (MicroData Instrument Inc., S. Plainfield, NJ, USA). Embryos exhibiting normal development and strong, uniform incorporation of the morpholinos were used for experiments and dosed as described in section 2.2.

2.4. *In vivo* EROD assay for equimolar potency

At 96 hpf, CYP1 activity was measured via a modified *in vivo* EROD assay (Nacci, Coiro et al. 1998; Billiard, Timme-Laragy et al. 2006). The CYP1 enzymes metabolize 7-ER into a fluorescent product, resorufin, which is visible predominantly in the gastrointestinal tract in zebrafish (Billiard, Timme-Laragy et al. 2006). Fluorescence was measured under 50× magnification using a rhodamine red filter set (Zeiss Axioskop, Thornwood, NY, USA) and quantified by IPLab software (Scanalytics Inc., Fairfax, VA, USA). EROD values in equimolar potency experiments are expressed as a percentage of the mean fluorescence relative to control embryos.

2.5. Deformity assessment

At 96 hpf, eleutheroembryo zebrafish were screened for cardiac deformities via measurement of pericardial edema (Billiard et al., 2006). Fish were rinsed with 30% Danieau and anesthetized with MS-222. Fish were then placed in a left lateral orientation in 3% methylcellulose on depression slides and imaged under 50× magnification (Zeiss Axioskop, Thornwood, NY, USA). The two-dimensional image of the pericardial area was manually traced and then quantified using IPLab software (Scanalytics Inc., Fairfax, VA, USA). Deformity values are expressed as a percentage of the two-dimensional pericardial area of non-injected (NI) control embryos.

2.6. Statistical analysis

Co-exposure experiments assessing deformities were repeated three times with 3 vials of 5 embryos per experiment ($n = 3$ per experimental replicate, three experimental reps for a total $n = 9$). Equimolar potency was determined in the same fashion ($n = 3$ per experimental rep, three experimental replicates for a total $n = 9$). Both experiments with CYP1A morpholino and AHR2 morpholino were repeated seven times ($n = 3$ per experimental rep, experiment total $n = 21$). Statistical analyses were performed using JMP 10.1.1 (SAS Institute Inc., Cary, NC, USA). For deformity and EROD analysis, data were analyzed via one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post-hoc test. For morpholino experiments, the data were analyzed via two-way ANOVA to determine an overall effect of the morpholino injection and dose followed by a Tukey-Kramer post-hoc test. No differences between experimental replicates were observed for any test. Data are represented as mean $\pm$ standard error of the mean (SEM). Values are considered significantly different at $p < 0.05$.

3. Results

3.1. Equimolar potency assessment

The rank order of CYP1A activity was Carb>3MI>2MI>Phe = Indigo = Indirubin. All concentrations of Carb, 2MI, and 3MI caused significant EROD induction compared to 0.1 μ M DMSO ($p < 0.05$) (10 μ M percent mean induction $\pm$ SEM: 2MI $266 \pm 11\%$, and 3MI $351 \pm 8\%$ Carb $354 \pm 11\%$ DMSO control) (Figure 1). Indigo and indirubin exposure produced weaker induction of EROD activities that were not statistically different from DMSO controls. Higher concentrations of indigo and indirubin were not studied because of solubility limits.

3.2. Cardiac toxicity caused by co-exposure to weak AHR agonists and FL

Pericardial edema in embryos exposed to the individual weak agonists or to FL alone did not differ significantly from that of the DMSO-exposed zebrafish (Figure 2). However, co-exposure of embryos to Carb, 2MI, 3MI, and Phe with FL significantly increased pericardial edema relative to DMSO controls ($p < 0.05$) (percent mean $\pm$ SEM: Carb $189 \pm 10\%$, 2MI $154 \pm 5\%$, 3MI $199 \pm 8\%$, Phe $172 \pm 6\%$). Indigo and indirubin did not cause pericardial edema greater than controls even when co-exposed with FL at concentrations nearing solubility limits (data not shown).

