AOP ID and Title:

SNAPSHOT

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AOP 57: AhR activation leading to hepatic steatosis

Short Title: AhR activation to steatosis

Authors

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Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite		1.29	Under Development

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	18	Activation, AhR (https://aopwiki.org/events/18)	Activation, AhR
2	KE	450	Suppression, VLDL secretion (https://aopwiki.org/events/450)	Suppression, VLDL secretion
3	KE	451	Inhibition, Mitochondrial fatty acid beta-oxidation (https://aopwiki.org/events/451)	Inhibition, Mitochondrial fatty acid beta-oxidation
4	KE	327	Accumulation, Fatty acid (https://aopwiki.org/events/327)	Accumulation, Fatty acid
5	KE	216	Decreased, PCK1 expression (control point for glycolysis/gluconeogenesis pathway) (https://aopwiki.org/events/216)	Decreased, PCK1 expression (control point for glycolysis/gluconeogenesis pathway)
6	KE	291	Accumulation, Triglyceride (https://aopwiki.org/events/291)	Accumulation, Triglyceride
7	KE	54	Up Regulation, CD36 (https://aopwiki.org/events/54)	Up Regulation, CD36
8	KE	465	Increased, FA Influx (https://aopwiki.org/events/465)	Increased, FA Influx
9	KE	466	Up Regulation, LDLR (low density lipoprotein receptor) (https://aopwiki.org/events/466)	Up Regulation, LDLR (low density lipoprotein receptor)
10	KE	467	Increased, LDL uptake (https://aopwiki.org/events/467)	Increased, LDL uptake
11	KE	80	Up Regulation, CYP1A1 (https://aopwiki.org/events/80)	Up Regulation, CYP1A1
12	KE	462	Up Regulation, SCD-1 (https://aopwiki.org/events/462)	Up Regulation, SCD-1

Sequence	Type	Event ID	Title	Short name
13	AO	455	Accumulation, Liver lipid (https://aopwiki.org/events/455)	Accumulation, Liver lipid

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Suppression, VLDL secretion (https://aopwiki.org/relationships/471)	adjacent	Accumulation, Liver lipid	High	High
Accumulation, Triglyceride (https://aopwiki.org/relationships/474)	adjacent	Accumulation, Liver lipid	High	High
Inhibition, Mitochondrial fatty acid beta-oxidation (https://aopwiki.org/relationships/475)	adjacent	Accumulation, Fatty acid	High	High
Activation, AhR (https://aopwiki.org/relationships/495)	adjacent	Up Regulation, CD36	High	High
Activation, AhR (https://aopwiki.org/relationships/499)	adjacent	Decreased, PCK1 expression (control point for glycolysis/gluconeogenesis pathway)	High	High
Up Regulation, CD36 (https://aopwiki.org/relationships/501)	adjacent	Increased, FA Influx	High	
Accumulation, Fatty acid (https://aopwiki.org/relationships/502)	adjacent	Accumulation, Liver lipid	High	High
Increased, FA Influx (https://aopwiki.org/relationships/505)	adjacent	Accumulation, Fatty acid		
Activation, AhR (https://aopwiki.org/relationships/506)	adjacent	Up Regulation, LDLR (low density lipoprotein receptor)	High	
Up Regulation, LDLR (low density lipoprotein receptor) (https://aopwiki.org/relationships/507)	adjacent	Increased, LDL uptake		
Increased, LDL uptake (https://aopwiki.org/relationships/508)	adjacent	Accumulation, Fatty acid		
Activation, AhR (https://aopwiki.org/relationships/19)	adjacent	Up Regulation, CYP1A1	High	
Activation, AhR (https://aopwiki.org/relationships/1656)	adjacent	Up Regulation, SCD-1	Moderate	
Up Regulation, SCD-1 (https://aopwiki.org/relationships/1657)	adjacent	Accumulation, Triglyceride	High	
Activation, AhR (https://aopwiki.org/relationships/473)	non-adjacent	Inhibition, Mitochondrial fatty acid beta-oxidation	Moderate	Moderate
Decreased, PCK1 expression (control point for glycolysis/gluconeogenesis pathway) (https://aopwiki.org/relationships/503)	non-adjacent	Accumulation, Fatty acid		
Activation, AhR (https://aopwiki.org/relationships/509)	non-adjacent	Suppression, VLDL secretion		

Overall Assessment of the AOP

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

References

Appendix 1

List of MIEs in this AOP

Event: 18: Activation, AhR (https://aopwiki.org/events/18)

