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LIGAND SELECTIVITY AND EVOLUTIONARY DIVERGENCE OF THE HUMAN ARYL HYDROCARBON RECEPTOR

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor of the basic region helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) homology super family. The AHR was first identified as the primary mediator of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity and regulator of xenobiotic metabolism. Further investigation revealed AHR possesses a promiscuous ligand binding domain (LBD) that is able to bind to an array of exogenous compounds, such as polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs). Following ligand-binding, the AHR forms a functional heterodimeric transcription factor with AHR nuclear translocator (ARNT) and regulates expression of target genes via binding of dioxin response elements (DREs) within their promoters.

The physiological activity of the AHR has expanded to include pivotal roles within immune regulation, mucosal barrier function, cell cycle progression, organogenesis, circadian rhythm, and cellular differentiation pathways. However, these functions often require the ligand activated form of the receptor and are known to occur in the absence of exogenous ligand. This finding led to the premise and eventual characterization of endogenous AHR ligands. The catabolism of the amino acid tryptophan by commensal microorganisms, endogenous enzymes, and radical oxidation is a known source of numerous AHR ligands.

The studies presented in here build upon previously published data establishing microbial metabolism as a reservoir of endogenous ligand production. In our research we have identified the microbial tryptophan metabolite indole as a human AHR selective agonist through SAR studies and *in silico* modeling of AHR-ligand molecular docking. *In silico* molecular docking models support observations of differential indole responsiveness between species through a unique bi-molecular (2:1) binding stoichiometry that is sterically permissive within the human

AHR, but not its mouse homolog. The capacity for host cells to sense microbial indole through the AHR may be paramount to the establishment of an inter-kingdom signaling pathway that affects maintenance of intestinal homeostasis.

Molecular functionalities of the AHR, such as heterodimerization with ARNT and binding of DREs, are conserved among vertebrate and invertebrate species. However, ligand specificity of the AHR can vary between species. Comparative studies have shown that the human AHR displays reduced binding capacity with regard to prototypical PAH agonists when compared to rodent homologs. Such differences suggest that divergence of the AHR ligand binding domain (LBD) structure during the course of mammalian evolution facilitated interspecies variation in ligand selectivity.

Here we present evidence supporting the hypothesis that selective desensitization of the human AHR to PAHs arose during speciation of *Homo sapiens*. Interspecies AHR analyses, through utilization of ligand binding assay, DNA binding assay, and AHR-dependent gene expression identified a single amino acid substitution (*A381V*) as the determinant in reduced PAH affinity exhibited by *H. sapiens* relative to their *Homo neanderthalensis* (Neanderthal) and primate counterparts. Notably, *H. sapiens* AHR responsiveness to endogenous ligands derived from tryptophan and indole metabolism remains unperturbed. These findings reveal that a functionally significant mutation in the AHR occurred uniquely in humans that would attenuate the response to many environmental pollutants, including chemicals present in smoke.

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ABBREVIATIONS

1MI	1-methylindole
2MI	2-methylindole
3-MC	3-methylcloranthrene
3MI	3-methylindole
A381	AHR alanine residue 381
AHH	Aryl hydrocarbon hydroxylase
AHR	Aryl hydrocarbon receptor
Ahr$^{-/-}$	AHR null
Ahr$^{b/b}$	AHR *b-1*-allele
Ahr$^{d/d}$	AHR *d*-allele
AHRR	Aryl hydrocarbon receptor repressor
AR	Androgen receptor
ARNT	AHR nuclear translocator
ATP	Adenosine triphosphate
B(*a*)P	Benzo(*a*)pyrene
BA	Benzanthracene
bHLH-PAS	Basic-Helix-Loop-Helix-Per-ARNT-Sim
BNF	β-napthoflavone
BPDE	Benzo(*a*)pyrene-7,8-dihydrodiol-9,10-epoxide
CA	Cinnabarinic acid
ChREBP	Carbohydrate-responsive element-binding protein
CKD	Chronic kidney disease
CREBH	cyclic adenosine monophosphate response element binding protein
CYP450	Cytochrome P450
Cyp1a1	Cytochrome P450 1a1
Cyp1a2	Cytochrome P450 1a2
Cyp1b1	Cytochrome P450 1b1
Cyp2e1	Cytochrome P450 2e1
DDT	Dichlorodiphenyltrichloroethane
DHNA	1,4-dihydroxy-2-napthoic acid
DIM	Diindolylmethane
DLU	Digitized light units
DRE	Dioxin Response Elements
EC_{50}	Effective concentration 50%
ER	Estrogen receptor
FBS	fetal bovine serum
FGF21	Fetal growth factor 21
FICZ	6-formylindolo[3,2-*b*]carbazole

hAHR	Human AHR
HAHs	Halogenated aromatic hydrocarbons
HDAC	Histone deacetylase complex
HIF-1a	Hypoxia-inducible factor 1-alpha
HIF-2a	Hypoxia-inducible factor 2-alpha
HSP90	Heat shock protein 90
I3ACN	Indole-3-acetonitrile
I3C	Indole-3-carbinol
I3S	Indoxyl-3-sulfate
IAA	Indole-3-acetic acid
Iald	Indole-3-aldehyde
IAM	Indole-3-acetamide
IBD	Inflammatory bowel disease
ICZ	Indolo[3,2-*b*]carbazole
IDO1	Indoleamine-2,3-dioxygenase 1
IEL	Intraepithelial lymphocytes
IL1B	Interleukin 1 beta
IL22	Interleukin 22
IL6	Interleukin 6
ILC	Innate lymphoid cells
ILC3s	Group 3 innate lymphoid cells
IND	Indole
IR	Indirubin
ITE	2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester
K554	AHR lysine residue 554
LBD	Ligand-binding domain
Ltr-1	2-(indol-3-ylmethyl)-3,3'-diindolylmethane
mAHR	Mouse AHR
Mφ	Macrophage
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
P23	Heat shock protein 90 co-chaperone p23
PAH	Polycyclic aromatic hydrocarbon
PAL	Photo-affinity ligand
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyls
PPARα	Peroxisome proliferator-activated receptor alpha
PPIases	*Cis*-trans peptidyl-prolyl isomerases
PVDF	Polyvinylidene fluoride
R554	AHR arginine residue 554
RB	Retinoblastoma protein

SAR	Structure-activity relationship
TAD	Transactivation domain
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachloro-dibenzofuran
TDO2	Tryptophan-2,3-dioxygenase 2
T_{H17}	T Helper 17 Cells
TnaA	Tryptophanase
TPR	Tetratricopeptide repeat
T_{Reg}	Regulatory T-cells
UV	Ultra-violet
V381	AHR valine residue 381
VEGF	Vascular endothelial growth factor
XAP2	X-associated protein 2

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CHAPTER 1: INTRODUCTION

1.1 DISCOVERY OF THE ARYL HYDROCARBON RECEPTOR

The objective of toxicological research is to better understand the adverse effects of chemical, biological, and physical agents upon living organisms and ecosystems. All forms of life endure a multitude of insults at the molecular level, and through induction of intracellular biochemical defense mechanisms, are able to efficiently metabolize and excrete toxicants. Recognition and detoxification of such molecules rely on integrated biological processes facilitated by membrane bound transporters, receptors, small molecules, enzymatic reactions, and repair mechanisms.

Over the past two hundred years industrialization and population expansion have drastically increased environmental and occupational exposures to hazardous pollutants. Industrial or occupational incidents resulting in the accidental release and exposure of individuals to toxic polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) have occurred on multiple occasions[1-4]. In 1949, an accidental release of HAHs, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), occurred from a Monsanto chemical plant resulting in several exposure related casualties and reports of porphyria, liver disease, and 121 reported cases of chloracne[2, 5]. Chloracne is a skin condition characterized by edema of the face, dermal lesions, and atrophy of sebaceous glands[6]. Characteristic symptoms of TCDD exposure are long-lived due to the stability and highly lipophilic nature of TCDD, which has a half life of 7-11 years within the human body[7]. A mechanistic understanding of molecular pathways responsible for the toxicity of HAHs and PAHs would not be established until characterization of the aryl hydrocarbon receptor (AHR) in 1976.

Prior to the identification of the AHR, responsiveness to HAHs/PAHs was determined by measurement of Benzo(*a*)pyrene (B(*a*)P) hydroxylase activity by quantification of the enzymatic

conversion of B(*a*)P to 3-hydroxybenzo(*a*)pyrene[8]. This function was later redefined as aryl hydrocarbon hydroxylase activity (AHH) by Dr. Daniel Nebert of the National Institutes of Health, when it was found that additional PAHs induced synonymous enzymatic activity[9]. The Nebert lab also observed the capacity for 3- methylchloranthrene (3-MC) to induce differential AHH activity in dissimilar mouse strains. Following chemical exposure, C57BL/6 mice were found to be "highly responsive", and DBA/2 mice "nonresponsive"[9, 10]. Cross-breeding strains of mice that differ in their ability to induce AHH activity revealed that increased AHH responsiveness segregates as an autosomal dominant phenotype. This discovery was the first characterization of the *Ah* locus[11]. Mice that are "highly responsive" to PAH treatment possess the dominant $Ahr^{b/b}$ allele, while "nonresponsive" mice possess the recessive $Ahr^{d/d}$ allele. Research by Dr. Alan Poland found that administration of TCDD, an extremely potent inducer of AHH activity relative to 3-MC, in $Ahr^{d/d}$ mice was able to surmount the "nonresponsive" phenotype and induce AHH activity[12, 13]. This indicates that the molecular signaling pathways required for induction of AHH activity are not absent from in $Ahr^{d/d}$ mouse strains. However, the observed variance of AHH activity induced in $Ahr^{b/b}$ and $Ahr^{d/d}$ mice following TCDD exposure is dependent upon the dose utilized[14]. A saturating dose of TCDD will elicit equivalent AHH activity in both strains, however in depth dose-response assessments indicate $Ahr^{b/b}$ mice to possess a 10-fold lower ED_{50}[12]. The source of strain-specific AHH inducibility was postulated to be caused by a mutation present in the *Ah* locus of non-responsive mice. Further comparative studies of the $Ahr^{b/b}$ and $Ahr^{d/d}$ alleles identified the determinant of TCDD/PAH sensitivity to be the presence of alanine ($Ahr^{b/b}$) or valine ($Ahr^{d/d}$) encoded at position 375 of the LBD[15]. Taken together, these results led to the hypothesis that the *Ahr* locus encodes a peptide capable of

binding TCDD or 3-MC, and that upon recognition of ligand, would mediate induction pathways necessary for xenobiotic metabolism.

The advent of radiolabeled TCDD synthesis permitted cell fractionation experiments that identified a 95 kDa protein within hepatic cytosol of C57BL6 mice capable of directly binding TCDD[16]. Notably, receptor-ligand interactions positively correlated with a corresponding induction of AHH activity. This study was the first to identify the AHR as the translated product of the *Ahr* locus, which acts as an inducible receptor capable of binding TCDD and mediating its toxic effects.

1.2 AHR BASIC-HELIX-LOOP-HELIX PERN-ARNT-SIM HOMOLOGY

Following its identification, cloning, and sequencing, the murine $Ahr^{b/b}$ allele was found to encode an 805 amino acid peptide with structural homology to that of basic-helix-loop-helix-Pern/Arnt/Sim (bHLH-PAS) family of transcription factors (**Figure-1.1**)[17]. The bHLH domain is defined by a basic DNA binding motif with an adjacent helix-loop-helix domain required for dimerization with binding partners. Proper function of both domains is paramount to generating a functional transcription factor complex. Notably, bHLH super family peptides without a PAS domain target the E-box core enhancer element (CANNTG) in the promoter of regulated genes[18]. This group includes peptides involved in neurogenesis (Neurogenin) and hematopoiesis (TAL1/TCL5)[19, 20].

A subclass of bHLH transcription factors also contain a PAS domain, characterized by two adjacent degenerate repeats, each containing 130 amino acids termed PAS-A and PAS-B. Transcription factors with bHLH-PAS homology are evolutionarily conserved and can mediate host responsivity to various environmental or endogenous stimuli. Members of bHLH-PAS homology family have been shown to sense oxygen tension (HIF-1a, HIF-2a), regulate circadian

rhythm in response to light-dark cycles (BMAL1, BMAL2), promote organ development (SIM), and sense xenobiotic exposure (AHR)[17, 21-25]. Both bHLH and PAS domains are required for ligand dependent AHR signaling.

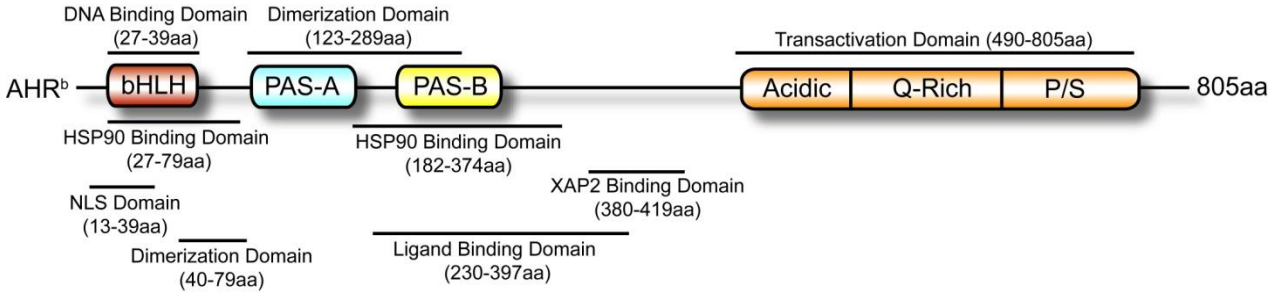


Figure 1.1. Homology domains and interacting regions of the AHR.
The bHLH, PAS and Transactivation (Acidic, Glutamine Rich (Q-Rich), and Proline/Serine/Threonine Rich (P/S)) domain regions of the AHR are illustrated above. Regions necessary for functional AHR signaling and interaction with cytosolic/nuclear binding partners are labeled accordingly.

1.3 THE CLASSICAL AHR SIGNALING PATHWAY

Structure-activity relationship (SAR) experiments utilizing radiolabeled AHR ligands have established a theoretical model of AHR molecular signaling reminiscent of members of the type I nuclear receptor family [26]. Under basal physiologic conditions the unliganded receptor is retained in the cytosol. Upon exposure to ligands, the AHR would translocate to the nucleus and initiate transcription of genes responsible for mediating AHH activity.

Building upon this hypothesis, Dr. Poland identified that heat-inactivated hepatic cytosol attenuated the accumulation of [^{3}H]-TCDD within nuclei protein extracts[27]. The nuclear shuttling of [^{3}H]-TCDD preceded a significant increase in 7-Ethoxycoumarin O-deethylation, which is indicative of AHR mediated induction of cytochrome P450 (Cyp450) activity. Translocation of [^{3}H]-TCDD could also be inhibited by co-treatment with non-radiolabeled ligands of the AHR[27]. Taken together, these results suggest that AHR is a cytosolic receptor, which upon exposure to ligand, is able to translocate into the nucleus and facilitate induction of xenobiotic metabolism[27, 28]. The sub-cellular localization of AHR was further characterized by immuno-fluorescent techniques utilizing a recently engineered targeted antibody[29, 30]. Alterations in AHR complex characteristics following ligand exposure, as evidenced by sucrose gradient sedimentation analysis, suggested additional roles of AHR chaperone proteins and binding partners in mediating AHR dependent gene transcription[31, 32].

The AHR classical signaling pathway, its principle protein components, and the mechanism of AHH activity induction were eventually established **(Figure 1.2)**. Initiation of the prototypic AHR signaling pathway is induced by receptor-ligand interaction and facilitates enhanced transcription of genes involved in xenobiotic metabolism, such as mixed function oxidase enzymes of the Cyp450 superfamily, and additional components of Phase I/II/III drug

metabolism[33, 34]. In the absence of ligand, AHR resides in a stable cytoplasmic complex composed of two molecules of heat shock protein 90 (HSP90), one molecule of X-associated protein 2 (XAP2), and one molecule of the heat shock protein 90 co-chaperone p23 (P23)[35-37]. Following agonist binding, the AHR undergoes a conformational change in protein structure that facilitates recognition of a minimum bipartite nuclear localization sequence (NLS) present within the amino terminus of the peptide (amino acids 13-39)[38]. Following NLS recognition, the AHR cytoplasmic complex is actively transported across the nuclear membrane. Once in the nucleus, AHR is able to heterodimerize with its DNA binding partner the ARNT to form a functional DNA-binding transcription factor. The AHR-ARNT heterodimer recognizes *cis*-acting DNA sequences termed dioxin response elements (DREs). Characterization of the AHR dependent transcriptional regulation upon the promoter of prototypic target gene Cytochrome P4501a1 (*Cyp1a1*) were critical in the identification of the consensus DNA sequence that defines a DRE[33]. Sequencing of the *Cyp1a1* proximal promoter and molecular approaches such as enhancer/promoter driven reporter and electrophoretic mobility shift assays facilitated identification of the consensus DRE sequence, 5'-KNKCGTGMKWR-3'[39, 40]. Binding of the AHR-ARNT heterodimer to DREs was found to enhance transcriptional activity at adjacent TATA-box promoters. This enhanced transcriptional effect was found to be synergistically enhanced by the presence of multiple DRE regulatory elements[33]. Consistent with this concept is the observation of strict AHR regulatory control of *Cyp1a1* induction, and the presence of eight consensus DREs within the gene promoter[33].

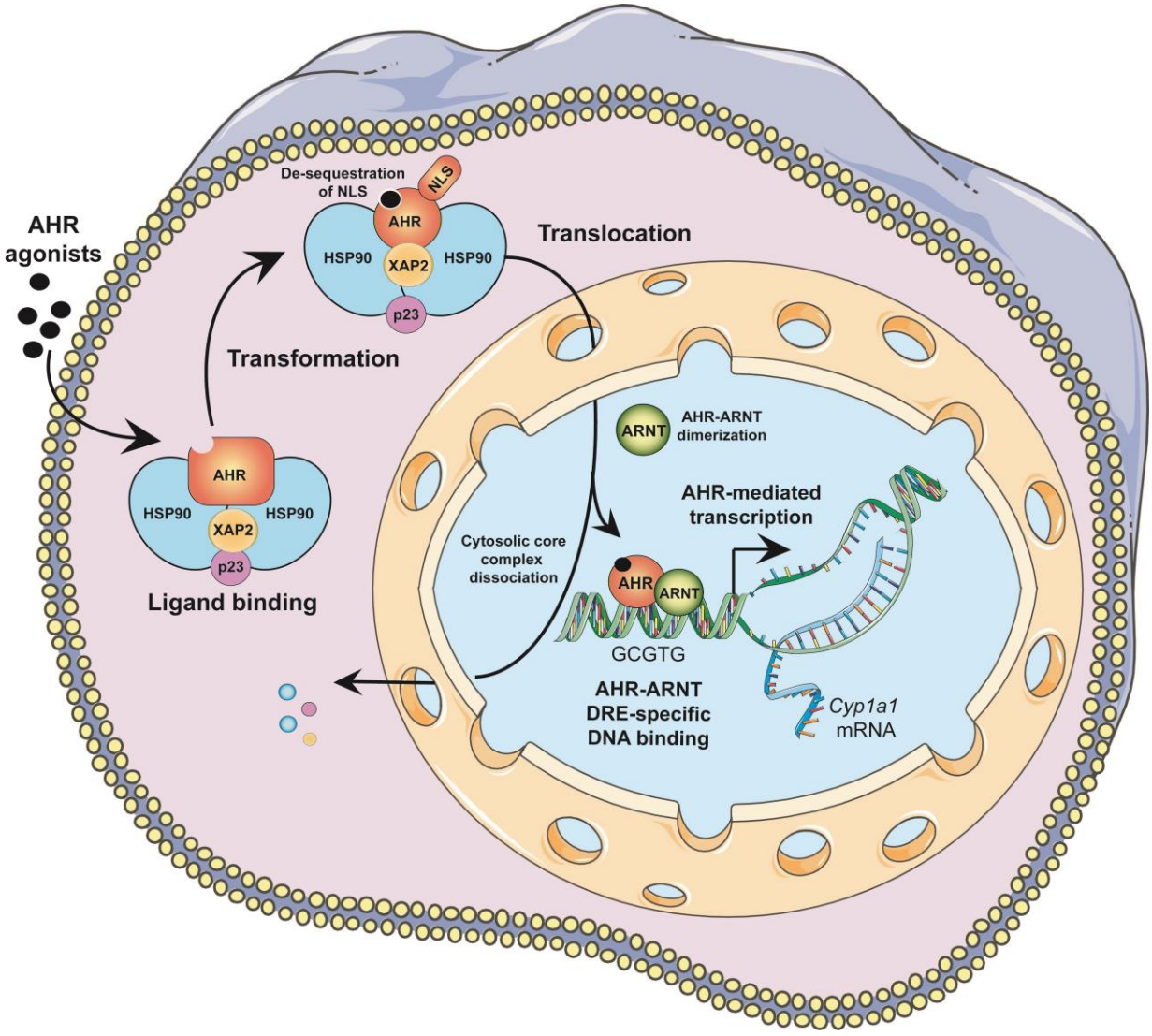


Figure 1.2: Canonical pathway of ligand-mediated AHR transcriptional activity.
Graphical display of inactive AHR residing in its cytoplasmic complex with HSP90, XAP2, and P23, followed by agonist mediated transformation, nuclear translocation, and heterodimerization with nuclear binding partner ARNT. Functional AHR/ARNT heterodimers are able to bind to DREs within promoter region of target genes to mediate assembly of transcriptional complex.

1.4 THE ROLE OF AHR CYTOPLASMIC CHAPERONE PROTEINS

Sucrose gradient analyses found the cytoplasmic AHR to reside within a 9S peak with a relative molecular mass of 285,000, indicative of the AHR residing within a large multimeric protein complex[41]. Initial techniques aimed at purification of the AHR proved insufficient due to the inherent instability and rapid turnover of the receptor. Development of enhanced AHR specific antibodies, synthesis of photo-affinity ligand 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin (PAL), and novel purification techniques eventually led to the characterization of the minimal cytoplasmic the cytoplasmic complex[42-44]. The minimal unliganded AHR core complex exists as a pentamer composed of AHR, two molecules of HSP90, XAP2, and P23.

HSP90

The molecular chaperone HSP90 is a highly conserved peptide that participates in maintenance of protein folding, stability, and is ubiquitously expressed in all cells. HSP90 is highly conserved and exists as two major isoforms α and β, which can constitute one to three percent of the cellular protein content[45]. Proper function of HSP90 is critical to cellular processes within vertebrates as evidenced by induced embryonic lethality within knockout mouse models[46]. HSP90 possesses two domains, an N-terminal and C-terminal domain, that preferentially interact with client peptides[47, 48]. The stability and ligand inducibility of steroid receptors, such as the estrogen receptor, progesterone receptor, and glucocorticoid receptor are dependent upon the interaction with HSP90[49]. The stability of HSP90 interaction with client proteins is enhanced upon molybdate supplementation through binding to its adenosine triphosphate (ATP) binding site[50].

