OECD Series on Adverse Outcome Pathways No. 15

**Adverse Outcome Pathway
on Aryl hydrocarbon
receptor activation leading
to uroporphyrria**

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Foreword

This Adverse Outcome Pathway (AOP) on Aryl hydrocarbon receptor activation leading to uroporphyrin, has been developed under the auspices of the OECD AOP Development Programme, overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), which is an advisory group under the Working Group of the National Coordinators for the Test Guidelines Programme (WNT). The AOP has been reviewed internally by the EAGMST, externally by experts nominated by the WNT, and has been endorsed by the WNT and the Working Party on Hazard Assessment (WPHA) in May 2019.

Through endorsement of this AOP, the WNT and the WPHA express confidence in the scientific review process that the AOP has undergone and accept the recommendation of the EAGMST that the AOP be disseminated publicly. Endorsement does not necessarily indicate that the AOP is now considered a tool for direct regulatory application.

The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to declassification of this AOP on 26 June 2019.

This document is being published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

The outcome of the internal and external reviews are publicly available respectively in the [AOP Wiki](#) and the [eAOP Portal of the AOP Knowledge Base](#) at the following links: [\[internal review\]](#) [\[external review\]](#).

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*Adverse Outcome Pathway on Aryl hydrocarbon receptor
activation leading to uroporphyrin Short Title: AHR
activation-uroporphyrin*

Short Title: AHR activation-uroporphyrin

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Abstract

Hepatic uroporphyria is a disorder where the disturbance of heme biosynthesis results in accumulation and excretion of uroporphyrin, heptacarboxyl- and hexacarboxyl porphyrin: collectively referred to as highly carboxylated porphyrins (HCPs)^{[1][2][3]}. The disorder is due to a homozygous mutation in uroporphyrinogen decarboxylase (UROD), an enzyme involved in the heme biosynthesis pathway ^[4], or may be chemically induced, which involves the inhibition of UROD. This adverse outcome pathway (AOP) describes the linkages leading to chemically induced porphyria through the activation of the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor. AHR activation leads to the induction of cytochrome P450 1A2, a phase I metabolizing enzyme, which in turn results in excessive oxidation of uroporphyrinogen. This oxidation produces a UROD inhibitor, preventing the conversion of uroporphyrinogen to coproporphyrinogen and increasing the synthesis of the UROD inhibitor in a positive feedback loop. The accumulation of uroporphyrinogen leads to its preferential oxidation and accumulation of HCP in various organs (Uroporphyria). This AOP was developed in accordance with OECD guidelines and demonstrates a high degree of confidence as a qualitative AOP. The quantitative understanding of this AOP however is not yet complete, preventing the accurate prediction of uroporphyria from lower level key events.

Background

Heme is a cyclic tetrapyrrole cofactor containing Fe^{2+} porphyrin-containing ferroprotein that forms various hemoproteins such as hemoglobin, cytochromes and catalases [62]. Its biosynthesis mostly occurs in the liver and involves 8 separate steps. Porphyria is a disorder in which the disturbance of any of the steps of heme biosynthesis results in accumulation and excretion of porphyrins[2]. A variety of porphyrias exist depending on which enzyme in the pathway is deficient. This AOP describes a situation in which the 5th step of heme biosynthesis, uroporphyrinogen decarboxylase (UROD), which converts uroporphyrinogen to coproporphyrinogen, is inhibited.

Hepatic uroporphyria is viewed somewhat differently by clinicians and toxicologists. **For the former** it is mostly a sporadic disease (porphyria cutanea tarda; PCT) occurring sometimes in patients exposed to a variety of insults such as alcohol, estrogens, hepatitis viruses, HIV and on dialysis. Importantly, very early on it was found that lowering body iron stores by bleeding or now chelators causes remission [61]. In some northern European and US patients, carrying the hemochromatosis mutation is a risk factor but in other patients other iron susceptibility genes may contribute. Carrying a UROD mutation (lowering activity) is also a risk factor but still dependent on other susceptibility factors to see porphyria. To reproduce these findings experimentally has proved challenging but now possible. **For toxicologists** hepatic uroporphyria has mostly been seen as a toxic, but unique and curious endpoint of polychlorinated ligands of the AHR. Experimentally, TCDD in mice is the most potent agent consistent with AHR mode of action but is more difficult in rats and other organisms. Hexachlorobenzene (HCB) has been greatly studied for its porphyria-inducing abilities and a large incident of porphyria in some young people in Turkey 60 years ago was ascribed to susceptible individuals who had consumed HCB. It is controversial whether HCB is a weak AHR ligand. Evidence of porphyria in people exposed accidentally or occupationally to accepted AHR ligands such as TCDD and PCBs is thin. Importantly, iron status can profoundly modify experimental uroporphyria induced by these chemicals especially in mice. In fact iron overload alone of mice will eventually produce a strong hepatic uroporphyria which is markedly genetically determined and toxicity can be ameliorated by chelators resembling PCT. Thus hepatic porphyria could alternatively be viewed as an iron AOP. At an overall level hepatic uroporphyria in animals and patients is the outcome of complex genetic traits and external stimuli in which in some traditional toxicological circumstances binding of a chemical to the AHR may have a major contribution[67] but in others may not.

Graphical Representation

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	Activation, AhR
2	KE	850	Induction, CYP1A2/CYP1A5	Induction, CYP1A2/CYP1A5
3	KE	844	Oxidation, Uroporphyrinogen	Oxidation, Uroporphyrinogen
4	KE	845	Inhibition, UROD	Inhibition, UROD
5	KE	846	Accumulation, Highly carboxylated porphyrins	Accumulation, Highly carboxylated porphyrins
6	AO	369	Uroporphyrin	Uroporphyrin

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation, AhR	adjacent	Induction, CYP1A2/CYP1A5	High	High
Induction, CYP1A2/CYP1A5	adjacent	Oxidation, Uroporphyrinogen	Moderate	Low
Oxidation, Uroporphyrinogen	adjacent	Inhibition, UROD	Moderate	Low
Inhibition, UROD	adjacent	Accumulation, Highly carboxylated porphyrins	Moderate	Moderate
Accumulation, Highly carboxylated porphyrins	adjacent	Uroporphyrin	High	High

Stressors

Name	Evidence
Dibenzo-p-dioxin	High
Polychlorinated biphenyl	High
Hexachlorobenzene	High
Polycyclic aromatic hydrocarbons (PAHs)	Moderate

Dibenzo-p-dioxin

2,3,7,8-tetrachlorodibenzo-p-dioxin causes porphyrin accumulation in mice (Smith et al. 2001; Davies et al. 2008) and chickens (Lorenzen and Kennedy 1995).

Smith, A. G., Clothier, B., Carthew, P., Childs, N. L., Sinclair, P. R., Nebert, D. W., and Dalton, T. P. (2001). Protection of the Cyp1a2(-/-) null mouse against uroporphyrin and hepatic injury following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol. Appl. Pharmacol.* 173, 89-98.

Davies, R., Clothier, B., Robinson, S. W., Edwards, R. E., Greaves, P., Luo, J., Gant, T. W., Chernova, T., and Smith, A. G. (2008) Essential role of the AH receptor in the dysfunction of heme metabolism induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Chem. Res. Toxicol.* 21 (2), 330-340.

Lorenzen, A., and Kennedy, S. W. (1995). Sensitivities of Chicken and Pheasant Embryos and Cultured Embryonic Hepatocytes to Cytochrome P4501A Induction and Porphyrin Accumulation by TCDD, TCDF and PCBs. *Organohalogen Compounds* 25, 65-68.

Polychlorinated biphenyl

Some polychlorinated biphenyls (namely non-ortho substituted congeners) cause porphyrin accumulation in mice (Hahn et al. 1988; Gorman et al. 2002) and chicken (Lorenzen et al. 1997; Lorenzen and Kennedy 1995; Goldstein et al. 1976).

