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Mono-substituted isopropylated triaryl phosphate, a major component of Firemaster 550, is an AHR agonist that exhibits AHR-independent cardiotoxicity in zebrafish

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ABSTRACT

Firemaster 550 (FM550) is an additive flame retardant mixture used within polyurethane foam and is increasingly found in house dust and the environment due to leaching. Despite the widespread use of FM550, very few studies have investigated the potential toxicity of its ingredients during early vertebrate development. In the current study, we sought to specifically investigate mono-substituted isopropylated triaryl phosphate (mITP), a component comprising approximately 32% of FM550, which has been shown to cause cardiotoxicity during zebrafish embryogenesis. Previous research showed that developmental defects are rescued using an aryl hydrocarbon receptor (AHR) antagonist (CH223191), suggesting that mITP-induced toxicity was AHR-dependent. As zebrafish have three known AHR isoforms, we used a functional AHR2 knockout line along with AHR1A- and AHR1B-specific morpholinos to determine which AHR isoform, if any, mediates mITP-induced cardiotoxicity. As *in silico* structural homology modeling predicted that mITP may bind favorably to both AHR2 and AHR1B isoforms, we evaluated AHR involvement *in vivo* by measuring CYP1A mRNA and protein expression following exposure to mITP in the presence or absence of CH223191 or AHR-specific morpholinos. Based on these studies, we found that mITP interacts with both AHR2 and AHR1B isoforms to induce CYP1A expression. However, while CH223191 blocked mITP-induced CYP1A induction and cardiotoxicity, knockdown of all three AHR isoforms failed to block mITP-induced cardiotoxicity in the absence of detectable CYP1A induction. Overall, these results suggest that, while mITP is an AHR agonist, mITP causes AHR-independent cardiotoxicity through a pathway that is also antagonized by CH223191.

1. Introduction

In 2004, the commercial polybrominated diphenyl ether (PBDE) mixture known as PentaBDE – a widely used brominated flame retardant (FR) – was voluntarily phased out in the United States due to concerns about persistence, bioaccumulation, and toxicity (Rahman et al., 2001; Tullo, 2003). As a result of strict fire safety standards set for low-density polyurethane foam in residential furniture and baby products, brominated and aryl phosphate ester (APE) components of the replacement FR mixture formulation known as Firemaster 550 (FM550) have been detected at concentrations comparable to and, in some cases, higher than total PBDE concentrations in household dust (Meeker and Stapleton, 2010; Springer et al., 2012; Stapleton et al., 2008, 2009). Moreover, brominated components of FM550 have been found in municipal sewage (Davis et al., 2012), urban and rural air samples (Ma et al., 2012), and marine mammal tissue (Lam et al., 2009). The high detection of FM550 components suggests that chronic human exposure following migration from treated end-use products is common within the US. Therefore, similar to concerns about PBDEs (Costa and Giordano, 2007; Herbstman et al., 2010), a better understanding of the potential effects of prenatal FM550 exposure

Abbreviations: AHR, aryl hydrocarbon receptor; CYP1A, cytochrome P450 1A; FM550, Firemaster 550; IHC, immunohistochemistry; LBD, ligand-binding domain; mITP, mono-substituted isopropylated triaryl phosphate; MO, morpholino; PAH, polycyclic aromatic hydrocarbon; qPCR, quantitative polymerase chain reaction; PE, pericardial edema; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TPP, triphenyl phosphate.

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resulting from maternal ingestion of contaminated house dust is needed since these stages may be more susceptible relative to later periods of development.

Using zebrafish (*Danio rerio*) as a model, McGee et al. (2013) recently evaluated the potential developmental toxicity of brominated and APE components present within FM550. The brominated component of FM550 consists of 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB, ~30%) and bis-(2-ethylhexyl)-tetrabromophthalate (TBPH, ~8%), whereas the APE component (~62%) consists of triphenyl phosphate (TPP, ~17%) and isopropylated triaryl phosphates (ITPs, ~45%) (McGee et al., 2013). The ITP component is a complex mixture of *ortho*-, *meta*-, and *para*-substituted isomers of mono-, di-, tri-, and tetra-ITPs comprising approximately 32%, 10%, 2.4%, and 0.4% of FM550, respectively (McGee et al., 2013). Within their study, McGee et al. (2013) demonstrated that exposure to TPP and mono-ITP (mITP) – but not TBB, TBPH, di-ITP, nor tri-ITP – resulted in severe pericardial edema (PE) and blocked normal looping of the atrium and ventricle, resulting in a "tube heart" phenotype.

Using an aryl hydrocarbon receptor (AHR) antagonist (CH223191), McGee et al. (2013) reported that mITP-induced – but not TPP-induced – cardiac abnormalities and cytochrome P450 1A (CYP1A) expression within zebrafish embryos were aryl hydrocarbon receptor (AHR)-dependent. While mammals have only one AHR, zebrafish have three AHR isoforms: AHR1A, AHR1B, and AHR2 (Andreasen et al., 2002; Karchner et al., 2005; Tanguay et al., 1999). AHR2 is the functional AHR paralog in zebrafish that mediates toxicity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Prasch et al., 2003), 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) (Jönsson et al., 2012), and many polycyclic aromatic hydrocarbons (PAHs) (Incardona et al., 2006). Although the functionalities of AHR1B and AHR1A are not well characterized, emerging evidence suggests that some PAH mixtures, PCB126, and the non-classical AHR agonist leflunomide can activate AHR1A and/or AHR1B, induce CYP1A expression, and mediate developmental toxicity (Garner et al., 2013; Goodale et al., 2012; Incardona et al., 2006, 2011). Therefore, McGee et al. (2013) began investigating the AHR-isoform dependence of mITP in zebrafish using an AHR2-specific translation-blocking morpholino (MO). However, AHR2 knockdown failed to block mITP-induced cardiac abnormalities, suggesting that this phenotype was mediated through an AHR2-independent pathway.