3.3. CYP1A knockdown has mixed effects

Based on the co-exposure experiments and previous studies, we sought to determine if weak AHR agonists and CYP1A knockdown by morpholino could produce similar pericardial edema. The effect of CYP1A knockdown only replicated the effects of FL and 2MI, and differed from FL's effect on 3MI, Carb, and Phe (Figure 3). Toxicity (edema) was more severe for 3MI, Phe, and Carb in combination with FL compared to CYP1A knockdown. CYP1A knockdown in 2MI and 3MI caused pericardial edema that was significantly higher than the effusion in non-injected (NI) DMSO controls and in NI embryos exposed to the weak agonists alone ($p < 0.05$). CYP1A knockdown in 2MI exposed embryos caused the same pericardial edema severity as NI embryos exposed to 2MI + FL (2MI >150%

compared to DMSO controls). 3MI and Phe exhibited enhanced pericardial edema with CYP1A knockdown relative to controls, but did not reach the levels observed in FL co-exposed embryos (3MI >150% and Phe > 125% effusion relative to DMSO controls). Carb exposure and CYP1A knockdown did not cause significantly different pericardial edema compared to controls. This lack of effect in Carb exposed CYP1A knockdown embryos is unique from the pericardial edema caused by Carb + FL co-exposure. CYP1A knockdown also did not affect the toxicity of indigo and indirubin. This corresponds to the absence of response in co-expose with FL.

3.4. AHR2 knockdown does not rescue pericardial edema caused by weak agonist and FL co-exposure

AHR2-morpholino knockdown did not differ from pericardial edema found in weak agonist + FL co-exposed embryos across treatment groups (Figure 4).

4. Discussion

Morphological defects caused by exposure to strong AHR agonist have been well-characterized and demonstrate consistencies in cardiac abnormalities among fish species (Walker, Spitsbergen et al. 1991; Henry, Spitsbergen et al. 1997; Marty, Short et al. 1997; Middaugh, Shelton et al. 1998; Carls, Rice et al. 1999; Vines, Robbins et al. 2000; Andreasen, Spitsbergen et al. 2002). In this study, four weaker AHR agonists significantly increased pericardial edema (Figure 2: Carb, 2MI, 3MI, and Phe) when coupled with FL. Of those compounds, three showed significant EROD induction in the dose response experiment (Figure 1: Carb, 2MI, and 3MI). Interestingly, 2MI and 3MI caused significant pericardial edema following CYP1A morpholino-injection ($p < 0.05$), but Carb did not (Figure 3). AHR2-morpholino knockdown did not rescue FL co-exposed embryos across all treatment groups. Looking at the compounds on a case-by-case basis highlights the mechanistic complexity of the relationship between AHR agonism and CYP1A inhibition.

Both 2MI and 3MI are members of the indole family and are tryptophan derivatives. 3MI is a pneumotoxicant found in cigarette smoke that is known to cause DNA adducts (Weems, Cutler et al. 2009). 2MI is used as an intermediate for synthesizing dyes, pigments, optical brighteners, and pharmaceuticals. Indoles have been shown to interact with the AHR-complex (Chen, Riby et al. 1995; Wei, Helleberg et al. 1998). Some indoles, such as 2MI and 3MI, have been shown to increase expression of monooxygenases associated with the AHR pathway and competitively bind to the AHR (Song, Clagett-Dame et al. 2002; D'Agostino, Zhuo et al. 2009; Weems, Cutler et al. 2009). In this study, 2MI and 3MI induced CYP1A activity, caused cardiac deformities when combined with FL, and caused pericardial edema in CYP1A-morpholino embryos. However, AHR2-morpholino was not able to rescue pericardial edema in exposed embryos.