Hahn, M.E., Gasiewicz, T.A., Linko, P., Goldstein, J.A. (1988) The role of the Ah locus in hexachlorobenzene-induced porphyria: Studies in congenic C57BL/6J mice. *Biochem. J.* 254, 245-254.

Gorman, N., Ross, K. L., Walton, H. S., Bement, W. J., Szakacs, J. G., Gerhard, G. S., Dalton, T. P., Nebert, D. W., Eisenstein, R. S., Sinclair, J. F., and Sinclair, P. R. (2002) Uroporphyrin in mice: thresholds for hepatic CYP1A2 and iron. *Hepatology* 35 (4), 912-921.

Lorenzen, A., Kennedy, S. W., Bastien, L. J., and Hahn, M. E. (1997) Halogenated aromatic hydrocarbon-mediated porphyrin accumulation and induction of cytochrome P4501A in chicken embryo hepatocytes. *Biochemical Pharmacology* 53 (3), 373-384.

Lorenzen, A., and Kennedy, S. W. (1995). Sensitivities of Chicken and Pheasant Embryos and Cultured Embryonic Hepatocytes to Cytochrome P4501A Induction and Porphyrin Accumulation by TCDD, TCDF and PCBs. *Organohalogen Compounds* 25, 65-68.

Goldstein, J. A., McKinney, J. D., Lucier, G. W., Hickman, P., Bergman, H., and Moore, J. A. (1976) Toxicological assessment of hexachlorobiphenyl isomers and 2,3,7,8-tetrachlorodibenzofuran in chicks. II. Effects on drug metabolism and porphyrin accumulation. *Toxicol. Appl. Pharmacol.* 36 (1), 81-92.

Hexachlorobenzene

Hexachlorobenzene exposure induces porphyria in mice (Hahn 1988), rats (Mylchreest and Charbonneau 1997) and humans (Cripps et al. 1984). A review by Smith and Elder (2010) includes numerous examples of hexachlorobenzene induced porphyria.

Hahn, M. E., Gasiewicz, T. A., Linko, P., and Goldstein, J. A. (1988) The role of the Ah locus in hexachlorobenzene-induced porphyria. Studies in congenic C57BL/6J mice. *Biochem. J.* 254 (1), 245-254.

Mylchreest, E., and Charbonneau, M. (1997) Studies on the mechanism of uroporphyrinogen decarboxylase inhibition in hexachlorobenzene-induced porphyria in the female rat. *Toxicol. Appl. Pharmacol.* 145 (1), 23-33.

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.

Smith, A.G. and Elder, G.H. (2010) Complex Gene-Chemical Interactions: Hepatic Uroporphyrin As a Paradigm. *Chem. Res. Toxicol.*, **23**, 712-723.

1. Polycyclic aromatic hydrocarbons (PAHs)

High and repeated doses of non-chlorinated AhR polycyclic ligands administered to AHRb mice with iron overload induce a marked hepatic uroporphyrin (Francis et al., 1987).

Francis, J. E., & Smith, A. G. (1987). Polycyclic aromatic hydrocarbons cause hepatic porphyria in iron-loaded C57BL/10 mice: comparison of uroporphyrinogen decarboxylase inhibition with induction of alkoxyphenoxazone dealkylations. *Biochemical and biophysical research communications*, **146**(1), 13-20.

Overall Assessment of the AOP

Overall, this AOP can most accurately be applied to mammalian species past the embryonic and infant stage of development. It is also representative of a solid toxicity pathway in avian species, however the contribution of the defining key event (UROD inhibition) is not as well understood; it is not as dramatically and consistently inhibited as it is with mammals. There is minimal evidence supporting the applicability of this AOP in fish, and none in alternate species. Details and supporting evidences are summarized below.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult	High
Juvenile	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
chicken	Gallus gallus	Moderate	NCBI
herring gull	Larus argentatus	Moderate	NCBI
Japanese quail	Coturnix japonica	Low	NCBI
Common Starling	Common Starling	Moderate	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage Applicability, Taxonomic Applicability, Sex Applicability

Elaborate on the domains of applicability listed in the summary section above. Specifically, provide the literature supporting, or excluding, certain domains.

Life Stage Applicability: Uroporphyrin occurs following chemical exposure in juvenile or adult individuals. Fetal exposure to dioxin-like compounds causes developmental abnormalities and embryolethality rather than HCP accumulation^{[15][16][17][18][19]}. Turkish children under the age of two that were exposed to HCB through breastmilk passed away from a condition called "pink sore"^[20].

Taxonomic Applicability: Although the AHR is highly conserved in evolution^[21], chemical-induced uroporphyrin has only been detected in birds^{[1][2][3]} and mammals^[22], including an accidental outbreak in humans due to hexachlorobenzene-contaminated grain

in the 1950s^[20]. Fish are less susceptible to chemical-induced uroporphyrin, but elevated levels of HCP have been documented in highly contaminated environments^[23].

Sex Applicability: Although this AOP applies broadly to both males and females, sexual dimorphism for uroporphyrin has been observed in rats exposed to hexachlorobenzene (HCB). Hepatic uroporphyrin III was markedly increased in female rats exposed to HCB whereas exposed males showed levels of hepatic porphyrins similar to controls^[24].

Essentiality of the Key Events

Molecular Initiating Event Summary, Key Event Summary

Provide an overall assessment of the essentiality for the key events in the AOP. Support calls for individual key events can be included in the molecular initiating event, key event, and adverse outcome tables above.

Every Key event in this AOP is absolutely essential for downstream events to occur. A summary of evidence for essentiality of each key event is given below.

Molecular Initiating Event: AHR activation (Essentiality=strong)

- Mice with a high-affinity Ahr allele (C57BL/6J) are much more sensitive to uroporphyrin than mice with low-affinity Ahr allele (DBA/2)^{[25][26][27][28][29]};
- The Ah locus influences the susceptibility of C57BL/6J mice to HCB-induced porphyria^[30];
- Ahr knockout mice (C57BL/6) are resistant to development of porphyria, even in the presence of iron loading^[25];
- Primary hepatocytes of avian species indicate that species that are highly sensitive to AHR activation are more sensitive to uroporphyrin accumulation than species with lower sensitivity to AHR activation^[31].

Key Event 1: CYP1A2/Cyp1A5 induction (Essentiality=strong)

- CYP1A2 knockout in mice prevents chemical-induced uroporphyrin^{[32][33][34]};
- CYP1A2 knockout prevents porphyria in genetically predisposed mice (Hfe-/-, Urod-/-) that normally develop porphyria in absence of external stimuli^[35];
- CYP1A2 levels are correlated with the extent of uroporphyrin accumulation in mice^[36];
- 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and non-ortho substituted PCBs that are potent inducers of CYP1A4/5 cause accumulation of only HCPs in chicken embryonic hepatocytes cultures, whereas PCBs that do not induce CYP1A4/5 cause a porphyrin pattern that is not consistent with inhibition of UROD^[37];
- Common tern (*Sterna hirundo*) embryonic hepatocyte cultures, which are ~50 to > 1600 times less sensitive than chicken embryonic hepatocyte cultures to CYP1A5 induction by TCDD and PCBs, do not accumulate HCPs upon chemical exposure^[31].

It should be noted that a recent study by Davies et al.^[25] found that both C57BL/6J mice (susceptible to chemical-induced porphyria) and DBA/2 mice (resistant to porphyria due to polymorphism in AHR gene) showed increased expression of CYP1A2 when exposed to TCDD, even though the DBA/2 strain did not develop porphyria. Furthermore AHR-/- mice showed a mild uroporphyrin response in the presence of iron loading and 5-aminolevulinic acid (a heme precursor). These findings suggest that the induction of CYP1A2 is not crucial for chemical-induced porphyria, but a basal level of expression is absolutely essential.

Key Event 2: Uroporphyrinogen oxidation (UROX) (Essentiality=strong)

- Uroporphyrin is characterized biochemically by increased formation of HCPs derived by oxidation of the porphyrinogen substrates of uroporphyrinogen decarboxylase (UROD); secondary to decreased activity of this enzyme in the liver^[22];
- Uroporphomethane, derived from oxidizing a single carbon bridge in uroporphyrinogen, has been identified as the UROD inhibitor that leads to chemically- and genetically-induced uroporphyrin in mice^[38];
- UROX activity is positively correlated with uroporphyrin levels in mice^[36].

