Published in final edited form as:

Curr Opin Toxicol. 2018 ; 11-12: 10–20. doi:10.1016/j.cotox.2018.11.005.

Aryl Hydrocarbon Receptor (AhR) Ligands as Selective AhR Modulators: Genomic Studies

Stephen Safe^a, Huajun Han^{b,c,d}, Jennifer Goldsby^{c,d}, Kumaravel Mohankumar^a, Robert S. Chapkin^{b,c,d}

^aDepartment of Veterinary Physiology and Pharmacology

^bDepartment of Biochemistry & Biophysics

^cDepartment of Nutrition & Food Science

^dProgram in Integrative Nutrition & Complex Diseases, Texas A&M University, College Station, TX, USA

Abstract

The aryl hydrocarbon receptor (AhR) binds structurally diverse ligands that vary from the environmental toxicant 2,3,7,8-tetrachlorodibenzo-*B*-dioxin (TCDD) to AhR- active pharmaceuticals and health-promoting phytochemicals. There are remarkable differences in the toxicity of TCDD and related halogenated aromatics (HAs) vs. health promoting AhR ligands, and genomic analysis shows that even among the toxic HAs, there are differences in their regulation of genes and pathways. Thus, like ligands for other receptors, AhR ligands are selective AhR modulators (SAhRMs) which exhibit variable tissue-, organ- and species-specific genomic and functional activities.

Keywords

AhR; ligands; structure-activity genomic differences

1. Introduction

The aryl hydrocarbon receptor (AhR) was initially discovered as the intracellular protein that mediated the toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally-related halogenated aromatic (HAs) compounds including halogenated dibenzo-*p*-dioxins, dibenzofurans and polychlorinated biphenyls (PCBs) [1–3]. This receptor also bound polynuclear aromatic hydrocarbons (PAHs) [4, 5]; however, it was recognized that although PAHs and HAs induced some common responses such as induction of cytochrome P450A1

To whom correspondence should be addressed: Stephen Safe, Department of Veterinary Physiology and Pharmacology, Texas A&M University, 4466 TAMU, College Station, TX 77843-4466 Tel: 979-845-5988 / Fax: 979-862-4929, ssafe@cvm.tamu.edu.

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Conflict of Interest: There are no conflicts of interest to declare.

(CYP1A1), the toxicities induced by these AhR ligands were different [6–8]. TCDD and related HAs typically induce chloracne, reproductive and developmental toxicities, porphyria, immunotoxicity, thymic and body weight loss. Induction of these responses are species-, strain-, sex- and age- dependent, and the lethality of TCDD is also highly variable with different species. LD₅₀ values vary by >1000 from highly sensitive (guinea pig) to less sensitive (hamster) species [1, 3]. Mechanistic studies using ligand (TCDD)-dependent induction of CYP1A1 gene expression as a model demonstrated a pathway similar to that observed for steroid hormone receptors. The ligand initially binds to the cytosolic AhR which subsequently forms a nuclear heterodimer with the AhR nuclear translocator (Arnt), which binds c/s-acting dioxin response elements (DREs) in target gene promoters [9,10]. This canonical pathway of the AhR has subsequently expanded to include multiple genomics and non-genomic AhR-dependent pathways for activation of gene expression and function [11–14]. Genetic studies showed that both the AhR and Arnt are members of the basic helix-loop-helix - PER - ARNT - SIM (bHLH-PAS) family of transcription factors and knockout of the AhR in mouse models resulted in loss of TCDD-induced toxicity [15–17].

2. AhR Functions in Cellular Homeostasis and Disease

Development of AhR-knockout (AhRKO) and subsequent tissue-specific AhRKO mice has demonstrated that the AhR has multiple functions in maintaining cellular homeostasis and disease. For example, loss of the AhR in mice results in failure of developmental closure of the ductus venosus in the liver, decreased fertility and liver size, reproductive tract deficits, ocular motor deficits, portal duct fibrosis, formation of uric acid stones in the bladder, and disruption of stem cell development [18–32]. Subsequent and ongoing research demonstrates a role for the AhR in immunity and autoimmunity, barrier functions in the gut, important roles in multiple tissues, and in carcinogenesis [33–39]. For example, the AhR exhibits tumor suppressor-like activity in the gut and in pituitary adenomas and is a tumor promoter for head and neck and lung cancers [40–45]. The detection and function of the AhR in multiple tissues and diseases also implies that AhR ligands other than TCDD are potential chemotherapeutic agents for treating both cancer and non-cancer diseases [39].