As mITP-induced cardiotoxicity and CYP1A induction were blocked by CH223191 but not AHR2 knockdown, one of the key questions arising from this study was whether cardiotoxicity is mediated by an AHR1A and/or AHR1B-dependent pathway. Therefore, we first used *in silico* structural modeling – an approach previously used to predict AHR ligand-binding (Bisson et al., 2009; Goodale et al., 2012) – to determine whether mITP would dock favorably with the AHR2, AHR1A, and AHR1B ligand-binding domains (LBDs). *In vivo* studies using zebrafish were then conducted to determine whether AHR1A and/or AHR1B contribute to mITP-induced cardiotoxicity during early embryonic development. In order to eliminate the potential for incomplete AHR2 knockdown with transient MOs, and to better investigate the individual roles of AHR1A and AHR1B isoforms, a functional zebrafish AHR2 knockout line was utilized.

2. Materials and methods

2.1. Chemicals

mITP (≥90%) (Fig. 1) was originally provided as FM550 (Chemtura) via Dr. Susan Klosterhaus (Cradle to Cradle Products Innovation Institute, San Francisco, CA) and was purified by Wellington Laboratories (Guelph, Ontario, Canada) as previously described (McGee et al., 2013). Due to the lack of commercially available analytical standards for mITPs, exact ratios of mITP congeners (*ortho*, *meta*, *para*) within our mITP fraction are unknown. The AHR antagonist, 2-methyl-2*H*-pyrazole-3-carboxylic acid (2-methyl-4-*o*-tolylazo-phenyl)-amide (CH223191, >99%) (Fig. 1), was purchased from Tocris Bioscience (Ellisville, MO). All stock solutions were dissolved in ACS–grade dimethyl sulfoxide (DMSO).

2.2. Zebrafish lines and embryos

All adult zebrafish were housed in accordance to approved Institutional Animal Care and Use Committee (IACUC) protocols at Oregon State University on a recirculating water system at 28 ± 1 °C with a 14-h light/10-h dark schedule. Embryos from adult 5D zebrafish were obtained from group spawns as previously described (Reimers et al., 2006). All *ahr2*^{hu3335} embryos were obtained from gated crosses with ~1:1 ratio of male to female. All embryos were collected within 1-h post fertilization (hpf), rinsed, and kept in petri dishes with E2 embryo medium at 28 ± 1 °C until treatment. The *ahr2*^{hu3335} line were mutagenized via Targeting Induced Local Lesions IN Genomes as previously described (Goodale et al., 2012).

2.3. Molecular modeling and docking

Homology models of human AHR (hAHR), and zebrafish AHR2, AHR1A, and AHR1B LBDs in the *apo* conformation were initially built as previously reported (Bisson et al., 2009; Goodale et al., 2012). TCDD, CH223191, and *ortho*, *meta*, and *para* mITP congeners were docked into hAHR, zebrafish AHR2, AHR1A, and AHR1B. *In vivo* studies have shown TCDD is a strong agonist for only AHR2 and AHR1B (Andreasen et al., 2002; Goodale et al., 2012; Karchner et al., 2005). The co-bound models were then submitted to 10^5 steps ligand-protein side chain optimization through Monte Carlo (MC) simulations in the internal coordinate space with Molsoft ICM (Katritch et al., 2012; manuscript submitted). The highest rank energy complex obtained from each simulation was used for molecular docking. Both tautomerization states (HisD and HisE) of human residue His 291 and zebrafish AHR2 and AHR1B His 296 homologues were considered.

Each receptor was represented by five types of interaction potentials: (i) van der Waals potential for a hydrogen atom probe; (ii) van der Waals potential for a heavy-atom probe (generic carbon of 1.7 Å radius); (iii) optimized electrostatic term; (iv) hydrophobic terms; and (v) lone-pair-based potential, which reflects directional preferences in hydrogen bonding. The energy terms were based on the Merck molecular force field (MMRF) to account for solvation free energy and entropic contribution (Totrov and Abagyan, 2001). Modified intermolecular terms such as soft van der Waals, hydrogen bonding, and hydrophobicity were added. Conformational sampling was based on the biased probability Monte Carlo (BPMC) procedure, which randomly selects a conformation in the internal coordinate space and then initiates a step to a new random position independent of the previous one – but according to a predefined continuous probability distribution – since, after each random step, full local minimization greatly improves the efficiency of the procedure. In the ICM–VLS (Molsoft ICM) screening procedure, the ligand scoring was optimized to obtain maximal separation between binders and non-binders. Based on fit within the receptor, each compound was assigned an ICM score that accounts for continuum and discreet electrostatics as well as hydrophobicity and entropy parameters (Totrov and Abagyan, 2001; Abagyan et al., 1994).