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AOP 21: Activation of the aryl hydrocarbon receptor (AhR) leading to early life stage mortality

Short Title: AhR activation leading to early life stage toxicity in fish

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Status

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Abstract

This adverse outcome pathway details the linkage between activation of the aryl hydrocarbon receptor (AhR) and early life stage mortality in fishes. Fishes represent the most sensitive taxa to adverse effects resulting from activation of the AhR. Embryos are more sensitive than juvenile or adult fishes based on endpoints that could be of significance to population trends. The AhR is critically involved in development of the heart and vasculature. Activation of the AhR can result in altered cardiovascular development and function in early life stages. Initial development of this AOP draws heavily on evidence collected from zebrafish (*Danio rerio*) through mechanistic investigations by use of targeted knockdown of genes and through use of selective agonists and antagonists. However, comparable manifestations of activation of the AhR have been recorded across freshwater and marine teleost and non-teleost fishes, as well as birds. Therefore, this AOP might be broadly applicable across oviparous vertebrate taxa. Despite this conservation, great differences in sensitivity exist both among and within taxonomic groups. Therefore, this AOP has utility in support of application toward the mechanistic understanding of adverse effects of chemicals that act as agonists of the AhR, particularly with regard to cross-species, cross-chemical, and

cross-taxa extrapolation.

Background

- The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor in the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) family of proteins (Okey 2007). The AhR is a highly conserved and ancient protein with homologs having been identified in most major animal groups, apart from the most ancient lineages, such as sponges (Porifera) (Hahn et al 2002).
- Investigations of invertebrates possessing early homologs of the AhR suggest that the AhR evolutionarily functioned in regulation of the cell cycle, cellular proliferation and differentiation, and cell-to-cell communications (Hahn et al 2002). However, critical functions in angiogenesis, regulation of the immune system, neuronal processes, metabolism, development of the heart and other organ systems, and detoxification have emerged sometime in early vertebrate evolution (Duncan et al 1998; Lahvis and Bradfield 1998; Emmons et al 1999).
- Activation of the AhR by anthropogenic pollutants that act as agonists can result in a range of adverse biological effects. These effects can include hepatotoxicity, histological lesions, hemopoiesis, suppression of immune responses and healing, impaired reproductive and endocrine processes, teratogenesis, carcinogenesis, wasting syndrome, and mortality (Kleeman et al 1988; Spitsbergen et al 1986; Walter et al 2000; Giesy et al 2002; Spitsbergen et al 1988a; 1988b).
- Despite the AhR being a highly conserved protein, differences in relative sensitivity to adverse effects both among and within vertebrate taxa are greater than 1000-fold (Cohen-Barnhouse et al 2011; Doering et al 2013; Hengstler et al 1999; Korkalainen et al 2001).
- Differences in binding affinity and transactivation of the AhR have been implicated as a key mechanism contributing to differences in sensitivity to agonists of the AhR among species and taxa. However, the precise mechanisms are not fully understood for all taxa.
- High-throughput, next-generation 'OMICS' technologies have identified hundreds to thousands of different genes that are regulated, either directly or indirectly, by the AhR (Brinkmann et al 2016; Doering et al 2016; Huang et al 2014; Li et al 2013; Whitehead et al 2010). These genes include Phase I and Phase II biotransformation enzymes, such as cytochrome P450 1A (CYP1A). Expressions and activities of CYP1A are routinely used as biomarkers of exposure to anthropogenic pollutants that act as agonists of the AhR (Whyte et al 2008).
- Exposure to mixtures of agonists of the AhR during the 1950's, 1960's, and 1970's has been implicated in early life stage mortality of Lake Ontario lake trout (*Salvelinus namaycush*) leading to population collapse (Cook et al 2003). However, populations of mummichog (*Fundulus heteroclitus*) and Atlantic tomcod (*Microgadus tomcod*) exposed to lethal concentrations of agonists of the AhR have evolved tolerance through several mechanisms which has protected against population collapse (Nacci et al 2010; Wirgin et al 2011).

Summary of the AOP

Stressors

Name	Evidence
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	Strong
Polycyclic aromatic hydrocarbons (PAHs)	Strong

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

- The AhR is activated by numerous planar aromatic hydrocarbons. Specifically, chemicals with molecular dimensions of 12 Å x 14 Å x 5 Å are potential ligands of the AhR (Waller & McKinney 1992; 1995).
- However, the AhR is best known as the molecular target of dioxin-like compounds (DLCs). DLCs include polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and planar polychlorinated biphenyls (PCBs). A total of seven PCDDs, ten PCDFs, and 12 PCBs are considered 'dioxin-like' because they bind to the AhR with relatively great affinity (Denison & Heath-Pagliuso 1998; Van den Berg et al 1998).
- The prototypical and most potent DLC is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

Molecular Initiating Event

Title	Short name
Activation, AHR	Activation, AHR

18: Activation, AHR

Short Name: Activation, AHR

AOPs Including This Key Event

AOP ID and Name	Event Type
21: Activation of the aryl hydrocarbon receptor (AhR) leading to early life stage mortality	MolecularInitiatingEvent
57: AhR activation leading to hepatic steatosis	MolecularInitiatingEvent
131: AhR activation leading to uroporphyrin	MolecularInitiatingEvent
150: Aryl hydrocarbon receptor activation leading to embryoletality via cardiotoxicity	MolecularInitiatingEvent

Stressors

Name
Benzidine
Dibenzo-p-dioxin
Polychlorinated biphenyl

Polychlorinated dibenzofurans

Hexachlorobenzene

Evidence for Perturbation of this Molecular Initiating Event by Stressor

The AHR can be activated by several structurally diverse chemicals, but binds preferentially to planar halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons. Dioxin-like compounds (DLCs), which include polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and certain polychlorinated biphenyls (PCBs), are among the most potent AHR ligands^[38]. Only a subset of PCDD, PCDF and PCB congeners has been shown to bind to the AHR and cause toxic effects to those elicited by TCDD. Until recently, TCDD was considered to be the most potent DLC in birds^[39]; however, recent reports indicate that 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) is more potent than TCDD in some species of birds.^{[40][13][41][21][42][43]} When screened for their ability to induce aryl hydrocarbon hydroxylase (AHH) activity, dioxins with chlorine atoms at a minimum of three out of the four lateral ring positions, and with at least one non-chlorinated ring position are the most active^[44]. Of the dioxin-like PCBs, non-ortho congeners are the most toxicologically active, while mono-ortho PCBs are generally less potent^{[45][9]}. Chlorine substitution at ortho positions increases the energetic costs of assuming the coplanar conformation required for binding to the AHR^[45]. Thus, a smaller proportion of mono-ortho PCB molecules are able to bind to the AHR and elicit toxic effects, resulting in reduced potency of these congeners. Other PCB congeners, such as di-ortho substituted PCBs, are very weak AHR agonists and do not likely contribute to dioxin-like effects^[9].

- Contrary to studies of birds and mammals, even the most potent mono-ortho PCBs bind to AhRs of fishes with very low affinity, if at all (Abnet et al 1999; Doering et al 2014; 2015; Eisner et al 2016; Van den Berg et al 1998).

The role of the AHR in mediating the toxic effects of planar hydrophobic contaminants has been well studied, however the endogenous role of the AHR is less clear^[1]. Some endogenous and natural substances, including prostaglandin PGG2 and the tryptophan derivatives indole-3-carbinol, 6-formylindolo[3,2-b]carbazole (FICZ) and kynurenic acid can bind to and activate the AHR.^{[6][46][47][48][49]} The AHR is thought to have important endogenous roles in reproduction, liver and heart development, cardiovascular function, immune function and cell cycle regulation^{[50][38][51][52][53][54][46][55][56][57]} and activation of the AHR by DLCs may therefore adversely affect these processes.

Biological Organization

Level of Biological Organization

Molecular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebra danio	Danio rerio	Strong	NCBI

Fundulus heteroclitus	Fundulus heteroclitus	Strong	NCBI
Gallus gallus	Gallus gallus	Strong	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong
development	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

The AHR structure has been shown to contribute to differences in species sensitivity to DLCs in several animal models. In 1976, a 10-fold difference was reported between two strains of mice (non-responsive DBA/2 mouse, and responsive C57BL/6 14 mouse) in CYP1A induction, lethality and teratogenicity following TCDD exposure^[3]. This difference in dioxin sensitivity was later attributed to a single nucleotide polymorphism at position 375 (the equivalent position of amino acid residue 380 in chicken) in the AHR LBD^{[30][19][31]}. Several other studies reported the importance of this amino acid in birds and mammals^{[32][30][22][33][34][35][31][36]}. It has also been shown that the amino acid at position 319 (equivalent to 324 in chicken) plays an important role in ligand-binding affinity to the AHR and transactivation ability of the AHR, due to its involvement in LBD cavity volume and its steric effect^[35]. Mutation at position 319 in the mouse eliminated AHR DNA binding^[35].