The capability of HSP90 to exist in complex with AHR was initially described by two different labs in 1988[35, 51]. This was later confirmed by the Perdew lab through utilization of

PAL-binding studies, chemical cross-linking, and polyacrylamide gel electrophoresis to describe the relative molecular weights of components within the 9S AHR complex to be 96, 88, and 46 kDa from mouse hepatoma cells (Hepa1)[52]. Immunoprecipitation studies and western blot analyses confirmed the stoichiometry of AHR:HSP90 complex to be 1:2 and to contain two isoforms of HSP90, HSP86 and HSP84, that represent the previously described 96 and 88 kDa components respectively[53]. The binding interactions of HSP90 and AHR have been mapped to residues 217-617 of HSP90 and the bHLH and PAS domains of AHR (27-79aa and 182-374)[54]. The interaction of AHR and HSP90 appear to be necessary for stability and proper folding of the ligand activated receptor. AHR binding of ligand promotes down-regulation of AHR protein levels within cultured cells[55]. This would suggest that the ligand bound AHR confirmation may destabilize HSP90:AHR binding, causing rapid turnover of AHR. This concept is supported by the rapid proteolytic turnover of AHR in cells treated with geldamycin, a chemical compound that restricts ATP binding by HSP90[56]. HSP90 dependent influences upon the AHR LBD structure and ligand selectivity have been shown to alter AHR capacity to respond to its' prototypic ligand TCDD, in a species dependent manner[57]. *In vitro* interspecies AHR analyses reveal that dissociation of AHR-HSP90 complexes facilitate loss of TCDD responsiveness in mice and humans. In contrast, species such as rabbit and guinea pig retain AHR dioxin sensitivity in the absence of HSP90 interactions, indicating interspecies variation in the requirement of HSP90 for stability and ligand binding activity of AHR[57]. In particular, human AHR (hAHR) is known to be unstable within the cytoplasmic complex relative to its murine *Ahr*$^{b/b}$ allele counterpart as a consequence of differential interactions with HSP90[58]. This hypothesis is further supported by the observation of elevated cytosolic human AHR proteins levels following media supplementation with sodium molybdate[50]. Additional roles of HSP90

and its role in AHR nuclear translocation and activation of transcription remain to be determined. Notably, AHR does appear to remain bound to HSP90 during translocation to the nucleus[59]. Once in the nucleus, AHR is able form a functional heterodimeric transcription factor with its binding partner ARNT. However, enhanced affinity between HSP90 and AHR can decrease dimerization efficiency of AHR:ARNT[60, 61]. This observation may partially explain the inherent elevated transcriptional activity of guinea pig AHR, which as stated before does not require HSP90 to respond to ligand[62]. Of note, it has not been established if AHR:HSP90 interaction is required in a cooperative dimerization mechanism with ARNT, or if AHR dissociates completely from HSP90 prior to interaction with ARNT. Additional variants of HSP90 have been identified that vary in ATPase activity[63]. Further studies are required to address ATPase function of HSP90 and how it may influence AHR stability or transcriptional activity.

XAP2

A cytosolic protein with a molecular weight of 38 kDa was also found to associate with the chemically cross-linked 9S AHR complex[53]. This protein was identified as XAP2, an immunophilin-like peptide with homology similar to that of 52 kDa FK506 binding protein (FKBP52)[64]. Immunophilins are *cis*-trans peptidyl-prolyl isomerases (PPIases) that were initially characterized for their role in mediating the immunosuppressive effects of drugs, such as cyclosporin and rapamycin[65]. These peptides have been demonstrated to perform a multitude of roles, including function of calcium ion channels, PPIase activity, and acting as a co-chaperone within steroid receptor complexes[65]. The transcriptional activity of the glucocorticoid receptor have been found to be enhanced or repressed via interaction with FKBP52 or homolog FKBP51, respectively[66]. Treatment with the immunophilin ligand cyclosporine negatively regulates androgen receptor (AR) signaling and decreases proliferation of prostate cancer cells *in vitro*[67].

Observed protein-protein interactions involving high molecular weight immunophilins are dependent upon the presence of tetratricopeptide repeat (TPR) domains. TPR domains consist of a degenerate 34 amino acid sequence, often found in tandem repeats containing numerous motifs[68]. Repetitive TPR motifs form scaffolding structures that mediate protein-protein interactions and assembly of multi-protein complexes that are required for interactions with HSP90[69, 70]. HSP90 in complex with immunophilin peptides have been demonstrated to stabilize various steroid hormone receptors such as the glucocorticoid receptor, AR, and progesterone receptor complexes, facilitating receptor confirmations conducive to binding ligands[71-73].

The 38 kDa component of the cytosolic AHR complex was identified as XAP2 through use of a yeast two-hybrid reporter experiment by three independent research groups[36, 74, 75]. The identified protein was synonymous to the previously characterized Hepatitis B Virus x-associated protein 2, that is able to repress transcriptional activity of Hepatitis B Virus X-Protein[76]. The interaction with AHR led additional classification of XAP2 under additional aliases, such as AHR-interacting protein, and AHR-associated protein 9. XAP2 shares homology with immunophilins, however, it is considered to be only immunophilin-like due to its inability to bind immunosuppressants like FK506 and rapamycin[64]. XAP2 contains various structural domains, such as a peptidylprolylisomerase domain, and three TPR motifs located near the carboxy-terminus. Within XAP2, the repetitive TPR domains and flanking amino acids adopt a α-helical confirmation, which are conducive to protein-protein interactions. Analyses within COS-1 cells identified the b-domain within the most c-terminal TPR motif is required for XAP2 integration within the AHR:HSP90 cytosolic complex[77]. Within the cytoplasmic complex XAP2 is able to form interactions with both AHR and HSP90[54]. Deletion studies upon the AHR confirm that XAP2 stably binds to the central portion of the receptor (131-492aa), which

contains the LBD[54, 75]. The location of this interaction has lead to speculation that XAP2 may impact the ligand selectivity of the AHR through alteration of the AHR LBD structure; however this has yet to be confirmed. Notably knockdown of XAP2 does not prevent ligand dependent AHR transcriptional activity, suggesting that XAP2 is dispensable with regard to ligand binding capacity of the AHR[77].

Studies utilizing a fluorescently conjugated AHR have shown that the AHR cytoplasmic complex undergoes dynamic nucleocytoplasmic shuttling in the absence of ligand treatment. This observation suggests that under basal physiologic conditions, the bipartite nuclear localization and export signals within AHR are functional and not sequestered in the absence of ligand. XAP2 overexpression studies in COS-1 cells have shown the capacity for XAP2 to hinder basal nuclear shuttling of the AHR complex and increase its cytosolic retention. Such observations suggest that XAP2 integration within the AHR cytoplasmic complex produces a stabilizing effect that may protect the complex from proteolytic degradation and inhibit recognition of NLS by nuclear importins[78, 79]. Enhanced ligand mediated AHR transcription has been observed following enhanced expression of XAP2[36]. Interpretation of such results suggests that stabilization of the cytosolic receptor pool enhances the availability of unliganded AHR leading to enhanced transcription of AHR target genes. Enhanced AHR stability within the cytoplasmic complex as a result of XAP2 integration could impact nuclear translocation rates and ARNT heterodimerization potential. While the mechanism by which XAP2 enhances transcriptional activity is not fully elucidated, its impact upon AHR complex stability is well founded.

P23

P23 is a evolutionarily conserved peptide originally characterized for its interaction with HSP90[80]. HSP90 with bound P23 is highly stable and more conducive to binding ATP than its P23-unbound form[81]. Through its interaction with HSP90, P23 is able to complex with many HSP90 client peptides such as the progesterone receptor and AHR[82, 83]. AHR ligand binding, localization to DREs, and transcription of target genes have been found to be increased by P23 expression[37, 84]. A study using *in vitro* translated proteins found that heterodimerization of AHR and ARNT in the absence of ligand is decreased by the addition of P23[37]. This result is likely mediated by enhanced stability of AHR within the HSP90/XAP2/P23 complex, which results in a lower probability of spontaneous favorable binding interactions with ARNT. However, like XAP2, P23 is dispensable with regard to AHR ligand-mediated signaling as evidenced by studies utilizing P23 null mice[85]. In summary, P23 is a stimulatory component that functions within AHR and additional client protein complexes likely through interactions with HSP90 to influence complex stability and activation potential.

1.5 AHR REGULATION OF TRANSCRIPTION AHR TRANSLOCATION

In the absence of exogenous ligand, the AHR resides within the cytoplasm in its previously described oligomeric protein complex. Upon ligand stimulation, the 9S AHR complex, containing a bipartite NLS (13-39aa), is recognized by two components of the nuclear pore targeting complex, importin α/β, which mediate nuclear translocation[86]. This active transport of AHR was originally thought to be mediated via interaction with ARNT, hence the name AHR nuclear transporter. However, this classification is considered a misnomer as evidenced by the observed nuclear translocation of AHR in ARNT$^{-/-}$ Hepa 1 C-4 cells[30]. Additional regulatory mechanisms, such as the phosphorylation state of serine residues in close

proximity to the NLS of AHR, can inhibit ligand mediated nucleocytoplasmic shuttling[87]. Once in the nucleus, the AHR proceeds along two distinct molecular pathways. The first being active export from the nucleus through recognition of a leucine-rich nuclear export signal (55-75aa)[38]. Alternatively, the AHR can heterodimerize with ARNT, forming a functional transcription factor capable of modulating transcriptional activity by targeting of their cognate response element.

ARNT

The distinctive physiochemical properties of nuclear AHR relative to its cytosolic form suggest additional factors may contribute to the specificity of AHR-DNA interactions[88]. This concept was further established through use of mutant mouse hepatoma cells deficient in their capacity to respond to prototypical AHR ligands and inability for AHR nuclear translocation. Screening of cDNA libraries by reconstitution experiments within the mutant cell line led to the discovery of a 110 kDa human peptide capable of rescuing AHR nuclear translocation and signaling[89].

ARNT is also a member of the bHLH-PAS protein super-family. Like AHR, ARNT contains a bHLH DNA binding domain within its N-terminal region and tandem PAS domains, and a transactivation domain within its C-terminal region[90]. However, in contrast to AHR, ARNT does not bind ligands and is a class II bHLH-PAS member, allowing it to heterodimerize with an array of class I members and form homodimers[91]. This diversity in potential client protein partners allows for ARNT to participate in the transcriptional regulation of a diverse array of genes, independent of AHR activation. ARNT actively triggers response to hypoxic conditions through heterodimerization with HIF-1a, to form the functional transcription factor hypoxia-inducible-factor 1. Hypoxia-inducible-factor 1 is able to bind hypoxic response elements (HREs) within the promoters of genes and regulate expression of factors involved in

angiogenesis and oxygen transport, such as vascular endothelial growth factor a (VEGFA) and erythropoietin[22]. Additional ARNT heterodimer partners include single minded proteins (SIM1, SIM2) and Cardiovascular Helix-Loop-Helix Factor 1 (CHF1), which participate in signaling pathways involved in neurogenesis and cardiovascular development[92, 93].

AHR-ARNT HETERODIMERIZATION AND DNA-BINDING

The dimerization and DNA-binding activities of AHR-ARNT is contingent upon the interaction of the minimal bHLH domains present within each peptide as evidenced by peptide fragmentation studies[94]. However, in the absence of the AHR PAS-A domain, specificity for ARNT heterodimerization was lost, as evidenced by formation of AHR bHLH homodimers and interactions with additional factors[94]. The function of the PAS-A domain of the AHR was found to be critical in dictating specificity of AHR-ARNT heterodimerization and DRE binding capacity. These studies confirm the importance of the PAS-A domain in conferring conformational regulation of the adjacent bHLH domain that facilitates heterodimerization potential and DNA response element specificity specific to AHR-ARNT function[94]. Formation of the AHR/ARNT heterodimer can also be influenced by the phosphorylation status of ARNT, but not AHR[95]. These data suggest multiple levels of molecular regulation that dictate AHR transcriptional activity prior to the binding of DNA.

After heterodimerization with ARNT, the functional AHR/ARNT transcription factor target response elements defined by a minimal 5'-GCGTG-3' motif within the major groove of DNA[96]. DNA cross-linking studies indicate the ARNT contact the downstream 5'-GTG-3', while the AHR binds the neighboring upstream 5'-TNGC-3' half site[97]. The binding of DNA by AHR/ARNT is facilitated by primary contacts with amino acids within the basic region of both peptides[98]. However, the PAS domains of both AHR and ARNT also modulate DNA binding

capacity, possibly through manipulation of DNA binding domain tertiary structure. While the minimum recognition site is well defined, studies suggest that flanking sequences may play a major, yet still undetermined role in AHR/ARNT binding to DNA[99]. The presence of DREs within the promoter/enhancer regions of genes suggests possible positive or negative transcriptional regulation by the AHR.

AHR REGULATION OF *CYP1A1* TRANSCRIPTION

The AHR is a principal regulator of *Cyp1a1* expression[33]. The *Cyp1a1* promoter exhibits low constitutive expression and robust induction potential, making it a useful surrogate for analyses of compound libraries for AHR agonists. The sensitivity of AHR-dependent induction of *Cyp1a1* is due in large part to the presence of multiple consensus DREs within the proximal promoter and enhancer regions, upstream of the transcriptional start site[39]. The presence of the AHR/ARNT heterodimer upon the promoter was confirmed by electrophoretic mobility shift assays utilizing nuclear extracts from cells lacking expression of either AHR or ARNT[100]. The promoter of *Cyp1a1* typically is sequestered within a nucleosomal configuration, which inhibits access of transcriptional machinery and is the reason for its low basal expression. Upon exposure to AHR ligands, the AHR/ARNT heterodimer binds to DREs within the enhancer region of *Cyp1a1* causing a restructuring of the chromatin configuration that enhances accessibility of the promoter region. C-terminal AHR mutants lacking the C-terminal transactivation domain were able to bind DREs within the *Cyp1a1* promoter and alter enhancer nucleosome organization. However, the ability to alter chromatin structure at the downstream promoter was abolished[101]. This evidence indicates that recruitment of additional factors via the AHR transactivation domain, such as acetyltransferases, histone modifiers, and coactivators comprise a critical component of transcriptional initiation at the *Cyp1a1* promoter.

ADDITIONAL MECHANISMS OF AHR TRANSCRIPTIONAL REGULATION

Transcriptional regulation of genes can be influenced by an array of competing molecular signals that ultimately dictate the level of expression and inducibility of genes. While AHR is capable of enhancing gene expression, this effect can often be promoter-specific and depend upon crosstalk with other competing signaling pathways.

Recently, our lab confirmed AHR regulation of hepatic fibroblast growth factor 21 (FGF21). FGF21 is induced during fasting and functions to induce gluconeogenesis, ketogenesis, and torpor. The promoter of *Fgf21* contains overlapping DNA response elements for peroxisome proliferator-activated receptor alpha (PPARα), carbohydrate response element binding protein (ChREBP), cyclic adenosine monophosphate response element binding protein (CREBH), and AHR/ARNT. *Fgf21* expression is enhanced following circumstances of PPARα, ChREBP, and CREBH promoter binding. However, activation of AHR was found to mediate repression of *Fgf21* transcription. This activity is thought to be mediated by competitive exclusion of stimulatory factors from the promoter due to overlapping DNA response elements[102]. This type of regulation would suggest that not only the combination of molecular signals received, but also the sequence in which they occur, can drastically affect the transcriptional outcome.

Other factors such as histone methylation and DNA acetylation patterns may influence chromatin structure and subsequent gene expression. The promoter of interleukin 6 (*IL6*) is strongly regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[103]. Studies within human cancer lines indicate activation of the AHR in combination with NF-κB signaling promotes a synergistic increase in *IL6* expression. The nature of the AHR-dependent increases in *IL6* promoter transcription involves the reorganization of promoter bound repressive

complexes. These repressive mediators were identified as histone deacetylase complexes (HDAC1/HDAC3)[104]. The presence of AHR upon the *IL6* promoter does little to enhance transcriptional activity. However, in combinatorial regulation with NF-κB, AHR represses HDAC association with the promoter, which increases promoter acetylation and subsequent recruitment of coactivators that enhance NF-κB-dependent transcription[104].

AHR-dependent regulation of transcription can occur in the absence of DRE-binding by direct interactions with other transcription factor complexes. Anti-estrogenic effects of TCDD exposure suggest crosstalk between AHR and estrogen receptor (ER) signaling[105]. Concurrent activation of both ER and AHR signaling pathways within breast cancer cells by their respective ligands 17β-estradiol and TCDD, results in the attenuation of both ER and AHR dependent transcription. Transrepression of ER/AHR signaling is mediated by tethering of ER and AHR/ARNT at the promoter of their responsive target genes[106, 107]. These physical interactions inhibit the transactivation potential of AHR/ER transcriptional complexes by disruption of prototypical co-activator recruitment and/or enhancement of co-repressor activity.

Similar to that of the ER, the crosstalk between AHR and NF-κB is also bidirectional and thought to be mediated by protein-protein interactions between both factors[108, 109]. Proinflammatory signaling molecules that promote dissociation of NF-κB from its cytosolic inhibitory complex and nuclear accumulation have been demonstrated to suppress AHR mediated transcription of prototypical target genes. Likewise, AHR activation by agonists in combination with induction of inflammatory signaling have been shown to attenuate NF-κB dependent transcriptional activity[110]. Limitation of RelA/RelB signaling by AHR occurs through mechanisms disparate from characterized AHR/ER cooperative signaling. Data suggests a limitation of RelA nuclear translocation and enhanced degradation of RelB protein following

proinflammatory stimulus in combination with AHR agonist treatment[111, 112]. Crosstalk between AHR and signaling pathways implicated in cell cycle progression, immune cell differentiation, and carcinogenesis suggest additional mechanisms of physiologic regulation, genomic and non-genomic, exerted by this pleotropic factor.

DOWN-REGULATION OF AHR-DEPENDENT SIGNALING

While AHR is required for normative cellular responses, supra-physiological levels of AHR activation can be detrimental as evidenced by toxicity associated with TCDD exposure. Down-regulation of AHR signaling occurs through two distinct mechanisms; a negative feedback loop mediated by the expression of the aryl hydrocarbon receptor repressor (AHRR) and targeted protein degradation of the AHR.

As stated previously, agonist activated AHR translocates to the nucleus where it interacts with its binding partner ARNT and alters transcription of responsive genes. Expression of the target gene *Ahrr* is enhanced following AHR agonist stimulation. AHRR possesses nearly identical structural homology with the N-terminal bHLH of AHR, but lacks conservation of its C-terminal region[113]. Following translation, the AHRR is actively transported to the nucleus, where it is able to heterodimerize with ARNT and bind DREs[113]. However, the C-terminal domain of AHRR does not promote transactivation of AHR target gene expression, but instead acts as a potent repressor of transcription[114]. As such, the generation of AHRR following AHR activation serves as an important negative feedback mechanism to limit persistent AHR signaling. Competitive exclusion of AHR from DREs by AHRR sequestration of ARNT serves to decrease AHR transcriptional activity. However, by limiting the formation of a stable heterodimer with ARNT, the bio-availability of AHR susceptible to targeted proteasomal degradation is increased.

Following treatment with a potent agonist, such as TCDD, a time-dependent decrease in AHR protein levels is observed. The turnover and subsequent restoration of AHR protein levels mediates a cyclic pattern of AHR activity in response to agonist treatment. When bound within its cytosolic complex, the AHR is less susceptible to proteasomal degradation. Once in the nucleus, AHR participates in the assembly of a CUL4B ubiquitin ligase that promotes self ubiquitination and targeted degradation[115]. Notably, AHR is thought to play an important role in the assembly of this complex and also possess ligand-dependent E3 ubiquitin ligase activity. The presence of AHR within the ubiquitin ligase complex and its propensity to interact with additional transcription factors suggests AHR may further influence molecular signaling by targeted proteasomal degradation of other factors. This mechanism is thought to play a critical role in AHR-dependent effects upon sex-hormone signaling by the estrogen and androgen receptors. In response to treatment with 3MC or the presence constitutively active AHR, a drastic decrease in ER and AR proteins levels were observed without a significant alteration of basal mRNA levels[116]. Further studies utilizing the proteasome inhibitor MG-132 indicate that increased AHR activation was coupled to increased ubiquitination of ER[117]. The ubiquitin ligase activity of the AHR highlights an additional non-genomic mechanism of crosstalk/regulation of molecular signaling pathways. Both the negative feedback-loop and protein degradation pathways serve as critical pathways to down-regulate AHR signaling. Such mechanisms are necessary to repress AHR signaling by persistent ligands, such as TCDD, that are not degraded by AHR-induced CYP-P450 metabolism.

1.6 PHYSIOLOGICAL IMPLICATIONS OF THE AHR KNOCKOUT MOUSE

The development of targeted gene manipulation in mammalian systems allowed for the generation of AHR knockout (*Ahr*$^{-/-}$) mouse models to examine AHR regulation of *in vivo* physiological functions in the absence of xenobiotic exposure [118]. The *Ahr*$^{-/-}$ mouse, unlike its *Arnt*$^{-/-}$ counterpart, does not cause embryonic lethality[119]. Mice lacking the AHR were found to be resistant to TCDD/PAH toxicity and failed to significantly express AHR target genes, such as *Cyp1a1* and *Cyp1a2* [120, 121]. The absence of the AHR signaling during neonatal development leads to a multitude of phenotypic abnormalities[118, 122]. Hepatic abnormalities, such as patent *ductus venosus*, were identified within AHR null mice developed by Dr. Christopher Bradfield [123]. Furthermore, aged AHR null mice exhibit numerous detrimental phenotypes including decreased liver size, impaired hepatic vascular development, cardiac hypertrophy, gastric lesions, and dermal fibrosis[122]. The decreased fitness of AHR null mice suggests extensive physiological roles for the AHR in developing mammals and supports the hypothesis that constitutive physiological AHR function in the absence of exogenous ligands is mediated through signaling by endogenous ligands.

AHR IMPLICATIONS IN IMMUNE FUNCTION

The role of the AHR within immune regulation has expanded beyond activities specific to innate immunity to include multifaceted functions within the adaptive immune response. Suppression of the humoral response through decreased development and maturation of B-cells and inhibited differentiation of IgG secreting plasma cells is observed following TCDD administration[124]. The influences of ligand-mediated AHR signaling pathways upon differentiation and expansion of specific T cell subpopulations has been the subject of thorough investigation. The differentiation of naïve $CD4^+$ T cells to either regulatory T cells (T_{Reg}) or T-

helper-17 (T_{H17}) cells were found to be dependent upon the specific AHR ligand administered[125]. In animal models of experimental autoimmune encephalitis, administration of the prototypical AHR ligand TCDD ameliorates disease severity by increasing the resident population of anti-inflammatory T_{Reg} cells. However, in the same model, administration of the AHR ligand *6*-formylindolo(3,2-*b*)carbazole (FICZ) facilitates an expansion of proinflammatory T_{H17} cells, further enhancing disease severity. However, this differential effect upon differentiation by FICZ was dependent upon the mode of ligand administration. Divergence of T cell lineages is thought to occur through ligand specific receptor confirmations that dictate differential receptor DRE dependent and independent activity. Evidence suggests that dioxin-bound AHR modulates T cell differentiation via expression of microRNAs and epigenetic regulation, such as increased methylation of CpG islands of Foxp3 and simultaneous demethylation of the *Il17* promoter. Thereby mediating a phenotypic switch from T_{H17} to T_{Regs}[126, 127]. Interestingly, there is no difference in AHR-dependent T_{H17} differentiation between $Ahr^{b/b}$ and $Ahr^{d/d}$ mouse strains[128]. Modulation of inflammatory signaling by selective AHR ligand treatments may have therapeutic applications. However, further studies are needed to assess ligand dose dependent levels of AHR signaling required to mediate the desired therapeutic outcome and minimize the inherent risk of prolonged receptor activity.

AHR AND INTESTINAL HOMEOSTASIS

Maintenance of intestinal homeostasis depends on complex interactions between the host microbiome, the intestinal epithelium, and the host immune system. Dysfunction within any one component may contribute to the etiology of chronic intestinal disorders such as IBD[129]. The physiological significance of AHR signaling within the gastrointestinal tract is evident in models of chemically-induced intestinal colitis. In these models, AHR null mice exhibit increased severity of

disease symptoms (weight loss, colon shortening) and lethality relative to their AHR competent counterparts[130, 131]. This effect was recapitulated by administration of TCDD in mice, suggesting AHR expression and ligand induced activity are protective in dextran sodium sulfate induced colitis models [132]. However, a more significant reduction in the colitis disease severity index was observed in AHR heterozygous compared to AHR homozygous mice, suggesting overstimulation of the AHR may have mixed consequences[131].

The AHR contributes to the maintenance of intraepithelial lymphocytes (IELs) and innate lymphoid cells (ILCs) cell populations, two critical components of gut-associated lymphoid tissue[133-135]. Located between the basolateral surfaces of the epithelial cells, IELs act to survey the intestinal epithelial surface for microbes or barrier disruption[136]. Studies using AHR null mice suggest AHR signaling is required to promote IEL maturation and survival in post-natal development[137]. Interestingly, increased IEL populations were observed following administration of dietary AHR ligands, suggesting increased levels of AHR activity can contribute to the maintenance intestinal homeostasis[138].

ILCs, specifically those classified as group 3 (ILC3s), are a recently identified component of the gut associated lymphoid tissue that expresses high levels of the AHR[139]. These cells lack cell surface markers associated with typical cellular components of the adaptive or innate immune response. Lack of AHR signaling during neonatal development and resultant attenuation of ILC3 differentiation have been found to associate with the absence in formation of intestinal secondary lymphoid tissues, such as lymphoid follicles and cryptopatches[140, 141]. Depletion of ILC3 populations leads to a significant reduction in interleukin 22 (IL22) production[140]. IL22 signaling within the intestinal tract promotes intestinal homeostasis through promotion of epithelial barrier function, enhanced mucin secretion by goblet cells, and production of

antimicrobial peptides such as β-defensin and lipocalin 2[142]. Notably, lack of IL22 dependent signaling within epithelial cells was detrimental in models of *C. rodentium* infection[141].