Carbaryl (Carb) is a widely used carbamate insecticide that has been shown to induce CYP1A1 gene transcription in transfected mouse, rat, guinea pig, and human cells. Carb can cause in vitro AHR-dependent luciferase expression, bind to the AHR complex, and compete with TCDD for binding (Denison and Nagy 2003; Bohonowych and Denison 2007). In addition to causing neurotoxicity via inhibition of acetylcholinesterase (Fukuto

1990), acute exposure to Carb can be teratogenic and/or affect physiological functions in aquatic organisms causing pericardial deformities (Lin, Hui et al. 2007; Clark and Di Giulio 2012). Casado et al. (2006) showed that Carb could activate the AHR in rat H4IIE cells and demonstrated that AHR activation could occur following Carb exposure in a cell-free *in vitro* system. Pericardial edema and bradycardia have been observed in zebrafish exposed to carbaryl (10 µg/ml) and bradycardia in zebrafish (Casado, Alonso et al. 2006; Lin, Hui et al. 2007). Carb has been shown to induce significant CYP1 expression at 1 mg/L and cardiac deformities at 10 mg/L in killifish (Clark and Di Giulio 2012). Studies in PAH-adapted and non-adapted killifish suggest that Carb is able to weakly stimulate cytochrome P450s; however, the observed cardiotoxicity was not fully attributable to an AHR dependent mechanism (Clark and Di Giulio 2012). Heart malformations, irregular heartbeat and oscillating blood in the heart have also been observed in Japanese medaka, *Oryzias latipes* following exposure to Carb (Solomon and Weis 1979). While co-exposure to Carb plus FL caused cardiac effects in zebrafish, the same toxicity was not observed when CYP1A-morpholino embryos were exposed to Carb. This suggests that while Carb can weakly activate CYP1A1, knockdown of CYP1A does not enhance Carb toxicity. These findings in concert with the observed enhancement of pericardial edema following Carb and FL coexposure imply that FL's contribution to toxicity may be more complicated than CYP1A inhibition alone. Thus for carbaryl, although it may induce a low level of CYP1A, it does not appear that CYP1A is the major detoxifying pathway, otherwise CYP1A morphants exposed to carb would have pericardial edema. This is further supported by the failure of AHR2 knockdown to rescue pericardial edema following co-exposure.

Phe did not significantly induce EROD activity in the equimolar assay; however, deformities were observed in embryos co-exposed to Phe in combination with FL. While Phe did not induce CYP1A activity in the trout liver cell line RTL-W1 (Billiard, Timme-Laragy et al. 2006) Phe weakly induced CYP1A response in zebrafish heart vessels proximal to the heart (Incardona, Carls et al. 2005). Incardona et al. (2005) also showed that isolated 28 µM Phe exposure (which is above solubility limits for Phe and higher concentrations used in this study) caused pericardial edema and that the phenotype could not be rescued by AHR2-morpholino. Mu et al. (2012) found that concentrations >50 µg/L Phe are necessary to begin detecting significant increases in EROD response in the marine medaka (*Oryzias melastigma*), with cardiac deformities occurring at higher concentrations (100 and 500 µg/L). However, when embryonic medaka were exposed to Phe and the CYP1A inhibitor α -naphthoflavone (ANF), cardiac deformities increased synergistically (50 µg/L Phe and 100 µg/L ANF) (Mu, Wang et al. 2012). Our results with Phe and FL co-exposure (6 µM Phe + 500 µg/L FL) were similar to the Phe and ANF co-exposure results reported by Mu et al. (2012). Our study suggests that CYP1A metabolism is detoxifying AHR agonists and that inhibition and/or alteration of CYP1A metabolism can enhance toxicity. Lastly, Phe and FL induced pericardial edema could not be rescued by AHR2-morpholino.

Indigoids have been isolated from human urine and bovine serum, and are plant derivatives used for textile coloring. Indigo is suspected to enter aquatic environments through improper disposal of dyeing agents, and a number of spills have been observed in Chinese blue jeans factories (Zeller, 2011). Indigo and indirubin have been suggested to be endogenous AHR