The first study that attempted to elucidate the role of avian AHR1 domains and key amino acids within avian AHR1 in avian differential sensitivity was performed by Karchner *et al.*^[22]. Using chimeric AHR1 constructs combining three AHR1 domains (DBD, LBD and TAD) from the chicken (highly sensitive to DLC toxicity) and common tern (resistant to DLC toxicity), Karchner and colleagues^[22], showed that amino acid differences within the LBD were responsible for differences in TCDD sensitivity between the chicken and common tern. More specifically, the amino acid residues found at positions 324 and 380 in the AHR1 LBD were associated with differences in TCDD binding affinity and transactivation between the chicken (Ile324_Ser380) and common tern (Val324_Alal380) receptors^[22]. Since the Karchner *et al.* (2006) study was conducted, the predicted AHR1 LBD amino acid sequences were been obtained for over 85 species of birds and 6 amino acid residues differed among species^{[14][37]}. However, only the amino acids at positions 324 and 380 in the AHR1 LBD were associated with differences in DLC toxicity in ovo and AHR1-mediated gene expression in vitro^{[14][37][16]}. These results indicate that avian species can be divided into one of three AHR1 types based on the amino acids found at positions 324 and 380 of the AHR1 LBD: type 1 (Ile324_Ser380), type 2 (Ile324_Alal380) and type 3 (Val324_Alal380)^{[14][37][16]}.

- Little is known about differences in binding affinity of AhRs and how this relates to sensitivity in non-avian taxa.
- Low binding affinity for DLCs of AhR1s of African clawed frog (*Xenopus laevis*) and axolotl (*Ambystoma mexicanum*) has been suggested as a mechanism for tolerance of these amphibians to DLCs (Lavine *et al* 2005; Shoots *et al* 2015).
- Among reptiles, only AhRs of American alligator (*Alligator mississippiensis*) have been investigated and little is known about the sensitivity of American alligator or other reptiles to DLCs (Oka *et al* 2016).
- Among fishes, great differences in sensitivity to DLCs are known both for AhRs and for embryos among

species that have been tested (Doering et al 2013; 2014).

- Differences in binding affinity of the AhR2 have been demonstrated to explain differences in sensitivity to DLCs between sensitive and tolerant populations of Atlantic Tomcod (*Microgadus tomcod*) (Wirgin et al 2011).
- Information is not yet available regarding whether differences in binding affinity of AhRs of fishes are predictive of differences in sensitivity of embryos, juveniles, or adults (Doering et al 2013).

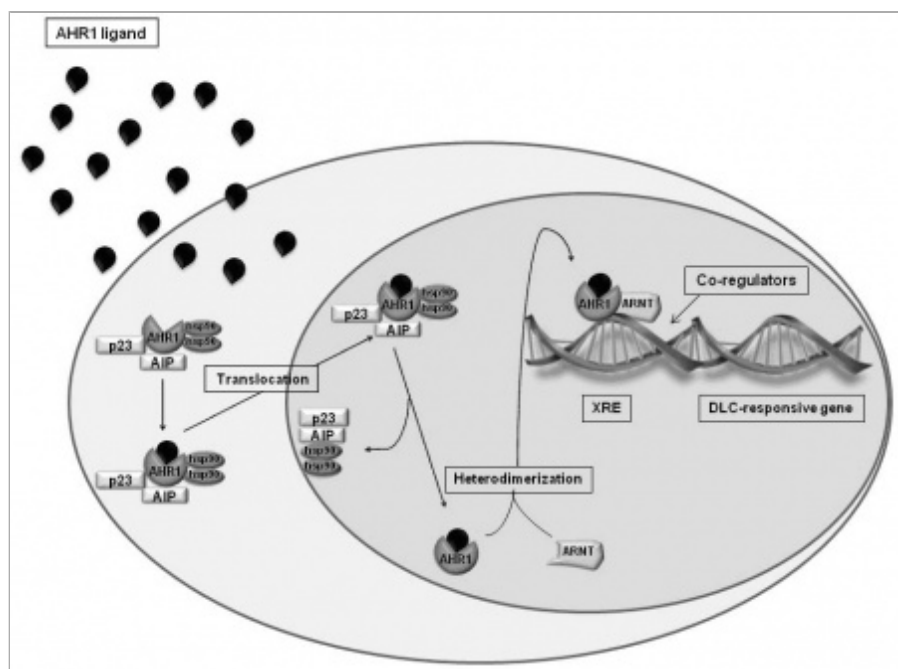
How this Key Event Works

The AHR Receptor

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that belongs to the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) superfamily and consists of three domains: the DNA-binding domain (DBD), ligand binding domain (LBD) and transactivation domain (TAD)^[1]. Other members of this superfamily include the AHR nuclear translocator (ARNT), which acts as a dimerization partner of the AHR^{[2][3]}; Per, a circadian transcription factor; and Sim, the “single-minded” protein involved in neuronal development^{[4][5]}. This group of proteins shares a highly conserved PAS domain and is involved in the detection of and adaptation to environmental change^[4].

- Investigations of invertebrates possessing early homologs of the AhR suggest that the AhR evolutionarily functioned in regulation of the cell cycle, cellular proliferation and differentiation, and cell-to-cell communications (Hahn et al 2002).
- However, critical functions in angiogenesis, regulation of the immune system, neuronal processes, metabolism, development of the heart and other organ systems, and detoxification have emerged sometime in early vertebrate evolution (Duncan et al., 1998; Emmons et al., 1999; Lahvis and Bradfield, 1998).

The molecular Initiating Event



The molecular mechanism of activation of gene expression by AHR1.

The molecular mechanism for AHR-mediated activation of gene expression is presented in the figure to the right. In its unliganded form, the AHR is part of a cytosolic complex containing heat shock protein 90 (HSP90), the HSP90

co-chaperone p23 and AHR-interacting protein (AIP)^[6]. Upon ligand binding, the AHR migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with ARNT^[7]. The AHR-ARNT complex then binds to a xenobiotic response element (XRE) found in the promoter of an AHR-regulated gene and recruits co-regulators such as CREB binding protein/p300, steroid receptor co-activator (SRC) 1, SRC-2, SRC-3 and nuclear receptor interacting protein 1, leading to induction or repression of gene expression^[6]. Expression levels of several genes, including phase I (e.g. cytochrome P450 (CYP) 1A, CYP1B, CYP2A) and phase II enzymes (e.g. uridine diphosphate glucuronosyl transferase (UDP-GT), glutathione S-transferases (GSTs)), as well as genes involved in cell proliferation (transforming growth factor-beta, interleukin-1 beta), cell cycle regulation (p27, jun-B) and apoptosis (Bax), are regulated through this mechanism ^{[6][8][7][9]}.

AHR Isoforms

- Over time the AhR has undergone gene duplication and diversification in vertebrates, which has resulted in multiple clades of AhR, namely AhR1, AhR2, and AhR3 (Hahn 2002).
- Fishes and birds express AhR1s and AhR2s, while mammals express a single AhR that is homologous to the AhR1 (Hahn 2002; Hahn et al 2006).
- The AhR3 is poorly understood and known only from some cartilaginous fishes (Hahn 2002).
- Little is known about diversity of AhRs in reptiles and amphibians (Hahn et al 2002).
- In some taxa, subsequent genome duplication events have further led to multiple isoforms of AhRs in some species, with up to four isoforms of the AhR (α , β , δ , γ) having been identified in Atlantic salmon (*Salmo salar*) (Hansson et al 2004).
- Although homologs of the AhR have been identified in some invertebrates, compared to vertebrates these AhRs have differences in binding of ligands in the species investigated to date (Hahn 2002; Hahn et al 1994).

Two AHR isoforms (AHR1 and AHR2) have been identified in the black-footed albatross (*Phoebastria nigripes*), great cormorant (*Phalacrocorax carbo*) and domestic chicken (*Gallus gallus domesticus*)^[10]. AHR1 mRNA levels were similar in the kidney, heart, lung, spleen, brain, gonad and intestine from the great cormorant but were lower in muscle and pancreas. AHR2 expression was mainly observed in the liver, but was also detected in gonad, brain and intestine. AHR1 levels represented a greater proportion (80%) of total AHR levels than AHR2 in the cormorant liver^[10], and while both AHR isoforms bound to TCDD, AHR2 was less effective at inducing TCDD-dependent transactivation compared to AHR1 in black-footed albatross, great cormorant and domestic chicken^{[11][10]}.

Roles of isoforms in Birds:

- AhR1 and AhR2 both bind and are activated by TCDD *in vitro* (Yasui et al 2007).
- AhR1 has greater binding affinity and sensitivity to activation by TCDD relative to AhR2 (Yasui et al 2007).
- AhR1 is believed to mediate toxicities of DLCs, while AhR2 has no known role in toxicities (Farmahin et al 2012; Farmahin et al 2013; Manning et al 2012).