Dysfunction between the host signaling and the resident microbiota play a significant role in the pathogenesis of intestinal diseases. The microbiome is composed of trillions of microbes that conduct many beneficial functions for the host, including breakdown of indigestible plant materials, production of short-chain fatty acids, routine priming of the immune system, and the repression of pathogenic colonization[143, 144]. Genetic analyses of 75,000 IBD cases and controls have identified 163 host susceptibility loci[145]. Notably these loci are enriched for signaling pathways that monitor environmental stimuli, such as bacterial components or metabolites. Recent studies suggest that the presence of AHR signaling or it overt activation by exogenous compounds affects the composition of the microbiome. Absence of AHR signaling was found to associate with higher prevalence of segmented filamentous bacteria and elevated inflammatory tone within the intestinal tract, relative to heterozygous littermates[146]. Exposure of mice to the potent AHR agonist, 2,3,7,8-tetrachloro-dibenzofuran (TCDF) was found to skew the *firmicutes*:*bacteroidetes* ratio, which associated with altered bile acid metabolism, bacterial fermentation, and inflammatory signaling within the host[147]. Further studies are needed to elucidate mechanistic understanding of AHR contributions to intestinal homeostasis, and further evaluate its viability as a therapeutic target in intestinal disease.

AHR IMPLICATIONS IN CELL CYCLE PROGRESSION AND CANCER

Complex and conflicting pro-tumorigenic and anti-tumorigenic activities have been found to associate with AHR signaling through its involvement within various aspects of cell cycle progression, tumorigenesis, and metastasis. Cellular proliferation events are highly regulated and composed of three distinct checkpoints at the M1/S-phase, M2/M-phase, and

Metaphase transition points. These checkpoints consist of networks of regulatory proteins that monitor DNA-repair, duplication of organelles, and macromolecule generation to signal continuation of mitosis or promotion of apoptosis. The AHR has been shown to promote cellular proliferation through enhancement of mitotic initiation by direct transcriptional activation of mitogenic factors such as *VEGFA*, platelet-derived growth factor (*PDGF*), and epiregulin (*EREG*)[148-150]. However, there are many examples in which AHR activity is able to inhibit cell cycle initiation/progression. Notably, AHR limits proliferative signaling by the Wnt/β-catenin signaling pathways through enhanced proteasomal degradation of β-catenin by its previously defined ubiquitin ligase activity[151]. Agonist-activated AHR attenuates phosphorylation of retinoblastoma protein (RB) through direct protein-protein interactions and transcriptional regulation of cyclin-dependent kinase inhibitor 1B (CDKI1B), also known as P27, which inhibits cyclin-dependent kinase 4 phosphorylation of RB[152-154]. Lack of RB phosphorylation enhances the stability with E2F, which attenuates E2F-dependent gene expression required for initiation of S-phase and leads to G_1-arrest. Interestingly, antagonism of AHR signaling by the ligand, Stemreginin, promotes a 50-fold expansion of *ex-vivo* treated hematopoietic stem cells, further suggesting a significant role of AHR in cellular proliferative signaling[155]. AHR-dependent effects upon proliferation are often convoluted due to opposing effects that often vary due to the *in vitro* or *in vivo* model utilized.

Additional AHR dependent effects upon inflammatory signaling and cell cycle progression suggests implications within various stages of tumorigenesis[156-158]. For example, high levels of constitutive receptor activity and corresponding nuclear localization have been observed within aggressive tumors and tumor cell lines[159-161]. Studies in mice that over express a constitutively active form of the AHR show increased incidence of hepatic and stomach tumors,

suggesting AHR activation may be pro-oncogenic. Unexpectedly, AHR null mice also exhibit increased colonic tumor incidence and elevated sensitivity to carcinogen induced hepatic tumor initiation, indicating that overall loss of receptor activity can also enhance pro-tumorigenic signaling. The measurement of constitutive AHR activity has been investigated as a possible diagnostic marker of cancer aggressiveness[156]. A positive or negative correlative effect between AHR activity and poor prognoses has been found to vary by the type of cancer or tissue of origin. Research suggests increased AHR activity is associated with diminished tumor-aggressiveness of hormone-dependent breast cancers, likely due to AHR transrepression of proliferative estrogen receptor signaling[162]. Conversely, elevated AHR transcriptional activity is observed with non-small cell lung cancer and correlates with increased malignancy[163]. Heightened AHR activity is associated with decreased expression of adherence peptides such as cadherins molecules, and increased expression of matrix metalloproteinases that enhance cytoskeletal rearrangement and metastatic potential of tumors[164, 165]. The dichotomous roles of AHR in pro/anti-tumorigenic activities are often model and ligand-dependent. Distinct AHR-associated effects upon potentiation of anti-inflammatory or anti-tumorigenic signaling are ligand dependent[166]. This suggests that targeting of the receptor through use of AHR ligands that exhibit specific desired activities may be of therapeutic interest[156, 158].

1.7 AHR LIGANDS

The AHR is considered promiscuous as evidenced by the wide array of ligand structures that it is able to accommodate within its LBD. Ligands can be classified within two distinct categories; exogenous compounds, which are considered synthetic in nature and are synthesized via anthropogenic activities and endogenous compounds, that are formed through natural biochemical processes.

EXOGENOUS AHR AGONISTS

The AHR was first identified as a regulator of xenobiotic metabolism due to its ability to the great affinity by which it bound TCDD and subsequently induced transcription of the CYP450 gene family. Additional environmental toxicants with physiochemical properties similar to that of dioxin, such as HAHs, and PAHs are also capable of mediating ligand-dependent activation of the AHR **(Figure 1.3)**.

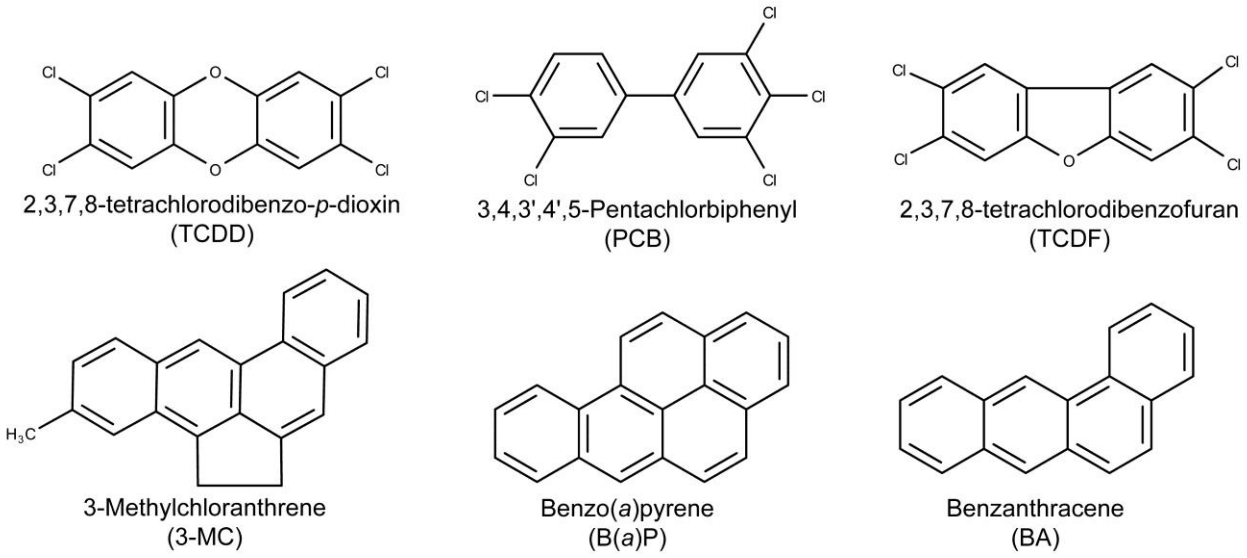


Figure 1.3: Exogenous AHR Ligand Structures.

HAHs are characterized as highly stable environmental contaminants with structures typified by polychlorinated and polybrominated dibenzo-*p*-dioxins, dibenzofurans, and bi-phenyls. By 1977, over 1 billion pounds of commercial-grade polychlorinated bi-phenyls (PCBs) had been manufactured in the U.S. for an array of industrial applications. These processes often yielded significant amounts of halogenated dibenzofurans or dibenzo-*p*-dioxins contaminants. HAHs exhibit similar binding affinity for the AHR, relative to TCDD[167]. Structure-activity relationship studies suggest that compound affinity for the AHR directly correlates with toxic outcomes. Due to the designation of TCDD as the most potent/toxic AHR ligand, compounds are compared via a scale relative to TCDD, referred to as Toxic-Equivalency Factors[167]. This scale was considered instrumental in the evaluation of environmental samples containing mixed populations of toxic congeners[168].

PAHs are non-halogenated compounds composed of multiple conjoining aromatic ring structures. These compounds are often derived from the incomplete combustion of organic matter. Predominant generation and exposure to PAHs are a result of human activities such as wood burning, cigarette smoking, and consumption of fossil fuels. PAHs diverge further from the halogenated structure of prototypical AHR ligand TCDD, and therefore possess lower binding affinities than HAHs. PAHs such as B(*a*)P and benzanthracene (BA), possess decreased AHR affinity, but due to high levels of environmental exposure pose significant risks to human health. Unlike TCDD, PAHs are notoriously genotoxic as evidenced by the metabolic activation of the pro-carcinogen B(*a*)P.

B(*a*)P METABOLISM

Environmental exposures to PAHs have been associated with carcinogenesis for centuries. In 1775, Sir John Percivall Pott observed increased incidence of scrotal cancer among

London chimney sweeps exposed to large amounts of coal soot, which contains high levels of PAHs. Occupational exposure among workers involved in the processing of coal tar showed heightened incidence of cancer relative to the general population. The causative agent within coal tar was eventually identified as B(*a*)P the "ultimate carcinogen". B(*a*)P is able to induce its own metabolism through activation of the AHR and subsequent initiation of phase I drug metabolism enzymes *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* **(Figure 1.4A)**[169]. Cyp-450 targeted oxidation of B(*a*)P yields benzo(*a*)pyrene-7,8-epoxide, a reactive intermediate with increased aqueous solubility. The epoxide substituent is labile for cleavage by resident epoxide hydrolase enzymes to yield benzo(*a*)pyrene-7,8-dihydrodiol. The B(*a*)P-diol moiety is subject to second Cyp-450-catalyzed epoxidation, generating Benzo(*a*)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), which is a highly electrophilic intermediate capable of forming adducts with protein, lipids, and DNA to facilitate cellular toxicity and initiate carcinogenesis[170, 171]. The major DNA adduct observed following B(*a*)P treatment is a *trans*-adduct between the N_2 atom of guanine bases and BPDE[172]. This alteration in DNA structure from intercalated BPDE will hinder DNA interactions with replication machinery and mediate a G->T transversion mutation[173]. *In vivo* studies of B(*a*)P mediated toxicity and cancer produce varied phenotypic observations that may be dependent upon dose, presentation, and route of exposure. Intraperitoneal injection of B(*a*)P at high concentrations result in toxicity of the liver, immune system, bone marrow, spleen, adipose tissue, thymus and arterial cell walls[174-177]. However, acute oral B(*a*)P administration causes immunotoxicity, but negligible effects in other tissues[178, 179].

Initial characterization studies of the xenobiotic metabolizing enzyme activities in the 1950's suggested that phase I drug metabolism are essential for detoxification of PAHs, such as B(*a*)P. A few years later, B(*a*)P metabolism was shown to unequivocally facilitate generation of

reactive intermediates capable of covalently attaching to proteins ,lipids, and DNA[180]. This observation led to the ongoing concept that phase I drug metabolism was notoriously detrimental due to production of reactive intermediates and their resultant toxicities, while phase II drug metabolism was considered extremely beneficial due to conjugation reactions that detoxify and enhance excretion of metabolites. Phase II conjugation of B(*a*)P was found to occur through mechanisms of sulfate, glutathione, and glucuronide conjugations in isolated rat hepatocytes[181]. This group also found that levels of reactive B(*a*)P intermediates and resultant DNA adducts were inversely correlated with rates of phase II metabolism. These results suggest that intricate coupling of phase I and phase II drug metabolism pathways are critical to limit cellular stress and toxicity as a result of PAH exposure **(Figure 1.4B)**.

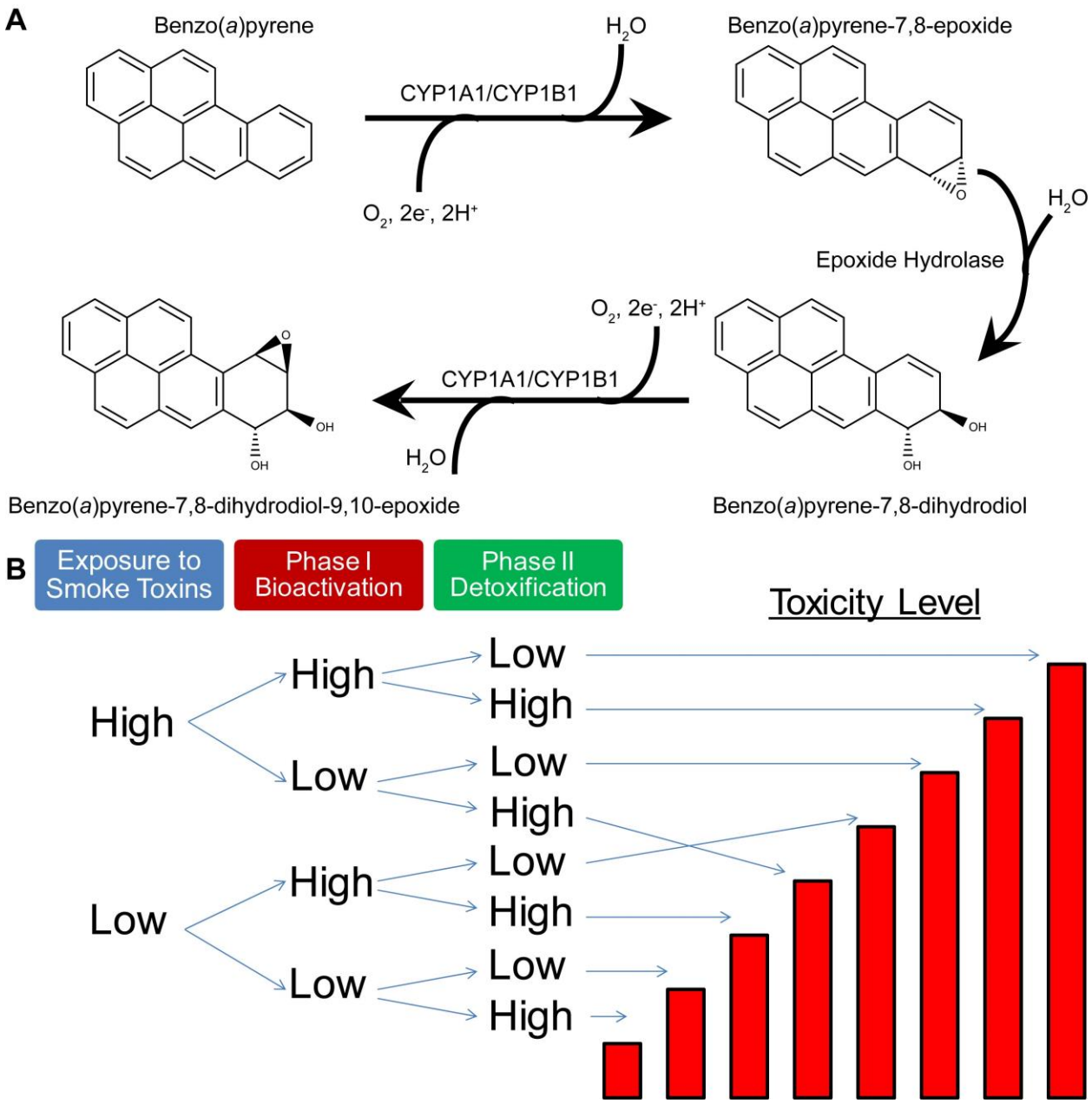


Figure 1.4: Benzo(*a*)pyrene metabolism and associated toxicity.
(A) B(*a*)P metabolic bioactivation to the ultimate carcinogen benzo(*a*)pyrene-7,8-dihydrodiol-9,10-epoxide by AHR mediated induction of phase I drug metabolism enzymes. **(B)** Graphical representation of resultant toxicity from coupling/decoupling of phase I and phase II drug metabolism pathways. Adapted from previous publication [182].

ENDOGENOUS AHR LIGANDS

Research with the goal of identifying putative endogenous AHR ligands and their physiological roles has been an expanding area of interest in the field of AHR biology[183, 184]. Non-exogenous routes of AHR activation may occur through diverse mechanisms, such as dietary intake, metabolism by host commensal microorganisms, free radical formation, and synthesis by host enzymatic activity. Numerous compounds have been proposed as putative endogenous AHR activators, many of which are generated through metabolic bio-activation of the amino acid tryptophan[185, 186]. Identification of novel routes of endogenous ligand synthesis is critical to understanding mechanisms of host constitutive and inducible receptor activity. Knowledge of non-exogenous routes of AHR activity may aid in the identification critical AHR-dependent physiological signaling activities or a viable therapeutic target.

DIETARY AHR LIGANDS

Consumption of AHR ligands could play a significant role in AHR mediated effects on intestinal homeostasis and the local metabolism of dietary and endogenous compounds. Thus, dietary intake of AHR agonists would provide a diverse array of potential ligands of varying potency and metabolic half-lives that could mediate systemic or localized receptor activation **(Figure 1.5)**.

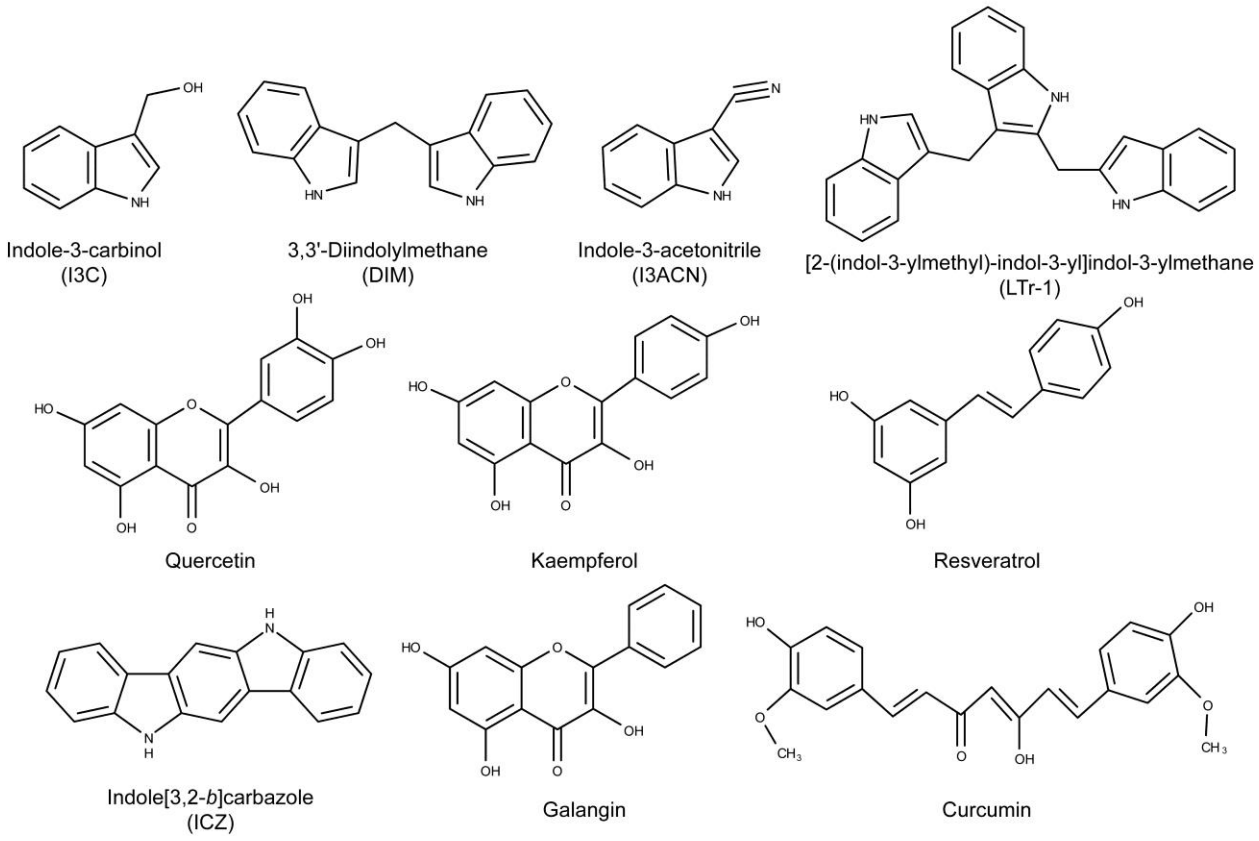


Figure 1.5: Diet-derived AHR ligand structures.

Biological analyses of extracts from herbal supplements, fruits, and vegetables has revealed significant activation of DRE-driven cell-based reporter systems, target gene expression, and DNA-binding functions, indicative of the presence of AHR activators[187]. Cruciferous vegetables, such as broccoli and brussels sprouts have been found to be a rich source of glucobrassicin, which can be bioactivated to an array of AHR ligands **(Figure 1.6)**. Consumption and subsequent gastric digestion promotes the enzymatic cleavage of glucobrassicin by myrosinases to yield indole-3-carbinol (I3C) and indole-3-acetonitrile (I3ACN), both of which exhibit the capacity to bind and activate the AHR, albeit weakly[188-191]. Interestingly, the *in vivo* potency of I3C with regard to AHR activation has been demonstrated to be dependent upon the mode of exposure. Intraperitoneal administration of I3C hinders endogenous AHR activation potential when compared to oral gavage[192]. In the presence of gastric acid both I3C and I3ACN undergo non-enzymatic acid condensation reactions to generate a number of additional AHR ligands, including 3,3'diindolylmethane (DIM), 2-(indol-3-ylmethyl)-3,3'-diindolylmethane (Ltr-1), and indolo[3,2-*b*]carbazole (ICZ)[191]. Among the array of glucobrassicin-derived compounds, ICZ exhibits the highest affinity and activation potential for the AHR and thus represents a significantly physiological relevant glucobrassicin-derived AHR ligand. Through the use of a competitive binding assay, ICZ was found to have a K_d of 190 pM, 27-fold less potent than TCDD[191, 193]. ICZ activated AHR is highly resistant to antagonism by prototypical antagonist GNF-351 and therefore likely interacts with the AHR LBD through a mechanism distinct from TCDD[102]. As a result of this unique binding dynamic, ICZ-activated AHR could be resistant to attenuation by additional AHR antagonists and continue to mediate AHR-activity in the presence of exogenous or endogenous antagonists.