3. AhR Ligands and the Concept of Selective AhR Modulators

TCDD is a high affinity AhR ligand and several structurally-related HAs containing 3 or 4 lateral chlorine substituents also bind the receptor and induce many of the same toxic and biochemical responses [1–3]. Subsequent studies have identified many other structural classes of compounds with modest to low AhR binding activity that induce or inhibit AhR-dependent genes/pathways and these responses can be both ligand-, tissue- and gene-specific (rev. in [6–8, 46]). These selective AhR modulators (SAhRMs) include a number of endogenous AhR ligands such as 6-formylindolo[3,2- b]carbazole (FICZ) and tryptophan metabolites including indole-3-acetate, indole-3- acetaldehyde, tryptamine, serotonin and kynurenic acid [47–51]. Other microbiota- derived compounds, phytochemicals including indole-3-carbinol and related compounds, flavonoids, curcumin, other natural products and pharmaceuticals (e.g. omeprazole) also activate the AhR (rev. in [6–8, 46]). The structural diversity of AhR ligands (Fig. 1) is similar to that reported for other receptors including steroid hormone receptors such as the estrogen receptor (ER) which are major druggable

targets [51–54]. The tissue- and ligand-specific effects of selective receptor modulators are due to several genomic and non-genomic factors including tissue-specific differences in expression of receptor cofactors, ligand structure-dependent receptor conformations, and non-genomic changes in histones and target gene promoters [55] (Fig. 2). Evidence for SAhRMs include (a) the identification of several compounds that exhibit partial response-specific AhR agonist and antagonist activities and these include 6-methyl-1,3,8-trichlorodibenzofuran, substituted flavonoids, CH223191, GNF351 [56–61], and (b) differences in the effects of TCDD and FICZ in an animal model of multiple sclerosis where the former compound protects and the latter exacerbates the disease [34]. The induction of CYP1A1 by structurally diverse AhR ligands is observed in most tissues and cell lines and is commonly used as an end point for screening for AhR-active compounds; however, a recent study demonstrated selectivity among several flavonoids, even for this response. For example, luteolin (5,7,3',4'-tetrahydroxyflavone) exhibited minimal AhR agonist activity as an inducer of CYP1A1 in Caco2 colon cancer cells and antagonized TCDD-induced CYP1A1 gene expression. In contrast, both luteolin (10–50 μ M) and TCDD (10 nM) induced comparable fold induction of UGT1A1 at very different dose levels [62]. The examples noted above and other key studies [58, 63–66] support the overall concept that structurally diverse AhR ligands are SAhRMs, and their biochemical and functional effects are also dependent on the animal species, strain and tissue/organ which differentially express nuclear cofactors and exhibit chromatin variability on target genes. The designation of AhR ligands as SAhRMs also has other implications; namely, like other selective receptor modulators, these compounds can be developed as therapeutics and for any specific ligand, it will be difficult to predict its activities without testing the compound. There is evidence that genomics can be a highly sensitive indicator of the function of a compound [67, 68] and specific patterns of gene expression are routinely used to identify effective therapeutic regimens [69, 70]. Therefore, it should also be possible to use a genomic approach not only to confirm that AhR ligands are SAhRMs but also (in the future) to predict activities and in the following section, the genomic effects associated with the AhR and its ligand will be examined.

4. AhR-regulated Gene Expression by TCDD and Related Compounds

(i). TEFs and Dioxin Equivalents:

The toxicology of TCDD and related compounds is characterized by their induction of a well-defined subset of toxic and biological responses and also induction of common genes including CYP1A1 and other drug metabolizing enzymes. Based on their common AhR-mediated mechanism of action, HAs are regulated by a toxic equivalency factor approach where the dioxin or toxic equivalents (TEQs) of a mixture of dioxin-like compounds (DLCs) can be defined by the equation,

$$\text{TEQ} = [\text{DLC}_i] \times [\text{TEF}_i]$$

where DLC_i is the concentration of the individual compound and TEF_i is the relative potency of the individual compound compared to TCDD ($\text{TEF} = 1.0$) [71–73]. This approach for risk assessment and risk management of DLCs has been extensively used by

regulatory agencies and has contributed to the dramatic decreases in environmental and human levels of these toxicants. Nevertheless, studies comparing potencies of individual DLCs to TCDD give highly variable TEFs which are animal species/strain-, tissue- and response- dependent [3, 71–73], and this holds true even for induction of CYP1A1 which is induced by TCDD in most cells [74]. As indicated below, genomic analysis of DLCs suggests that even though these compounds induce a similar pattern of toxic responses, there is evidence that these compounds are also SAhRMs.