Roles of isoforms in fishes:

- AhR1 and AhR2 both bind and are activated by TCDD *in vitro* (Bak et al 2013; Doering et al 2014; 2015; Karchner et al 1999; 2005).
- AhR1 has greater sensitivity to activation by TCDD than AhR2 in red seabream (*Pagrus major*), white sturgeon (*Acipenser transmontanus*), and lake sturgeon (*Acipenser fulvescens*) (Bak et al 2013; Doering et al 2014; 2015).
- AhR2 has greater binding affinity or activation by TCDD than AhR1 in zebrafish (*Danio rerio*) and mummichog (*Fundulus heteroclitus*) (Karchner et al 1999; 2005).
- AhR2 is believed to mediate toxicities in fishes, while AhR1 has no known role in toxicities. Specifically, knockdown of AhR2 protects against toxicities of dioxin-like compounds (DLCs) and polycyclic aromatic hydrocarbons (PAHs) in zebrafish (*Danio rerio*) and mummichog (*Fundulus heteroclitus*), while knockdown of

AhR1 offers no protection (Clark et al 2010; Prasch et al 2003; Van Tiem & Di Giulio 2011).

Roles of isoforms in amphibians and reptiles:

- Less is known about AhRs of amphibians or reptiles.
- AhR1 is believed to mediate toxicities in amphibians (Hahn 2002; Lavine et al 2005; Oka et al 2016; Shoots et al 2015). However, all AhRs of amphibians that have been investigated have very low affinity for TCDD (Hahn 2002; Lavine et al 2005; Oka et al 2016; Shoots et al 2015).
- Both AhR1s and AhR2 of American alligator (*Alligator mississippiensis*) are activated by agonists with comparable sensitivities (Oka et al 2016). AhRs of no other reptiles have been investigated.

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Transactivation Reporter Gene Assays (recommended approach)

Transient transfection transactivation

Transient transfection transactivation is the most common method for evaluating nuclear receptor activation^[12]. Full-length AHR cDNAs are cloned into an expression vector along with a luminescent reporter gene construct (chimeric luciferase, P-lactamase or CAT reporter vectors containing the appropriate response elements for the gene of interest). There are a number of commercially available cell lines that can serve as recipients for these vectors (CV-1, HuH7, FLC-7, LS174T, LS180 MCF-7, HEC1, LLC-PK1, HEK293, HepG2, and Caco-2 cells)^[12]. The greatest advantage of using transfected cells, rather than primary cell cultures, is the assurance that the nuclear receptor of interest is responsible for the observed induction. This would not be possible in a primary cell culture due to the co-regulation of different receptors for the same target genes. This model makes it easy to compare the responsiveness of the AHR across multiple species under the same conditions simply by switching out the AHR clone. One disadvantage to the transient transfection assay is the inherent variability associated with transfection efficiency, leading to a movement towards the use of stable cell lines containing the nuclear receptor and reporter gene linked to the appropriate response elements^[12].

Luciferase reporter gene (LRG) assay

- Luciferase reporter gene (LRG) assays have been used to investigate activation of AhRs of mammals (Abnet et al 1999), birds (Farmahin et al 2012; 2013; Manning et al 2013), reptiles (Oka et al 2016), amphibians (Lavine et al 2005; Shoots et al 2015; Ohi et al 2003), and fishes (Andreasen et al 2002; Abnet et al 1999; Bak et al 2013; Doering et al 2014; 2015; Evans et al 2005; Hansson & Hahn 2008; Karchner et al 1999; Tanguay et al 1999; Wirgin et al 2011).

For demonstrative purposes, a luciferase reporter gene assay used to measure AHR1-mediated transactivation for avian species is described here. A monkey kidney cell line (Cos-7) that has low endogenous AHR1 expression was transfected with the appropriate avian AHR1 clone, cormorant ARNT1, a CYP1A5 firefly luciferase reporter construct and a *Renilla* luciferase vector to control for transfection efficiency. After seeding, the cells were exposed to DLC and luciferase activity was measured using a luminometer. Luminescence, which is proportional to the extent of AHR activation, is expressed as the ratio of firefly luciferase units to *Renilla* luciferase units^[13]. This particular assay was modified from its original version to increase throughput efficiency; (a) cells were seeded in 96-

well plates rather than Petri dishes or 48- well plates, (b) DLCs were added directly to the wells without changing the cell culture medium, and (c) the same 96-well plates were used to measure luminescence without lysing the cells and transferring to another plate. Similar reporter gene assays have been used to measure AHR1 activation in domestic and wild species of birds, including the chicken, ring-necked pheasant (*Phasianus colchicus*), Japanese quail (*Coturnix japonica*), great cormorant, black-footed albatross and peregrine falcon (*Falco peregrinus*).^{[14][13][15][11][16][17]}

Transactivation in stable cell lines

Stable cell lines have been developed and purified to the extent that each cell contains both the nuclear receptor and appropriate reporter vector, eliminating the variability associated with transfection^[12]. A stable human cell line containing a luciferase reporter driven by multiple dioxin response elements has been developed that is useful in identifying AhR agonists and antagonists^[18]. An added benefit of this model is the potential to multiplex 3 assays in a single well: receptor activation, cell viability and enzyme activity^[12]. Such assays are used extensively in drug discovery due to their high throughput efficiency, and may serve just as useful for risk assessment purposes.

Ligand-Binding Assays

Ligand binding assays measure the ability of a test compound to compete with a labeled, high-affinity reference ligand for the LBD of a nuclear receptor. It is important to note that ligand binding does not necessitate receptor activation and therefore cannot distinguish between agonists and antagonists; however, binding affinities of AHR ligands are highly correlated with chemical potencies^[19] and can explain differences in species sensitivities to DLCs^{[20][21][22]}; they are therefore worth mentioning. Binding affinity and efficacy have been used to develop structure-activity relationships for AHR disruption^{[20][23]} that are potentially useful in risk-assessment. There has been tremendous progress in the development of ligand-binding assays for nuclear receptors that use homogenous assay formats (no wash steps) allowing for the detection of low-affinity ligands, many of which do not require a radiolabel and are amenable to high throughput screening^{[24][12]}. This author however was unable to find specific examples of such assays in the context of AHR binding and therefore some classic radioligand assays are described instead.

Hydroxyapatite (HAP) binding assay

The HAP binding assay makes use of an *in vitro* transcription/translation method to synthesize the AHR protein, which is then incubated with radiolabeled TDCPP and a HAP pellet. The occupied protein adsorbs to the HAP and the radioactivity is measured to determine saturation binding. An additional ligand can also be included in the mixture in order to determine its binding affinity relative to TCDD (competitive binding)^{[25][22]}. This assay is simple, repeatable and reproducible; however, it is insensitive to weak ligand-receptor interactions^{[22][21][26]}.

Whole cell filtration binding assay

Dold and Greenlee^[27] developed a method to detect specific binding of TCDD to whole mammalian cells in culture and was later modified by Farmahin et al.^[21] for avian species. The cultured cells are incubated with radiolabeled TCDD with or without the presence of a competing ligand and filtered. The occupied protein adsorbs onto the filter and the radioactivity is measured to determine saturation binding and/or competitive binding. This assay is able to detect weak ligand-receptor interactions that are below the detection limit of the HAP assay^[21].

Protein-DNA Interaction Assays

The active AHR complexed with ARNT can be measured using protein-DNA interaction assays. Two methods are described in detail by Perez-Romero and Imperiale^[28]. Chromatin immunoprecipitation measures the interaction of proteins with specific genomic regions *in vivo*. It involves the treatment of cells with formaldehyde to crosslink neighboring protein-protein and protein-DNA molecules. Nuclear fractions are isolated, the genomic DNA is

sheared, and nuclear lysates are used in immunoprecipitations with an antibody against the protein of interest. After reversal of the crosslinking, the associated DNA fragments are sequenced. Enrichment of specific DNA sequences represents regions on the genome that the protein of interest is associated with *in vivo*. Electrophoretic mobility shift assay (EMSA) provides a rapid method to study DNA-binding protein interactions *in vitro*. This relies on the fact that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments. The protein-DNA complex components are then identified with appropriate antibodies. The EMSA assay was found to be consistent with the LRG assay in chicken hepatoma cells dosed with dioxin-like compounds^[29].

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Key Events

Title	Short name
dimerization, AHR/ARNT	dimerization, AHR/ARNT
Increase, COX-2 expression	Increase, COX-2 expression
Altered, Cardiovascular development/function	Altered, Cardiovascular development/function

944: [dimerization, AHR/ARNT](#)

Short Name: dimerization, AHR/ARNT

AOPs Including This Key Event

AOP ID and Name	Event Type

150: Aryl hydrocarbon receptor activation leading to embryo lethality via cardiotoxicity	KeyEvent
21: Activation of the aryl hydrocarbon receptor (AhR) leading to early life stage mortality	KeyEvent

Biological Organization

Level of Biological Organization
Molecular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
chicken	Gallus gallus	Strong	NCBI
zebrafish	Danio rerio	Strong	NCBI
mouse	Mus musculus	Strong	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong
development	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

- ARNTs have been identified in all tetrapods investigated to date (Drutel et al 1996; Hirose et al 1996; Hoffman et al 1991; Lee et al 2007; Lee et al 2011).
- ARNTs have been identified in a great phylogenetic diversity of fishes, including early fishes (Doering et al 2014; 2016).
- ARNT has been identified in investigated invertebrates (Powell-Coffman et al 1998).