Key Event 3: Uroporphyrinogen decarboxylase (UROD) inhibition (Essentiality=strong)

- Mutations in the UROD gene that reduce or eliminate UROD activity lead to porphyria in mammals; a decrease in hepatic UROD activity of at least 70% is necessary to observe symptoms from overproduction of porphyrins^[22];
- A marked progressive decrease in UROD enzyme activity is a common feature in animal models of chemical-induced porphyria^{[22][34][39][40][41]};
- Liver cytosol UROD activity in female rats exposed to HCB was decreased more than 70% and correlated with elevated hepatic uroporphyrin levels, whereas male rats, which did not develop porphyria, showed UROD activity similar to controls^[24];
- UROD activity is inversely proportional to uroporphyrin levels in mice^[36];
- In chicken hepatocytes, the strongest inducers of porphyrin accumulation were also the strongest inhibitors of UROD activity^[41];
- Reduced UROD enzyme activity, not protein levels, is characteristic of uroporphyrin in humans and rats^{[24][42][43]}.

Key Event 4: Highly carboxylated porphyrin (HCP) accumulation (Essentiality=strong)

- Under normal heme biosynthesis, porphyrins are only present in trace amounts in the liver; however, in the absence of UROD activity, the oxidation of Uroporphyrinogen to uroporphyrins dominates, leading to an accumulation of HCPs;
- Porphyrins are strongly fluorescent compounds resulting in a characteristic red fluorescence of hepatic tissue under UV light that is proportional to the level of porphyrins^{[44][45]}. Increased urinary excretion of porphyrins is also indicative of their accumulation and can lead to dark red/brown urine^[22]. HCPs also accumulate in the skin causing solar hypersensitivity and increased skin fragility^[46];
- HCP accumulation was observed in avian embryo hepatocyte cultures following exposure potent AHR agonists (dioxin-like compounds)^{[37][47][48][49]} and in the livers of Japanese quails and chickens exposed to PCBs^{[50][51][52]};
- HCP accumulation was evident in mice treated with polyhalogenated aromatic compounds^[36] or TCDD^[25].

Weight of Evidence Summary

Dose concordance

Table 1 demonstrates that upstream KEs (monooxygenase activity/quantity) are significantly affected at lower doses than downstream KEs (porphyrin levels). After a 6 month recovery period, CYP450 and hepatic porphyrin levels were dramatically reduced, however, they did not return to normal. Furthermore, urinary porphyrin excretion remained maximally elevated^[53]

Table 1: Porphyria in Female Sprague-Dawley rats chronically exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) over 16 weeks.

Total exposure (µg/kg)	AHH activity	Hepatic CYP450 (nmol/g)	Relative porphyrin level (hepatic)	Incidence of porphyria
0		22		
0.16	3-fold	26	1	0/8
1.6	16-fold*	34*	5	1/8
16	48-fold*	42*	1000* (urinary uroporphyrin ↑ 120-fold*)	7/8
16 (after 6 month recovery)	2-fold*	20* (Control=25)	100* (urinary uroporphyrin = 100-fold*)	

*p<0.05 relative to control; AHH= aryl hydrocarbon hydroxylase; general measure of monooxygenase activity.
(Goldstein et al. 1982)

Temporal Concordance

Table 2 demonstrates that upstream KEs (CYP1A2 expression and UROD inhibition) are significantly affected at earlier time-points than downstream KEs (porphyrin levels). These studies also show that upstream KEs are more sensitive to change than downstream KEs; ddY mice showed a 44% reduction in UROD activity but did not develop uroporphyrin^{[25][54]}.

Table 2. Characterization of porphyria over time susceptible (C57BL/6 and C57BL/6J) or resistant (ddY and DBA/2) mice.

and DBA/2 mice.					
Strain	Time (weeks)	CYP1aA2 expression fold change	UROD (nmol/h/g)	Relative Uroporphyrin level	Reference
C57BL/6	Control		21.4	1	(Seki <i>et al.</i> , 1987)
	1		7.4	9	
	2		8.5	16	
	3		4.4	2100	
ddY	Control		32.8	1	
	1		30.3	3	
	3		31.8	3	
	6		21.1	14	
	10		18.4	13	
DBA/2	0.5 (3 days)	~39		<1	(Davies <i>et al.</i> , 2008)
	2	~27		<1	
	5	~15		<1	
C57BL/6J	0.5 (3 days)	~49		<1	
	2	~50		56 (20 ^a nmol/g liver)	
	5	~25		677 (270 ^a nmol/g liver)	

C57BL/6 and ddY mice were exposed to 500 ppm of Kanechlor-500 and DBA-2 and C57BL/6J mice were exposed to 75 µg/kg TCDD. ^aValues estimated from figure.

Key Events Relationships

Table 3 shows a sampling of the literature that demonstrates changes in KEs at multiple levels of organization leading to uroporphyrin. The use of animal models resistant to porphyria (low AHR affinity or AHR/CYP1A2 knockout) illustrates the essentiality of these KEs in for downstream effects.

Table 3: Sampling of literature supporting essentiality of key events and demonstrating key events relationships

	MROD (pmol/ min per mg	MDOD/ EROD	CYP1A2 (relative protein level)	UROX (pmol/ min per mg	UROD (pmol/ min per mg	Hepatic Porphyrins (nmol/ g)	Liver Toxicity	Ref.
Female C57BL/6J wild-type mice 500 mg Fe/kg + 300 ppm of 3,4,5,3',4',5'-hexachlorobiphenyl in feed for 3 weeks								(Hahn et al. 1988)
weeks						Hepatic	Urinary (nmol/ day)	
8							6.3	
9						75.4±57.6	12	
10							30	
11							60	
12							140	
13							145	
14							180	
15			12-fold		30%	1110±393	265	
Female C57BL/6J AHR non-responsive mice								
13							3	
14							6	
15			4-fold		80%	17.6±14.5	6.3	
16							10	
17						48.5±31.6	13	
Male C57BL/6J Wild-type mice pre-treated with Iron								(Sinclair et al. 1998)
Control	71±7	1.9±0.1		8.1±0.9		3-310 (162±111)		
75 mg/kg MC +ALA	240±210	1.3±0.6		15±6		50-260		
100 mg/kg HCBZ			2-3-fold			270-350		(Sinclair et al. 2000)
Male C57BL/6J CYP1A2(-/-) mice pre-treated with Iron								(Sinclair et al. 1998)
Control	18±2	0.4±0.02		3.4±0		<0.8		
75 mg/kg MC +ALA	300±150	0.2±0.2		2.6±0.7		<0.8		
100 mg/kg HCBZ	ND		NC			<0.8		(Sinclair et al. 2000)
C57BL/6J Wild-type mice pre-treated with Iron								(Smith et al. 2001)
Control	54±6	1±0	ND		100%	0.4±0.1	-	
75 mg/kg TCDD	800±170	0.50±0.1	↑		10%	1150±690	+++	
C57BL/6J CYP1A2(-/-) mice pre-treated with Iron								(Smith et al. 2001)
Control	8±3	0.18±0.02	ND		100%	0.6±0.6 0.2	-	
75 mg/kg TCDD	336±30	0.13±0.01	ND		95%	0.3±0.2	+	
Male C57BL/6J mice 75 µ/kg TCDD + 800 mg Fe/kg + 2 mg/mL drinking water ALA								(Davies et al. 2008)
AHR(+/-)	860	0.38				400 (964 fold)	+++	
AHR(+/-)	940	0.38				10 (60 fold)	+	
AHR(-/-)	40	0.4				<1 (0 fold)	-	
Male C57BL/6J mice 500 mg Fe/kg + 2 mg/mL drinking water ALA								(Gorman et al. 2002)
						4 wks	8 wks	
CYP1A2(-/-)	10±2			3.4±0.4		<1	<1	
CYP1A2(-/+)	48±5			5.5±0.7		<1	19±9	
CYP1A2(+/-)	65±10			9.0±1.0		33±30	194±88	
CYP1A2(+/-) /PCB 126	140±35			19±3.9		205±81	NA	

Liver Toxicity=hepatocellular hypertrophy and degeneration, necrosis, and focal chronic inflammation; Fe= iron;
ALA= 5-aminolevulinic acid; ND=Not detected; NC= No change; ↑=Induced relative to control.