(ii). Genomic Effects of DLCs:

Genomic analysis of DLCs provides evidence for both common and divergent effects with respect to gene sets and pathways [75–78]. One study investigated the dose-response effects of 3 DLCs, namely TCDD, 2,3,4,7,8 pentachlorodibenzofuran and 2,3,7,8-tetrachlorodibenzofuran (TCDF), on primary rat hepatocytes using an Agilent rat whole genome 4×44K array [75]. This approach could assess concentration-dependent changes in gene expression and hierarchical clustering, illustrating similarities in the heat maps for each compound; however, analysis of the results demonstrated variability and significant compound-dependent differences in gene expression. Using a false discovery adjusted p-value (<0.05) and a fold-change of 1.5, there were 3283 differentially expressed genes associated with at least one concentration for one congener. Only 399 of these genes were significant for both concentration and congener effects, and these genes exhibited expected differences in potencies but parallel concentration response curves. TEF or relative potencies (REP) values varied for each congener were both pathway- and gene- dependent. Studies of the genomic differences and similarity of TCDD, TCDF and 3,3',4,4',5-pentachlorobiphenyl (PCB126) were also determined in C57BL/6 mouse liver using a dose-response *in vivo* protocol [76, 77]. Differences in REP data were also observed; however, it was apparent that among the 3 DLCs, there were major differences in gene expression as determined using Agilent 4×44K microarrays. TCDD, TCDF and PCB126 induced changes in expression of 3280, 2343 and 1411 genes, respectively, and depending on the stringency of the statistical cutoff, the number of overlapping genes for all 3 compounds was 202 (high) or 1156 (low stringency). Although these compounds induced many of the same hepatotoxic responses, it was concluded that along with pharmacokinetic considerations, the potencies of gene regulation by these compounds were influenced by “differences in the ability of the liganded aryl hydrocarbon receptor:AhR nuclear translocator complex to elicit differential hepatic gene expression” [76]. A similar dose-response study of TCDD and TCDF in mouse liver [77] showed that TCDD and TCDF induced dose response curves for 1027 and 837 genes, respectively; however, only 373 genes were induced in common by both compounds. These results demonstrate that even among DLCs that there were significant differences in their induction of gene expression, and these observations are consistent with their designation as SAhRMs.

(iii). Effects of TCDD: Species, Strain and Tissue Differences:

The effects of TCDD alone in tissues of various rodent species and human-derived cells has also been investigated and compared [79], and a recent paper summarizes TCDD-mediated transcriptomic data sets from mammalian studies [80]. This included 20 studies from human, mouse and rat which varied with respect to target tissue, dose and duration of

treatment with TCDD. A toxicogenomic approach was used to investigate the comparative effects of TCDD in rat and human hepatocytes [80]; in one study, TCDD induced 1547 genes in rat and 475 genes in human with 158 genes in common. Further analysis of the TCDD-induced GeneGo pathways showed that 42 and 37 pathways were induced in rat and human hepatocytes, respectively, and only 12 pathways were induced in common, including an “AhR signaling” pathway. A second study compared the effects of TCDD and PCB126 on changes in gene expression in rat and human hepatocytes and this study also observed highly divergent induction responses that were species-dependent (rat vs. human) and also ligand-dependent where PCB126 exhibited decreased Ah-responsiveness in human cells [81]. cDNA microarrays were used to investigate the dose-dependent effects of TCDD (10–40 µg/kg) on hepatic gene expression in Ah-responsive Sprague-Dawley rats and C57BL/6 mice over multiple time points [82]. The custom arrays used in this study contained 3087 orthologous genes, and TCDD induced 201 and 238 genes in rat and mouse liver, respectively, and only 33 genes were induced in both species. Another study by the same group using expanded microarrays showed that TCDD induced 563 and 922 genes in rat and mouse liver, respectively, and only 70 orthologous genes were induced by TCDD in both species [83]. Moreover, analysis of TCDD-induced genes in liver after treatment for 1, 3 or 5 days also exhibited highly variable gene expression patterns in the same species [83].