This mechanism is conserved across species. Mammals possess a single AHR, whereas birds and fish express multiple isoforms, and all three express multiple ARNT isoforms. Not all of the isoforms identified are functionally active. For example, killifish AHR1 and AHR2 are active and display different transcription profiles, whereas zebrafish AHR2 and ARNT2 are active in mediating xenobiotic-mediated toxicity and AHR1 is inactive (Hahn et al. 2006; Prasch et al. 2006).

How this Key Event Works

Structure and Function of ARNT

- The aryl hydrocarbon receptor nuclear translocator (ARNT) is a member of the Per-Arnt-Sim (PAS) family of proteins (Gu et al 2000).
- PAS proteins share highly conserved PAS domains (Gu et al 2000).
- PAS proteins act as transcriptional regulators in response to environmental and physiological cues (Gu et al 2000).

Isoforms of ARNT:

- Over time ARNT has undergone gene duplication and diversification in vertebrates, which has resulted in three clades of ARNT, namely ARNT1, ARNT2, and ARNT3.
- Each clade can include multiple isoforms and splice variants (Hill et al 2009; Lee et al 2007; Lee et al 2011; Powel & Hahn 2000; Tanguay et al 2000).
- ARNT1s have been demonstrated to function predominantly through heterodimerization with the aryl hydrocarbon receptor (AhR) and hypoxia inducible factor 1 α (HIF1 α) (Prasch et al 2004; 2006; Wang et al 1995).
- ARNT2s are believed to function predominantly through heterodimerization with Single Minded (SIM) (Hirose et al 1996).
- ARNT3s, which are also known as ARNT-like (ARNTL), Brain and Muscle ARNT-like-1 (BMAL1), or Morphine Preference 3 (MOP3), are believed to function predominantly through heterodimerization with Circadian Locomotor Output Cycles Kaput (CLOCK) (Gekakis et al 1998).
- ARNTs have numerous key roles in vertebrates related to responses to developmental and environmental cues.

Roles of ARNTs in mammals:

- ARNT1 functions in normal vascular and hematopoietic development (Kozak et al 1997; Maltepe et al 1997; Abbott & Buckalew 2000).
- ARNT2 functions in development of the hypothalamus and nervous system (Hosoya et al 2001; Keith et al 2001).
- ARNT3 functions in biological rhythms (Gekakis et al 1998).

Roles of ARNTs in other taxa:

- ARNTs have been demonstrated to have roles in development of the heart, brain, liver, and possibly the peripheral nervous system in zebrafish (*Danio rerio*) (Hill et al 2009).
- Roles of ARNTs in other taxa have not been sufficiently investigated to date.

Interaction with AHR

Upon ligand binding, the aryl hydrocarbon receptor (AHR) migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with AHR nuclear translocator (ARNT) (Mimura and Fujii-Kuriyama 2003). The AHR-ARNT complex then binds to a xenobiotic response element (XRE) found in the promoter of an AHR-regulated gene and recruits co-regulators such as CREB binding protein/p300, steroid receptor co-activator (SRC) 1, SRC-2, SRC-3 and nuclear receptor interacting protein 1, leading to induction or repression of gene expression (Fujii-Kuriyama and Kawajiri 2010). Expression levels of several genes, including phase I (e.g. cytochrome P450 (CYP) 1A, CYP1B, CYP2A) and phase II enzymes (e.g. uridine diphosphate glucuronosyl transferase (UDP-GT), glutathione S-transferases (GSTs)), as well as genes involved in cell proliferation (transforming growth factor-beta, interleukin-1 beta), cell cycle regulation (p27, jun-B) and apoptosis (Bax), are

regulated through this mechanism (Fujii-Kuriyama and Kawajiri 2010; Giesy et al. 2006; Mimura and Fujii-Kuriyama 2003; Safe 1994).

How it is Measured or Detected

The active AHR complexed with ARNT can be measured using protein-DNA interaction assays. Two methods are described in detail by Perez-Romero and Imperiale (Perez-Romero and Imperiale 2007). Chromatin immunoprecipitation measures the interaction of proteins with specific genomic regions *in vivo*. It involves the treatment of cells with formaldehyde to crosslink neighboring protein-protein and protein-DNA molecules. Nuclear fractions are isolated, the genomic DNA is sheared, and nuclear lysates are used in immunoprecipitations with an antibody against the protein of interest. After reversal of the crosslinking, the associated DNA fragments are sequenced. Enrichment of specific DNA sequences represents regions on the genome that the protein of interest is associated with *in vivo*. Electrophoretic mobility shift assay (EMSA) provides a rapid method to study DNA-binding protein interactions *in vitro*. This relies on the fact that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments. The protein-DNA complex components are then identified with appropriate antibodies. The EMSA assay was found to be consistent with the luciferase reporter gene assay (in chicken hepatoma cells dosed with dioxin-like compounds (Heid et al. 2001).

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1269: Increase, COX-2 expression

Short Name: Increase, COX-2 expression

AOPs Including This Key Event

AOP ID and Name	Event Type
21: Activation of the aryl hydrocarbon receptor (AhR) leading to early life stage mortality	KeyEvent

Biological Organization

Level of Biological Organization
Molecular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Danio rerio	Danio rerio	Strong	NCBI
Oryzias latipes	Oryzias latipes	Strong	NCBI
Gallus gallus	Gallus gallus	Strong	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

- There is a high level of conservation of COX-2, as well as its function, especially across vertebrates (Havird et al 2008; 2015), indicating that numerous vertebrate taxa might be susceptible to up-regulation in COX-2.
- Typically, teleost fish genomes contain more than one COX -2 gene, likely a result of genome duplication after divergence of teleosts from tetrapods (Ishikawa et al 2007; Havird et al 2015). In zebrafish there is COX-

2a and COX-2b (Teraoka et al 2014).

- In invertebrates, COX is found in most crustaceans, the majority of molluscs, but only in specific lineages within Cnidaria and Annelida. COX genes are not found in Hemichordata, Echinodermata, or Platyhelminthes. Insecta COX genes lack in homology, but might function as COX enzymes based on structural analyses (Havird et al 2015).

How this Key Event Works

- Prostaglandin-endoperoxide synthase (PTGS; KEGG ID E.C. 1.14.99.1) is an enzyme that has two catalytic sites.
- Cyclooxygenase site (COX) catalyzes conversion of arachidonic acid into endoperoxide prostaglandin G2 (Simmons et al 2004).
- Peroxidase active site converts PGG2 to PGH2 (KEGG reactions 1599, 1590). PGH2 is a precursor for synthesis of other prostaglandins (PGEs, PGFs), prostacyclin, and thromboxanes (Simmons et al 2004; Botting & Botting 2011).
- There are two isoforms, COX-1 and COX-2
- COX-2 is inducible by certain chemical exposures, inflammation, during discrete stages of gamete maturation, and more (Green et al 2012).

How it is Measured or Detected

- COX-2 has been measured as mRNA by use of quantitative real-time polymerase chain reaction (q-RT PCR) (Dong et al 2010; Fujisawa et al 2014; Teraoka et al 2008; 2014).

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317: Altered, Cardiovascular development/function

Short Name: Altered, Cardiovascular development/function

AOPs Including This Key Event

AOP ID and Name	Event Type
150: Aryl hydrocarbon receptor activation leading to embryoletality via cardiotoxicity	KeyEvent
21: Activation of the aryl hydrocarbon receptor (AhR) leading to early life stage mortality	KeyEvent

Biological Organization

Level of Biological Organization
Organ

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
chicken	Gallus gallus	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
zebrafish	Danio rerio	Strong	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong
development	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

- Some form of cardiovascular system is present in members of the clade Bilateria (Bishopric 2005). This clade includes most animal phyla, except for sponges (Porifera), jellyfishes and corals (Cnidaria), placozoans (Placozoa), and comb jellies (Ctenophora).
- Differences in cardiovascular systems are present among taxa. Vertebrates have closed circulatory systems, while some invertebrate taxa have open circulatory systems (Kardong 2006).

Birds, fish and mammals are all susceptible to cardiotoxicity following embryonic chemical exposure.

Cardiovascular development and function altered by dioxin-like compounds (DLCs):

- Applicable to all teleost and non-teleost fishes so far investigated (Buckler et al 2015; Elonen et al 1998; Huang et al 2012; Johnson et al 1998; Park et al 2014; Tillitt et al 2016; Toomey et al 2001; Walker et al 1991; Yamauchi et al 2006; Zabel et al 1995). However, altered cardiovascular development and function has not been investigated in jawless fishes, such as lamprey (Petromyzontiformes) and hagfish (Myxiniiformes), or in cartilaginous fishes (Chondrichthyes).
- Applicable to birds (Heid et al 2001).
- Altered cardiovascular development and function by DLCs has not been sufficiently investigated in amphibians, reptiles, or invertebrates that possess a cardiovascular system.

How this Key Event Works

This key event applies to the disruption of cardiogenesis early enough in embryogenesis to result in gross morphological alterations leading to reduced cardiac function.

With respect to dioxin-like compounds that are strong AHR agonists, the malformations that have been observed following embryonic exposure are summarized in table 1.