Uncertainties

CYPs other than CYP1A2 are able of catalyzing uroporphyrinogen oxidation, raising doubts on the essentiality of CYP1A2 for this pathway. For instance, Phillips et al.^[35] were able to generate mild uroporphyrin in a Cyp1A2^{-/-} mouse model that is genetically predisposed (Hfe^{-/-}, Urod^{-/+}) to develop porphyria.

The essentiality of CYP1A2 induction in human porphyria cutanea tarda is unclear. UROX activity in human liver microsomes was not correlated with CYP1A2 content^[66]. Furthermore, there is contradictory evidence regarding the association between CYP1A2 polymorphism and susceptibility to porphyria cutanea tarda^[63-64]. It may be possible that in patients with a genetic variation in UROD causing an inherent reduction in activity, the activity of CYP1A2 is less important.

UROD inhibition is not always observed and/or is less pronounced in avian models of porphyria, mainly in quail^[47]. It is suggested that a mechanism other than UROD inhibition explain the extent of porphyrin accumulation in birds. Therefore, the applicability of this AOP to avian remains uncertain.

The characterization of the UROD inhibitor isolated by Phillips^[38] has been criticized by Danton^[66]. Therefore, UROD inhibitor has yet to be identified.

AhR binding stressors under certain conditions do not lead to adverse effect in particular mammalian strains^[25].

Quantitative Consideration

Provide an overall discussion of the quantitative information available for this AOP.

Support calls for the individual relationships can be included in the Key Event

Relationship table above.

The overall quantitative understanding of this AOP is moderate for mammals and poor for alternate species. Quantitative models have been developed that predict the AHR transactivation potential of various compounds^{[55][56][57]}, but the extent of AHR activation necessary to produce porphyria is not known. It has been established that a reduction in UROD activity of at least 70% is required to lead to overt uroporphyrin in mammals^{[58][24][54]}. Additionally, numerous in vitro systems have been developed to study porphyrin accumulation and UROD inhibition simultaneously; therefore, this KER provides the most feasible target for a predictive, quantitative model. However, care must be taken when reading across to other species; UROD inhibition is not always observed in avian models of porphyria, and when it is, it is less pronounced^{[59][60][47]}.

Considerations for Potential Applications of the AOP (optional)

This AOP was developed with the intended purpose of chemical screening as well as ecological risk assessment. There are numerous in vitro assays for each key event up to the level of UROD activity. There is sufficient evidence that a 70% inhibition of UROD activity significantly increases the risk of developing uroporphyrin in mammals, making it a promising target assay in the battery of chemical screening tools. Furthermore, there has recently been significant advances in the understanding of differences in avian sensitivity to AHR agonists, and a similar effort is underway for fish. Sequencing the AHR ligand binding domain of any bird species (and potentially fish species) allows for its classification as low, medium or high sensitivity, which aids in the chemical risk assessment of DLCs and other AHR agonists. There is also potential use for this AOP in risk management, as minimum allowable environmental levels can be customized to the sensitivity of the native species in the area under consideration.

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Appendix 1

List of MIEs in this AOP

Event: 18: Activation, AhR

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	MolecularInitiatingEvent
Aop:57 - AhR activation leading to hepatic steatosis	MolecularInitiatingEvent
Aop:131 - Aryl hydrocarbon receptor activation leading to uroporphyrin	MolecularInitiatingEvent
Aop:150 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF	MolecularInitiatingEvent

Stressors

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

Biological Context

Level of Biological Organization
Molecular

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 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.

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 potent 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
Gallus gallus	Gallus gallus	High	NCBI
Pagrus major	Pagrus major	High	NCBI
Acipenser transmontanus	Acipenser transmontanus	High	NCBI
Acipenser fulvescens	Acipenser fulvescens	High	NCBI
rainbow trout	Oncorhynchus mykiss	High	NCBI
Salmo salar	Salmo salar	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
Ambystoma mexicanum	Ambystoma mexicanum	High	NCBI
Phasianus colchicus	Phasianus colchicus	High	NCBI
Coturnix japonica	Coturnix japonica	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
Microgadus tomcod	Microgadus tomcod	High	NCBI

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_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).
- 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

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-footed albatross, great cormorant and domestic chicken^{[11][10]}.

- 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

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 (*Oncorhynchus 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 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 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 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: 850: Induction, CYP1A2/CYP1A5

Short Name: Induction, CYP1A2/CYP1A5

Key Event Component

Process	Object	Action
gene expression	cytochrome P450 1A2	increased
gene expression	cytochrome P450 1A5 (chicken)	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:131 - Aryl hydrocarbon receptor activation leading to uroporphyrin	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
chicken	Gallus gallus	High	NCBI
zebrafish	Danio rerio	High	NCBI
Haliaeetus leucocephalus	Haliaeetus leucocephalus	High	NCBI
Ardea herodias	Ardea herodias	High	NCBI
Double-crested cormorant	Double-crested cormorant	High	NCBI
Nycticorax nycticorax	Nycticorax nycticorax	High	NCBI
osprey	Pandion haliaetus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

CYP1A expression has been measured in chicken^[11] as well as in wild bird species, including bald eagles (*Haliaeetus leucocephalus*)^[15], great blue herons (*Ardea herodias*)^[16], double-crested cormorants (*Phalacrocorax auritus*)^[17], black-crowned night herons (*Nycticorax nycticorax*)^[18] and ospreys (*Pandion haliaetus*)^[19]. It's also been measured in a number of mammalian and piscine species including humans, rats^[21], mice^[20] and zebrafish^[30].

Key Event Description

The Cyp1A2/Cyp1A5 gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. The protein encoded by this gene localizes to the endoplasmic reticulum and its expression is induced by some polycyclic aromatic hydrocarbons (PAHs), some of which are found in cigarette smoke. The enzyme's endogenous substrate is unknown; however, it is able to metabolize some PAHs to carcinogenic intermediates. Other xenobiotic substrates for this enzyme include caffeine, aflatoxin B1, and acetaminophen. ^[4]

The CYP1A subfamily of enzymes is very well studied and is often used as a biomarker of Dioxin-like compound (DLC) exposure and toxicity^{[5][6][7][8]}. CYP1A5 is the avian isoform and is orthologous to the mammalian CYP1A2^[9]. CYP1A5 is expressed in avian heart, liver and kidney tissues^{[10][11]}, and has been measured in avian hepatocyte and cardiomyocyte cultures^{[12][13][10][14]}. Mouse CYP1A2 is only constitutively expressed in the liver, but is inducible in the liver, lung, and duodenum^[20].

How it is Measured or Detected

Enzyme activity

There are a number of substrates that are preferentially metabolized by Cyp1A2 and CYP1A5 allowing for CYP1A activity to be measured as a function metabolite formation. Methoxyresorufin O-demethylation (MROD) is a classic marker of Cyp1A2/5 activity^[21] and is often used due to the ease of fluorometric techniques; however, Burke *et al.*^[21] suggest that a ratio of MROD to ethoxyresorufin O-demethylation (EROD) is a better measure of CYP1A2 activity due to the contribution of CYP1A1 to MROD. CYP1A2/5 activity can also be measured as the metabolic rate of arachidonic acid^[11], oroporphyrinogen^[22], acetanilide 4-hydroxylase and caffeine^[23]. Caffeine metabolism has been used in clinical studies as a biomarker for CYP1A2 activity in humans^[24].