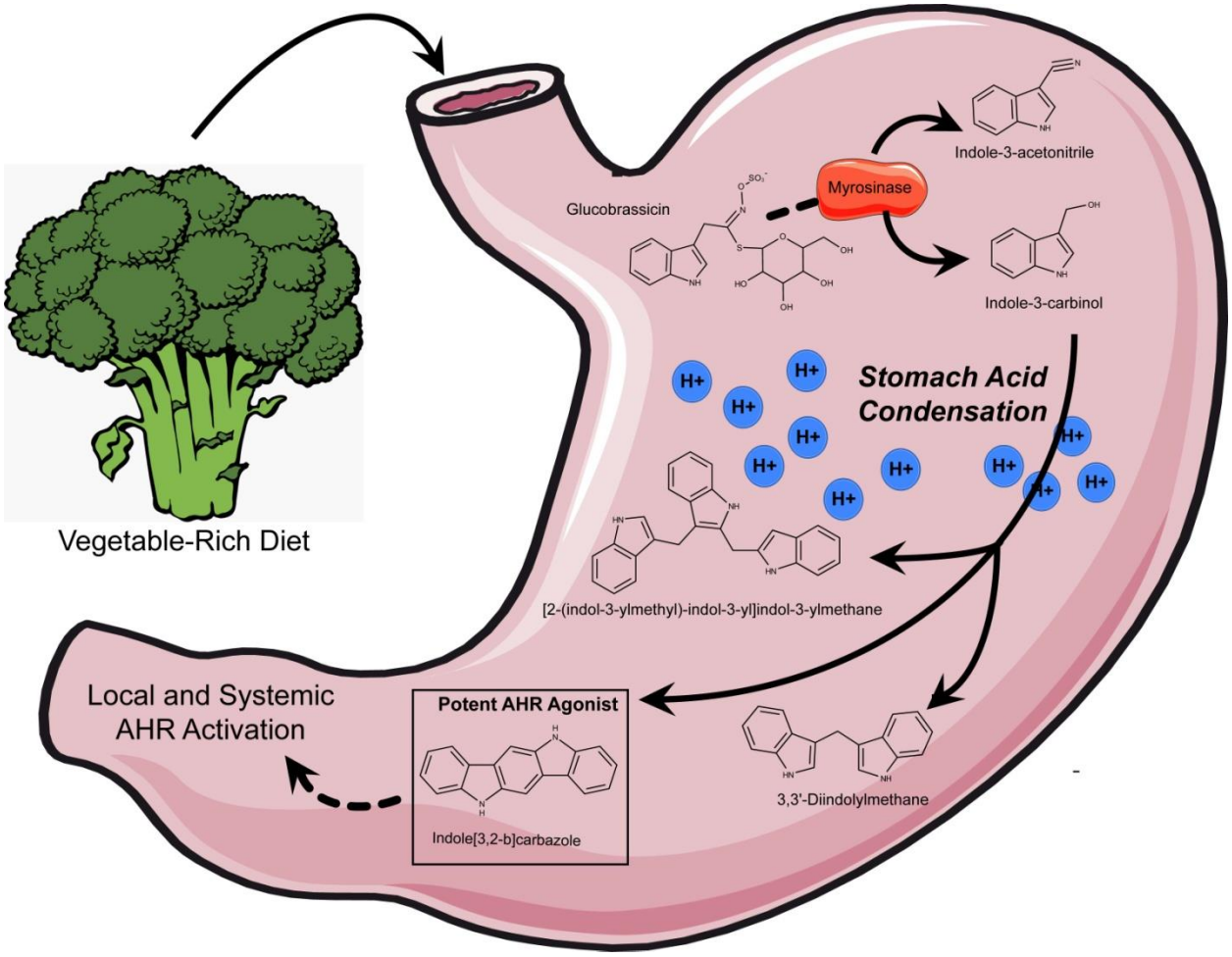


Figure 1.6: Diet derived AHR ligands via stomach acid condensation.

The beneficial effects of I3C and its condensation products are wide ranging. Dietary I3C is reported to suppress *in vivo* models of prostate carcinogenesis by inducing cell cycle arrest and apoptosis of prostate cancer cells[194-196]. Dietary I3C or its condensation products have displayed therapeutic activity in mouse models of multiple sclerosis and dextran sodium sulfate induced colitis [197, 198]. Both murine disease models facilitate the induction of detrimental inflammatory states, which were alleviated through the AHR-dependent promotion of anti-inflammatory T_{Reg} cell differentiation. The dietary metabolite I3C has been shown to limit pro-inflammatory responses through additional mechanisms such as suppression of NF-κB signaling pathways[199]. Additionally, I3C or its metabolites exhibit anti-microbial properties, such as inhibition of *Escherichia coli* and *Staphylococcus aureus* biofilm formation[200]. These effects may have significant implications upon host intestinal microbiota composition and may be a deterrent to opportunistic infection.

Despite these beneficial effects of I3C and its condensation products, long term treatment studies in rodents indicate, that consistent with its activity as an AHR agonist, I3C may be procarcinogenic. Investigation by the National Toxicology Program division of NIH indicated that I3C administration to rodents correlate with an increase in incidence of hepatocarcinogenesis [201]. This effect may be due to systemic overstimulation of the AHR facilitated by excessive amounts of ligand. Currently, I3C is available as a dietary supplement at dosages exceeding levels achievable with normal consumption of vegetables. Dietary levels of I3C most likely would facilitate beneficial localized activation of the AHR within the intestinal tract, while avoiding the potentially detrimental effects of systemic activation.

Flavonoids are a large group of polyphenolic derivatives common in edible plants and constitute additional sources of possible dietary AHR ligands. Dietary flavonols quercetin and kaempferol were both shown to modulate *Cyp1a1* induction within MCF-7 breast-cancer cell

lines[202]. Quercetin is an AHR agonist; however, the loss of a single hydroxyl group in its structure to yield kaempferol, an AHR antagonist. A similar flavonol, galangin, also functions to antagonize AHR signaling[203]. Structure activity relationship studies of using these compounds may allow dissection of critical AHR-ligand interactions required to mediate agonist or antagonist activities by the human AHR. Additional plant-derived components such as the AHR agonist curcumin, found in the spice turmeric, and the AHR antagonist resveratrol, found in grapes, present viable contributors to dietary AHR activity in their consumers[204, 205].

MICROBIOTA DERIVED AHR LIGANDS

The gastrointestinal tract is composed of a complex microbial ecosystem that contributes significantly to the first-pass metabolism of dietary constituents. The co-evolutionary commensalism between host and microbes may contribute to the catabolism of dietary or endogenous molecules to AHR ligands **(Figure 1.7)**. Interkingdom signaling pathways may have evolved to promote the maintenance of intestinal health through the sensing of bacterial metabolites at the epithelial interface. In contrast, the excessive or limited production of AHR ligands could play a role in the etiology of various intestinal disease states. This hypothesis is further supported by identification of pigmented virulence factors pyocyanin, 1-hydroxyphenazine, and phthiocol, derived from pathogenic *P. aeruginosa* and *M. tuberculosis*, as AHR ligands[206]. Interestingly, the AHR-dependent responsiveness to these molecules promoted increased expression of cytokine/chemokine signaling that resulted in enhanced bacterial clearance within respiratory infection models[206]. Microflora mediated activation of the AHR has been observed in murine models following oral gavage of heat killed *Lactobacillus bulgaricus* OLL1811, which was found to attenuate dextran sodium sulfate induced colitis[207]. The metabolite responsible for the observed therapeutic activity remains to firmly established. Screening of chemical libraries derived from

probiotic bacterial strains led to the identification of the AHR agonist, 1,4-dihydroxy-2,-napthoic acid (DHNA)[208]. *In vivo* administration of DHNA was associated with increased expression of anti-microbial peptides and protection in models of chemically induced colitis[208]. This concept is of further interest in the field of prebiotics and probiotics, where elucidation of the mechanisms facilitating their beneficial effects remains to be fully explored.

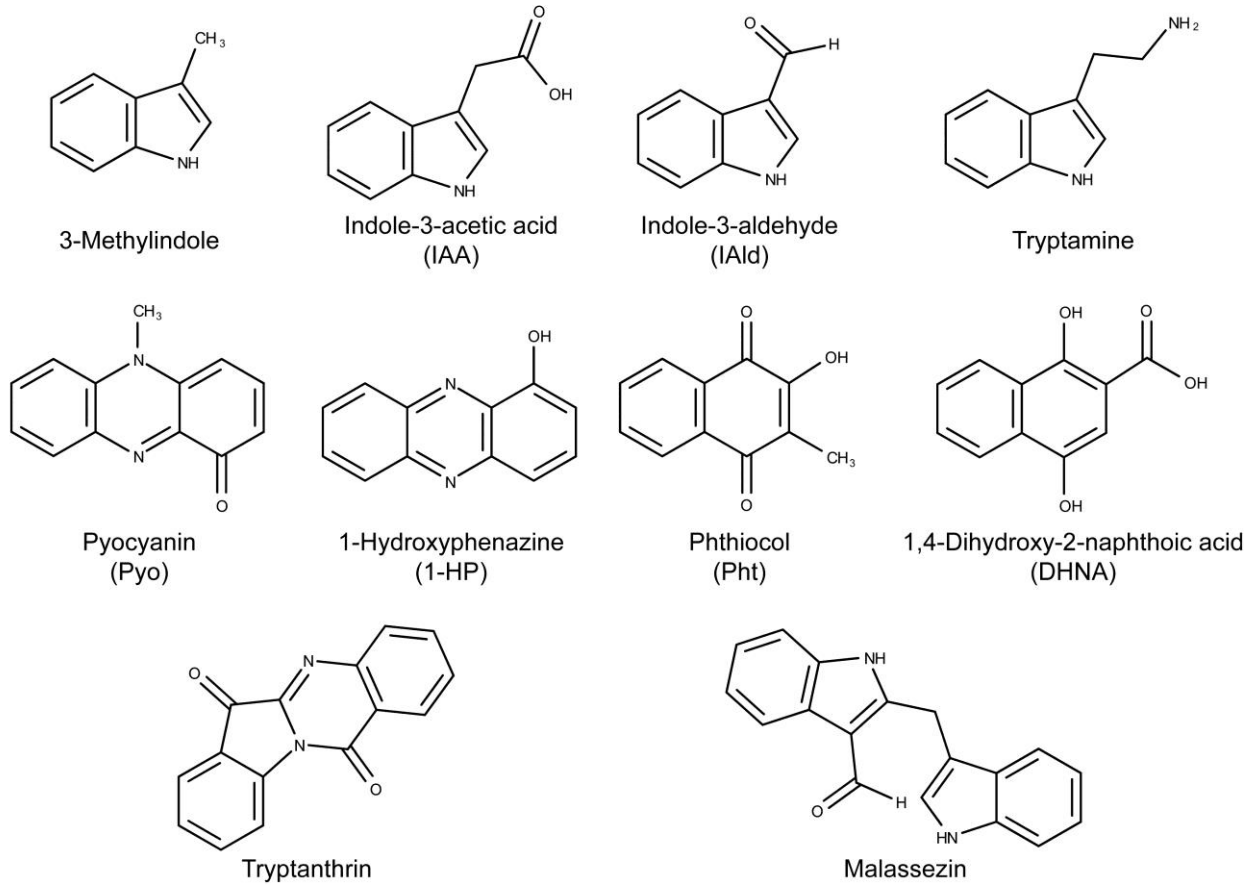


Figure 1.7: Microbiota derived AHR ligand structures.

Microbial metabolism of the amino acid tryptophan is recognized as a source for the generation of a number of AHR ligands **(Figure 1.8)**. Tryptophan is an essential amino acid, and can only be acquired through the diet due to its inability to be synthesized *de novo*. Resident microbes, such as *E.coli* may utilize tryptophan as a source of nitrogen and contain up to three distinct permeases responsible for its transport, *Mtr*, *AroP*, and *TnaB*[209]. Tryptophan can be converted to mono-substituted indole compounds, such as indole acetic acid (IAA) and tryptamine, which activate the AHR in a yeast DRE-dependent reporter assay and to stimulate target gene expression within human colon-carcinoma (Caco2) cell line[210, 211]. The EC_{50} of IAA and tryptamine were determined to be 0.5 mM and 0.2 mM respectively, using a [^{3}H] TCDD competition assay, indicating that they bind to the ligand binding domain with low affinity[212]. The monoamine alkaloid, tryptamine, is derived from the direct decarboxylation of tryptophan. Tryptophan is converted to IAA via the enzymes tryptophan monooxygenase (*IaaM*) and indole-3-acetamide hydrolase (*IaaH*) that constitute the indole-3-acetamide (IAM) pathway[213]. The common fecal metabolite 3-methyl indole (skatole), is generated by decarboxylation of IAA and has been shown to facilitate transcription of AHR target genes in human bronchial and colonic epithelial cells[214, 215].The physiological implications of AHR activation by tryptamine, IAA, or skatole remain to be established.

The catabolism of tryptophan by commensal lactobacilli has been shown to hinder colonization of the intestinal tract by pathogenic *Candida albicans* through AHR dependent expression of *IL22*[216]. Targeted metabolomic analysis of *L. reuteri* and *L. johnsonii* tryptophan catabolism identified the indole derivative indole-3-aldehyde (Iald), which is generated through the indole pyruvate pathway, catalyzed by aromatic amino acid aminotransferase. *In vivo* treatments with Iald were found to activate the AHR and to promote intestinal homeostasis through induction of *IL22*[216]. The manipulation of pathways that promote Iald production has

demonstrated therapeutic promise; although more evidence is needed to address IL22 mediated activity in human models. It will be important to quantitatively assess the level of AHR activation required to mediate its healthful benefits upon intestinal homeostasis. Establishing a threshold of AHR activity will help to limit detrimental over-stimulation of AHR signaling and off target effects of administered ligands. This can be followed by microbial reconstitution studies by lactobacillus species in order to assess the level of colonization required to produce adequate amounts of AHR ligand to stimulate beneficial intestinal signaling.

The lack of intestinal homeostasis has been shown to be a contributing factor to the pathology of many disorders, including inflammatory bowel disease, Crohn's disease, ulcerative colitis, diabetes, obesity and cancer. Maintenance of intestinal homeostasis solely by dietary AHR ligands would be transient and vary greatly by the types of ligands consumed, their respective bioavailability and half-lives. Synthesis of putative AHR ligands by the intestinal commensal microflora may provide a more consistent source of *in vivo* AHR stimulation. Such AHR stimulation arising from microbial activity may represent a mechanism by which the AHR acts as a pseudo-pattern recognition receptor of the microbiota community and through its now established role as a modulator of immune function maintain host-microbe homeostasis. The physiological implications of microbial AHR ligands, the species that produce them and their effects upon intestinal disease require further investigation.

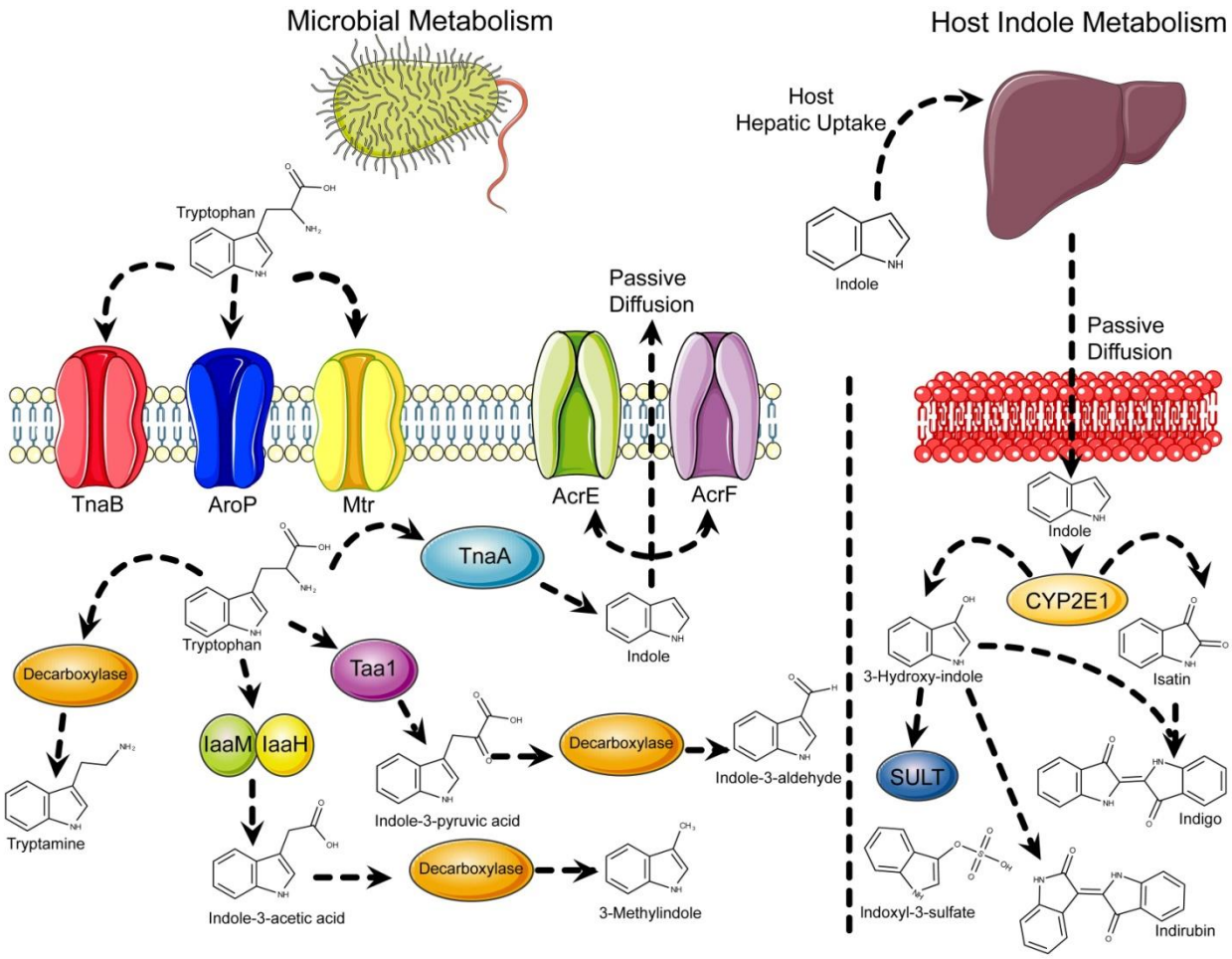


Figure 1.8: Microbial synthesis of AHR ligands and host indole metabolism.

HOST BIOACTIVATION OF INDOLE

Bacterial metabolism of tryptophan by the tryptophanase pathway has been shown to generate indole. Indole is shuttled in and out of bacteria by passive diffusion or active transport by AcrEF-TolC and Mtr transporters[209, 217, 218]. Interspecies signaling by indole regulates many physiological functions, such as biofilm formation, motility, plasmid stability, virulence and antibiotic resistance in neighboring microflora[219-221]. Metabolic analysis of human fecal samples have shown indole to be found at elevated concentrations of indole (250-1100 μM)[222, 223]. Such elevated *in vivo* concentrations of the metabolite suggest it may facilitate a mutually beneficial relationship with the host. In fact, human intestinal epithelial cells (HCT-8) were shown to increase epithelial barrier function and attenuate inflammatory markers upon treatment with indole[224].

Prevailing evidence suggests that the bioavailability of indole is dependent upon microbial metabolic activity[225]. Absorption of indole by the host occurs via passive diffusion through the colonic epithelia. Hepatic uptake and metabolism of indole is an additional source for the generation of potent AHR ligands **(Figure 1.8)**. For example, phase I metabolism of indole by cytochrome P450 2E1 (Cyp2e1) in rat liver microsomes catalyzes the hydroxylation of indole to form 3-hydroxy-indole (indoxyl) and isatin. This activity has been confirmed using yeast models of recombinant human CYP450 enzyme expression[226, 227]. *In vivo* generation of the indigoids, indigo and indirubin, have been proposed to occur through radical redox mechanisms involving the unstable intermediates, indoxyl and isatin[227]. Indigo and indirubin have been identified within human urine and found to be potent activators of the AHR[228-230]. Indirubin was found to be a more potent human AHR agonist when compared to the mouse AHR [231]. Furthermore, in a yeast DRE-driven β-galactosidase reporter expressing the human AHR indirubin was found to possess an EC_{50} of ~0.2 nM, 45-fold less than

TCDD[228]. Whether significant quantities of indirubin or indigo are synthesized in the liver capable of activating the AHR has not been established.

Hepatic phase II drug metabolism of indoxyl via sulfation by sulfotransferases generates the uremic toxin indoxyl-3-sulfate (I3S). I3S is recognized as a human AHR agonist with 500-fold greater potency in human versus murine hepatoma cell lines[232]. Increased serum concentrations of I3S are correlated with vascular dysfunction in chronic kidney disease (CKD) patients[233]. Assessment of serum from dialysis patients indicates an accumulation of I3S to high micro-molar concentrations. Failed excretion and subsequent accumulation of I3S may contribute to the overt toxicity seen in dialysis patients and has been shown to contribute to increased risk of tumor growth[234]. Antagonism of the AHR or targeted inhibition of I3S synthesis may have a beneficial therapeutic effect in CKD patients[235]. Oxidative stress from the accumulation of I3S has also been shown to increase the risk of cardiovascular disease in CDK patients through oxidative modification of low density lipoproteins[236].

The *in vitro* oxidation of low density lipoproteins by indoxyl radicals has been shown to generate the AHR activator tryptanthrin[237]. Tryptophan metabolism by the *Candida lipolytica* and *Malassezia* yeast species can also generate the potent AHR ligands such as tryptanthrin and malassezin[238, 239]. Furthermore, the production of AHR agonists within the epidermis by *Malassezia* yeasts have been shown to contribute to seborrhoeic dermatitis disease progression[240]. However, production of tryptanthrin appears to be localized to the skin, limiting its *in vivo* relevance as a systemic endogenous regulator.

The metabolic profiling of tissue extracts has led to the identification of endogenous AHR ligands that contain indolyl moieties. Organic extractions of porcine lung tissue led to the discovery of the AHR ligand 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE),

which was shown to be a direct high affinity ligand for human, mouse and fish receptors[241]. However, the presence of ITE *in vivo* has not been established and thus it is possible that the generation ITE may be an artifact of the extraction process in which the porcine lung methanol extract was heated to high temperatures. Nevertheless, ITE was found to inhibit the onset of symptoms in mouse models of experimental autoimmune uveitis through AHR-mediated T_{Reg} cell differentiation[242]. Recently, ITE was found to enhance differentiation of human T_{Reg} cells and be protective against chemically induced colitis within humanized AHR mouse models[243]. ITE has also been shown to possess AHR independent biological activity, such as the inhibition of TGFβ1 mediated myofibroblast differentiation, which prevents fibrosis within human tissue[244]. The AHR ligand ITE has many biological activities; however its classification as an "endogenously" derived compound remains to be established.

HOST TRYPTOPHAN METABOLISM GENERATES AHR LIGANDS

Enzymatic reactions that account for greater than 90% of the peripheral metabolism of tryptophan in mammals are found in the kynurenine pathway[245]. The kynurenine pathway is composed of multiple enzymatic steps that facilitate the metabolism of tryptophan to nicotinamide adenine dinucleotide and multiple intermediate byproducts, some of which have been characterized as activators of the AHR **(Figure 1.9)**. The kynurenine pathway plays a pivotal role in cancer-immunity through signaling cascades that promote immune suppression or tolerance. The depletion of tryptophan and its subsequent bioactivation to AHR ligands have been shown to induce $CD4^+$ T-helper cell differentiation to favor an anti-inflammatory T_{Reg} rather than a T_{H17} phenotype[125, 246]. This attenuation of adaptive immunity responsiveness functions to hinder immune surveillance within the tumor microenvironment. As such, increased expression of the enzymes in this pathway have been observed within tumors as evidenced by a

increase in the kynurenine:tryptophan ratio, and has been found to correlate with cancer aggressiveness [247, 248].