Table 1: Cardiotoxic effects of strong AHR-agonists

Zebrafish Embryo	Chicken Embryo	Mouse
• Reduced extension of common cardinal vein • Reduced blood flow • Reduced heart rate • Disrupted erythropoiesis • Decreased heart volume • Pericardial edema • Overt heart malformations	• Enlarged left ventricle • Increased heart rate • Increased myosin content • Reduced β-adrenergic responsiveness • Increased ANF mRNA • Arrhythmia • Increased apoptosis • Reduced myocyte proliferation • Pericardial edema • Overt heart malformations	Embryo/Fetus • Reduced heart-to-body weight • Reduced myocyte proliferation • Vascular remodeling 21 Days old • Increased heart-to-body weight • Increased left ventricle weight • Reduced heart rate • Cardiac hypertrophy • Increased ANF mRNA • Increased risk of heart disease

ANF= cardiac atrial natriuretic factor; an indicator of cardiac stress. Source: (Kopf and Walker 2009)

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Adverse Outcomes

Title	Short name
Increase, Mortality	Increase, Mortality

350: Increase, Mortality

Short Name: Increase, Mortality

AOPs Including This Key Event

AOP ID and Name	Event Type
21: Activation of the aryl hydrocarbon receptor (AhR) leading to early life stage mortality	AdverseOutcome

Biological Organization

Level of Biological Organization
Individual

Scientific evidence supporting the linkages in the AOP

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation, AHR	directly leads to	dimerization, AHR/ARNT	Strong	Moderate
dimerization, AHR/ARNT	directly leads to	Increase, COX-2 expression	Strong	Moderate
Increase, COX-2 expression	directly leads to	Altered, Cardiovascular development/function	Moderate	Moderate
Altered, Cardiovascular development/function	directly leads to	Increase, Mortality	Moderate	Strong

[Activation, AHR leads to dimerization, AHR/ARNT](#)

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	Strong	NCBI
Danio rerio	Danio rerio	Strong	NCBI

Life Stage Applicability

Life Stage	Evidence
all life stages	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

- The aryl hydrocarbon receptor (AhR) and aryl hydrocarbon receptor nuclear translocator (ARNT) are highly conserved and ancient proteins with homologs having been identified in most major animal groups, apart from the most ancient lineages, such as sponges (Porifera) (Hahn et al 2002).
- *In vitro* dimerization of AhRs and ARNTs have been demonstrated in mammals, birds, reptiles, amphibians, teleost and non-teleost fishes, and some invertebrates (Butler et al 2001; Emmons et al 1999; Hahn et al 2002; Powell-Coffman et al 1998).

How Does This Key Event Relationship Work

Upon ligand binding, the aryl hydrocarbon receptor (AHR) migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with AHR nuclear translocator (ARNT) (Mimura and Fujii-Kuriyama 2003). The AHR-ARNT complex then binds to a xenobiotic response element (XRE) found in the promoter of an AHR-regulated gene and recruits co-regulators such as CREB binding protein/p300, steroid receptor co-activator (SRC) 1, SRC-2, SRC-3 and nuclear receptor interacting protein 1, leading to induction or repression of gene expression (Fujii-Kuriyama and Kawajiri 2010). Expression levels of several genes, including phase I (e.g. cytochrome P450 (CYP) 1A, CYP1B, CYP2A) and phase II enzymes (e.g. uridine diphosphate glucuronosyl transferase (UDP-GT), glutathione S-transferases (GSTs)), as well as genes involved in cell proliferation (transforming growth factor-beta, interleukin-1 beta), cell cycle regulation (p27, jun-B) and apoptosis (Bax), are regulated through this mechanism (Fujii-Kuriyama and Kawajiri 2010; Giesy et al. 2006; Mimura and Fujii-Kuriyama 2003; Safe 1994).

Roles of AhR and ARNT isoforms:

- AhRs can heterodimerize with ARNT1 and ARNT2 isoforms in order to activate reporter constructs in transfected cells and recognize response elements in gel shift assays in all investigated vertebrates, including birds, fishes, and reptiles (Abnet et al 1999; Andreassen et al 2002a; 2002b; Bak et al 2013; Doering et al 2014; Doering et al 2015; Farmahin et al 2012; 2013; Hansson & Hahn 2008; Karchner et al 1999; 2006; Lavine et al 2005; Oka et al 2016; Shoots et al 2015; Tanguay et al 1999; 2000; Wirgin et al 2011).

Weight of Evidence

Biological Plausibility

The mechanism of AHR-mediated transcriptional regulation is well understood (Fujii-Kuriyama and Kawajiri 2010).

- Numerous PAS proteins are known to interact with each other in response to environmental and developmental cues through dimerization at their PAS domains (Pohjanvirta 2012).

Empirical Support for Linkage

- Unliganded AhR exists as a cytosolic 9S form, while in the presence of a ligand the AhR exists as a nuclear 6S form. ARNT exists as a nuclear 6S form (Okey 2007).
- The 6S form of AhR is approximately 210 kDa. Liganded AhR is approximately 100 kDa and ARNT is approximately 110 kDa (Elferink et al 1990; Swanson et al 1993).
- Dimerization of AhRs with ARNTs has been demonstrated in all invertebrate and vertebrate species so far investigated (Butler et al 2001; Emmons et al 1999; Hahn et al 2002; Powell-Coffman et al 1998).
- Heterodimers are not formed on response elements in gel shift assays in the absence of AhR and/or ARNT (Tanguay et al 2000).

ARNT is a necessary dimerization partner for the transcriptional activation of AHR regulated genes (Hoffman et al. 1991; Poland et al. 1976). The AHR/ARNT complex was confirmed following in vitro exposure to halogenated aromatic hydrocarbons using an electrophoretic mobility shift assay; a dose-dependent supershift in DNA-binding was observed using specific antibodies in chicken and human cell lines (Heid et al. 2001).

Uncertainties or Inconsistencies

- There are uncertainties in the precise physiological and toxicological roles of different AhR clades (AhR1, AhR2, AhR3) and isoforms (α , β , δ , γ).
- There are uncertainties in the precise physiological and toxicological roles of different ARNT clades (ARNT1, ARNT2, ARNT3) and isoforms (a, b, c).
- Nothing is known about differences in binding affinity of AhR for ARNT and of the AhR/ARNT heterodimer for DNA among species and taxa.

Quantitative Understanding of the Linkage

- Strong quantitative relationships are known for exposure to ligands and interaction with DREs on the DNA by use of transfected COS-7 cells and gel shift assays (Abnet et al 1999; Andreassen et al 2002a; 2002b; Bak et al 2013; Doering et al 2014; Doering et al 2015; Farmahin et al 2012; 2013; Hansson & Hahn 2008; Karchner et al 1999; 2006; Lavine et al 2005; Manning et al 2012; Oka et al 2016; Shoots et al 2015; Tanguay et al 1999; 2000; Wirgin et al 2011).
- Specifically, greater concentrations of ligands or greater potency ligands cause greater interaction with DREs on the DNA.
- Numerous ligands of the AhR are rapidly metabolized and only cause transient activation of the AhR. These ligands do not result in sustained interaction with DREs and do not cause downstream effects (Farmahin et al 2016).
- There is uncertainty in whether anthropogenic contaminants that act as ligands of the AhR and lead to dimerization of AhR with ARNT in vertebrates also act as ligands in invertebrates.

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dimerization, AHR/ARNT leads to Increase, COX-2 expression

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Danio rerio	Danio rerio	Strong	NCBI
Oryzias latipes	Oryzias latipes	Strong	NCBI
Gallus gallus	Gallus gallus	Weak	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong
development	Strong

Sex Applicability

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Sex	Evidence
Unspecific	Strong

- Dimerization of AhR/ARNT leading to increased expression of COX-2 has only been investigated in zebrafish, Japanese medaka, and chicken (Dong et al 2010; Teraoka et al 2008; 2014; Fujisawa et al 2014).
- Due to the presence of a functional AhR/ARNT pathway and COX-2 genes among all vertebrate taxa, it is acknowledged that this key event relationship is likely applicable to vertebrates in general and possibly some invertebrates.

How Does This Key Event Relationship Work

- The AhR/ARNT heterodimer is able to interact with dioxin-responsive elements (DREs) on the DNA causing the up-regulation in dioxin-responsive genes (Whitlock et al 1996).
- DREs in the promoter region of COX-2 allow the AhR/ARNT heterodimer to up-regulate expression of COX-2 (Degner et al 2007; Jonsson et al 2012).

Weight of Evidence

Biological Plausibility

- Putative DREs have been identified in the promoter region of COX-2 in zebrafish and presumably exist in other species and taxa (Degner et al 2007; Jonsson et al 2012).
- DREs are well characterized and numerous other genes that have DREs in their promoter region are known to be up-regulated by the AhR/ARNT heterodimer (Denison et al 1988).