Quantitative polymerase chain reaction (QPCR)

Levels of CYP1A2/5 messenger RNA can be measured using QPCR. This technique monitors the amplification of a targeted gene during PCR as accumulative fluorescence^[25]. For example, Head and Kennedy^[26] developed a multiplex QPCR assay utilizing dual-labeled fluorescent probes to measure CYP1A4 and CYP1A5 mRNA levels simultaneously

from samples already analyzed for EROD activity. QPCR has high throughput capability and a low detection limit relative to other methods.

Luciferase reporter gene (LRG) assay

An LRG assay can be used to measure AHR1-mediated transactivation of a target gene. This assay is particularly useful as it can measure CYP1A4/5 induction exclusively caused by activation of the AHR, through which many DLCs exert their toxic effects. This assay is easily modified to measure AHR1-mediated transactivation in various species, simply by transfecting the desired AHR cDNA clone and reporter gene construct (containing the appropriate reporter gene) into the chosen cell line. This has been demonstrated to be an efficient high throughput method in various avian and mammalian studies. [\[27\]\[28\]\[29\]](#)

LC/MS-MS

The European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) is working on a human hepatic in vitro metabolically competent test systems to evaluate CYPs induction. Cryopreserved human HepaRG® or cryopreserved human primary hepatocytes are incubated in presence of a potential CYP1A2 inducer and the identity and abundance of CYP1A2 product is evaluated using analytical HPLC (High Performance Liquid Chromatography) coupled with mass spectrometry (MS). HPLC is applied for concentration and purification of the product to be detected, whereas MS is applied for its specific quantification [\[31\]](#).

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Event: 844: Oxidation, Uroporphyrinogen

Short Name: Oxidation, Uroporphyrinogen

Key Event Component

Process	Object	Action
	uroporphyrinogen	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:131 - Aryl hydrocarbon receptor activation leading to uroporphyrinogen	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
hepatocyte

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
chicken	Gallus gallus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	High

UROX has been measured in chicken^[3], mouse^[4], rat^[4] and human^[6] microsomes.

Key Event Description

Figure 2: Oxidation of the heme precursor uroporphyrinogen III to uroporphyrin III due to inhibition of UROD. UROD: uroporphyrinogen decarboxylase. (Modified from Smith and Elder (2010) *Chem. Res. Toxicol.* **23** (4), 712-723.

Uroporphyrinogen III is the first cyclic metabolic intermediate in the biosynthesis of heme. Under normal conditions, it is converted into coproporphyrinogen III by the enzyme uroporphyrinogen decarboxylase (UROD), and subsequently processed to heme following three further steps^[1]. In the event that UROD activity is reduced (due to genetic disorders or chemical inhibition) uroporphyrinogen III, and other porphyrinogen substrates of UROD, are oxidized to highly stable porphyrins, which accumulate and lead to a heme disorder known as porphyria (Figure 2)^[2].

How it is Measured or Detected

Porphyrins fluoresce red when exposed to UV light; therefore, uroporphyrinogen oxidation (UROX) can be directly measured as uroporphyrin fluorescence in a spectrophotofluorimeter. UROX has been measured spectrofluorimetrically in avian^[3] and mammalian^[4] species.

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Event: 845: Inhibition, UROD

Short Name: Inhibition, UROD

Key Event Component

Process	Object	Action
catalytic activity	uroporphyrinogen decarboxylase	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:131 - Aryl hydrocarbon receptor activation leading to uroporphyrin	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
chicken	Gallus gallus	High	NCBI
Japanese quail	Coturnix japonica	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified
Adult	High

Sex Applicability

Sex	Evidence
Unspecific	High

UROD inhibition has been measured in mouse^[10] rat^[11] and human liver^[1], Japanese quail kidney^[12] and chicken erythrocytes^[13] and hepatocytes^[14].

Key Event Description

Figure 3: Disruption of the normal heme biosynthesis pathway by uroporphyrinogen decarboxylase (UROD) inhibition. Formation of the inhibitor (suggested as being uroporphomethene) is thought to require the action of the phase I metabolizing enzyme, CYP1A2. Synergistic induction of ALA synthase 1 and increases in oxidative stress (reactive oxygen species (ROS)), caused by alcohol, estrogens and xenobiotics, potentiate the accumulation of porphyrins and therefore the porphyric phenotype. (Modified from Caballes (2012) *Liver Int.* **32** (6), 880-893.)

Uroporphyrinogen decarboxylase (UROD) is the fifth enzyme in the heme biosynthesis pathway and catalyzes the step-wise conversion of uroporphyrinogen to coproporphyrinogen. Each of the four acetic acid substituents is decarboxylated in sequence with the consequent formation of hepta-, hexa-, and pentacarboxylic porphyrinogens as intermediates^[1]. Impairment of this enzyme, either due to heterozygous mutations in the UROD gene or chemical inhibition of the UROD protein, leads to accumulation of uroporphyrins (and other highly carboxylated porphyrins)^[2], which are normally only present in trace amounts.

How it is Measured or Detected

Due to the high instability of porphyrinogens, they must be synthesized as an integral part of the enzyme assay for use as a substrate. Uroporphyrinogen can either be generated by enzymatic synthesis or chemical reduction^[7]. The former makes use of bacterial porphobilinogen deaminase to prepare the porphyrinogen substrate and the latter often utilizes sodium amalgam or sodium borohydride under an inert gas. Chemical reduction however often involves large quantities of mercury or extremely alkaline conditions and requires significant purification before the enzyme assay can be performed. Bergonia and colleagues^[8] suggest palladium on carbon (Pd/C) to be the most efficient and environmentally friendly chemical preparation of porphyrinogens as Pd/C is more stable

than sodium amalgam and can easily be removed by filtration, eliminating the need for laborious purification.

Once uroporphyrinogen is synthesized it is co-incubated with UROD under standardized conditions. The reaction is then stopped, reaction products and un-metabolized substrate are esterified, and the porphyrin esters are separated and quantified using high performance liquid chromatography^[7]. This enzyme assay classically utilizes milliliter quantities but has been modified to a microassay, minimizing cost and enhancing sensitivity^[9].

Another method based on reverse-phase HPLC was developed^[15]. This assay system uses either uroporphyrinogen III or pentacarboxyporphyrinogen I as substrate and liver homogenate in sucrose treated with a suspension of cellulose phosphate as enzyme source.

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Event: 846: Accumulation, Highly carboxylated porphyrins

Short Name: Accumulation, Highly carboxylated porphyrins

Key Event Component

Process	Object	Action
	porphyrins	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:131 - Aryl hydrocarbon receptor activation leading to uroporphyrin	KeyEvent

Biological Context

Level of Biological Organization
Organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI
chicken	Gallus gallus	High	NCBI
Japanese quail	Coturnix japonica	High	NCBI
herring gull	Larus argentatus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Juvenile	High
Adults	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Elevated porphyrins have been reported in mouse^[4], rat^[5], Japanese quail and chicken liver^[6] and in clinical diagnosis of humans^[2]. Elevated HCPs (highly carboxylated porphyrins) have been measured in Herring gulls from highly contaminated Great Lakes colonies^[7].

Key Event Description

Under normal conditions, the heme biosynthesis pathway is tightly regulated and porphyrins (other than protoporphyrin) are only present in trace amounts^[1]. However, when the regulatory process is disturbed, a variety of porphyrin precursors of heme accumulate in various organs including the liver and urinary and fecal excretion is elevated^[2]. The pattern of porphyrin accumulation in chicken and rodents is similar following exposure to a variety of chemicals, and can be used to identify which enzyme in the heme pathway is predominately affected^[1].

How it is Measured or Detected

The hepatic and urinary/fecal porphyrin patterns can be determined using a high-performance liquid chromatograph equipped with a fluorescence detector. Kennedy *et al.*^[3] describe the method for tissue extraction and porphyrin quantification in detail, which is rapid and highly sensitive.