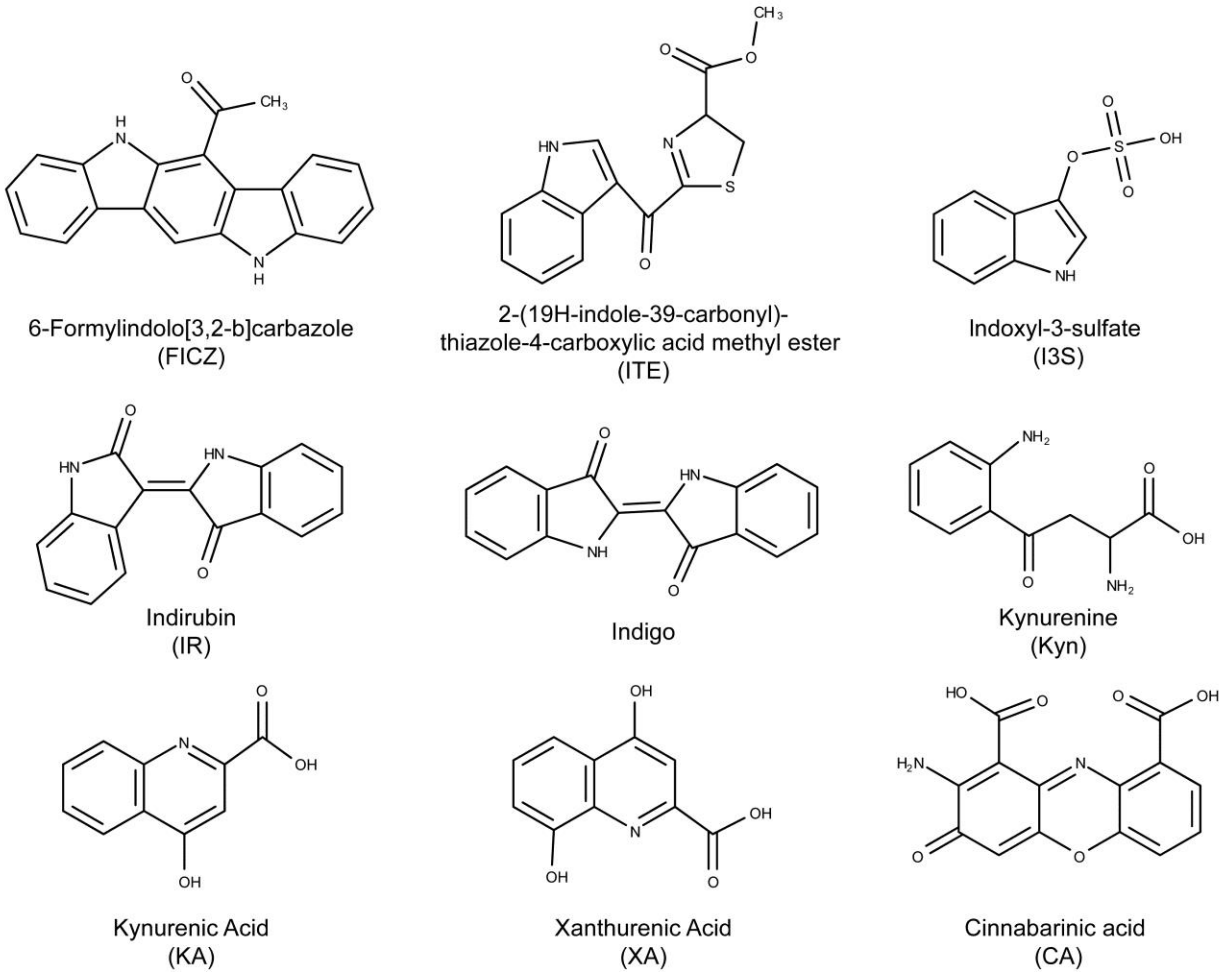


Figure 1.9: Endogenously-derived AHR ligand structures.

The kynurenine pathway is initiated by host metabolism of tryptophan via indoleamine-2,3-dioxygenase (IDO1) and Tryptophan-2,3-dioxygenase (TDO2). Both enzymes perform the analogous catabolic conversion of tryptophan to kynurenine, but are structurally divergent and exhibit distinct expression patterns *in vivo*[249]. Type I interferon signaling has been shown to regulate *IDO1* expression via activation of a JAK/STAT signaling in cell types of epithelial or monocytic lineage[250, 251]. *IDO1* induction was also found to be dependent upon AHR transcriptional regulation within bone-marrow derived dendritic cells, indicating a autocrine signaling cascade that would significantly deplete local tryptophan and increase the bio-availability of AHR agonists[251]. More recent studies have identified TDO2 enzymatic activity as an additional mechanism of tryptophan depletion occurring within hepatocytes and neurons. The upregulated expression of *TDO2* in glioblastoma tissues has displayed a positive correlation with the induction of the AHR target gene *CYP1B1*, which supports the hypothesis of AHR ligand generation within the kynurenine pathway[252]. The metabolism of tryptophan by IDO/TDO in glioma cell lines yields elevated levels of kynurenine, a low affinity ligand for the AHR[252, 253]. Further metabolism of kynurenine can yield more potent AHR ligands that may have an increased impact upon immune regulation and tumor malignancy **(Figure 1.10)**. The metabolite kynurenic acid, generated by the irreversible transamination of kynurenine by kynurenine aminotransferases (KATs), was found to be a relatively potent endogenous ligand for the human AHR[254,255]. KA can be detected in serum at a concentration of ~5 μM in some CKD patients[256]. KA at a physiologically relevant dose of 100 nM mediated activation of the AHR and was found to induce prototypical AHR target genes and synergistically increase the expression of *IL6* in the presence of inflammatory signaling[255]. This investigation also found that the kynurenine metabolite, xanthurenic acid, displayed agonist potential in an AHR dependent luciferase

reporter in human hepatoma cells. Xanthurenic acid is generated by transamination of the kynurenine metabolite, 3-hydroxykynurenine. Recently, the kynurenine metabolite cinnabarinic acid (CA) has been identified as an additional endogenous AHR agonist that mediates AHR dependent transcription of *IL22* within human and murine $CD4^+$ T cells[257]. CA is generated by a condensation of 3-hydroxyanthranilic acid, which is produced by kynureninase from 3-hydroxykynurenine. Recently, CA has also been shown to play a cytoprotective role in endoplasmic reticulum or oxidative stress induced apoptosis through AHR dependent induction of stanniocalcin-2[258]. Further investigation of the kynurenine pathway may lead to the identification of additional endogenous AHR ligands.

The identity of the metabolite(s) primarily responsible for AHR activation within tumor tissue has not yet been confirmed. Increased levels of AHR activity in tumors, characterized by increased *CYP1B1* mRNA expression, have been shown to be a predictive marker of poor survival in glioma patients[252]. Studies examining metabolite concentrations within pancreatic adenocarcinomas identified a positive correlation between kynurenic acid levels and tumor size[259]. Such evidence supports the notion that AHR stimulation may increase tumor aggressiveness through metabolites of the kynurenine pathway. Targeted antagonism of the AHR or the kynurenine pathway enzymes responsible for synthesis of higher affinity ligands may be of therapeutic interest as a supplement to traditional chemotherapy. In support of this notion, pharmaceutical inhibition of IDO function is currently in the initial stages of clinical trials.

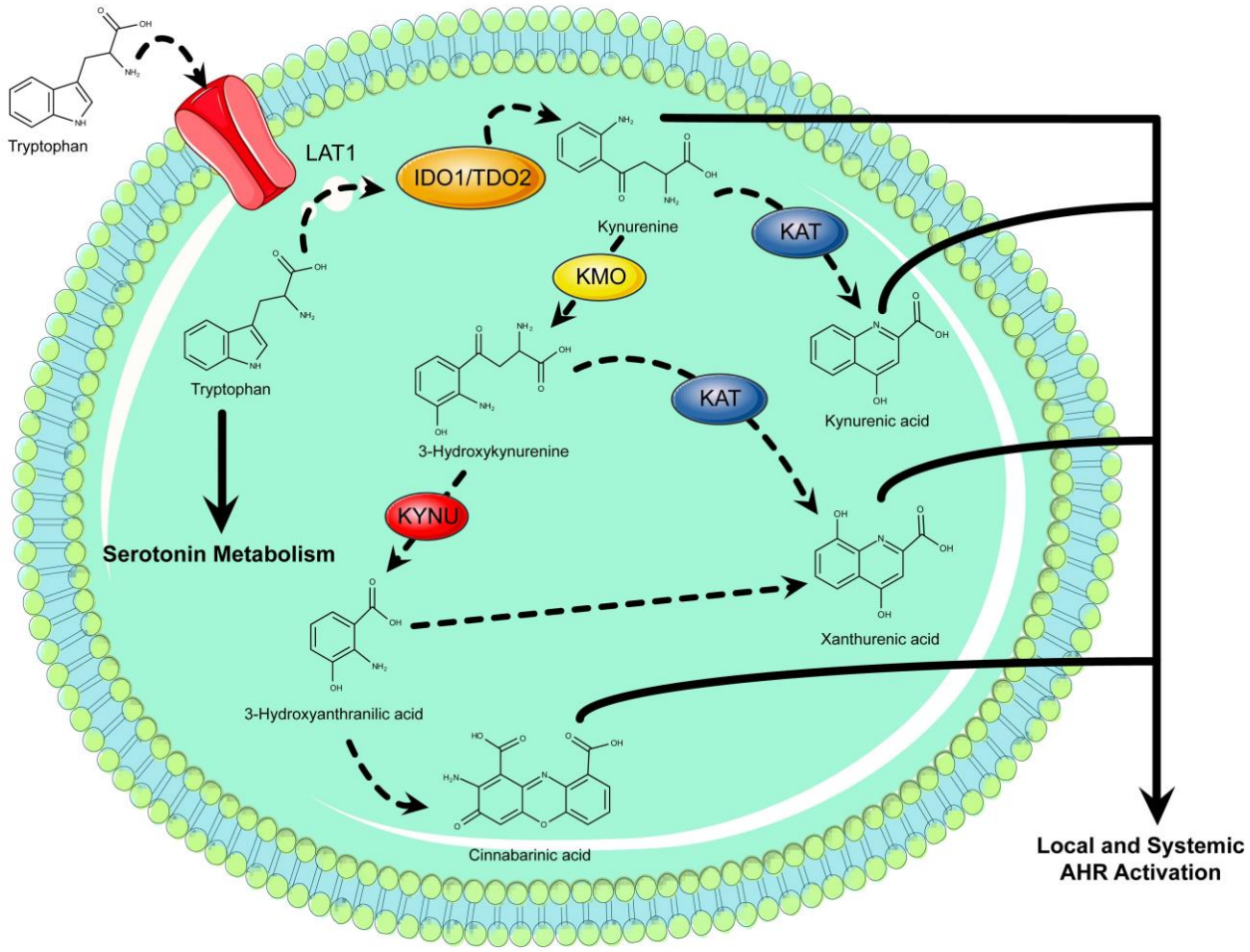


Figure 1.10: Endogenous tryptophan metabolism via the kynurenine pathway generates AHR ligands.

UV-CATALZYED GENERATION OF AHR LIGANDS

Studies have found that exposure of culture media to ultraviolet (UV) light leads to increased AHR activation and induction of cytochrome P450 target genes within cultured cells[260]. The photo-oxidation of tryptophan generates FICZ[261]. FICZ has been established as a potent AHR ligand with an affinity similar to that of TCDD, as demonstrated by competitive ligand binding assays[262]. Treatment of FICZ within a murine autoimmune encephalitis model displayed an increase in the severity of symptoms, contradictory to what is observed with TCDD[125, 246]. Further investigation found that FICZ induced differentiation of T_{H17} cells and expression of proinflammatory *Il17* rather than anti-inflammatory T_{Reg} cells. This unique physiological activity of FICZ suggests that it may exhibit selective AHR activity relative to other AHR ligands. However, FICZ dependent modulation of immune signaling has been found to greatly depend upon the route of administration within *in vivo* models.

The endogenous relevance of FICZ remains to be firmly established. Epidermal UV-irradiation of both human skin or keratinocyte cultures was found to increase AHR target monooxygenases, but this apparently did not depend on FICZ formation[263, 264]. The metabolic analysis of human urine indicated the presence of multiple FICZ conjugates, suggesting it may be an endogenous high affinity regulator of the AHR[265]. However, confirmation of sub-dermal synthesis of FICZ has not yet occurred. Studies using murine hepatoma cells indicate a negative feedback loop in which FICZ induces *Cyp1a1* expression that in turn leads to its rapid degradation[266]. Thus, limited *in vivo* synthesis and rapid degradation make FICZ unlikely to be a significant contributor to constitutive AHR activity within the context of an entire organism. However, photo-oxidation of tryptophan to FICZ within cell culture media should be considered in studies hoping to evaluate ligand-independent functions of AHR. Nevertheless, FICZ may

have significant implications within the context of AHR activity of the skin, where constant UV-exposure may generate sufficient quantities of the ligand to activate the AHR. Clinical trials assessing pharmacological intervention in patients with lower limb ulcers indicated topical tryptophan mediated significantly enhanced re-epithelialization and decreased pain relative to control groups[267]. However, further studies are needed to assess whether production of AHR agonists were required for this effect and if so, are ligands produced endogenously, by resident skin microbes, or radically by UV-oxidation mediating this beneficial effect.

1.8 THE AHR LIGAND BINDING DOMAIN

The AHR activation by various classes of structurally distinct ligands indicates the presence of a promiscuous LBD. Ligand-binding capacity of the AHR is dependent upon structural confirmation of the PAS-B domain, which can be significantly affected by its interaction with HSP90. Deletion of the LBD or disruption of HSP90-dependent cytoplasmic complex stability mediates constitutive activation of the AHR, similar to that following exposure to a potent agonist, such as dioxin. Knowledge of molecular mechanisms that dictate AHR-ligand binding and resultant activities remain to be established due to the lack of an X-ray crystal structure. Current homology models of AHR-ligand docking within the LBD are derived from NMR spectrometric analyses of the PAS-B domain of human HIF-2α[268]. Homology modeling of AHR binding of TCDD, in combination with AHR mutagenic studies, identifies multiple structural and chemical requirements for ligand binding. These combinatorial analyses suggest that residues A375 and I319 are necessary for molecular recognition of TCDD, residues F289, Y316, and F345 stabilize interactions with aromatic regions, and polar residues T283 and H285 mediate electrostatic stabilization with polar regions of TCDD[269]. Indeed, *in silico* modeling can be used as a predictive assessment for identification of novel AHR ligands, but these models are

often considered incomplete due to their inability to identify ligands that possess an atypical structure or size. These LBD models are also considered incomplete due to their inability to account for HSP90-AHR interactions that may affect the accessibility or size of the ligand-binding cavity. Analysis of AHR ligand-binding kinetics suggest that binding of potent "irreversible" ligands, such as TCDD, may disrupt AHR-HSP90 interactions, leading to the collapse of the ligand-binding pocket[270, 271]. However, studies suggest that binding-kinetics of AHR with structurally diverse ligands may occur through distinct mechanisms. This hypothesis is supported by the observation that the AHR antagonist CH223191 preferentially competes for AHR occupancy with HAHs, but is unable to block binding of PAHs, flavonoids, or indirubin[272]. Further mutagenesis studies identify the residues within the AHR LBD that are vital in selective responsiveness to various classes of ligands.

1.9 INTERSPECIES DIFFERENCES IN AHR LIGAND SELECTIVITY AND TRANSCRIPTIONAL ACTIVITY

The evolutionary conservation of AHR homologs in invertebrates such as *Caenorhabditis elegans* to vertebrates, including *Homo sapiens,* indicates that the ancestral gene arose over 500 million years ago[273]. Invertebrate AHR homologs are incapable of binding to TCDD, suggesting that activity may be modulated by unknown endogenous ligands or constitutive activity that is regulated by context-specific AHR expression. Interspecies comparison of mammalian AHR constructs has revealed wide variances in TCDD sensitivity between different organisms. Identification of amino acid differences between AHR homologs that dictate differential ligand sensitivity between species is an expanding area of research. These structure activity relationship studies were used to identify the determinant for reduced ligand sensitivity between $Ahr^{b/b}$ and $Ahr^{d/d}$ mice as a valine substitution at position 375[15]. The human AHR and mouse $Ahr^{b/b}$ share

85% sequence homology within their respective N-terminal regions, but demonstrate differential ligand-binding preferences. The human AHR possesses a corresponding valine substitution at position 381 similar to *Ahr*$^{d/d}$ mice, which mediate an approximately 10-fold decreased affinity for TCDD. However, the human AHR readily binds indirubin, indoxyl sulfate, and kynurenic acid with increased potency relative to mouse homologs[232, 255, 274]. Interestingly, the guinea pig AHR is found to be highly sensitive to TCDD exposure relative to other mammalian homologs, but shares increased sequence homology with the human AHR[275].

Interspecies variations in AHR mediated signaling and toxicity is not just a result of differential ligand binding kinetics, but additional parameters such as discrepancies in transactivation potential. Both vertebrate and invertebrate AHR homologs are able to interact with ARNT and bind consensus DREs within promoters of target genes[276]. While the regulation of monooxygenase expression by the AHR appears to be conserved, many differences in target gene expression occur following exposure to ligand. Heterogeneity in the hepatic transcriptomic response was observed between mice and rats following TCDD exposure using high throughput microarray platforms[277]. Notably, mouse and rat AHR exhibit similar affinity for TCDD, but vary in the composition of their transactivation domain (TAD). Alterations in the TAD can have profound effects upon promoter occupancy, recruitment of coactivators/co-repressors, and assembly of the transcriptional complex. However, transcriptional differences may be due to lack of conservation of DRE localizations within promoters of target genes. Within our lab a different model utilizing hepatocytes from *Ahr*$^{b/b}$ and transgenic humanized AHR mice was utilized to assess variances in TCDD mediated transcription[274]. This elegant model eliminates differences in DRE promoter occupancy and allows careful dissection of structure dependent differences in AHR-mediated transcription. The number of differentially expressed genes varied between AHR

homologs, 2852 genes in murine hepatocytes and 1965 genes in murine hepatocytes with functional human AHR. Pathway analyses identified a greater number of genes involved in metabolism and transport regulated by mouse AHR, whereas the human AHR targeted genes involved in proliferation and immune function. Such data indicates that a compendium of AHR-dependent targets exhibit a large degree of overlap, however, species-specific targets do exist and are likely a consequence of inherent differences in AHR structure.

1.10 OVERVIEW AND SIGNIFIGANCE

In depth critical analyses of AHR literature identified multiple ongoing trends in AHR research. The role of the AHR in the mediation of TCDD toxicity was paramount to its identification. However, current research has shifted away from xenobiotic toxicity and refocused on the physiological implications of the AHR in various aspects of normative mammalian biology. As such, in the absence of exogenous chemical exposure, understanding of endogenous routes of AHR activation is critical to understanding basal signaling pathways that affect homeostatic AHR signaling. Due to structural differences between the human and mouse AHR it is imperative that human-specific ligands be identified, as they may be overlooked in compound library screenings that utilize non-human cell lines.

The biomedical implications of microbiome signaling are increasingly being identified as significant contributors to normal or pathogenic phenotypes within the host. Interkingdom signaling between resident microbes and the host likely contribute to maintenance of intestinal homeostasis and therefore constitute a vital aspect of a host-microbe commensalism. To this point many studies have identified microbial derived AHR ligands. However, few of these studies have identified whether such ligands are able to modulate human AHR activity or if these molecules can mediate AHR activation at concentrations that are relevant *in vivo*. The work

presented in chapter 2 characterizes the identification of the microbial tryptophan metabolite, indole, as a human specific AHR agonist. Homology modeling of AHR-indole ligand docking simulations suggests the possibility that the human AHR is permissive to simultaneous binding of two indole molecules.

Comparative studies have demonstrated that the human AHR exhibits increased binding capacity with regard to endogenous ligands derived from tryptophan and indole metabolism and decreased responsiveness to prototypical PAH agonists when compared to rodent homologues. Such differences suggest selective adaptations may have arisen during the course of mammalian evolution. However, due to limitations in the availability of genomic sequence data from archaic hominin species, the origin of reduced AHR PAH-sensitivity and its biological implications remain to be established. The work presented in chapter 3 presents evidence supporting the hypothesis that this selective desensitization of the human AHR to PAHs arose during or after speciation of *Homo sapiens* and discuss its implications with regard to Paleolithic fire use and resultant exposure to toxic constituents within smoke by *H. sapiens* and *Homo neandethalensis*.

Taken together my research broadens the current understanding of human AHR-ligand interactions to allow for enhanced therapeutic targeting of the receptor and generation of better mouse models to study the implications of the human AHR in disease models. Characterization of human AHR differential ligand responsiveness has identified an evolutionarily selected trait unique to the human lineage.

CHAPTER 2 : UNIQUE BINDING CHARACTERISTICS AND ADAPTATION OF THE HUMAN ARYL HYDROCARBON RECEPTOR TO SENSE MICROBIOTA-DERIVED INDOLES

2.1 ABSTRACT

Tryptophan is an essential amino acid, incapable of being synthesized *de novo* by humans. Healthy tryptophan levels are primarily maintained by consumption of various foods such as red meat, fish, and eggs. Tryptophan is required for protein translation and synthesis of numerous biological molecules such as hormones, neurotransmitters, pigments, and antioxidants. Resident intestinal microbes catabolize tryptophan to indole, an aromatic hetero-bicyclic compound, recently shown to promote enhanced epithelial barrier function within human intestinal cells. The recognition of indole by host cells may promote a mutualistic relationship through interkingdom signaling pathways. However, a mechanism for host indole recognition has not yet been determined. Here, we present experimental evidence in support of indole as a species-specific ligand that selectively binds and activates the human AHR. *In silico* modeling data suggests that the human AHR ligand binding cavity had adapted to optimally interact with indoleic compounds relative to mouse AHR and supports a novel bi-molecular binding mechanism. In this model two indole molecules are able to interact with a single human AHR protein. Our findings suggest that responsiveness to indole is conserved in the AHR of higher order primates, such as the chimpanzee. Such results support the notion that the human AHR has adapted during the course of mammalian evolution to act as a pseudo-microbe associated molecular pattern receptor, which may promote AHR-dependent maintenance of intestinal homeostasis.

2.2 INTRODUCTION

Indole is an aromatic heterocyclic compound composed of benzene conjugated to a pyrrole ring. This moiety is found abundantly in nature as the side chain that defines the hydrophobic amino acid, tryptophan. However, indoleic moieties serve as a structural base or functional group within numerous biological molecules with critical functions in plants, animals, and microbes. The indole metabolite auxin is plant signaling hormone required for normal establishment of growth polarity[278]. Within mammalian species, tryptophan metabolism yields indole-containing neurotransmitters such as the indoleamines, tryptamine, melatonin, and serotonin. Bacterial synthesis of indole is mediated by the enzymes present within the *Tna* operon, specifically the catabolic function of tryptophanase (TnaA) facilitates the conversion of L-tryptophan to indole[279-281]. In bacteria, indole and indolyl compounds including, isatin and various hydroxy-indole derivatives, function as intra- and inter-species signaling molecules across bacterial populations, where they are involved in biofilm formation, bacterial motility, plasmid stability, virulence and antibiotic resistance[221, 282-284].

The microbiome is a dynamic ecosystem composed of nearly 100 trillion diverse microbiota, containing between five hundred to one thousand different species, colonizing the human intestinal tract[285]. Through large scale collaborations, like the Human Microbiome Project, we have only begun to scratch surface of understanding these microbial communities, their composition, and how they may be of benefit or detriment to their host. The intestinal tract contains a somatic cell to bacterial ratio of approximately one:ten, with the comprehensive bacterial genome containing a compendium of genes 100 fold greater in number than entire human genome[285, 286]. The composition of the intestinal microbiome is not static, but constantly varying due to fluctuations in diet, anatomical location, and host genetics. Such discrepancies in

bacterial populations, transcriptional activity, and metabolism can influence the host in a multitude of capacities such as immune system development, displacement of pathogenic organisms, and energy utilization through metabolism of additional substrates[287-289].Therefore some would consider the intestinal microbiome as an additional vital organ of the human body and the metagenome an extension of the human genome[143].

Within the gastrointestinal tract there exist greater than 10^{12} enteric bacteria with the capacity to produce an array of bioactive molecules, such as indole. Indole is present at high micro-molar concentrations within the intestinal lumen and feces[222, 283]. Continual *in situ* generation of indole would mediate elevated exposure upon host intestinal epithelial cells. Recent evidence has demonstrated that indole treatment of human intestinal epithelial cells mediates modulation of inflammatory gene expression and the maintenance of epithelial barrier integrity[290-292]. The mechanism(s) by which intestinal epithelial cells are able to respond to bacterially generated has not yet been established.

Notably, the AHR is receptive to a number of indolyl metabolites including indoxyl-3-sulfate, FICZ, kynurenine, kynurenic acid, tryptamine and indole-3-acetate, thus implicating the AHR as a candidate indole receptor[210, 211, 260, 293-295]. The human AHR is acutely responsive to compounds containing indole moieties[231]. Additionally, recent studies have established the AHR as a critical component in the establishment/maintenance of intestinal homeostasis through promotion of epithelial barrier integrity, regulation of commensal microbial community composition, and protection from pathogenic insults[222, 296-300]. These observations support the notion that the AHR is a necessary component for maintenance of gastrointestinal health. Bacterially derived indolyl compounds represent an expanding class of endogenously produced AHR ligands. This prompted us to examine investigate indole as a putative ligand for the AHR.

Our findings demonstrate that indole exhibits species-specific AHR agonist activity, with regard to human, but not mouse AHR. *In silico* modeling of species-specific AHR-ligand docking simulations indicate that interspecies variances in indole responsiveness could be the consequence of a unique bimolecular (2:1) stoichiometry between indole and the ligand binding domain of human AHR. Furthermore, responsiveness to indole was found to be retained by chimpanzee AHR suggesting that this functionality has been conserved during the course of primate evolution. While the precise benefits of indole sensitivity are unknown, it is plausible that AHR binding of indole may represent an evolutionary adaptation within primates that established the AHR as a host sensor of the enteric bacteria. This host AHR/bacterial-indole axis may provide an additional link between the diet, gut microbiota, AHR, and gastrointestinal homeostasis.