Empirical Support for Linkage

- Expression of COX-2 is up-regulated in response to exposure to ligands that activate AhR causing dimerization with ARNT (Dong et al 2010; Teraoka et al 2008; 2014).
- Knockdown of ARNT1 prevents interaction of AhR with DREs and the up-regulation in dioxin-responsive genes (Antkiewicz et al 2006; Prasch et al 2004).
- Depletion of ARNT lessens or prevents interaction of AhR with DREs and the up-regulation in dioxin-responsive genes (Prasch et al 2004).

Uncertainties or Inconsistencies

- In chicken (*Gallus gallus*), and presumably other species of birds, COX-2 is believed to be up-regulated by the AhR through non-genomic mechanisms that are independent of the AhR/ARNT heterodimer (Fujisawa et al 2014). DREs are not believed to be present in the promoter region of COX-2 in chicken (Fujisawa et al 2014).
- However, nothing is known regarding the presence or absence of DREs in the promoter region of COX-2 in species or taxa other than zebrafish.
- Amounts of COX-2 protein have not been investigated and therefore only increases in expressions of transcript are known.

Quantitative Understanding of the Linkage

- Limited dose-response information is available regarding dimerization of AhR/ARNT leading to increased expression of COX-2.
- In Japanese medaka (*Oryzias latipes*), abundance of transcript of COX-2 followed a dose-dependent increase to waterborne concentrations of the AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Dong et al 2010).

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Increase, COX-2 expression leads to Altered, Cardiovascular development/function

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Danio rerio	Danio rerio	Strong	NCBI
Oryzias latipes	Oryzias latipes	Strong	NCBI
Gallus gallus	Gallus gallus	Strong	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong

development

Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

- Links between induction of COX-2 and alteration of cardiovascular development and function has only been demonstrated in zebrafish, Japanese medaka, and chicken (Dong et al 2010; Teraoka et al 2008; 2014).
- However, it is acknowledged that this key event relationship could be applicable to all vertebrate taxa and some invertebrate taxa based on presence of COX-2 genes and a cardiovascular system.

How Does This Key Event Relationship Work

- The precise role that COX-2 plays in altered cardiovascular development/function has not been investigated. However, the prostaglandin synthesis pathway, of which COX-2 is a rate limiting step, is known to have roles in development of the heart (Dong et al 2010; Huang et al 2007; Teraoka et al 2008; 2014).

Weight of Evidence

Biological Plausibility

- The prostaglandin synthesis pathway, of which COX-2 is a rate limiting step, is known to have roles in development of the heart and therefore altered levels of expression of COX-2 could be expected to result in altered development of the heart.

Empirical Support for Linkage

- Blocking induction of COX-2 through knockdown of COX-2 or through selective antagonists of COX-2 prevents alteration in cardiovascular development and function in zebrafish, Japanese medaka (*Oryzias latipes*), and chicken (*Gallus gallus*) (Dong et al 2010; Teraoka et al 2008; 2014).
- Knockdown of and selective antagonists of thromboxane A synthase 1 (CYP5A), which is down-stream of COX-2 in the prostaglandin synthesis pathway, prevents alteration in cardiovascular development and function by inducers of COX-2 (Teraoka et al 2008).

Uncertainties or Inconsistencies

- Intermediary steps between increased expression of COX-2 and altered heart development and function have not been investigated.
- The precise role of COX-2 and the prostaglandin synthesis pathway in early development of the heart is not known.

Quantitative Understanding of the Linkage

- There is an R^2 of 0.84 between relative COX-2 mRNA and altered heart development measured as pericardial area in embryos of Japanese medaka exposed to the COX-2 inducer, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Dong et al 2010).

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Altered, Cardiovascular development/function leads to Increase, Mortality

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Danio rerio	Danio rerio	Strong	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong
development	Strong

Sex Applicability

Sex	Evidence
Unspecific	Strong

- Altered cardiovascular development and function leading to mortality has been extensively demonstrated in numerous teleost and non-teleost fishes and to a lesser extent in birds (Brunstrom 1990; Brunstrom & Andersson 1988; Buckler et al 2015; Cohen-Barnhouse et al 2011; Elonen et al 1998; Hoffman et al 1996; 1998; Huang et al 2012; Johnson et al 1998; Park et al 2014; Powell et al 1998; Tillitt et al 2016; Toomey et al 2001; Walker et al 1991; Yamauchi et al 2006; Zabel et al 1995).
- Although limited studies are available, altered cardiac development and function leading to mortality is acknowledged to be applicable to reptiles, amphibians, and possibly some invertebrates based on presence of a cardiovascular system.

How Does This Key Event Relationship Work

- The cardiovascular system functions in the transport of nutrients, oxygen, carbon dioxide, and hormones to all parts of the organism and is required for survival (Kardong 2006).

Weight of Evidence

Biological Plausibility

A properly functioning cardiovascular system is required for survival of vertebrates and some invertebrates. Therefore, altered development leading to dysfunction would be expected to cause mortality.

Empirical Support for Linkage

- Pumping efficiency of the heart and measured blood cell perfusion rate decreases with increased severity of cardiovascular teratogenesis and increases percent mortality (Dong et al 2010; Teraoka et al 2008).
- Severe hemorrhage is observable in mortalities as a result of complete cardiac collapse due to cardiovascular teratogenesis (Buckler et al 2015; Elonen et al 1998; Johnson et al 1998; Park et al 2014; Tillitt et al 2016; Toomey et al 2001; Walker et al 1991; Yamauchi et al 2006; Zabel et al 1995).
- Organisms are significantly less sensitive to mortality due to alteration in cardiovascular development and function post-heart developmental stages (Carney et al 2004; Lanham et al 2012).

Uncertainties or Inconsistencies

- There are numerous different alterations in development or function that can result in mortality. The exact phenotypes that result in lethal versus sub-lethal outcomes is not well known.
- Nothing is known about differences in altered cardiovascular development and function leading to mortality in invertebrates with open circulatory systems or closed circulatory systems.

Quantitative Understanding of the Linkage

- There is strong quantitative understanding between incidence of and severity of cardiovascular teratogenicity and mortality (Buckler et al 2015; Elonen et al 1998; Johnson et al 1998; Park et al 2014; Tillitt et al 2016; Toomey et al 2001; Walker et al 1991; Yamauchi et al 2006; Zabel et al 1995).
- However, numerous different cardiovascular endpoints are investigated among studies making side-by-side comparisons difficult.

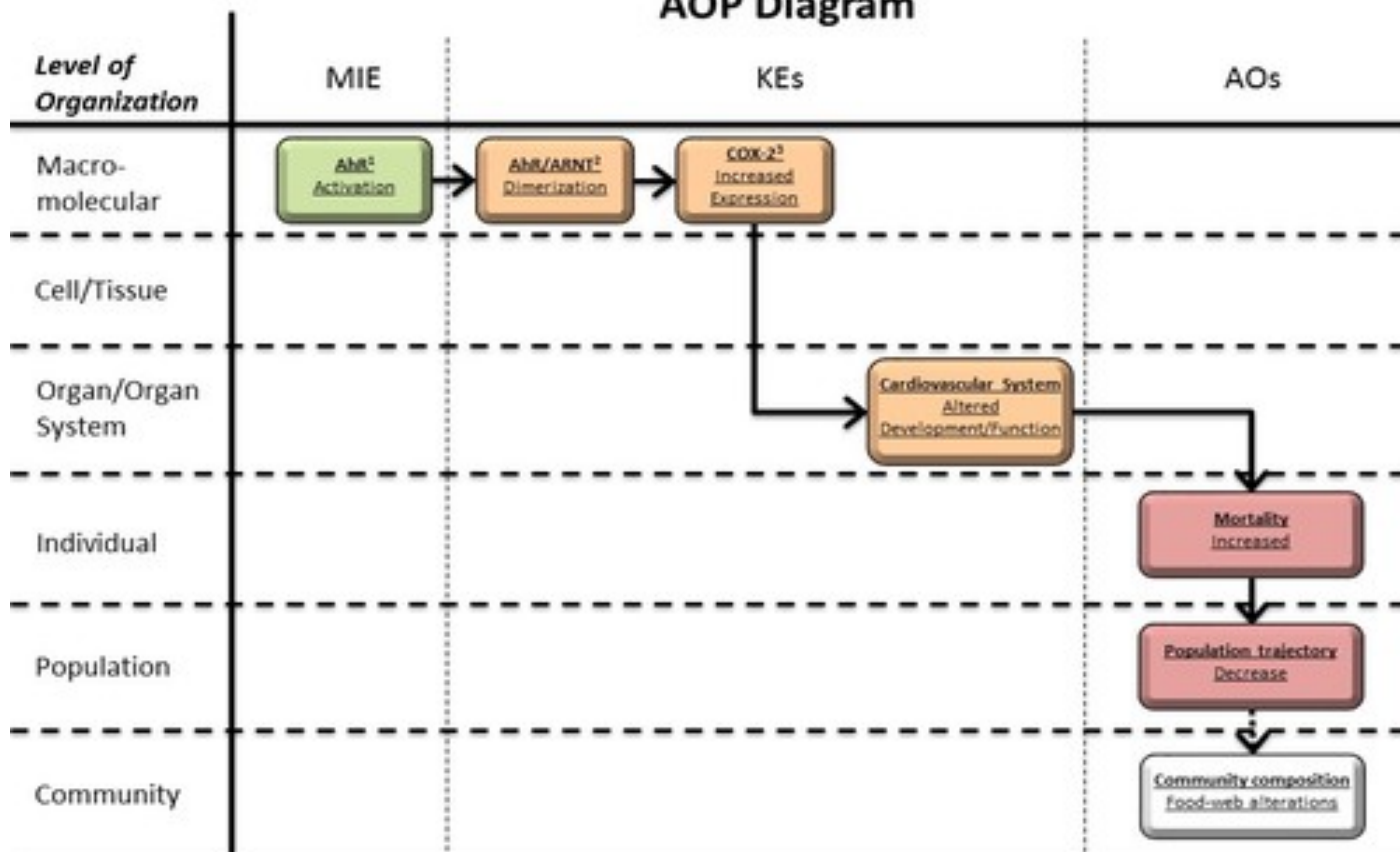
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Graphical Representation