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List of Adverse Outcomes in this AOP

Event: 369: Uroporphyrria

Short Name: Uroporphyrria

Key Event Component

Process	Object	Action
porphyria		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:131 - Aryl hydrocarbon receptor activation leading to uroporphyrria	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rats	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI
herring gull	Larus argentatus	High	NCBI
chicken	Gallus gallus	High	NCBI
Japanese quail	Coturnix japonica	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Juvenile	High
Adult	High

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Chemical-induced uroporphyrria has only been detected in birds^{[7][1][8]} and mammals^[5], including an accidental outbreak in humans due to hexachlorobenzen-contaminated grain

in the 1950s^[9]. Fish are less susceptible to chemical-induced uroporphyrin, but elevated levels of highly carboxylated porphyrins (HCP) have been documented in highly contaminated environments^[10].

Key Event Description

Figure 4: The heme biosynthetic pathway. Deficiency in a particular gene along the pathway results in the indicated form of porphyria: 8 separate disorders that are characterized by hepatic accumulation and increased excretion of porphyrins. Source: Frank, J., and Poblete-Gutierrez, P. (2010) Porphyria cutanea tarda--when skin meets liver. *Best. Pract. Res. Clin Gastroenterol.* **24** (5), 735-745.

Porphyria is a disorder in which the disturbance of heme biosynthesis results in accumulation and excretion of porphyrins^[1]. A variety of porphyrias exist depending on which enzyme in the pathway is deficient (Figure 4). In the case of chemically induced uroporphyrin, uroporphyrinogen decarboxylase (UROD), which converts uroporphyrinogen to coproporphyrinogen, is inhibited. In humans, this disorder is known as porphyria cutanea tarda and may be caused by chemical exposure or a hereditary deficiency in UROD^[4]. The accumulation of porphyrins in the liver causes cirrhosis, mild fatty infiltration, patchy focal necrosis, and inflammation of portal tracts. When the activity of UROD is reduced to less than 30% of normal, the disorder manifests as an overt skin disease; the accumulation of porphyrins in the skin causes photosensitization that is characterized by fragile skin, superficial erosions, sub-epidermal bullae, hypertrichosis, patchy pigmentation and scarring^[5].

How it is Measured or Detected

Porphyria is easily confirmed through a urinary or fecal analysis to measure the levels and pattern of excreted porphyrins. Samples are quantified using a high-performance liquid chromatograph equipped with a fluorescence detector^[6]. Frank and Poblete-Gutiérrez^[4] illustrate how the types of porphyria can be differentiated by the relative abundance of different porphyrins (Figure 2). Uroporphyrin is the animal model equivalent to human porphyria cutanea tarda^[5].

Figure 2: Biochemical characteristics of the porphyrias in urine, stool, and blood (plasma and erythrocytes). Source:

<http://www.mayomedicallaboratories.com/articles/communique/2015/03-porphyrin-testing/>; Accessed December 9, 2015

Type of Porphyrin	Enzyme Defect	Enzyme Assay Available	Biochemical Findings – Increased Levels Seen in Affected Patients			
			Urine	Feces	Erythrocytes	Plasma
Aminolevulinic acid dehydratase deficiency porphyria	Aminolevulinic acid dehydratase	Yes	Aminolevulinic acid, Coproporphyrin	NA	Zinc protoporphyrin	NA
Acute intermittent porphyria	Porphobilinogen deaminase	Yes	Porphobilinogen, Aminolevulinic acid, Uroporphyrin, Coproporphyrin*	Uroporphyrin*	NA	Uroporphyrin*
Hereditary coproporphria	Coproporphyrinogen oxidase	No	Coproporphyrin, Porphobilinogen, Aminolevulinic acid	Coproporphyrin III Coproporphyrin III/ Coproporphyrin I ratio	NA	Coproporphyrin*
Variegate porphyria	Protoporphyrinogen oxidase	No	Coproporphyrin, Porphobilinogen, Aminolevulinic acid	Protoporphyrin > Coproporphyrin III Coproporphyrin III/ Coproporphyrin I ratio	NA	Coproporphyrin, Protoporphyrin*
X-linked dominant protoporphyria	Aminolevulinic acid synthase 2	No	NA	Protoporphyrin*	Zinc protoporphyrin, Free protoporphyrin	Protoporphyrin
Congenital erythropoietic porphyria	Uroporphyrinogen III synthase	Yes	Uroporphyrin, Coproporphyrin	Coproporphyrin I	Uroporphyrin, Coproporphyrin, Zinc protoporphyrin	Uroporphyrin, Coproporphyrin
Porphyria cutanea tarda	Uroporphyrinogen decarboxylase	Yes	Uroporphyrin, Heptacarboxyl-porphyrin	Isocoproporphyrin, Heptacarboxyl-porphyrin III	NA	Uroporphyrin, Heptacarboxyl-porphyrin
Hepatoerythropoietic porphyria	Uroporphyrinogen decarboxylase	Yes	Uroporphyrin, Heptacarboxyl-porphyrin	Isocoproporphyrin, Heptacarboxyl-porphyrin III, Coproporphyrin I,	Zinc protoporphyrin	Uroporphyrin, Heptacarboxyl-porphyrin
Erythropoietic protoporphyria	Ferrochelatase	No	NA	Protoporphyrin	Free protoporphyrin	Protoporphyrin

Informative Biochemical Findings in Porphyrin

*sometimes

(Source: <http://www.mayomedicallaboratories.com/articles/communique/2015/03-porphyrin-testing/>; Accessed December 9, 2015.)

Regulatory Significance of the AO

Uroporphyrin is a disorder affecting multiple organs and can significantly decrease the quality of life in humans. The outbreak of porphyria in Turkish populations in the 1950's due to contaminated grain had significant, long-term health effects^[9].

Uroporphyrin has been detected in one wild animal population (Herring gulls in contaminated Great Lakes colonies^[8]); although the disorder is characterized by hepatotoxicity, it has not been shown to lead to death, and therefore is not expected to cause population decline. Elevated porphyrins however are apparent long before overt signs of toxicity are manifested, making it a sensitive biomarker of chemical exposure; monitoring porphyrin levels in at-risk wild populations would identify the need for remediation of contaminated sites before the occurrence of overt adverse effects.

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Appendix 2: List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 869: Activation, AhR leads to Induction, CYP1A2/CYP1A5

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to uroporphyrin	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI
chicken	Gallus gallus	High	NCBI
fish	fish	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Multiple AHR isoforms have been isolated and characterized in mammals, fish and birds^[17]. Mammals possess a single AHR that controls the expression of CYP1A2, while birds and fish possess 2 AHR isoforms (AHR-1 and AHR-2), with AHR-1 being homologous to the mammalian AHR. The avian orthologue to CYP1A2 is CYP1A5^[18]. Most fish species only express a single CYP1A gene^[19].

Key Event Relationship Description

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)^[1]. Upon ligand binding, the AHR migrates to the nucleus where it dissociates from

the cytosolic complex and forms a heterodimer with Ahr nuclear translocator (ARNT)^[2]. 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 of gene expression^[1].

Evidence Supporting this KER

WOE is strong for this KER.

Biological Plausibility

There is a strong mechanistic understanding of AHR-mediated induction of CYP1A genes^[1].

Empirical Evidence

Include consideration of temporal concordance here

It is well established that the extent of CYP1A induction is directly proportional to the strength of ligand binding to the AHR^{[3][4][5]}. Two sites within the ligand binding domain (LBD) of the AHR have been identified (positions 375 and 319 in mammals; equivalent to positions 380 and 324 in birds) as being responsible for the range of binding affinities of dioxin-like compound (DLCs) and their corresponding efficacy (transactivation potential).^{[4][3][6][7][8][9][10]} A similar investigation in sturgeon (fish) revealed that the residue at position 388 of the LBD of AHR2 was responsible for differences in sensitivity between White Sturgeon and Lake Sturgeon, both of which are endangered species^[11]. Furthermore, Hestermann et al.^[12] described that compounds with a high intrinsic efficacy demonstrate a 1:1 relationship between AHR binding affinities and CYP1A protein induction.

Uncertainties and Inconsistencies

There are no knowledge gaps or inconsistencies/conflicting lines of evidence for this KER.