agonists (Adachi et al. 2001), and studies show that they bind to the AHR and upregulate CYP1A1 monooxygenase activity (Guengerich, Martin et al. 2004; Sugihara, Kitamura et al. 2004). Indirubin, the more potent AHR agonist of the two, has an EC₅₀ for AHR activation of 0.2 nM when measured in a yeast reporter system (Adachi, Mori et al. 2001). However, the potency of indirubin was established to be 100-fold lower than that of TCDD in mammalian cells (Guengerich, Martin et al. 2004). In our study, indigo showed comparable dose-response AHR agonism (as indicated by EROD) to Phe, and indirubin elicited decreased EROD response relative to controls exposed to DMSO. Due to the CYP1A-mediated metabolism of this compound in mammalian systems (Spink, Hussain et al. 2003; Wu, Spink et al. 2003; Guengerich, Martin et al. 2004; Spink, Wu et al. 2008), the length of ligand exposure and cell type could contribute to an observed difference in AHR activation exhibited via the EROD assay. Embryonic zebrafish did not demonstrate strong AHR activation following exposure to indigo or indirubin. The lack of observable toxicity from indigo or indirubin coupled with FL in our study was peculiar because other studies have demonstrated AHR activity follow exposure. It would appear that the zebrafish AHR does not bind indigo and indirubin or that these compounds are not penetrating the chorion of the embryo.

CYP1A morpholino knockdown coupled with Carb, 2MI, and 3MI caused pericardial edema. These data suggest that activation of the cytochrome P450s is important to the detoxification process. When CYP1A is functional, these parent compounds are likely metabolized to less toxic metabolites and/or the metabolic process does not generate detrimental concentrations of metabolites. However, when CYP1A is knocked down, the metabolic process is potentially altered, generating more harmful metabolites and/or extending the half-life of the parent compound. AHR2-morpholino knockdown did not rescue pericardial edema observed in agonist + FL coexposed embryos.

The weak agonists that elicited higher CYP1A enzyme activities, such as Carb, 2MI, and 3MI, were more likely to cause cardiac deformities when combined with FL than agonists with lower potency, such as indigo and indirubin (Phe being the exception). This corroborates earlier research findings demonstrating that co-exposure to a PAH that is a moderate AHR ligand (such as BaP, BkF, or BNF) and one that is a CYP inhibitor (such as FL or α -ANF) can cause severe pericardial edema and the stringy heart (Wassenberg and Di Giulio 2004; Billiard, Timme-Laragy et al. 2006; Van Tiem and Di Giulio 2011; Mu, Wang et al. 2012). However, AHR2 morpholino knockdown did not rescue the deformities observed by combining weak AHR agonists with FL. Therefore, our study suggests that while some weak AHR agonists can generate pericardial edema phenotypes similar to stronger AHR agonists when combined with a CYP1A inhibitor, the mechanism underlying these deformities does not appear to be closely associated with the AHR pathway. The finding that CYP1A1 morpholino injection can enhance deformities supports the notion that CYP1A is necessary for detoxifying several of the weak agonists to less toxic forms and/or reducing their half-lives and/or changing the suite of metabolites produced. The initial characterization of FL as a CYP1A inhibitor (Willett, Wassenberg et al. 2001) suggested that it acted at the level of protein stability, and not as a true enzymatic active site inhibitor. Because morpholino knockdown actually acts at the level of protein translation, one would expect morpholino to be better at specifically targeting and inhibiting CYP1A than FL.

Although CYP1A knockdown may be sufficient to enhance deformities, FL co-exposure in this study suggests that FL may have additional mechanistic impacts that go beyond CYP1A inhibition.

Our study found no reduction in pericardial edema following AHR2-morpholino injection. These findings argue that either an AHR independent mechanism is accountable for the observed toxicity or that AHR2-morpholino knockdown may not have been enough to mitigate the consequences of these exposures. Morpholino injections are knockdowns and not knockouts. To fully evaluate this question, Western blots following AHR2-morpholino injection would need to be performed to see the level to which AHR is knocked down. Additionally, it is important to note that zebrafish have multiple copies of the AHR (Andreasen, Spitsbergen et al. 2002; Karchner, Franks et al. 2005; Garner, Brown et al. 2013). Therefore, future studies should consider investigating the role of other AHRs (both individually and in combination) in mitigating this observed pericardial edema following co-exposure to weak agonists and CYP1A inhibitors. In addition, other measures of cardiac function may provide more resolution than just the pericardial area measures of edema.