In addition to these species- and time-dependent differences in TCDD-induced gene expression, several reports show equally divergent expression of genes in different rat strains and particularly between relatively AhR-non-responsive Han/Wistar rats and other strains [83–90]. The Han/Wistar rat is an unusually TCDD-resistant animal that expresses a modified transactivation domain of the AhR due to aberrant RNA splicing [91]. The effects of TCDD on Han/Wistar rats vs. other rodents also results in differentially expressed genes in these animal models. In one study, the differences in TCDD-induced gene expression in the hypothalamus was investigated in Han/Wistar and Long Evans mice since there are differences in these species with respect to their timing of decreased food intake after treatment with TCDD. Despite the fact that the hypothalamus is an important regulator of food intake, TCDD modified expression of 15 and 6 genes in Han/Wistar and Long Evans rats, respectively, (only 3 genes in common) and it was concluded “that the hypothalamus is not the predominant site of initial events leading to hypophagia and associated wasting” caused by exposure to near lethal doses of TCDD [87]. A comparable study on white adipose tissue, another presumed target of TCDD, showed minimal overlap in TCDD-induced genes in Han/Wistar and Long Evans rats [89] and inter-strain heterogeneity among several rat strains was also observed in expression of hepatic genes after treatment with TCDD [90]. Figures 3A–3C are derived from studies investigating the effects of TCDD in various cells [83, 92–94]. Each column is generated from a different study and is based on the differentially expressed gene (FDR < 0.05), comparing TCDD to control. Only the top 25 pathways, GO terms or genes are shown, and the subset of studies selected had at least 70 differentially expressed genes at an FDR < 0.05. The differentially expressed genes from each study were uploaded to IPA (Qiagen) which determined the enrichment of certain qualities using z-scores. Within IPA, we then compared studies and list the top categories using z-scores. The results clearly demonstrate that the effects of TCDD are highly variable and species- and cell/organ context- dependent.

Flaveny and coworkers [95] compared the effects of TCDD on gene expression in hepatocytes derived from C57BL/6^{bb} mice expressing wild-type mouse AhR (mAhR) and hepatocytes derived from transgenic mice expressing human AhR (hAhR). Thus, this study evaluates the effects of AhR-ligand interaction and the subsequent interactions of the bound receptor interacting with a similar set of mouse nuclear cofactors required for transactivation. TCDD induced/repressed 1752/1100 genes in mAHR hepatocytes and 1186/779 genes in hAhR hepatocytes, and the number of common genes induced or repressed was 265 (18%) and 462 (49%), respectively. Gene function analysis confirmed that TCDD differentially modulated genes associated with the immune response and cell proliferation in hepatocytes expressing hAhR compared to mAHR. Thus, the effects of TCDD alone were highly variable and dependent on the source of the AhR (mouse, rat, human) in which there were species- and strain-dependent differences in the response to TCDD and there were also tissue- dependent differences in TCDD-induced gene expression. These genomic data for TCDD and DLCs suggest that despite their structural similarities, these compounds are also SAhRMs based on genomic profiles observed after induction. Comparisons of genomic profiles of DLCs and other structurally diverse AhR ligands have not been extensively investigated; however, a recent report showed that the AhR ligands TCDD, benzo[a]pyrene, benzo[k]fluoranthene, FICZ and 2(1-4-indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester induced both common and divergent set of genes in A549 lung cancer cells [96].

(iv). AhR-dependent Gene Regulation (Ligand-independent):

As indicated above, loss of the AhR in mouse models resulted in loss of several functions in various tissues, and AhR loss in various tumor or cancer cell lines demonstrated that the AhR exhibits target organ/tissue-specific tumor suppressor- or oncogene-like activity. Not surprisingly, loss of the AhR results in changes in gene expression [84, 92, 93, 97, 98]. Boutros and coworkers [84] examined tissue (liver/kidney)-specific differences in gene expression in AhR^{+/+} (wild-type) and AhR^{-/-} (knockout) mice and also the effects of TCDD. Results from AhR^{-/-} and AhR^{+/+} mice were also analyzed as outlined in Figure 3 and the data represent differentially expressed genes at FDR < 0.05 (648 and 480 genes for liver and kidney, respectively) [84, 94] (Fig. 4). There were markedly different patterns of gene expression, canonical pathways, and functions between kidney vs. liver since the former organ exhibited minimal Ah-responsiveness. Wild-type and AhRKO mice and differentially Ah-responsive Han/Wistar and Long Evan rats were used as models to examine tissue-specific differences in gene expression [97]. There were transcriptomic and pathway differences between tissues in the same species and in different species, and different fractions of the differentially expressed genes demonstrated constitutive AhR binding using a published ChIPseq data set [93].

(v). ChIPseq Analysis of AhR Binding Sites:

ChIPseq analysis of AhR binding sites has been determined in a limited number of studies include mouse hepatoma cells [93] (indicated above). Approximately 750 genes exhibited constitutive AhR binding which was determined by comparing AhR interactions with mouse hepatoma cells expressing wild-type and mutant (DNA binding) AhR. AhR-bound genes exhibited multiple functions which were consistent with constitutive activity of the AhR.