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Relationship: 868: Induction, CYP1A2/CYP1A5 leads to Oxidation, Uroporphyrinogen

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
<u>Aryl hydrocarbon receptor activation leading to uroporphyrin</u>	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
chicken	Gallus gallus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	High
Juvenile	High

Sex Applicability

Sex	Evidence
Unspecific	High

CYP1A2 catalyzes UROX in mice, rats and humans^{[1][2][11]}, as does CYP1A5 in chickens^[3].

Key Event Relationship Description

The oxidation of uroporphyrinogen to its corresponding porphyrin (UROX) is preferentially catalyzed by the phase one metabolizing enzyme, CYP1A2, in mammals^{[1][2]} and CYP1A5 in birds^[3]. Uroporphyrinogen, an intermediate in heme biosynthesis, is normally converted to coproporphyrinogen by uroporphyrinogen decarboxylase (UROD)^[4]; induction of CYP1A2 expression translates to increased protein levels and therefore an increased incidence of binding, and oxidation of uroporphyrinogen, preventing its normally dominant conversion to coproporphyrinogen.

Evidence Supporting this KER

WOE for this KER is moderate.

Biological Plausibility

Uroporphyrinogen has clearly been identified as a substrate of CYP1A2/5, which results in its oxidation to uroporphyrin^{[1][2][3]}.

Empirical Evidence

Include consideration of temporal concordance here

UROX activity is increased by inducers of the CYP1A subfamily^{[4][1]} and inhibited by substrates of CYP1A2^[2], indicating that uroporphyrinogen binds to the active site of CYP1A2. Furthermore, mice with a higher endogenous level of CYP1A2 are more susceptible to porphyrin accumulation^[5] and CYP1A2 knock-out prevents chemical-induced uroporphyrinuria all-together^{[6][7][8]}; therefore, CYP1A2 is essential for UROX. A mild porphyric response was observed in the presence of iron loading and 5-aminolevulinic acid (ALA; a heme precursor) in AHR-/- mice, indicating that CYP1A2 induction is not absolutely necessary, but that constitutive CYP1A2 levels are sufficient for UROX under certain conditions^[9].

Uncertainties and Inconsistencies

It is worth noting that Cyp1a2(-/-) knockout mice have up to 40% of the UROX activity of Cyp1a2(+/+) mice^[7], suggesting that some UROX activity is CYP1A2-independent. Likewise, transfection of human Cyp1a1, Cyp3a4, Cyp3a5, or Cyp2e1 in insect cells resulted in UROX activity^[10], suggesting that UROX can be catalyzed by other CYPs than CYP1A2 both in mouse and human. Additionally, iron overload or other induced pathways can potentially induce UROX^[13]. However, it was shown in mice that only CYP1A2-dependent UROX activity is associated with UROD inhibition^[7]. No such experiment was conducted in human, therefore, uncertainties remain for that species.

In mice, TCDD can elicit AhR-dependent, CYP1A1/A2-independent mitochondrial ROS production suggesting that general oxidative stress induced independently of CYP1A2 induction may contribute to the resulting overall UROX by TCDD^[14].

Phillips et al.^[11] were able to generate uroporphyrinuria in a Cyp1a2-/- mouse model that is genetically predisposed (Hfe-/-, Urod-/-, which translates into intrinsic iron-overload and reduced UROD activity) to develop porphyria in the absence of external stimuli; CYP1A2 knockout alone prevented porphyrin accumulation, but with the addition of iron and ALA to the triple knockout, modest porphyria was observed. Therefore, under extreme porphyric conditions, UROX leading to porphyria can occur in the absence of the CYP1A2 enzyme. Altogether, these results indicate that while CYP1A2 is a major catalysis of UROX activity, other CYPs and/or modulating factors are involved in the pathway.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

Correlation between total hepatic uroporphyrin accumulation and hepatic CYP1A2 activities in mice after exposure to TCDD (A), 4-PeCDF (B), PCB 126 (C), or PCB 118 (D).

Correlation between total hepatic uroporphyrin accumulation and hepatic CYP1A2 activities in mice after exposure to TCDD (A), 4-PeCDF (B), PCB 126 (C), or PCB 118 (D). (Source: van Birgelen *et al.* (1996). *Toxicol. Appl. Pharmacol.* **138** (1), 98-109.)

UROX is positively correlated with CYP1A2/5 activity^[12] but this relationship has not been quantitatively describes. It has been noted however, that a CYP1A2 induction of just 2-fold dramatically induces porphyrin accumulation in iron-loaded mice^[5].

Known modulating factors

Iron

Iron status can profoundly modify the level of uroporphyrin accumulation especially in mice. In fact iron overload alone of mice will eventually produce a strong hepatic uroporphyria which is markedly genetically determined and toxicity can be ameliorated by chelators^[15-16]. In human suffering from uroporphyrin accumulation, it was found that lowering body iron stores by bleeding or now chelators causes remission^[17].

Cycling between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) redox states allows Fe to catalyze the Haber-Weiss reaction, in which highly reactive $\cdot\text{OH}$ is generated from H_2O_2 and O_2^- . Thus, by catalyzing the formation of reactive oxygen species, it is suggested that iron can increase the rate at which uroporphyrinogen is oxidized to uroporphyrin and therefore enhance uroporphyrin formation^[18].

Ascorbic acid

Ascorbic acid (AA) can prevent uroporphyrin accumulation experimental uroporphyria, but only when hepatic iron stores are normal or mildly elevated^[19]. It was shown in chick embryo liver cells that AA could prevent uroporphyrin accumulation caused by treatment with 3,3',4,4'-tetrachlorobiphenyl and 5-aminolevulinate by competitively inhibiting microsomal CYP1A2-catalyzed oxidation of uroporphyrinogen^[20]. Oppositely, in a

spontaneous mutant rat that requires dietary AA, hepatic uroporphyrin accumulation caused by treatment with 3-methylcholanthrene or hexachlorobenzene was found to be enhanced when the animals were maintained on a very low AA dietary intake^[21].

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Relationship: 865: Oxidation, Uroporphyrinogen leads to Inhibition, UROD

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to uroporphyrin	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rats	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified
Adult	High
Juvenile	High

Sex Applicability

Sex	Evidence
Unspecific	High

A hepatically generated UROD inhibitor has been detected in porphyric mice^[7] and rats^[6], and humans with porphyria cutanea tarda^[1]).

Key Event Relationship Description

One of the oxidation products of uroporphyrinogen is believed to be a competitive inhibitor of uroporphyrinogen decarboxylase (UROD). This inhibitor binds to the active site of UROD preventing the normal synthesis of heme, allowing uroporphyrinogen oxidation to dominate and increasing accumulation of hepatic porphyrins^[1]. The formation of this inhibitor is increased by iron, a well-known oxidant, by activity of cytochrome P-450A2, by alcohol excess and by estrogen therapy^[2].

Phillips et al.^[1] identified this inhibitor as being uroporphomethene using a murine model for porphyria; however, their interpretation of the mass spectroscopy results has been criticized as inaccurate^[8], leaving the exact characterization of the UROD inhibitor unresolved.

A negative-feedback loop exists in which the end-product (heme) represses the enzyme ALA synthase 1 and prevents excess formation of heme. When UROD activity is low, the regulatory heme pool is potentially depleted, causing a repression of the negative feedback loop, thereby increasing levels of precursors and furthering the accumulation of porphyrins.

Evidence Supporting this KER

The WOE for this KER is moderate.

Biological Plausibility

Reduced UROD enzyme activity, not protein levels, is characteristic of uroporphyrinemia in humans and rats^{[3][4][5]}, indicating that disrupted decarboxylation is due to an enzyme inhibitor rather than a reduction in protein synthesis. Early reports confirmed the presence of a UROD inhibitor in porphyric animal models that was not present in animals resistant to chemical-porphyrinemia under the same conditions^{[6][7]}. The identity of this UROD inhibitor is not yet agreed upon, but there is a general consensus among the scientific community that it is an oxidation product of uroporphyrinogen or hydroxymethylbilane (the tetrapyrrole precursor of uroporphyrinogen)^[2].