2.3 MATERIALS AND METHODS

Animals: C57BL/6J, *AHR^{Ttr}Ahr$^{fx/fx}$Cre^{Alb}*, and Taconic© C57BL/6-*Ahrtm1.1(AHR)Arte* mice were housed on corncob bedding in a temperature- and light-controlled facility and given access to food and water *ad libitum*. Mice were maintained in a pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of the Pennsylvania State University. Adult (10–12 weeks) mice were used for macrophage isolation experiments.

Cell Culture: Hepa1, HepG2 and their respective AHR-reporter derivatives harboring the stably integrated pGudluc 1.1 were maintained in α-modified essential media (Sigma-Aldrich, St. Louis, MO) supplemented with 8% fetal bovine serum (Hyclone Laboratories, Logan,). The Caco-2 human colon carcinoma cell line were maintained in α-MEM with 20% fetal bovine serum. Primary peritoneal Mφ cells were maintained in DMEM (Gibco, Carlsbad, CA) supplemented with 10 % fetal bovine serum, 2 mM L-Glutamine, and 1 mM sodium pyruvate (Sigma, St. Louis, MO). Cells were cultured at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2 in the presence of 100 IU/ml penicillin/100 μg/ml streptomycin (Sigma-Aldrich).

Indole Recrystalization/Purification: Commercial indole was purified by recrystallization using chloroform/petroleum ether and the pure compound was characterized by ^{1}H and ^{13}C NMR. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.62 (s, 1H), 7.14-7.17 (t, 1H, *J* = 7.5 Hz), 7.20-7.26 (m, 2H), 7.40 (d, 1H, *J* = 8 Hz), 7.68 (d, 1H, *J* = 7.5 Hz), 8.09 (s, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 102.66, 111.04, 119.85, 120.75, 122.02, 124.13, 127.89, 135.81; mp = 52-54 ºC. Further, the purity was confirmed by reverse-phase HPLC (HPLC conditions: 4.6 mm × 25 cm vydac-C18

column eluted with a gradient from 30 to 100% CH_3OH in 30 min, the flow rate was 1 mL/min, and monitored at 254 nm, RT = 14.65 min. The purity level was > 99%.

Primary peritoneal macrophages isolation from mice: Mice (m*Ahr*^{b} and h*Ahr*) were injected with 3 ml of 3 % thioglycolate media intraperitoneally on day one. 72 h post thioglycolate injection, mice were euthanized. 1^0 Mφ were isolated by peritoneal lavage in ice-cold phosphate buffered saline (PBS). Cells were briefly spun and re-suspended in Mφ culture media for 4 h. After 4 h cells were briefly washed in warm PBS and incubated overnight in Mφ media[301]. Cells were treated the following day for 4 h.

PAL Ligand Competition assay: Characterization of competitive binding within the AHR ligand binding pocket between the AHR photo-affinity ligand, 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin and indole were completed as described previously[231]. To generate liver cytosol for ligand binding experiments, mouse livers were homogenized in buffer (25 mM MOPS, 2 mM EDTA, 0.02% NaN_3, and 10% glycerol, pH 7.4) containing 20 mM sodium molybdate and protease inhibitors (Sigma, St. Louis, MO) and centrifuged at 100,000*g* for 1 h. All binding experiments were conducted in a dark room until UV-mediated cross-linking of the PAL to the AHR LBD. In brief, a saturating amount of the PAL (0.21 pmol, 8×10^5 cpm per tube) was added to 150 μg of total protein of mouse liver cytosol from C57BL/6J, or humanized *AHR*^{Ttr}*Ahr*$^{fx/fx}$*Cre*^{Alb} along with increasing amounts of the competing ligands: Indole or 5,6-benzoflavone (β-naphthoflavone). Samples were then subjected to ligand binding conditions in which ligand-treated lysates were incubated at room temperature for 20 min and then photolyzed at 8 cm with 402 nm UV light. Dextran-coated charcoal (1%) was added to samples, which were then centrifuged at 3000*g* for 10 min to remove free ligand. Labeled samples were resolved using

8% acrylamide-tricine-SDS-PAGE, transferred to PVDF membrane, and visualized using autoradiography. Labeled AHR bands were excised and counted using a γ counter.

Luciferase Reporter Assays: The reporter cells (Hepa 1.1/Hep G2 40/6) were seeded in twelve-well plates and cultured to ~90% confluence. Cells were treated as indicated for 4 h then lysed in 400 µl of lysis buffer [25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% (v/v) glycerol, and 1% (v/v) Triton X-100]. Lysate (20 µl) was combined with 80 µl of Luciferase Reporter Substrate (Promega, Madison, WI), and luciferase activity was measured with a TD-20e luminometer (Turner Designs, Sunnyvale, CA).

Quantitative PCR Analysis: Total RNA was isolated from cells using TRI Reagent (Sigma-Aldrich), followed by reverse transcription using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD) was used to determine mRNA levels, and analysis was conducted using MyIQ software, in conjunction with a MyIQ-single-color PCR detection system (Bio-Rad Laboratories, Hercules, CA). All target genes were normalized to intra-sample *RPL13a*/*Rpl13a* levels.

Real time PCR Primers:

Human

CYP1A1-F-5'-TAC CTC AGC CAC CTC CAA GAT-3'

CYP1A1-R-5'-GAG GTC TTG AGG CCC TGA TT-3'

CYP1B1-F-5'-TGC CTG TCA CTA TTC CTC ATG CCA -3'

CYP1B1-R-5'-ATC AAA GTT CTC CGG GTT AGG CCA -3'

AHR-F-5'-GAA GAT GGT GAT GGG ATT TC-3'

AHR-R-5'-GAA GGT GAA GGT CGG AGT -3'

IL6-F-5'-AAA TTC GGT ACA TCC TCG ACG-3'

IL6-R-5'-AGT GCC TCT TTG CTG CTT TCA -3'

RPL13a-F-5'-CCT GGA GGA GAA GAG GAA AGA GA-3'

RPL13a-R-5'-GAG GAC CTC TGT GTA TTT GTC AA -3'

Mouse

Cyp1a1-F-5'-CTC TTC CCT GGA TGC CTT CAA-3'

Cyp1a1-R-5'-GGA TGT GGC CCT TCT CAA ATG -3'

Cyp1b1-F-5'- GCT AGC CAG CAG TGT GAT GAT ATT-3'

Cyp1b1-R-5'-GGT TAG CCT TGA AAT TGC ACT GAT -3'

Ahr-F-5'-GCG TCA GCT ACC TGA GGG CCA-3'

Ahr-R-5'-GGG CCA TGG GCT TCG TCC AC-3'

Rpl13a-F-5'-TTC GGC TGA AGC CTA CCA GAA AGT-3'

Rpl13a-R-5'-GCA TCT TGG CCT TTT TCC GTT-3'

ELISA: To quantify protein expression, media was collected from Caco-2 cells at 12 and 24 h post treatment and stored at -80°C. IL6 content of media samples was determined via LEGEND MAX™ Human IL6 ELISA kit with pre-coated plates (Biolegend, San Diego, Ca.) according to manufacturer's protocol.

CYP1A1 Activity Assay: Activity of CYP1A1 was determined using P450-Glo™ CYP1A1 Assay (Promega, Madison, WI). Caco-2 cells were cultured to ~90% confluence, then treated with ligand as indicated for 12h, followed by the addition of 5 μL (1:100 v/v) of Luc-CEE for an additional 3 h. This was followed by the addition of 150 μL of lysis buffer, chemiluminescence

was determined by mixing 50 μL of lysate plus 50 μL of luciferase reporter substrate and measured on TD-20e luminometer. The luciferase activity was normalized via protein content of the lysate as determined by BCA protein assay (Thermo Fisher Scientific, Waltham, MA).

AHR Nuclear Translocation Analysis: HepG2 and Hepa1 cells were treated with vehicle or indole (100 μM) were treated for two hours and then harvested using trypsin/EDTA and washed with PBS. Washed cells were homogenized in MENG (25 mM MOPS, 2 mM EDTA, 20 mM sodium molybdate, 10% Glycerol, pH 7.5) with 25 strokes in a Dura-Grind dounce tissue grinder (Wheaton instruments, Millville, NJ) on ice. The cytosolic supernatant was isolated following centrifugation at 17,000g for 30 min. The nuclear pellet was resuspended and washed three times in MENG with 50 mM NaCl. High salt nuclear extract was prepared by suspending nuclear pellet in MENG with 500 mM NaCl for 1 h on ice, followed by centrifugation at 100,000g for 1 h. Total protein in cytosolic and nuclear fractions were determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL). Protein samples were separated by SDS-PAGE on a 8.0% polyacrylamide tricine gel. Proteins were electrophoretically transferred to PVDF membrane (Immobilon P, Millipore, Bedford, MA). Transfer was done over 2 h at 26 v using transfer buffer (20 mM tris, 185 mM glycine, 20% methanol). Following transfer the membrane placed in blocking buffer (3% bovine serum albumin, 10 mM Na-phosphate, 150 mM NaCl, Tween-20 0.5%, pH 7.4) for 1 h at room temperature. Blots were incubated with αAHR mAb RPT-1 (0.5 μg/mL), B-actin(C4) mAb (Santa Cruz Biotechnology, Santa Cruz, CA) (1 μg/mL), Lamin A/C (H-110) rAb (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer for 1 h. Blots were rinsed with wash buffer (10 mM Na-Phosphate, 150 mM NaCl, 0.5% Tween 20, 0.1% BSA) as described previously[302]. Blots were then incubated with biotinylated goat anti-mouse or goat anti-rabbit IgG, followed by a 30 min incubation with 125I-labeled streptavidin in

was buffer, followed by three more 10 min incubations with wash buffer. Blots were visualized by autoradiography and quantified by was determined via Phosphoimager and OptiQuant software, and presented as digitized light units (DLU).

Electrophoretic Mobility Shift Assay: Gel retardation assays were performed using *in vitro* translated human AHR, mouse AHR and ARNT protein generated via TnT® Coupled Reticulocyte Lysate Systems according to manufacturer's protocol (Promega, Madison, WI). Human AHR *in vitro* translations were supplemented with sodium molybdate to a final concentration of 1.25 mM to enhance stability of the receptor. The AHR and ARNT proteins were combined at a 4 μL:4 μL, supplemented with 1.5 μL of HEDG buffer (25 mM HEPES, 1mM EDTA, 10mM sodium molybdate, and 10% glycerol), with the addition of indicated treatments (0.5 μL) and incubated at room temperature for 15 min. Following treatment incubation, $2x10^6$ cpm of ^{32}P labeled DRE oligonucleotides were added to each reaction and incubated at room temperature for an additional 15 min. Upon addition of 2 μL of 0.25% xylene cyanol in 20% (w/v) Ficoll to the reaction mixture, samples were loaded onto a 6% non-denaturing DNA retardation gel (Invitrogen, Carlsbad, CA) and separated by electrophoresis. The gels were fixed in a 7:1:1:1 water:methanol:acetic acid:glycerol solution for 20 min, dried for 60 min, and subjected to autoradiography for analysis.

<u>mCcl20_3.1kb DRE Oligonucleotide EMSA Sequence</u>

Ccl20-3.1kb DRE-F-5'-TTG TGT GTG TGC GTG TGT GCG TGT GTT AC-3'

Ccl20-3.1kb DRE-R-5'-TGT AAC ACA CGC ACA CAC GCA CAC ACA C-3'

In Silico Modeling: The homology model of mouse and human AhR-PASB-LBD based on the NMR *apo* of the HIF-2α-PASB (PDB 1P97) was prepared and optimized as recently described[303]. Indirubin was docked into the mouse and human optimized model. Then, the

complexes were submitted to $1.5x10^4$ steps MC ligand-protein side chain optimization to reach the most energetically favorable conformations. Molecular Docking was run as previously reported[304-307]. In the ICM-VLS (Molsoft ICM) screening procedure, the ligand scoring is optimized to obtain maximal separation between the binders and non-binders. Each compound is assigned a score according to its fit within the receptor; this ICM score accounts for continuum and discreet electrostatics, hydrophobicity and entropy parameter[308]. Surface energy of binding is based on atomic solvent-accessible surface using special water molecule probe radii designed for calculations of the solvation energy. This term based on "*atomic solvation*" is a product of atomic accessibilities by the atomic energy density parameters similar to those proposed in literature[309]. The non-hydrogen atomic accessible surfaces are calculated using a faster modification of the Shrake & Rupley algorithm[310]. The energy value calculated is the difference between the two molecules and the sum of the one molecule-free state (ΔH, kcal/mol).

Chimpanzee AHR cDNA synthesis: The codon-optimized chimpanzee AHR cDNA was synthesized by GenScript (Piscataway, NJ) and cloned into the HindIII and XhoI sites of the mammalian expression vector, pcDNA3.

Data Analysis: Data analysis was conducted using Prism 4 software, GraphPad Software Inc., (San Diego, CA). One Way ANOVA analysis was completed using Bonferroni post test. T-test parameters: Unpaired, Two-tailed. Parentheses surrounding letter denote the reference column, additional columns defined by a letter and asterisks indicate statistically significant differences between that column and the ascribed reference column. Data represent mean ± S.E.M. and are representative of three independent experiments p-value≤0.05 (*), p-value≤0.01 (**), p-value≤0.001 (***).

2.4 RESULTS

Human AHR is permissive for indole-mediated activation

To investigate the effect of indole **(Figure 2.1A)** upon AHR-mediated gene expression, human HepG2(40/6) cells stably transfected with the AHR responsive pGudluc6.1 luciferase reporter construct were incubated (4 h) with vehicle, 10 nM TCDD or increasing concentrations (1-100 μM) of re-crystallized indole as indicated (**Figure 2.1B**). Indole treatment resulted in a dose-dependent increase in luciferase reporter expression with an $EC_{50} \sim 3$ μM. A significant 2-fold induction over vehicle treated was observed at 1 μM and maximal 7-fold expression evident at 100 μM, the highest concentration examined and equivalent to the induction obtained with prototypical AHR agonist, TCDD at 10 nM. These data suggest that indole stimulates canonical DRE-dependent AHR-mediated gene expression in the context of human AHR.

The AHR is known to exhibit interspecies variability with regard to ligand sensitivity. We therefore examined the capacity of indole to influence AHR-mediated gene expression in mouse Hepa1.1 cells stably transfected with the AHR responsive pGudluc luciferase reporter construct. Hepa1.1 cells were incubated (4 h) with vehicle, 10 nM TCDD or increasing concentrations (1-100 μM) of indole as indicated (**Figure 2.1C**). Contrary to the human HepG2(40/6) cell line, exposure to indole resulted in a negligible dose-dependent increase in mouse hepatoma AHR-driven reporter activity. In contrast, mouse cells were highly sensitive to 10 nM TCDD, eliciting ~ 400-fold increase in reporter expression. Further examination of the interspecies indole sensitivity was assessed using the rat hepatoma H4-II-E1.1 luciferase reporter cell line. Indole treatments within the context of rat AHR-mediated gene expression were similar to mouse and therefore not sensitive to induction by indole (**Figure 2.1D**).

Contrary to these results, previous studies have established indole to act as an antagonist with regard to AHR-driven target gene expression[211]. In order to validate our observed agonist capacity of indole and eliminate the possibility of contamination of our indole source we re-examined the sensitivity of human HepG2(40/6) using re-crystallized, HPLC-purified and ^{1}H-NMR validated indole. AHR-mediated reporter expression in HepG2(40/6) cells exposed to 10 μM recrystalized or HPLC-purified indole were identical to that obtained in previous treatments, both inducing a significant 6-fold increase in luciferase reporter activity **(Figure 2.2A)**. However, no significant increase in human AHR-driven reporter activity was observed following treatment with commercially available indole **(Figure 2.2B)**. Such a result suggests that indole purity and possible inorganic contamination of commercial indole may account for observed discrepancies in the agonist potential of indole treatments. These initial observations suggest that human AHR is highly permissive to activation by indole, in direct contrast to rodent AHR homologs.

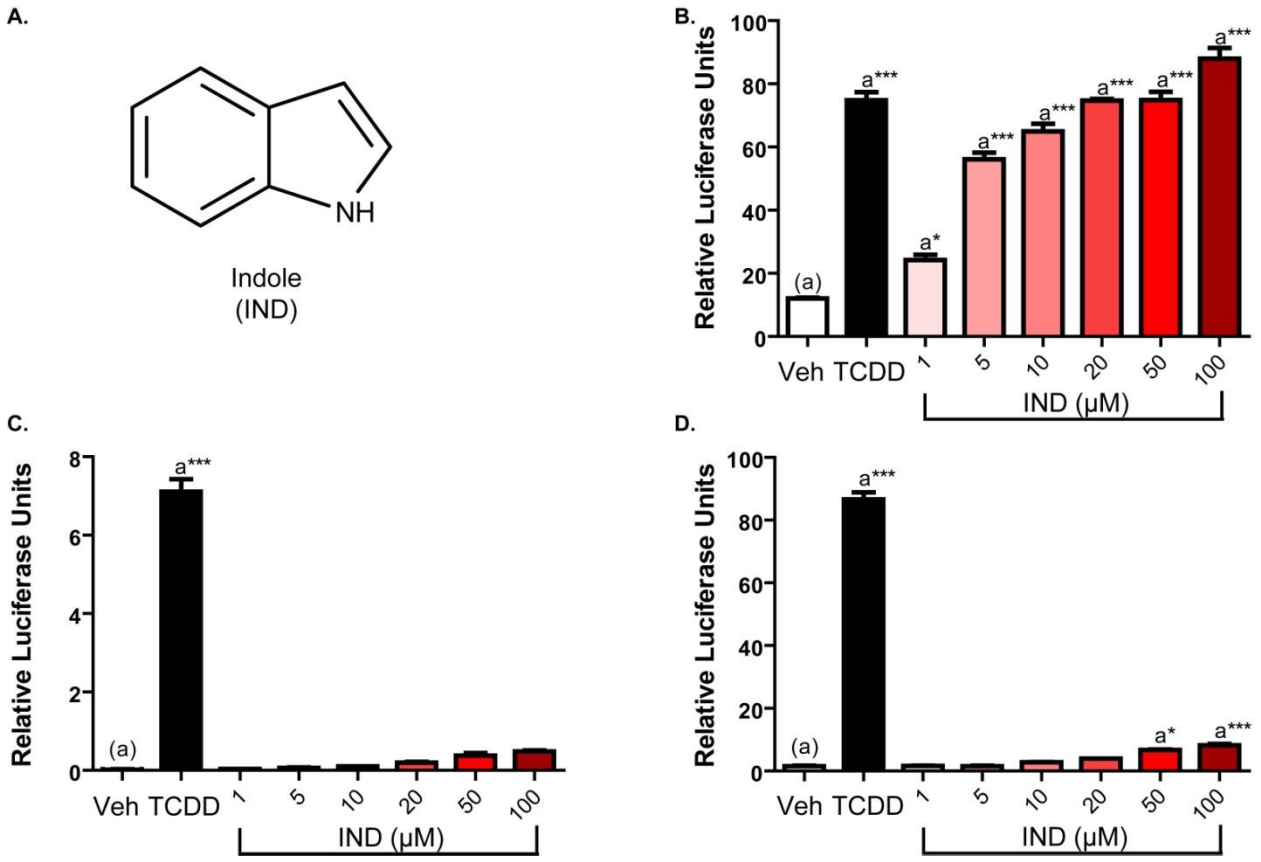


Figure 2.1: Interspecies analyses of AHR-dependent activity.
(A) Molecular Structure of Indole. **(B)**Human HepG2 (40/6) cells **(C)** Mouse Hepa 1.1 and **(D)** Rat H4IIE1.1 reporter cells were treated as indicated for 4h, followed by lysis and quantification of AHR-driven luciferase activity.

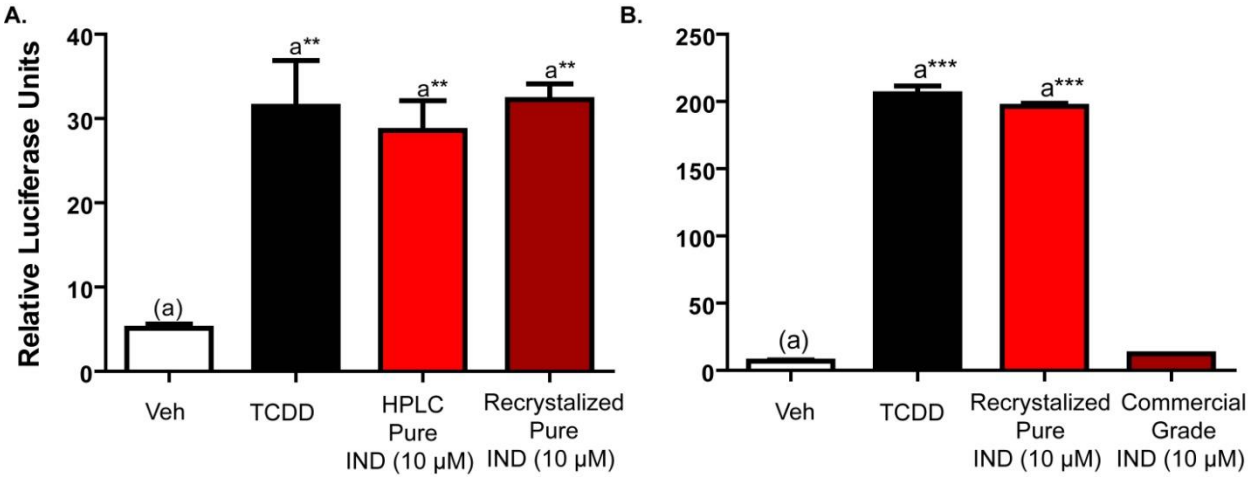


Figure 2.2: Purified indole induces AHR-dependent reporter activity.
(A) Comparison of AHR-dependent luciferase activity of HPLC purified and recrystalized and **(B)** Comparison of recrystalized versus commercial indole in human HepG2 (40/6) , cells were treated as indicated for 4h, followed by lysis and quantification of AHR-driven luciferase activity.

Indole stimulates human AHR-mediated target gene expression

To further establish indole as a human AHR agonist, quantitation of indole-mediated AHR target gene expression was quantitated by PCR in colonic epithelial Caco2 cells exposed (4 h) to vehicle, 10 nM TCDD, 20 and 100 μM indole. Induction of prototypical AHR target genes *CYP1A1* and *CYP1B1* by TCDD and indole was consistent with previously observed reporter cell line data with 20 μM indole displaying equivalent inductive capacity as 10 nM TCDD by eliciting significant 410 and 39-fold increases in *CYP1A1* and *CYP1B1* respectively **(Figure 2.3A).** Further analysis of *AHR* mRNA expression in response to indole exposure revealed no significant upregulation of *AHR* expression **(Figure 2.3A)**. Therefore induction of AHR target genes is not occurring through enhanced AHR expression via increased constitutive receptor activity. Reciprocal analyses of *Cyp1a1* and *Cyp1b1* expression performed using the mouse Hepa1 cell line failed to recapitulate the induction of *CYP1A1/B1* observed with human cells, further supporting a model of species-specific AHR activation by indole **(Figure 2.3B)**. Indole-mediated induction of prototypical target genes within Caco2 cells were shown to be dependent upon receptor activation through competitive antagonism by AHR antagonist GNF351, which suppressed ~88% of indole-dependent *CYP1A1* transcription **(Figure 2.3C)**[311]. Examination of human Caco2 CYP1A1 enzymatic activity using an EROD-based methodology established that indole-mediated functional upregulation of CYP1A1/CYP1B1 metabolism and not simply increased transcription of *CYP1A1/CYP1B1* mRNA **(Figure 2.3D)**. Following a 12 h treatment with 100 μM indole, CYP1A1 EROD activity is significantly enhanced ~4-fold over vehicle treated controls. Such indole-mediated induction of enzymatic activity proved to be reduced relative to that obtained by treatment with 10 nM TCDD. This difference may be attributed to the half-life of indole, which is likely metabolized at a faster rate than TCDD.