Short Name: Activation, AhR

Key Event Component

Process	Object	Action
aryl hydrocarbon receptor activity	aryl hydrocarbon receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:21 - aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2 (https://aopwiki.org/aops/21)	MolecularInitiatingEvent
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	MolecularInitiatingEvent
Aop:131 - Aryl hydrocarbon receptor activation leading to uroporphyria (https://aopwiki.org/aops/131)	MolecularInitiatingEvent
Aop:150 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF (https://aopwiki.org/aops/150)	MolecularInitiatingEvent

Stressors

Name
Benzidine
Dibenzo-p-dioxin
Polychlorinated biphenyl
Polychlorinated dibenzofurans
Hexachlorobenzene
Polycyclic aromatic hydrocarbons (PAHs)

Biological Context

Level of Biological Organization
Molecular

Level of Biological Organization

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

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

- 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.

Dibenzo-p-dioxin

Denison, M. S., Soshilov, A. A., He, G., DeGroot, D. E., and Zhao, B. (2011). Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol.Sci.* **124**, 1-22.

Polychlorinated biphenyl

Of the dioxin-like PCBs, non-ortho congeners are the most toxicologically active, while mono-ortho PCBs are generally less potent (McFarland and Clarke 1989; Safe 1994). Chlorine substitution at ortho positions increases the energetic costs of assuming the coplanar conformation required for binding to the AHR (McFarland and Clarke 1989). 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 (Safe 1994).

Safe, S. (1994). Polychlorinated biphenyls (PCBs): Environmental impact, biochemical and toxic responses, and implications for risk assessment. *Critical Reviews in Toxicology* **24**, 87-149.

McFarland, V. A., and Clarke, J. U. (1989). Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: Considerations for a congener-specific analysis. *Environ.Health Perspect.* **81**, 225-239.

Polychlorinated dibenzofurans

Denison, M. S., Soshilov, A. A., He, G., DeGroot, D. E., and Zhao, B. (2011). Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol.Sci.* **124**, 1-22.

Hexachlorobenzene

Cripps, D. J., Peters, H. A., Gocmen, A., and Dogramici, I. (1984) Porphyria turcica due to hexachlorobenzene: a 20 to 30 year follow-up study on 204 patients. *Br. J Dermatol.* **111** (4), 413-422.

Polycyclic aromatic hydrocarbons (PAHs)

PAHs are pontent AHR agonists, but due to their rapid metabolism, they cause a transient alteration in AHR-mediated gene expression; this property results in a very different toxicity profile relative to persistent AHR-agonists such as dioxin-like compounds (Denison et al. 2011).

Denison, M. S., Soshilov, A. A., He, G., DeGroot, D. E., and Zhao, B. (2011). Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol.Sci.* **124**, 1-22.

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebra danio	Danio rerio	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7955)
Gallus gallus	Gallus gallus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9031)
Pagrus major	Pagrus major	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=143350)
Acipenser transmontanus	Acipenser transmontanus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7904)
Acipenser fulvescens	Acipenser fulvescens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=41871)
rainbow trout	Oncorhynchus mykiss	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8022)
Salmo salar	Salmo salar	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8030)
Xenopus laevis	Xenopus laevis	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8355)
Ambystoma mexicanum	Ambystoma mexicanum	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8296)
Phasianus colchicus	Phasianus colchicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9054)
Coturnix japonica	Coturnix japonica	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=93934)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
Microgadus tomcod	Microgadus tomcod	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=34823)

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

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_Ala380) 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_Ala380) and type 3 (Val324_Ala380)[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).
    - This was attributed to the rapid evolution of populations in highly contaminated areas of the Hudson River, resulting in a 6-base pair deletion in the AHR sequence (outside the LBD) and reduced ligand binding affinity, due to reduces AHR protein stability.
- 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).

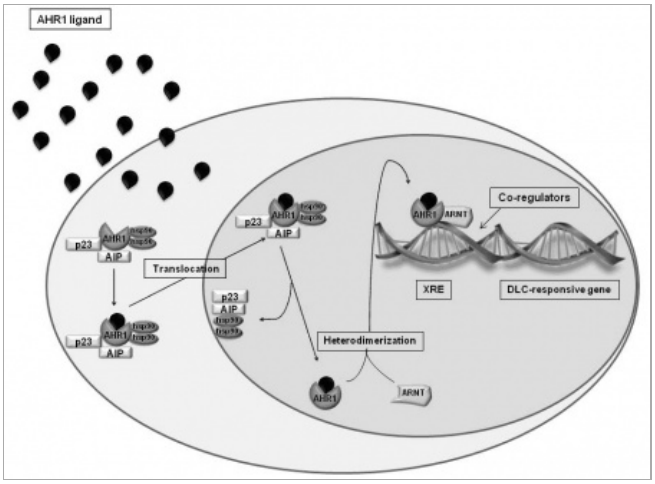
Key Event Description

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



(https://aopwiki.org/wiki/index.php/File:AHR_mechanism.jpeg)

Figure 1: The molecular mechanism of activation of gene expression by AHR.