AOP Diagram



¹AhR: Aryl Hydrocarbon Receptor

²ARNT: Aryl Hydrocarbon receptor Nuclear Translocator

³COX-2: Cyclooxygenase-2

Overall Assessment of the AOP

Consider the following criteria (may include references to KE Relationship pages): 1. concordance of dose-response relationships; 2. temporal concordance among the key events and adverse effect; 3. strength, consistency, and specificity of association of adverse effect and initiating event; 4. biological plausibility, coherence, and consistency of the experimental evidence; 5. alternative mechanisms that logically present themselves and the extent to which they may distract from the postulated AOP. It should be noted that alternative mechanisms of action, if supported, require a separate AOP; 6. uncertainties, inconsistencies and data gaps.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong
development	Strong

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
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Atlantic killifish	Fundulus heteroclitus	Moderate	NCBI
zebrafish	Danio rerio	Strong	NCBI
rainbow trout	Oncorhynchus mykiss	Moderate	NCBI
medaka	Oryzias latipes	Strong	NCBI
fathead minnow	Pimephales promelas	Moderate	NCBI
channel catfish	Ictalurus punctatus	Moderate	NCBI
Salvelinus namaycush	Salvelinus namaycush	Moderate	NCBI
Salvelinus fontinalis	Salvelinus fontinalis	Moderate	NCBI
Carassius carassius	Carassius carassius	Moderate	NCBI
Pagrus major	Pagrus major	Moderate	NCBI
Acipenser fulvescens	Acipenser fulvescens	Moderate	NCBI
Coregonus artedii	Coregonus artedii	Moderate	NCBI
Catostomus commersonii	Catostomus commersonii	Moderate	NCBI
Esox lucius	Esox lucius	Moderate	NCBI
Scaphirhynchus albus	Scaphirhynchus albus	Moderate	NCBI
Scaphirhynchus platyrhynchus	Scaphirhynchus platyrhynchus	Moderate	NCBI
Xenopus laevis laevis	Xenopus laevis laevis	Weak	NCBI
Gallus gallus	Gallus gallus	Strong	NCBI

Sex Applicability

Sex	Evidence
Unspecific	Strong

Sex: This AOP is only applicable to early life stages prior to sexual differentiation.

Life stages: This AOP is only applicable starting from embryonic development. In zebrafish, this critical window extends from fertilization to approximately 24 hours post fertilization (hpf) (Belair et al 2001; Goldstone & Stegeman 2008).

Taxonomic: The specific characteristics of altered cardiovascular development and function vary to some degree among taxonomic groups of vertebrates. However, reduction in myocyte proliferation and heart pumping efficiency is common to all investigated vertebrates (Antkiewicz et al 2005; Jones & Kennedy 2009; Thackaberry et al 2005).

This AOP is applicable to:

- All teleost and non-teleost fishes that have been investigated as embryos so far (Buckler et al 2015; Doering et al 2013; Elonen et al 1998; Johnson et al 1998; Park et al 2014; Tillitt et al 2016; Toomey et al 2001; Walker et al 1991; Yamauchi et al 2006; Zabel et al 1995).
- All birds (Canga et al 1993; Cohen-Barnhouse et al 2011; Fujisawa et al 2014; Heid et al 2001; Ivnitski et al 2001; Walker & Catron 2000). However, some details of the AOP might be different in birds as cyclooxygenase-2 (COX-2) is believed to be up-regulated through non-genomic mechanisms in these taxa based on investigations in chicken (*Gallus gallus*) (Fujisawa et al 2014).
- Amphibians and reptiles have insufficient mechanistic and early life stage mortality information to demonstrate applicability at this time. However, amphibians and reptiles express AhRs that are activated by agonists in a manner consistent with other vertebrates and express AhRs during embryonic development (Lavine et al 2005; Shoots et al 2015; Ohi et al 2003; Oka et al 2016). However, altered cardiovascular development and function and early life stage mortality have not been observed at any investigated concentration of DLC in amphibians studied to date (Jung et al 1997). This tolerance is believed to result from AhRs of amphibians having very low affinity for agonists (Lavine et al 2005; Shoots et al 2015). Therefore, it is acknowledged that this AOP is likely to be applicable to reptiles. However, it might not be applicable to amphibians due to their extreme tolerance to activation of the AhR.
- Cartilaginous fishes (Chondrichthyes) have insufficient mechanistic and early life stage mortality information to demonstrate applicability at this time. However, sharks and rays are known to express AhRs that are structurally comparable to AhRs of teleost fishes (Hahn 2002). Sharks and rays have also been shown to respond to exposure to agonists of the AhR through responses that are comparable to teleost fishes, specifically through induction of CYP1A (Hahn et al 1998). Therefore, it is acknowledged that this AOP is likely to be applicable to Chondrichthyes.

This AOP is not applicable to:

- Mammals because the cause of mortality of the young is primarily a result of wasting syndrome and not necessarily altered cardiovascular development and function (Kopf & Walker 2009). Further, studies of CYP1A1 and CYP1A2 null mice (*Mus musculus*) demonstrate that wasting syndrome and mortality are mediated by CYP1A1 in mammals (Uno et al 2004).
- Invertebrates because AhRs of invertebrates have less diverse functionalities relative to vertebrates, AhRs of invertebrates are not known to bind agonists that represent anthropogenic pollutants, and no critical adverse effects are known in invertebrates as a result of activation of the AhR (Hahn 2002; Hahn et al 1994).
- Jawless fishes, such as lamprey (Petromyzontiformes) and hagfish (Myxiniiformes), because of a lack of measurable AhR-mediated responses (Hahn et al 1998). Although additional information is necessary for this taxa, it is currently acknowledged that this AOP is likely not applicable to jawless fishes.

Essentiality of the Key Events

Support for essentiality for key events in the AOP was provided by a series of knockdown and targeted agonist and antagonist experiments. These investigations were conducted mainly with zebrafish as the model species and TCDD as the model agonist of the AhR.

Rationale for essentiality calls:

- AhR, activation: [Strong] Knockdown of AhR prevents TCDD induced alteration in cardiovascular development and function (Clark et al 2010; Hanno et al 2010; Karchner et al 1999; Prasch et al 2003; Van Tiem & Di Giulio 2011).

- AhR/ARNT, dimerization: [Strong] Knockdown of ARNT prevents TCDD induced alteration in cardiovascular development and function (Antkiewicz et al 2006; Prasch et al 2004). Depletion of ARNT lessens or prevents TCDD induced alteration in cardiovascular development and function (Prasch et al 2004).
- COX-2, increase: [Strong] Knockdown of COX-2 and selective antagonists of COX-2 prevent TCDD induced alteration in cardiovascular development and function (Dong et al 2010; Teraoka et al 2008; 2014). COX-2 inducers that are not agonists of the AhR cause altered cardiovascular development and function that is consistent with activation of the AhR (Huang et al 2007). Knockdown of and selective antagonists of thromboxane A synthase 1 (CYP5A), which is down-stream of COX-2 in the prostaglandin synthesis pathway, prevents TCDD induced alteration in cardiovascular development and function (Teraoka et al 2008). Exposure to the substrate for COX-2, arachidonic acid, causes an up-regulation in COX-2 and altered cardiovascular development and function that is consistent with exposure to TCDD (Dong et al 2010).
- Cardiovascular development and function, altered: [Strong] Isosmotic rearing solution prevents yolk sac edema, but has no effect on TCDD induced alteration in cardiovascular development and function or mortality (Hill et al 2004). This indicates that mortality is not caused by yolk sac edema. Knockdown of cytochrome P450 1A (CYP1A) or injection with antioxidants decreases oxidative stress but has no effect on TCDD induced alteration in cardiovascular development and function or mortality (Carney et al 2004; Scott et al 2011). This is suggestive that mortality is not caused by oxidative stress. Exposure to agonists of the AhR post-heart development lessens or prevents alteration in cardiovascular development, decreased blood flow, and cardiac failure (Carney et al 2004; Lanham et al 2012). Exposure to agonists of the AhR post-heart development dramatically reduces mortality (Carney et al 2004; Lanham et al 2012). This suggests that mortality is caused by circulatory failure as a result of cardiovascular teratogenesis.