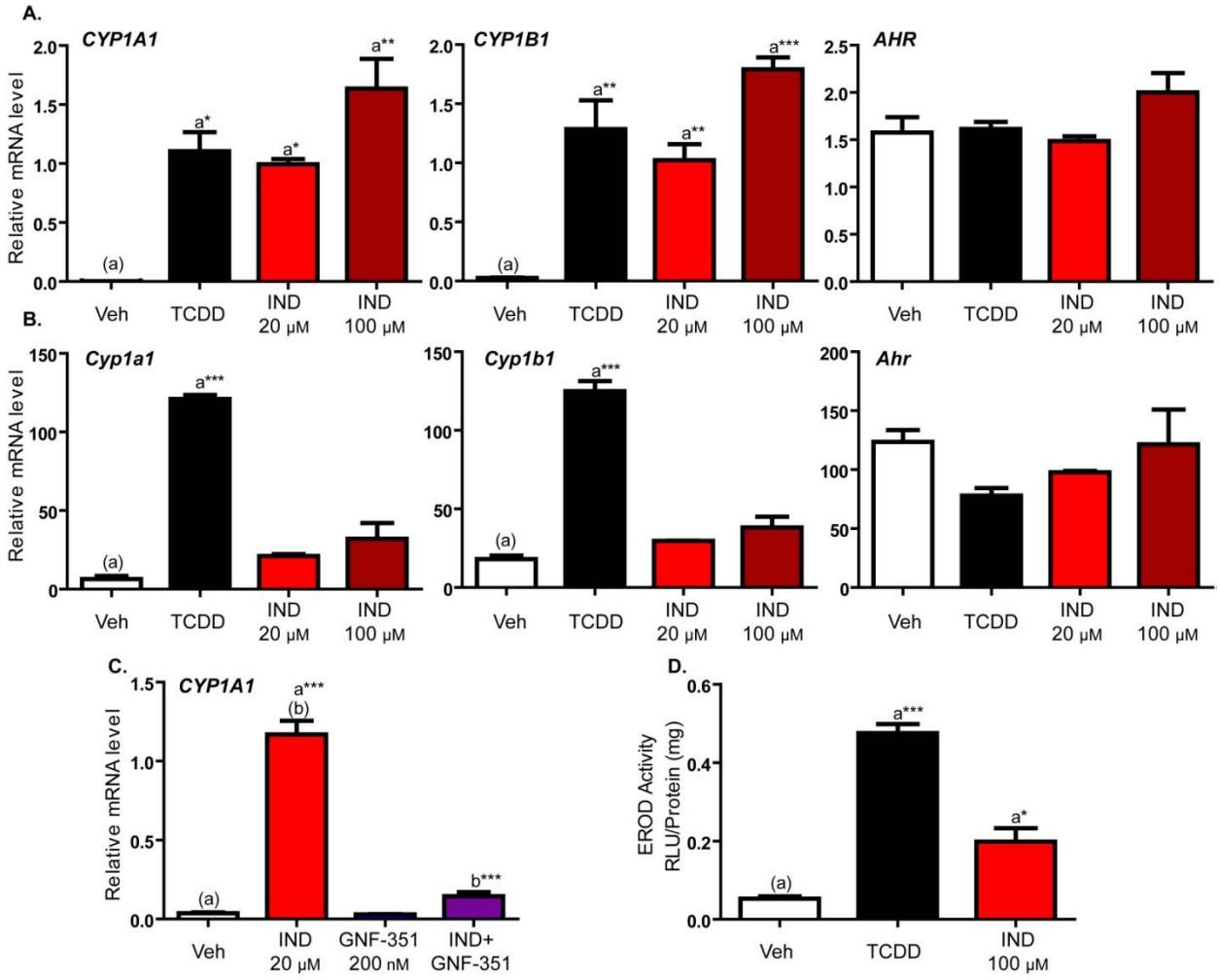


Figure 2.3: Indole stimulates human AHR gene expression.
(A) Expression of *CYP1A1*, *CYP1B1*, and *AHR* within human Caco2 cells and **(B)** Expression of *Cyp1a1*, *Cyp1b1*, and *Ahr* within mouse Hepa1 cells were determined by qPCR following 4h of treatment with vehicle, TCDD (10 nM), or indole (IND) at the indicated dose. **(C)** Expression of *CYP1A1* within Caco2 cells were determined by qPCR following 4 h treatment with vehicle or indole, with or without a 1 h pretreatment with AHR antagonist GNF-351. **(D)** Average CYP1A1/CYP1B1 enzymatic activity was assessed in human Caco2 cells following 12 h treatment with vehicle, TCDD (10 nM), or indole and 3 h incubation with luciferin-CEE reagent.

In addition to direct DRE-mediated induction of target genes, the AHR is known to combinatorially impact gene expression upon inflammatory co-stimulation. Previous results from our lab demonstrate synergistic induction of *IL6* following co-treatment with an AHR agonist and pro-inflammatory stimuli [312]. Therefore, in order to further characterize indole as a bonafide activator of the human AHR, we assessed its capacity to facilitate synergistic induction of *IL6.* Caco2 cells were exposed (4 h) to vehicle or 20 μM indole with or without the addition of Interleukin 1 Beta (IL1B) (10 ng/mL), followed by qPCR analysis of *IL6* mRNA expression **(Figure 2.4A)**. Treatments without the addition of IL1B failed to induce significant upregulation of *IL6*. However, combinatorial treatment with indole and IL1B prompted a synergistic 3-fold induction of *IL6* relative to cytokine treatment alone. To further support AHR dependency within the context of IL6 induction mediated by indole, we performed co-treatments with competitive AHR antagonist GNF-351[311]. Treatments were repeated as previously described within Caco2 cells with or without the addition of a 1 h pretreatment of GNF-351 (200 nM) **(Figure 2.4A)**. Indeed, treatment with GNF-351significantly attenuated indole/IL1B mediated induction of *IL6* by ~50%, while having a no discernible effect upon IL1B-alone stimulated transcription. These observations indicate that indole-specific effects upon IL6 expression are mediated through the human AHR. This was followed by ELISA analyses to establish that indole/IL1B mediated synergy upon the *IL6* promoter is reflected in protein expression in addition to transcriptional activity. Caco2 cells were treated with vehicle, TCDD (10 nM) or indole (100 μM) in isolation or in combination with IL1B (10 ng/mL), as indicated for 24 h **(Figure 2.4B)**. Following the treatment time course, conditioned media was collected and assayed for secreted IL6 levels. The results obtained coincided with previous qPCR observations, in that combinatorial administration

of IL1B with AHR agonists TCDD or indole stimulated a significant ~3-fold increase in IL6 levels relative to IL1B treatment alone.

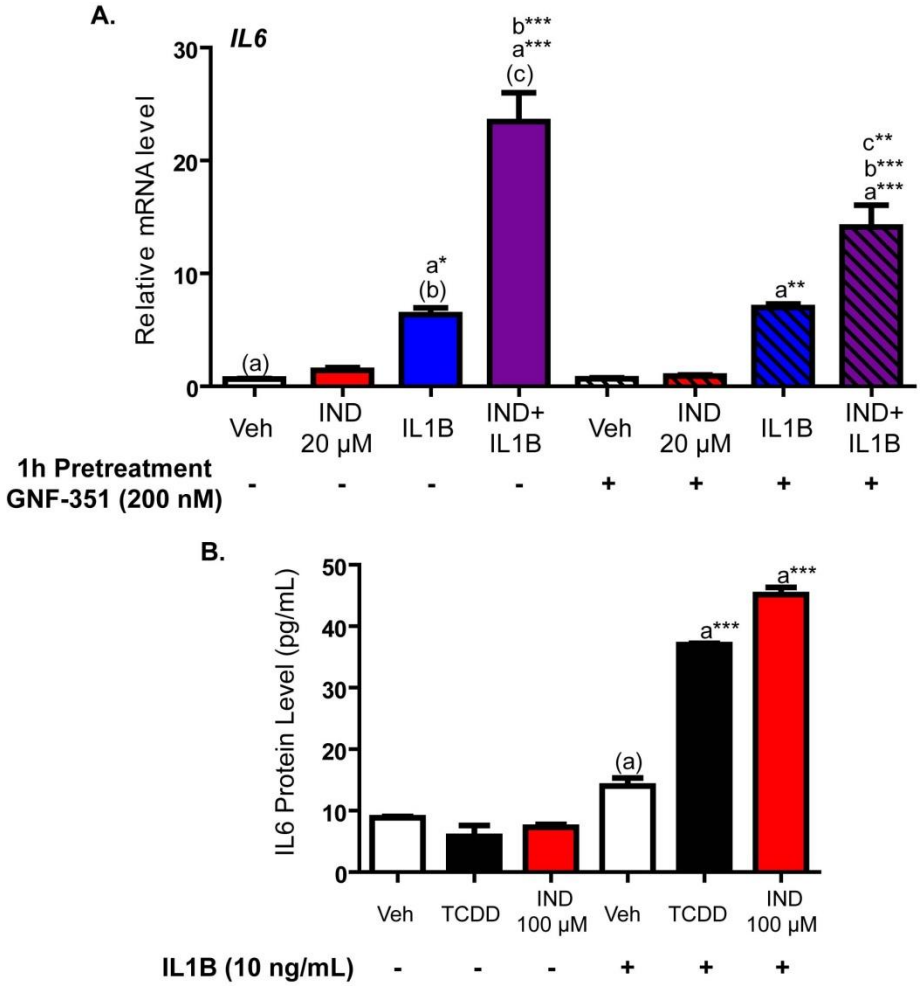


Figure 2.4: Indole stimulates synergistic induction of IL6.
Within human Caco2 cells **(A)** IL6 expression was assessed by qPCR following treatment with indole (20 µM) with or without the addition of IL1B (10 ng/mL), AHR dependency was evaluated by 1 h pretreatment with AHR antagonist GNF-351 (200 nM). **(B)** ELISA quantification of secreted IL6 following 24 h treatment with vehicle, TCDD (10 nM), or indole (100 µM), with or without the addition of IL1B (10 ng/mL).

In order to characterize human AHR activation by indole in non-transformed cell lines, we utilized primary peritoneal macrophages (Mø) isolated from commercially available 'humanized' AHR mice in comparison to C57BL6/J controls expressing mouse AHR. Hepatic expression of human and murine AHR by 'humanized' AHR and control mice respectively were established through AHR-specific immunoblot analyses (**Figure 2.5A**). The human AHR peptide (~96 kDa) is slightly larger than the mouse homolog (~90 kDA) allowing for differentiation of isoform expression. Expression of *Cyp1a1*was assessed by qPCR within isolated peritoneal Mø derived from 'humanized' and wild-type controls following a 4 h exposure to vehicle, 10 μM indole, or 500 pM indirubin **(Figure 2.5B)**. Exposure to indirubin elicited a significant 20-fold increase in *Cyp1a1* expression by 'humanized' AHR Mø, but failed to induce in wild-type Mø. This result is consistent with previous reports demonstrating human AHR-specific activation by indirubin, thus validating that 'humanized' AHR Mø are permissive for AHR-mediated transcription by human-specific ligands[231]. Indole treatments elicited a 5-fold increase in *Cyp1a1* expression in 'humanized' AHR Mø, but was unable to induce expression in identically treated wild-type Mø. These data further support indole as a selective interspecies AHR activator and suggest that our previous observations were not an artifact of the utilized cancer cell lines.

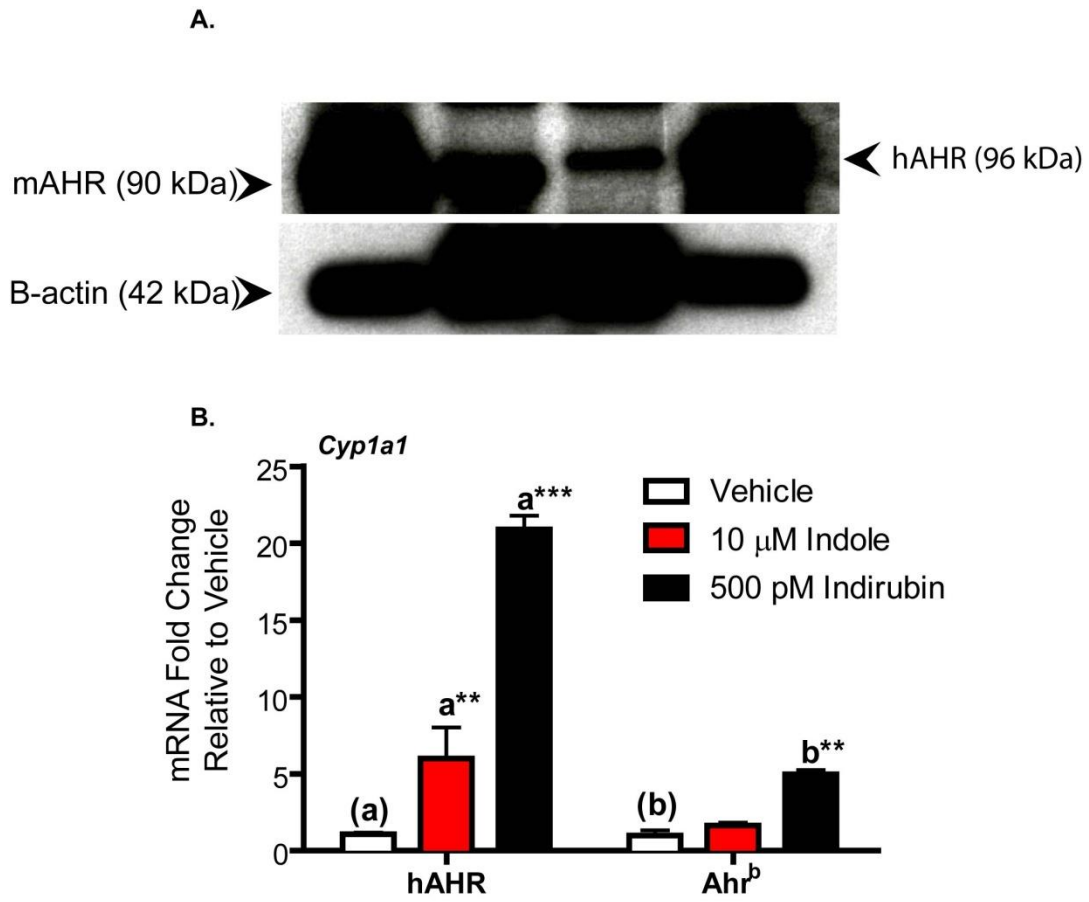


Figure 2.5: Indole mediates expression of *Cyp1a1*in humanized AHR peritoneal macrophages.

Within AHR humanized mice (hAHR) and wild-type C57BL6/J (mAHR) **(A)** Protein expression was assessed by Western blot analysis to validate AHR isoform expression within livers of humanized and wild-type mice. **(B)** Cyp1a1 gene expression within isolated peritoneal macrophages were evaluated by qPCR following 4 h treatment as indicated.

Activation of human AHR-mediated transcription by indole is a consequence of direct ligand binding

Species-specific activation of human AHR-mediated transcription indicated that indole is a putative AHR activator. However, AHR-activation by indole through a direct interaction with the AHR was not established in previous experiments. To establish indole a direct ligand for the human AHR, competitive ligand binding assays were performed. Hepatic cytosol isolated from mice expressing the human *AHR* transgene under the control of the hepatocyte-specific *albumin* promoter were compared to samples derived from wild-type C57BL6/J animals expressing the mouse AHR. Humanized AHR cytosol incubated under saturating conditions with the AHR photo-affinity radio-labeled ligand (PAL) and increasing concentrations of indole displayed a dose-dependent decrease in PAL binding **(Figure 2.6A)**. This result is consistent with competitive exclusion of PAL and indicates that indole is a direct ligand for the human AHR. The increased rate of PAL exclusion exhibited by the known AHR agonist beta-napthoflavone (BNF) suggests that the relative affinity of human AHR for indole is an order of magnitude lower than for βNF. Mouse AHR cytosol demonstrated similar activity with regard to binding of βNF, but indole displayed no capacity to hinder PAL binding to mouse AHR under all concentrations tested **(Figure 2.6B)**. Higher concentrations of indole could not be utilized in this assay due to possible non-specific effects upon PAL-AHR binding interactions. These data further indicate that indole is human AHR specific ligand.

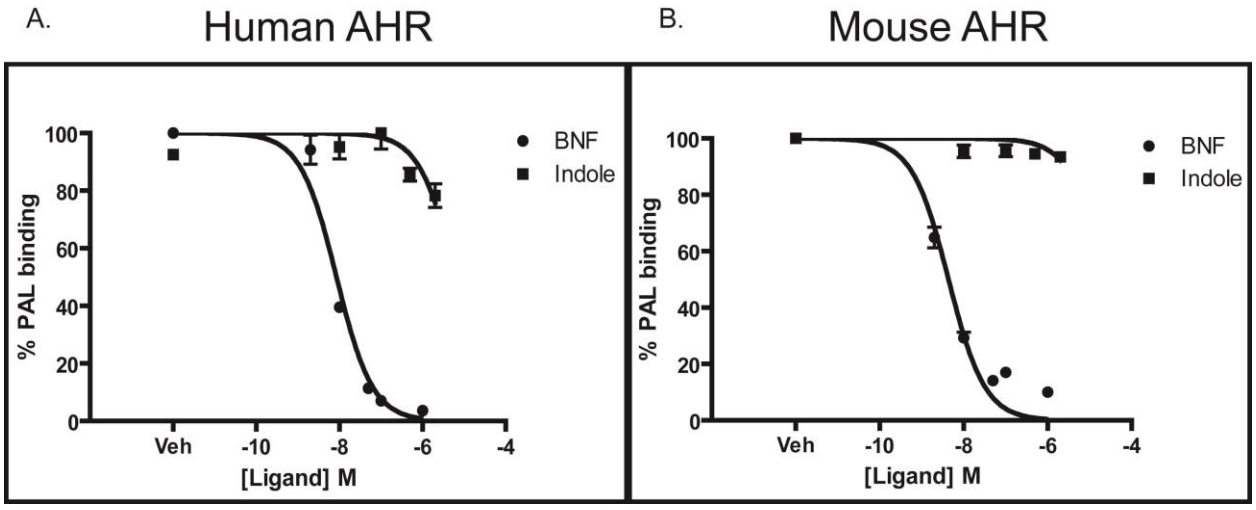


Figure 2.6: Indole is a human-specific AHR ligand.
Photoaffinity ligand binding competition assay in which increasing doses of β-napthoflavone (BNF) or indole were added to **(A)** Human AHR of **(B)** Mouse AHR hepatic cytosol in combination with a fixed saturating amount of the photoaffinity ligand (PAL) to evaluate relative binding competition between PAL and the compounds tested for occupancy within the AHR ligand binding domain.

Upon ligand activation, the AHR is able to translocate into the nucleus, interact with ARNT, and bind to DREs to mediate target gene transcription. The capacity for indole to mediate human specific AHR nuclear translocation and bind DREs was determined through use of nuclear translocation and DNA binding assays. Indole regulation of AHR transformation and sub cellular localization was assessed in human HepG2 and mouse Hepa1 cells following a1 h incubation with vehicle or indole (100 μM). After treatment nuclear isolates were assessed by Western blot analyses and confirmed an indole-dependent redistribution of AHR from the cytoplasm into the nucleus of human cells, but not mouse cells (**Figure 2.7A)**. In support of the observed human-specific nuclear redistribution of AHR following exposure to indole, DNA retardation assays exhibited a species-specific capacity of indole to facilitate binding of *in vitro* translated human AHR/ARNT, but not murine AHR/ARNT, to ^{32}P-labeled oligonucleotides containing its cognate DNA response element **(Figure 2.7B)**.

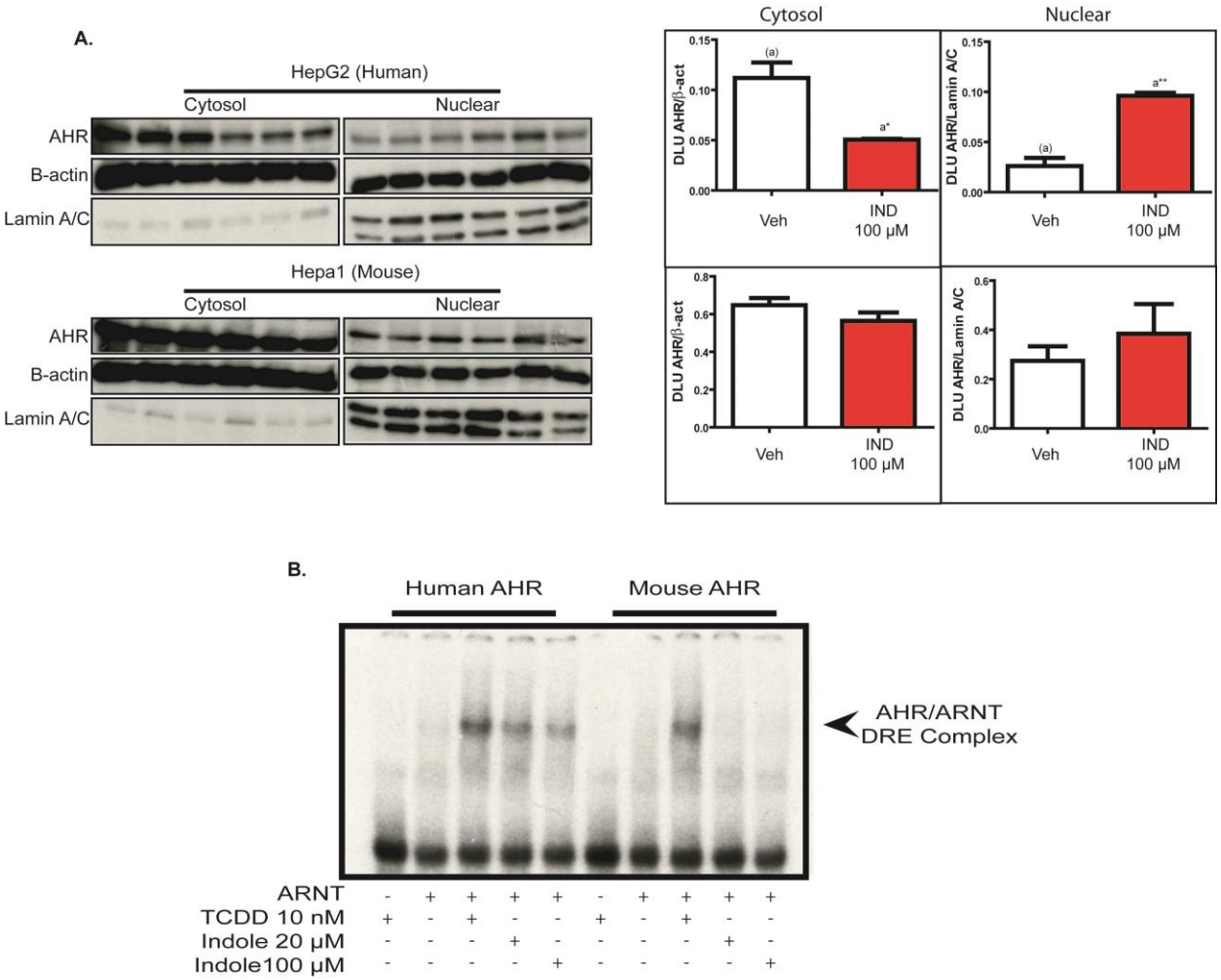


Figure 2.7: Indole mediates human AHR specific nuclear translocation and DRE binding capacity.

(A) Nuclear translocation of AHR was assessed via Western blot analyses following 1 h incubation with the indicated treatment in human HepG2 cells and mouse Hepa 1 cell lines. Relative levels of AHR (normalized to β-actin or Lamin A/C) was quantified via Phosphoimager and Optiquant software in digitized light units (DLU). **(B)** In vitro translated gel shift assay displaying indole capacity to induce human specific formation of AHR/ARNT/DNA complex.