The molecular mechanism for AHR-mediated activation of gene expression is presented in Figure 1. 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).

Roles of isoforms in birds:

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-

- 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 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

The described luciferase reporter gene (LRG) assays have been used to investigate activation of AhRs of:

- Humans (*Homo sapiens*) (Abnet et al 1999)
- Species of birds, namely chicken (*Gallus gallus*), ring-necked pheasant (*Phasianus colchicus*), Japanese quail (*Coturnix japonica*), and common tern (*Sterna hirundo*) (Farmahin et al 2012; Manning et al 2013), Mutant AhR1s with ligand binding domains resembling those of at least 86 avian species have also been investigated (Farmahin et al 2013). AhR2s of birds have only been investigated in black-footed albatross (*Phoebastria nigripes*) and common cormorant (*Phalacrocorax carbo*) (Yasio et al 2007).
- American alligator (*Alligator mississippiensis*) is the only reptile for which AhR activation has been investigated (Oka et al 2016), AhR1A, AhR1B, and AhR2 of American alligator were assayed (Oka et al 2016).
- AhR1 of two amphibians have been investigated, namely African clawed frog (*Xenopus laevis*) and salamander (*Ambystoma mexicanum*) (Lavine et al 2005; Shoots et al 2015; Ohi et al 2003),
- AhR1s and AhR2s of several species of fish have been investigated, namely Atlantic salmon (*Salmo salar*), Atlantic tomcod (*Microgadus tomcod*), white sturgeon (*Acipenser transmontanus*), rainbow trout (*Onchorhynchys mykiss*), red seabream (*Pagrus major*), lake sturgeon (*Acipenser fulvescens*), and zebrafish (*Danio rerio*) (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. However, comparable assays are utilized for investigating AHR1s and AHR2s of all taxa. 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 binging 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].

In silico Approaches

In silico homology modeling of the ligand binding domain of the AHR in combination with molecular docking simulations can provide valuable insight into the transactivation-potential of a diverse array of AHR ligands. Such models have been developed for multiple AHR isoforms and ligands (high/low affinity, endogenous and synthetic, agonists and antagonists), and can accurately predict ligand potency based on their structure and physicochemical properties (Bonati et al 2017; Hirano et al 2015; Sovadinova et al 2006).

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List of Key Events in the AOP

Event: 450: Suppression, VLDL secretion (https://aopwiki.org/events/450)

Short Name: Suppression, VLDL secretion

Key Event Component

Process	Object	Action
secretion	very-low-density lipoprotein	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
hepatocyte

Event: 451: Inhibition, Mitochondrial fatty acid beta-oxidation (https://aopwiki.org/events/451)

Short Name: Inhibition, Mitochondrial fatty acid beta-oxidation

Key Event Component

Process	Object	Action
fatty acid beta-oxidation	fatty acid	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent
Aop:58 - NR1I3 (CAR) suppression leading to hepatic steatosis (https://aopwiki.org/aops/58)	KeyEvent
Aop:61 - NFE2L2/FXR activation leading to hepatic steatosis (https://aopwiki.org/aops/61)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
hepatocyte

Event: 327: Accumulation, Fatty acid (https://aopwiki.org/events/327)

Short Name: Accumulation, Fatty acid

Key Event Component

Process	Object	Action
	fatty acid	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:36 - Peroxisomal Fatty Acid Beta-Oxidation Inhibition Leading to Steatosis (https://aopwiki.org/aops/36)	KeyEvent
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent
Aop:58 - NR1I3 (CAR) suppression leading to hepatic steatosis (https://aopwiki.org/aops/58)	KeyEvent
Aop:60 - NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis (https://aopwiki.org/aops/60)	KeyEvent

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
liver

Event: 216: Decreased, PCK1 expression (control point for glycolysis/gluconeogenesis pathway) (https://aopwiki.org/events/216)

Short Name: Decreased, PCK1 expression (control point for glycolysis/gluconeogenesis pathway)

Key Event Component

Process	Object	Action
gene expression	phosphoenolpyruvate carboxykinase, cytosolic [GTP]	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Event: 291: Accumulation, Triglyceride (https://aopwiki.org/events/291)

Short Name: Accumulation, Triglyceride

Key Event Component

Process	Object	Action
	triglyceride	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:34 - LXR activation leading to hepatic steatosis (https://aopwiki.org/aops/34)	KeyEvent
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
hepatocyte

Key Event Description

Leads to Fatty Liver Cells.