This study demonstrated that certain weak AHR agonists are capable of causing cardiac teratogenesis in zebrafish when combined with a CYP1A inhibitor. While the specific mechanisms underlying this observed toxicity remain unclear, the findings suggest that the toxicity is likely mediated by an AHR-independent mechanism. Furthermore, the results suggest that CYP1A metabolism is important for detoxification of weak AHR agonists and alteration of metabolism can lead to pericardial edema. Carb, 2MI, and 3MI induced CYPs and Phe, 2MI, and 3MI caused pericardial edema with CYP1A-morpholino knockdown. Further experiments should determine why the observed co-exposures with FL can cause cardiac teratogenesis that is similar to AHR mediated toxicity (observed with TCDD and some PCBs and PAHs). Other CYPs may also play a role in the observed teratogenesis, and metabolites of these compounds should be investigated. FL is clearly a compound in need of more mechanistic characterization, specifically when accompanying other exposures. Future studies should also investigate the role of other AHRs in mediating the observed toxicity as well as other plausible metabolic pathways. Lastly, continuing to study mixtures is vital given the sensitivity of the developing fish heart and the mechanistic complexity underlying cardiac teratogenesis.

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Figure 1. Dose response EROD expression in larval zebrafish following exposure to weak agonists: carbaryl (Carb), 2-methylindole (2MI), 3-methylindole (3MI), phenanthrene (Phe), indigo, and indirubin at 3 equimolar concentrations (0.1, 1, and 10 μM, the white, grey, and black bars respectively)

The EROD assay was performed at 96 hpf to compare cytochrome P450 activity in response to weak agonist exposure (measured by fluorescence and compared to DMSO controls). The EROD assay was repeated three times with 3 vials of 5 embryos per experiment ($n = 3$ per experiment, experiment total $n = 9$). Each n represents the average of five embryos. Asterisk indicates significantly different than DMSO EROD activity ($p < 0.05$).

Figure 2. Mean pericardial edema in larval zebrafish following exposure to weak agonists: Carb, 2MI, 3MI, Phe, indigo, and indirubin (white bars, numbers preceding treatments are μM concentrations)

The black bars represent agonists combined with 500 μg/L fluoranthene (FL). Values are expressed as percent DMSO pericardial edema ± SEM. The graph represents three experimental replicates with 3 vials of 5 embryos per experiment ($n = 3$ per experiment, experiment total $n = 9$). Each n represents the average of five embryos. Asterisks indicate that the treatment is statistically different than DMSO controls ($p < 0.05$).

Figure 3. Mean pericardial edema in larval zebrafish following exposure to weak agonists: Carb, 2MI, 3MI, Phe, indigo, and indirubin

White bars are non-injected (NI) embryos treated with isolated weak agonists. The grey bars are control morpholino (Co-mo) injected embryos. Black bars represent agonists combined with 500 μg/L fluoranthene (FL). Patterned bars are CYP1A-mo injected embryos that received exposure to individual agonists. Values are expressed as percent DMSO pericardial edema ± SEM. The graph represents three experimental replicates with 3 vials of 5 embryos per experiment (n = 3 per experiment, experiment total n = 9). Groups not sharing common letters are statistically different ($p < 0.05$).

Figure 4. Mean pericardial edema in larval zebrafish following exposure to weak agonists: Carb, 2MI, 3MI, Phe, indigo, and indirubin

Numbers preceding treatments are μM concentrations. White bars are non-injected (NI) embryos treated with isolated weak agonists. The grey bars are control morpholino (Co) injected embryos. Black bars are AHR2-mo knockdown embryos. Values are expressed as percent DMSO pericardial edema ± SEM. The graph represents three experimental replicates with 3 vials of 5 embryos per experiment (n = 3 per experiment, experiment total n = 9). Groups not sharing common letters are statistically different ($p < 0.05$).