Empirical Evidence

Include consideration of temporal concordance here

Phillips *et al.*^[1] identified uroporphomethene, a compound in which one bridge carbon in the uroporphyrinogen macrocycle is oxidized, as a potent UROD inhibitor derived from the liver of porphyric mice.

Uncertainties and Inconsistencies

The precise mechanism of UROD inhibition has yet to be identified. It could be a direct or indirect inhibition via an oxidized uroporphyrinogen generated by CYP1A2 or reactive oxygen species derived from iron overload, or other induced pathways.

The characterization of the inhibitor isolated by Phillips *et al.*^[1] has been criticized by Danton and Lim^[8]. Namely, they claim that the high-performance liquid chromatography/electrospray ionization tandem mass spectrometry results were interpreted incorrectly. They analyzed the fragmentation pattern themselves, and concluded that the compound is not a tetrapyrrole or an uroporphyrinogen or uroporphyrin related molecule, but rather a poly(ethylene glycol) structure. The expected chemical instability of the inhibitor – a partially oxidized porphyrinogens that bear unsubstituted methylene group(s) at the *meso* position – might play an important role in the difficulty to characterize it^[9].

Porphodimethene inhibitor 16 (PI-16), a synthetic inhibitor of UROD, was developed based on its similarity to coproporphyrinogen, uroporphyrinogen, and the previously suggested endogenous inhibitor^[9]. This molecule directly interacts with UROD to specifically and effectively inhibit its activity. PI-16 structural similarity to an oxidized uroporphyrinogen including the suggested endogenous inhibitor supports the hypothesis of an oxidized uroporphyrinogen as endogenous UROD inhibitor.

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

This linkage has not been quantitatively characterized.

Known Feedforward/Feedback loops influencing this KER

Induction of CYP1A2 increases its availability and consequently its ability to compete with UROD to oxidize uroporphyrinogen. At least one of these oxidation products is believed to be a competitive inhibitor of UROD. Therefore, UROD inhibition potentiates the oxidation of uroporphyrinogens by CYP1A2 to porphyrins leading to increased porphyrin accumulation and in turn UROD inhibition.

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Relationship: 1070: Inhibition, UROD leads to Accumulation, Highly carboxylated porphyrins

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to uroporphyrin	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
chicken	Gallus gallus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	High

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

Chemical induces porphyrin accumulation has been demonstrated in, rats, mice and chicken^{[18][4][2]}. Human porphyria cutanea tarda is also characterized biochemically by an increase in porphyrinogen oxidation leading to accumulation of porphyrins^[15]. The correlation between reduced UROD activity and HCP accumulation in mammals is well defined^{[15][16][17]} but is less consistent in avian models^[14].

Key Event Relationship Description

Through the normal heme biosynthesis pathway, uroporphyrinogen is converted to coproporphyrinogen by uroporphyrinogen decarboxylase (UROD)^[1]. In the event that UROD activity is reduced (due to genetic disorders or chemical inhibition) uroporphyrinogen, and other porphyrinogen substrates of UROD, are preferentially oxidized to highly stable porphyrins by the phase one metabolizing enzyme CYP1A2 (in mammals; CYP1A5 in birds)^{[2][3][4]}. Uroporphyrin and hepta- and hexa-carboxylic acid porphyrins (highly carboxylated porphyrins)^[5] accumulate in the liver, kidneys, spleen, skin and blood leading to a heme disorder known as porphyria^{[6][7]}.

Evidence Supporting this KER

The WOE for this KER is strong in mammals and Moderate in birds.

Biological Plausibility

Hepatic uroporphyrinogen accumulation versus inhibition of uroporphyrinogen decarboxylase activity from individual mice treated with iron and HCB. Control: ○, Treated: △. (Source: Lambrecht, R.W. *et al.* (1988) *Biochem. J.* **253** (1), 131-138.)

It is well established that porphyrin accumulation, which is a result of uroporphyrin oxidation (UROX), and UROD inhibition go hand in hand^[8]. Because CYP1A2/5 binds a broad range of substrates, significant UROX only occurs when there is an excess of uroporphyrinogen, which occurs when UROD is inhibited. Each of the four acetic acid substituents of porphyrinogen is decarboxylated in sequence with the consequent formation of hepta-, hexa-, and pentacarboxylic porphyrinogens as intermediates^[9]. Oxidation of these intermediates results in their corresponding, highly stable porphyrins.

Empirical Evidence

Include consideration of temporal concordance here

A number of studies have demonstrated that increased UROD inhibition results in higher hepatic porphyrin accumulation^{[10][11][12]}.

Uncertainties and Inconsistencies

Uroporphyrin accumulation in avian models is less consistently accompanied by decreased UROD activity, and when it does occur, it is less marked than in mammals^{[13][14]}. Although numerous studies show both a decrease in UROD activity and porphyrin accumulation in avian species, Lambrecht *et al.*^[14] reported the accumulation of porphyrins in chicken embryo hepatocytes and Japanese quail liver without a decrease in UROD activity. They also note that the modest reduction in UROD activity (often less than 50%) is not enough to explain the extent of porphyrin accumulation observed and suggests there may be another mechanism at play. Alternatively, the difference between avian and mammals in regard to UROD inhibition may lie in the time-course of the response rather than its mechanism^[19].

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

A reduction in UROD activity of at least 70% is required to achieve a makeable increase in hepatic porphyrins, in mammals.^{[15][16][17]}

Known Feedforward/Feedback loops influencing this KER

Induction of CYP1A2 increases its availability and consequently its ability to compete with UROD to oxidize uroporphyrinogen. At least one of these oxidation products is believed to be a competitive inhibitor of UROD. Therefore, UROD inhibition potentiates the

oxidation of uroporphyrinogens by CYP1A2 to porphyrins leading to increased porphyrin accumulation and in turn UROD inhibition.

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Relationship: 866: Accumulation, Highly carboxylated porphyrins leads to Uroporphyrin

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Aryl hydrocarbon receptor activation leading to uroporphyrin	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
chicken	Gallus gallus	High	NCBI
herring gull	Larus argentatus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	High
Juvenile	High

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

This relationship exists in birds^[3] and mammals, including humans^[4].

Key Event Relationship Description

Accumulation of porphyrins causes both physical and chemical damage to tissues, resulting in what is generally termed porphyria. The ability of porphyrins to absorb light of 400–410 nm (the Soret band) is the key factor in producing the photocutaneous lesions observed on sun exposed areas in affected individuals. The porphyrins absorb this light and enter a high energy state, which is then transferred to molecular oxygen resulting in reactive oxygen species (ROS). These ROS cause phototoxic damage and further catalyze the oxidation of porphyrinogens to porphyrins. Some porphyrins, mainly uroporphyrin and heptacarboxyl porphyrin, form needle-shaped crystals resulting in hydrophilic cytoplasmic inclusions^[1]. Porphyrins demonstrate a range of water solubilities, and therefore show unique tissue and cellular distributions, resulting in different patterns of phototoxic damage histologically and cytologically^[2].

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Violet light excites the delocalized electrons in porphyrins. If the energy is not given out as red fluorescent light, it is passed onto oxygen to form tissue damaging free radicals. (Source: Sarkany, R. P. (2008). Photodermatol. Photoimmunol. Photomed. 24(2), 102-108.)

Evidence Supporting this KER

The WOE for tyhis KER is strong.

Biological Plausibility

The mechanism by which porphyrins cause tissue damage is well understood^{[1][2]}

Empirical Evidence

Include consideration of temporal concordance here

Uroporphyrin is defined as the accumulation and excretion of uroporphyrin, heptacarboxyl- and hexacarboxyl porphyrin: collectively referred to as highly carboxylated porphyrins (HCPs)^[3]. It is the animal model equivalent to the human disorder, porphyria cutanea tarda^[4].

Uncertainties and Inconsistencies

No current inconsistencies to report.

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