Event: 54: Up Regulation, CD36 (https://aopwiki.org/events/54)

Short Name: Up Regulation, CD36

Key Event Component

Process	Object	Action
gene expression	platelet glycoprotein 4	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:34 - LXR activation leading to hepatic steatosis (https://aopwiki.org/aops/34)	KeyEvent
Aop:58 - NR1I3 (CAR) suppression leading to hepatic steatosis (https://aopwiki.org/aops/58)	KeyEvent
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent
Aop:60 - NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis (https://aopwiki.org/aops/60)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
hepatocyte

Key Event Description

Fatty acid translocase CD36 (FAT/CD36) is a scavenger protein mediating uptake and intracellular transport of long-chain fatty acids (FA) in diverse cell types [1], [2]. In addition, CD36 can bind a variety of molecules including acetylated low density lipoproteins (LDL), collagen and phospholipids [3]. CD36 has been shown to be expressed in liver tissue [4], [5]. It is located in lipid rafts and non-raft domains of the cellular plasma membrane and most likely facilitates LCFA transport by accumulating LCFA on the outer surface [6], [7], [8].

FAT/CD36 gene is a liver specific target of LXR activation [9]. Studies have confirmed that the lipogenic effect of LXR and activation of FAT/CD36 was not a simple association, since the effect of LXR agonists on increasing hepatic and circulating levels of triglycerides and free fatty acids (FFAs) was largely abolished in FAT/CD36 knockout mice suggesting that intact expression and/or activation of FAT/CD36 is required for the steatotic effect of LXR agonists [10], [11]. In addition to the well-defined pathogenic role of FAT/CD36 in hepatic steatosis in rodents the human up-regulation of the FAT/CD36 in NASH patients is confirmed [12]. There are now findings that can accelerate the translation of FAT/CD36 metabolic functions determined in rodents to humans [13] and suggest that the translocation of this fatty acid transporter to the plasma membrane of hepatocytes may contribute to liver fat accumulation in patients with NAFLD and HCV [14]. In addition, hepatic FAT/CD36 up-regulation is significantly associated with insulin resistance, hyperinsulinaemia and increased steatosis in patients with NASH and HCV G1 (Hepatitis C Virus Genotype1) with fatty liver. Recent data show that CD36 is also increased in the liver of morbidly obese patients and correlated to free FA levels [15].

References

1. ↑ Su & Abumrad 2009
2. ↑ He et al. 2011
3. ↑ Krammer 2011

4. ↑ Pohl et al. 2005
5. ↑ Cheung et al. 2007
6. ↑ Ehehalt et al. 2008
7. ↑ Pohl et al. 2005
8. ↑ Krammer 2011
9. ↑ Zhou 2008
10. ↑ Febbraio et al. 1999
11. ↑ Lee et al. 2008
12. ↑ Zhu et al. 2011
13. ↑ Love-Gregory et al. 2011
14. ↑ Miquilena-Colina et al. 2011
15. ↑ Bechmann et al. 2010

Event: 465: Increased, FA Influx (https://aopwiki.org/events/465)

Short Name: Increased, FA Influx

Key Event Component

Process	Object	Action
fatty acid transport	fatty acid	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent
Aop:60 - NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis (https://aopwiki.org/aops/60)	KeyEvent
Aop:58 - NR1I3 (CAR) suppression leading to hepatic steatosis (https://aopwiki.org/aops/58)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
hepatocyte

Event: 466: Up Regulation, LDLR (low density lipoprotein receptor) (https://aopwiki.org/events/466)

Short Name: Up Regulation, LDLR (low density lipoprotein receptor)

Key Event Component

Process	Object	Action
gene expression	low-density lipoprotein receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
hepatocyte

Event: 467: Increased, LDL uptake (https://aopwiki.org/events/467)

Short Name: Increased, LDL uptake

Key Event Component

Process	Object	Action
receptor-mediated endocytosis	low-density lipoprotein	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
hepatocyte