After treatment with either TCDD or benzop[a]pyrene, there were similarities and differences in ligand-dependent effects on AhR-gene promoter interactions. Matthews and coworkers investigated basal and induced AhR genomic interactions in mouse hepatic tissue and human breast cancer cells (T47D and MCF-7) [99–104]. In T47D breast cancer cells treated with TCDD, 412 regions of the genome were bound by the AhR [99]. A second study in the same cell line showed that TCDD and 3-methylcholanthrene (3MC) enhanced AhR binding at 413 and 241 regions, respectively [100]. TCDD and 3MC enhanced AhR binding at 127 common regions indicating a ligand-dependent effect in recruiting the AhR to chromatin regions. These studies used human promoter arrays (Affymetrix) which could assess binding, and 87% of the top 100 regions bound by the AhR after treatment with 3MC were also bound by AhR after treating cells with TCDD [100]. Other differences between TCDD and 3MC included their AhR and Arnt binding periodicity, chromatin changes and temporal differences in Ah-responsiveness. A subsequent study in MCF-7 breast cancer cells [103] showed that after treatment with TCDD, 2594 AhR-bound, 1352 Arnt-bound, and 882 AhR/Arnt-bound sites were identified. Of the 882 AhR/Arnt-bound sites, 60% (503) contained at least one DRE or Ah response element indicating that regulation of 40% of genes not containing these c/s-element occurs via a non-classical pathway. Similar results have been obtained for mouse hepatic binding sites [101, 102] and confirm the complexity of endogenous and ligand-induced Ah signaling and the ligand-dependent differences (i.e. TCDD vs. 3MC) observed using both genome-wide ChIP and gene expression assays.

5. Summary

Structurally diverse AhR ligands which exhibit tissue-specific AhR agonist, mixed agonist/antagonist and antagonist activities are SAhRMs and this is supported by both genomic and non-genomic studies. Ligand-activated AhR-dependent changes in gene expression results in activation of multiple genes and is a highly sensitive indicator of ligand structure-dependent differences in interactions of the AhR complex with promoter DNA and induction/repression of Ah-responsive genes. This dual approach demonstrates that even among TCDD and related HAs, there is evidence for differences between these compounds, suggesting that they are also SAhRMs; however, their potencies are species-/tissue- and response-specific. The genomic results for PCB126 and TCDD clearly demonstrate that, even among structurally similar compounds that act through the same receptor, there are many differences with respect to their regulation (induction or repression) of genes. The WHO-recommended TEF value for PCB126 is 0.1, whereas analysis of hepatic gene expression in C57BL6 mice gave a TEF value of 0.02 [76] and similar results were observed in primary rat hepatocytes [75]. Using CYP1A1 induction as an end-point, the TEF values for PCB126 in human keratinocytes and rat liver cancer cells are 0.0027 and 0.082, respectively [74], thus demonstrating species-/tissue-specific differences for TEFs as previously observed during the selection of these arbitrary values [71]. Since there is an increasing number of “natural” signaling molecules such as tryptophan metabolites that play critical roles in cellular homeostasis, the development of genomic approaches to determine their activities as SAhRMs may serve to define their gene signatures and facilitate development of bio-identical molecules for therapeutic applications.

Acknowledgements

We would like to thank Rachel C. Wright for generation of the gene expression heat maps. Funding was provided by Texas AgriLife Research, the Sid Kyle Chair Endowment, the Allen Endowed Chair in Nutrition & Chronic Disease Prevention, the Cancer Prevention Research Institute of Texas (RP160589); and the National Institutes of Health (R01-ES025713, R01-CA202697, R35-CA197707).

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Figure 1.
Structurally diverse AhR ligands.

Figure 2.
The AhR/Arnt function is dependent on multiple factors including ligand/ligand-induced receptor conformation, enhancing and inhibiting cofactors, and promoter modulation.

Figure 3.

Several studies comparing TCDD to control listed by species and tissue are shown. All analyses are comparing TCDD treatment to control across species/tissues. Only differentially expressed genes with an FDR < 0.05 were used in the individual IPA core analyses. Analyses were performed using IPA to compare the core genes across studies as shown in the provided heatmaps. (A) Lists top canonical pathways that are enriched across the five studies. Pathways with the combined highest Z-Scores are listed first. (B) Lists of

top upstream analysis factors that are predicted to be activated or inhibited. (C) Lists of top Diseases and Biofunctions that are increased or decreased within biological processes.

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Figure 4.

Two mouse studies comparing AhR knock out to AhR in liver and kidney. Only genes with an FDR < 0.05 were used in the individual IPA core analyses. See Figure 3 for legend details.