Weight of Evidence Summary

Biological Plausibility:

- In general, the biological plausibility and coherence linking activation of the AhR through early life stage mortality from COX-2 induced alteration in cardiovascular development and function is very strong.
- The AhR is known to have critical roles in development of the heart and therefore dysregulation of these roles would be expected to result in altered cardiac development.
- The prostaglandin synthesis pathway, of which COX-2 is a rate limiting step, is known to have roles in development of the heart (Dong et al 2010; Huang et al 2007; Teraoka et al 2008; 2014).
- A properly functioning circulatory system is widely acknowledged to be crucial for survival of vertebrates (Kardong 2006). General dysfunction of the heart or associated vasculature is widely documented to have the potential to result in mortality through cardiac failure, regardless of the mechanism of dysfunction.

Concordance of dose-response relationships:

- There is significant evidence showing concordance of dose-response for incidence and severity of alteration in cardiovascular development and function and subsequently mortality across at least 16 different species of fish (Buckler et al 2015; Elonen et al 1998; Huang et al 2012; Johnson et al 1998; Park et al 2014; Tillitt et al 2016; Toomey et al 2001; Walker et al 1991; Yamauchi et al 2006; Zabel et al 1995) and 8 different species of birds (Cohen-Barnhouse et al 2011; Brunstrom 1990; Brunstrom & Andersson 1988; Hoffman et al 1996; 1998; Powell et al 1998) for several PCDDs, PCDFs, planar PCBs, and PAHs. Concordance of dose-response has not been observed in amphibians studied to date because no elevated mortality or altered

cardiovascular development and function was observed at any tested concentration of agonist (Jung et al 1997).

- Less is known regarding concordance of dose-response relationships for COX-2. In Japanese medaka (*Oryzias latipes*), abundance of transcript of COX-2 is significantly greater than controls at concentrations of TCDD of 0.2 ppb and greater (Dong et al 2010). Likewise, incidence of cardiovascular development and heart area were both significantly different than controls at concentrations of TCDD of 0.2 ppb and greater (Dong et al 2010).

Temporal concordance among the key events and adverse effect:

- Alterations in cardiovascular development or function is first observable in zebrafish around 48 hours post fertilization (hpf), while mortality does not begin to occur until around 86 hpf (Goldstone & Stegeman 2006).
- AhR transcript and protein is first detectable in zebrafish around 24 hpf (Tanguay et al 1999).
- COX-2 transcript is first detectable in zebrafish around 6 hpf (Teraoka et al 2008). Expression of COX-2 was investigated in zebrafish exposed to TCDD at 55 and 72 hpf (Teraoka et al 2014). At 55 hpf there was a trend towards up-regulation of COX-2 (~ 1.5-fold), while at 72 hpf there was a significant up-regulation of COX-2 (~ 4.5-fold) (Teraoka et al 2014).
- Therefore, there is a general temporal concordance in this AOP.
- However, there is some uncertainty in the early manifestations of altered cardiovascular development and up-regulation of COX-2. It is possible that the first manifestations of altered cardiovascular development result from mechanisms other than COX-2. For example, sex determining region Y-box-9b (Sox9b) is first expressed in zebrafish at around 24 hpf and has been known to cause some altered cardiovascular phenotypes (Hofsteen et al 2013; Li et al 2002). However, no studies have yet investigated temporal concordance of regulation of Sox9b by the AhR prior to 72 hpf (Hofsteen et al 2013). It is also possible that temporal concordance of early increases in COX-2 is obscured by the relatively little fold-changes observed for COX-2. Additional investigations into up-regulation of COX-2 by activation of the AhR across developmental stages is warranted.

Consistency:

- There are no known AhR-mediated effects that occur at concentrations below those that cause alteration in cardiovascular development and function that result in early life stage mortality in fishes, amphibians, reptiles, or birds.
- There are no studies in which COX-2 and altered cardiovascular development or function were co-investigated in which altered cardiovascular development or function occurred without an up-regulation in COX-2.
- In TCDD exposure groups, some individuals do not manifest alterations in cardiovascular development or function (Dong et al 2010). TCDD exposed individuals of Japanese medaka that did not manifest alterations in cardiovascular development or function had expression of COX-2 that was not statistically different than controls, while individuals that did manifest alterations in cardiovascular development or function had increased expression of COX-2 (Dong et al 2010).
- There is also consistency in the TCDD-induced alterations in cardiovascular phenotype between distantly related oviparous taxa, namely fish and birds (Teraoka et al 2008; Fujisawa et al 2014). Likewise, COX-2 is known to be up-regulated in both these taxa (Teraoka et al 2008; Fujisawa et al 2014). Cardiovascular development and function and COX-2 have not been investigated in amphibians or reptiles.

Uncertainties, inconsistencies, and data gaps:

- There are several other pathways by which activation of the AhR could result in altered cardiovascular development and function in developing embryos. These include, but are not limited to, down-regulation in Sox9b, BMP-4, and genes in the cell cycle gene cluster (Hofsteen et al 2013; Jonsson et al 2007), oxidative stress (Goldstone & Stegeman 2008), and AhR cross-talk with hypoxia inducible factor 1 α (HIF1 α) (Goldstone & Stegeman 2008).
- However, investigations of knockdown and null strains for Sox9b in zebrafish do not result in the complete phenotype of altered cardiovascular development recorded in embryos following exposure to planar aromatic hydrocarbons (Hofsteen et al 2013). Injection of TCDD exposed embryos with Sox9b mRNA was able to prevent the Sox9b phenotype of cardiovascular development, however it did not prevent altered cardiovascular development altogether (Hofsteen et al 2013). Further, altered cardiovascular development as a result of down-regulation in Sox9b is not severe enough to cause complete cardiac failure and early life stage mortality in zebrafish (Hofsteen et al 2013).
- Oxidative stress as a result of induction in CYP1A has commonly been proposed as the mechanism of altered cardiovascular development and function and CYP1A follows dose- and temporal concordance with mortality across numerous investigations in fishes and birds (Goldstone & Stegeman 2008). Early studies of CYP1A knockdown in zebrafish demonstrated protection against alteration in cardiovascular development and function induced by exposure to TCDD (Teraoka et al 2003). However, more recent investigations have observed no protection (Carney et al 2006). This inconsistency has been proposed to result from the earlier studies only recording alteration in cardiovascular development and function at early stages when adverse effects are difficult to accurately observe (Carney et al 2006). In birds, COX-2 inhibitors have no effect on expression of CYP1A but protect against TCDD induced alteration in cardiovascular development and function suggesting that CYP1A is not involved in toxicities (Fujisawa et al 2014).
- For cross-species and cross-taxa extrapolation, there is uncertainty in whether COX-2 is up-regulated by AhR through genomic or non-genomic mechanisms. Specifically, there is no detailed analysis regarding how widespread COX-2 genes which contain DREs in the promoter region are among species and among taxa and whether non-genomic or genomic mechanisms of up-regulation in COX-2 are more ubiquitous.
- All mechanistic investigations into mechanisms of AhR-mediated alteration in cardiovascular development and function have been conducted in zebrafish, Japanese medaka, and chicken. Therefore, no mechanistic information is available to conclude cross-species extrapolation outside of a shared phenotype of altered cardiovascular development and function. There is no information about AhR-mediated alteration in cardiovascular development or function or up-regulation of COX-2 in early fishes (Petromyzontiformes; Myxiniiformes; Chondrichthyes), amphibians, or reptiles.

Quantitative Consideration

- There is a strong quantitative understanding between quantitative structure-activity relationship (QSAR) or binding affinity for the AhR and potency among PCDDs, PCDFs, and planar PCBs (Van den Berg et al 1998; 2006). Specifically, these studies have demonstrated that congeners with greater binding affinity have greater potency (Van den Berg et al 1998; 2006). This has partially contributed to the successful development of the toxic equivalency factor (TEF) methodology in risk assessment (Van den Berg et al 1998; 2006).
- There is also a strong quantitative understanding of differences in binding affinity of the AhR among species of birds and differences in sensitivity to early life stage mortality (Karchner et al 2006; Farmahin et al 2012; 2013; Manning et al 2013). Specifically, these studies demonstrate that species of birds with AhRs with greater affinity for DLCs have greater sensitivity than species with AhRs with lesser affinity for DLCs (Karchner et al 2006). These differences in sensitivity range by more than 40-fold for TCDD (Cohen-Barnhouse et al 2011). However, a quantitative understanding between differences in binding affinity of the AhR among species and differences in sensitivity to early life stage mortality is not yet available for other taxa

(Doering et al 2013).

- There is some quantitative understanding between up-regulation of COX-2 and incidence of and severity of cardiac deformities for medakafish exposed to TCDD (Dong et al 2010). This quantitative understanding includes a strong linear relationship ($R^2 = 0.88$) between abundance of COX-2 transcript and heart area (Dong et al 2010). However, this information is not available with regards to multiple investigations, species, taxonomic groups, or chemicals.
- There is strong quantitative understanding between incidence of and severity of cardiovascular deformities and mortality. However, numerous different cardiovascular endpoints are investigated among studies making side-by-side comparisons difficult.

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