Event: 80: Up Regulation, CYP1A1 (https://aopwiki.org/events/80)

Short Name: Up Regulation, CYP1A1

Key Event Component

Process	Object	Action
gene expression	cytochrome P450 1A1	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
hepatocyte

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Acipenser transmontanus	Acipenser transmontanus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=7904)
Oncorhynchus mykiss	Oncorhynchus mykiss	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=8022)

Event: 462: Up Regulation, SCD-1 (https://aopwiki.org/events/462)

Short Name: Up Regulation, SCD-1

Key Event Component

Process	Object	Action
gene expression	acyl-CoA desaturase	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:58 - NR1I3 (CAR) suppression leading to hepatic steatosis (https://aopwiki.org/aops/58)	KeyEvent
Aop:60 - NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis (https://aopwiki.org/aops/60)	KeyEvent
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
hepatocyte

List of Adverse Outcomes in this AOP

Event: 455: Accumulation, Liver lipid (https://aopwiki.org/events/455)

Short Name: Accumulation, Liver lipid

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:57 - AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	AdverseOutcome

Biological Context

Level of Biological Organization
Organ

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 471: Suppression, VLDL secretion leads to Accumulation, Liver lipid (https://aopwiki.org/relationships/471)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**High**	**High**

Relationship: 474: Accumulation, Triglyceride leads to Accumulation, Liver lipid (https://aopwiki.org/relationships/474)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**High**	**High**

Relationship: 475: Inhibition, Mitochondrial fatty acid beta-oxidation leads to Accumulation, Fatty acid (https://aopwiki.org/relationships/475)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**High**	**High**
NR1I3 (CAR) suppression leading to hepatic steatosis (https://aopwiki.org/aops/58)	**adjacent**		

Relationship: 495: Activation, AhR leads to Up Regulation, CD36 (https://aopwiki.org/relationships/495)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**High**	**High**

Relationship: 499: Activation, AhR leads to Decreased, PCK1 expression (control point for glycolysis/gluconeogenesis pathway) (https://aopwiki.org/relationships/499)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**High**	**High**

Relationship: 501: Up Regulation, CD36 leads to Increased, FA Influx (https://aopwiki.org/relationships/501)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**High**	
NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis (https://aopwiki.org/aops/60)	**adjacent**	**High**	
NR1I3 (CAR) suppression leading to hepatic steatosis (https://aopwiki.org/aops/58)	**adjacent**	**High**	**High**

Relationship: 502: Accumulation, Fatty acid leads to Accumulation, Liver lipid (https://aopwiki.org/relationships/502)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**High**	**High**

Relationship: 505: Increased, FA Influx leads to Accumulation, Fatty acid (https://aopwiki.org/relationships/505)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**		
NR1I3 (CAR) suppression leading to hepatic steatosis (https://aopwiki.org/aops/58)	**adjacent**	**High**	
NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis (https://aopwiki.org/aops/60)	**adjacent**	**High**	

Relationship: 506: Activation, AhR leads to Up Regulation, LDLR (low density lipoprotein receptor) (https://aopwiki.org/relationships/506)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**High**	

Relationship: 507: Up Regulation, LDLR (low density lipoprotein receptor) leads to Increased, LDL uptake (https://aopwiki.org/relationships/507)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**		

Relationship: 508: Increased, LDL uptake leads to Accumulation, Fatty acid (https://aopwiki.org/relationships/508)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**		

Relationship: 19: Activation, AhR leads to Up Regulation, CYP1A1 (https://aopwiki.org/relationships/19)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**High**	

Relationship: 1656: Activation, AhR leads to Up Regulation, SCD-1 (https://aopwiki.org/relationships/1656)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**Moderate**	

Relationship: 1657: Up Regulation, SCD-1 leads to Accumulation, Triglyceride (https://aopwiki.org/relationships/1657)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**adjacent**	**High**	

List of Non Adjacent Key Event Relationships

Relationship: 473: Activation, AhR leads to Inhibition, Mitochondrial fatty acid beta-oxidation (https://aopwiki.org/relationships/473)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**non-adjacent**	**Moderate**	**Moderate**

Relationship: 503: Decreased, PCK1 expression (control point for glycolysis/gluconeogenesis pathway) leads to Accumulation, Fatty acid (https://aopwiki.org/relationships/503)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**non-adjacent**		

Relationship: 509: Activation, AhR leads to Suppression, VLDL secretion (https://aopwiki.org/relationships/509)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)	**non-adjacent**		