Indole derivatives exhibit structure-specific capacity to activate the human AHR

The characterization of indole as a human-specific AHR agonist/ligand led to the speculation that additional physiologically relevant indole derivatives may also display human specific AHR activity. To address this hypothesis we examined the biological activity of other microbial derived indole derivatives within human AHR-driven reporter cells. Human HepG2(40/6) cells were treated for 4 h with vehicle, TCDD (10 nM), indole (10 μM), and 3-methyl indole, 2-oxindole or 3-indole propionic acid (1, 5, 10 μM), followed by quantification of luciferase activity **(Figure 2.8A)**. The results substantiate or hypothesis and demonstrate that 3-methyl indole and 2-oxindole are capable of activating the human AHR with both indole derivatives exhibiting a dose-dependent increase in luciferase reporter activity and produce a response equivalent to indole at matching concentrations. While, 3-indole propionic acid stimulated reporter activity, it failed to show dose-dependent responsiveness typical of AHR activators. Complementary studies conducted using mouse Hepa1.1 cells failed to identify any of the tested indole derivatives as AHR agonists **(Figure 2.8 B)**. The specificity of the human AHR for indole derivatives were further investigated using constitutional isomers of methyl indole. Human HepG2(40/6) cell luciferase reporter activity was determined following 4 h treatment with vehicle, TCDD (10 nM), indole (10 μM) or 1/2/3- substituted methyl indole (1, 5, 10 μM) **(Figure 2.9A)**. The data obtained revealed no significant increase in AHR activity upon treatment with either 1-methyl or 2-methyl indole, suggesting the interaction of human AHR with indole or 3-methyl indole is dictated by specific interactions within the LBD. All methyl-indole isomers tested showed no significant AHR-driven luciferase activity within mouse Hepa 1.1 cell lines **(Figure 2.9B)**. Identical structure activity associations were observed by analyzing *CYP1A1*/*B1* mRNA expression in Caco2 cells (**Figure 2.10A**). Whereas, only 3-methyl-indole

showed a slight increase in AHR target gene mRNA expression within mouse Hepa1 cells **(Figure 2.10B)**. The propensity for 3-methyl indole to mediate human AHR activation was further supported by significant induction of CYP1A1/CYP1B1 enzymatic activity **(Figure 2.10C)** and synergistic *IL6* mRNA/protein expression in Caco2 cells (**Figure 2.10D/E**). Transformation of human AHR to AHR/ARNT/DNA complex was mediated specifically by the 3-methyl-indole isomer within DNA retardation assays **(Figure 2.11A)**. However, no elevation of mouse AHR/ARNT/DNA complex formation was observed following treatment with all tested methyl-indole compounds **(Figure 2.11B)**.

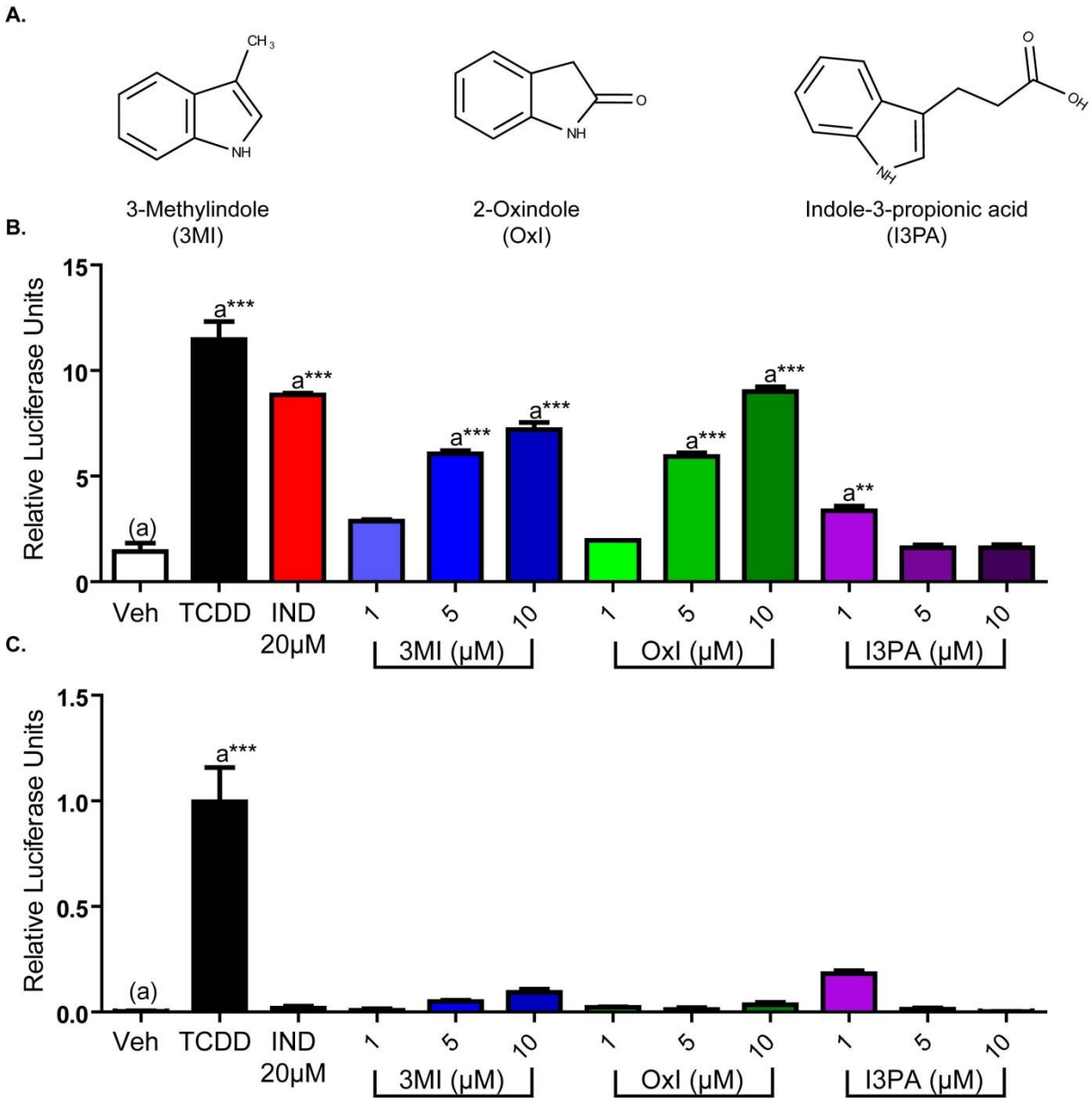


Figure 2.8: Ligand specificity of human AHR for microbiota-derived indoles.
(A) Structures of common indoles synthesized by microbes. **(B)** Human HepG2 (40/6) and **(C)** Mouse Hepa 1.1 cells were treated as indicated for 4h, after time course cells were lysed and luciferase activity quantified.

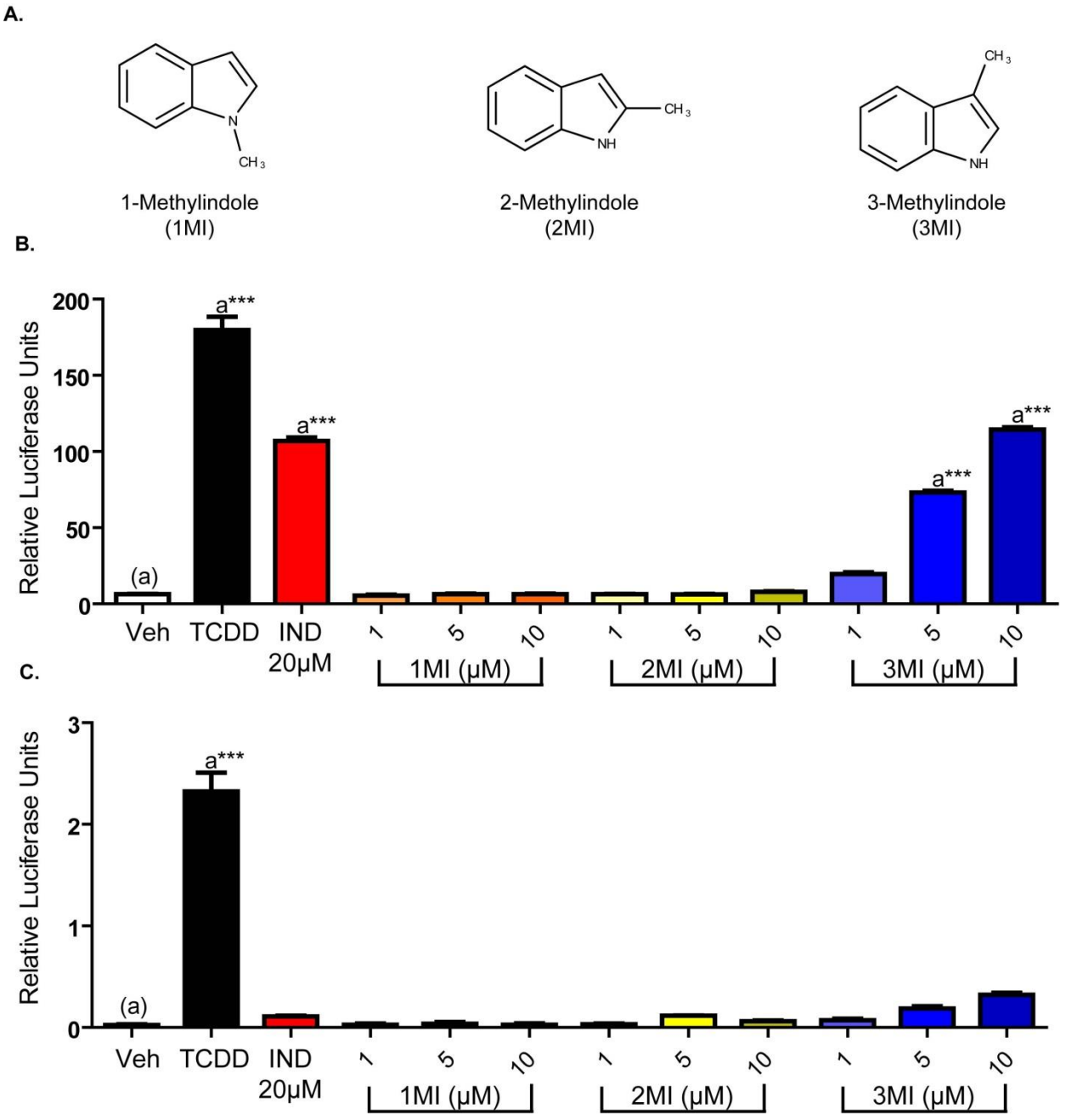


Figure 2.9: Methyl-indole isomers exhibit differential capacity to activate AHR.
(A) Structures of methyl-indole constitutional isomers analyzed. **(B)** Human HepG2 (40/6) and **(C)** Mouse Hepa 1.1 cells were treated as indicated for 4h, after time course cells were lysed and luciferase activity quantified.

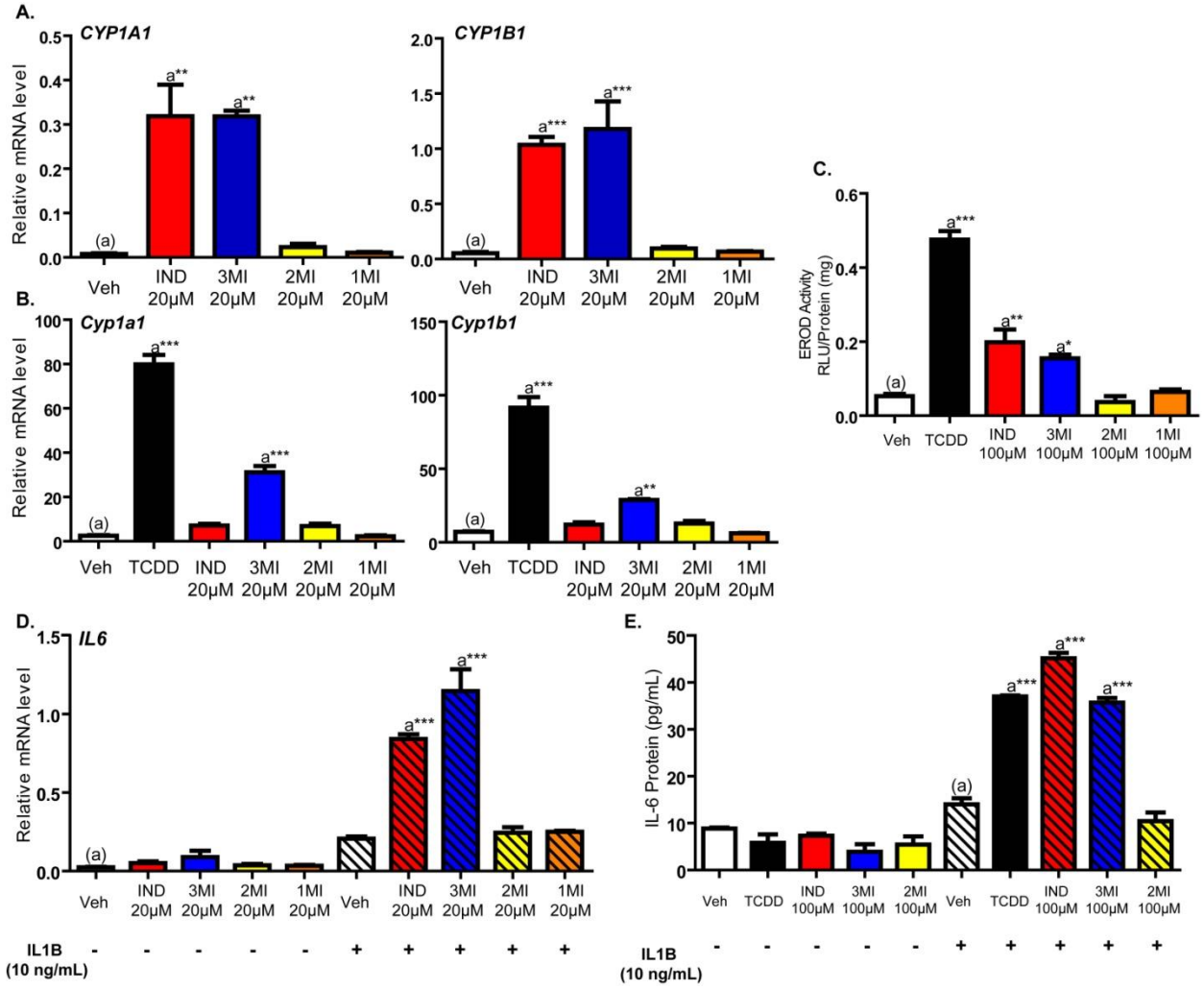


Figure 2.10: Indole stimulates human AHR gene expression.
(A) Expression of *CYP1A1* and *CYP1B1* within human Caco2 cells and **(B)** Expression of *Cyp1a1* and *Cyp1b1* within mouse Hepa1 cells were determined by qPCR following 4h of treatment with vehicle, TCDD (10 nM), indole (IND), 3-methyl indole (3MI), 2-methyl-indole (2MI), or 1-methyl-indole (1MI) at the indicated dose. **(C)** Average CYP1A1/CYP1B1 enzymatic activity was assessed in human Caco2 cells following 12 h treatment with vehicle, TCDD (10 nM), or indole and 3 h incubation with luciferin-CEE reagent. **(D)** IL6 expression was assessed by qPCR following treatments as indicated with or without the addition of IL1B (10 ng/mL) in Caco2 cells. **(E)** ELISA quantification of secreted IL6 following 24 h treatment with vehicle, TCDD (10 nM), or indole derivatives (100 μM), with or without the addition of IL1B (10 ng/mL).

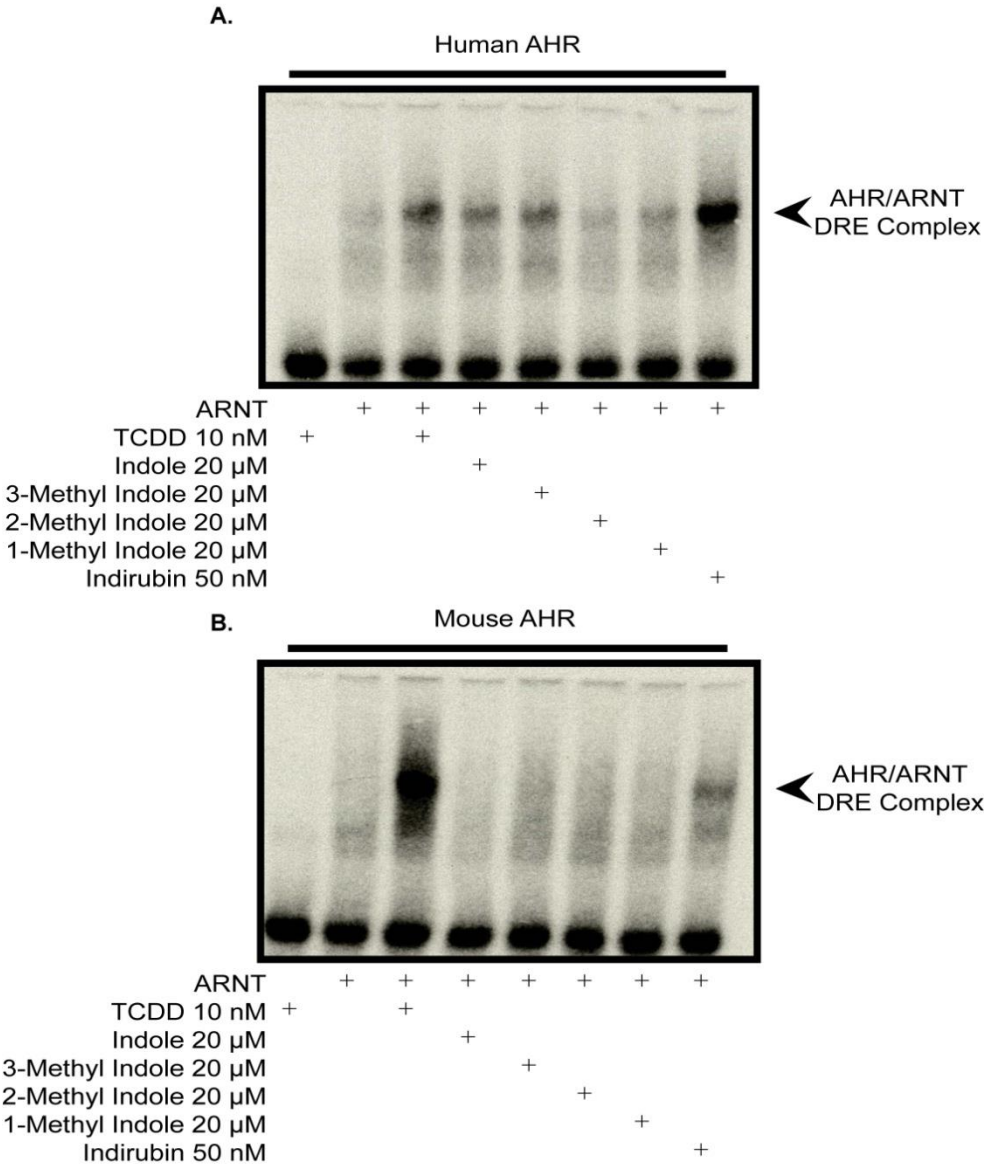


Figure 2.11: Indole mediates human AHR specific nuclear translocation and DRE binding capacity.

In vitro translated gel shift assay displaying methyl-indole isomer capacity to induce formation of AHR/ARNT/DNA complex through activation of **(A)** human AHR or **(B)** Mouse AHR.

***In silico* modeling predicts the structure-activity selectivity of indole and 3-methyl indole associated with human AHR**

In summary the data obtained suggest that the AHR-dependent activities associated with both indole and 3-methyl indole are restricted to the human homolog. In an effort to better dissect the molecular basis for the species specific nature of indole AHR agonism, we utilized *in silico* modeling of ligand docking within the human and murine AHR LBDs (amino acid residues 247-290 or 241-284, human or murine respectively). Ligand docking simulations of a single molecule within the LBD of the human AHR suggest little difference in favorable binding mechanics between indole, 3-methyl-indole, or 2-methyl indole based upon calculated ICM scores **(Figure 2.12 and Data Table 2.1)**. Homology modeling of human and murine AHR LBD docked with indirubin was used as an optimized model to establish the most energetically favorable LBD conformation for ligand binding (**Figure 2.13 A/B**). The previously established specificity of the human AHR for indirubin, which closely resembles two covalently-linked indole moieties, prompted us to investigate a unique AHR ligand binding mechanism in which the stoichiometry of human AHR/indole binding may be 1:2 rather than 1:1. The dual- indole binding hypothesis was assessed using the *in silico* LBD models of human or murine AHR. The models predicted a favorable conformation associated with two indole molecules within the human but not the murine AHR LBD (**Figure 2.13 C/D**). A similar favorable predicted binding mechanism was suggested in models of 3-methyl-indole binding within the human, but not mouse AHR LBD **(Figure 2.13E/F)**. These data support the dual-indole binding hypothesis, as evidence by a more favorable surface energy of binding was observed with human AHR in the binding of indole and 3-methyl indole relative to mouse AHR **(Data Table 2.2)**. Notably, homology models used to predict binding mechanics of two 2-methyl indole molecules proved to

be energetically unfavorable in the context of human AHR. Overall the *in silico* modeling of favorable species specific AHR-ligand interactions supports are congruent with our previous experimental observations and provide support for a novel dual-indole binding mechanism within the LBD of human AHR.

Data Table 2.1: Relative ICM score indicating energetic favorability of ligand docking within human AHR LBD.

Single Molecule Binding	ICM Score
Indole	-14.64
3-Methyl Indole	-12.72
2-Methyl Indole	-13.07

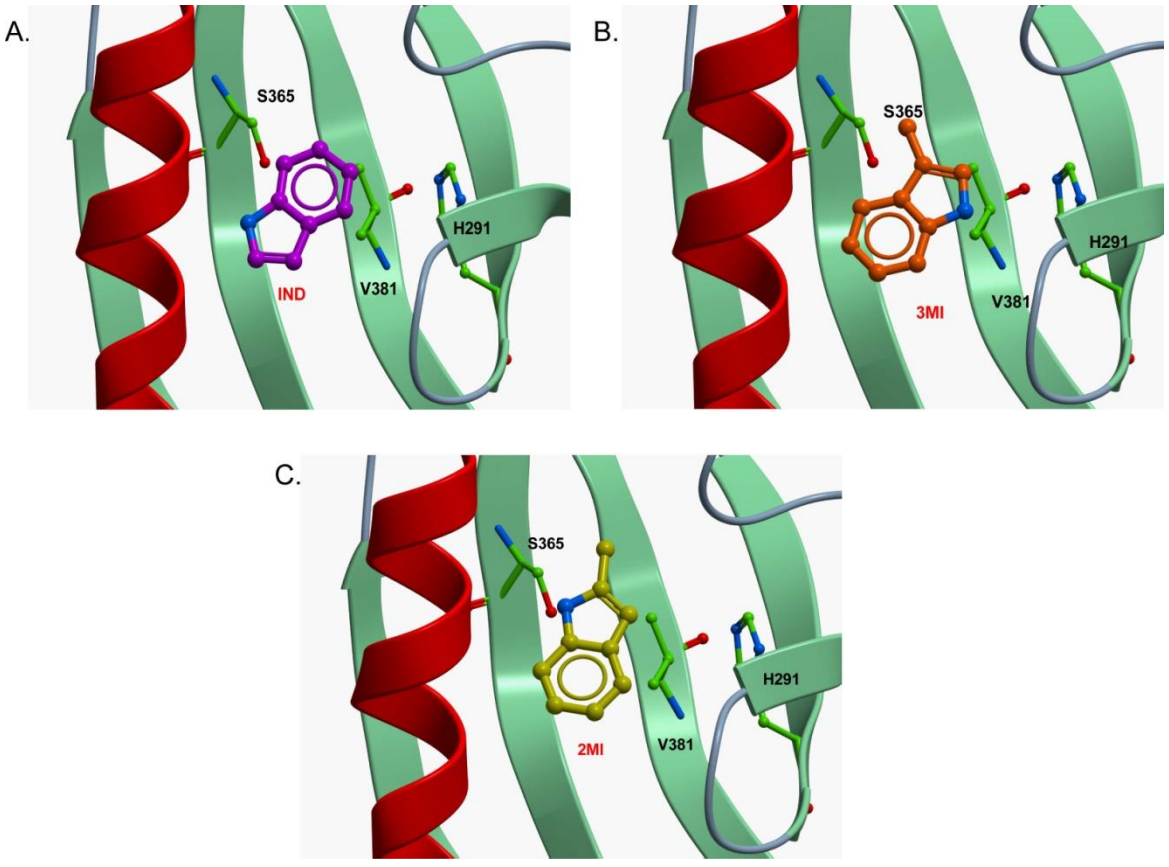


Figure 2.12: In silico modeling of human AHR binding of indole derivatives.
In silico modeling of single indole molecules binding within the LBD of the human AHR (A)Indole (B) 3-methyl-indole (C) 2-methyl-indole

Data Table 2.2: Surface energy of binding (ΔH, kcal/mol) for indole and 3-methyl indole molecules within the mouse and human AHR LBD after second round of docking.

Compound	Mouse AHR	Human AHR
Indole	**-0.09**	**-0.58**
3-Methyl Indole	**-0.37**	**-1.76**

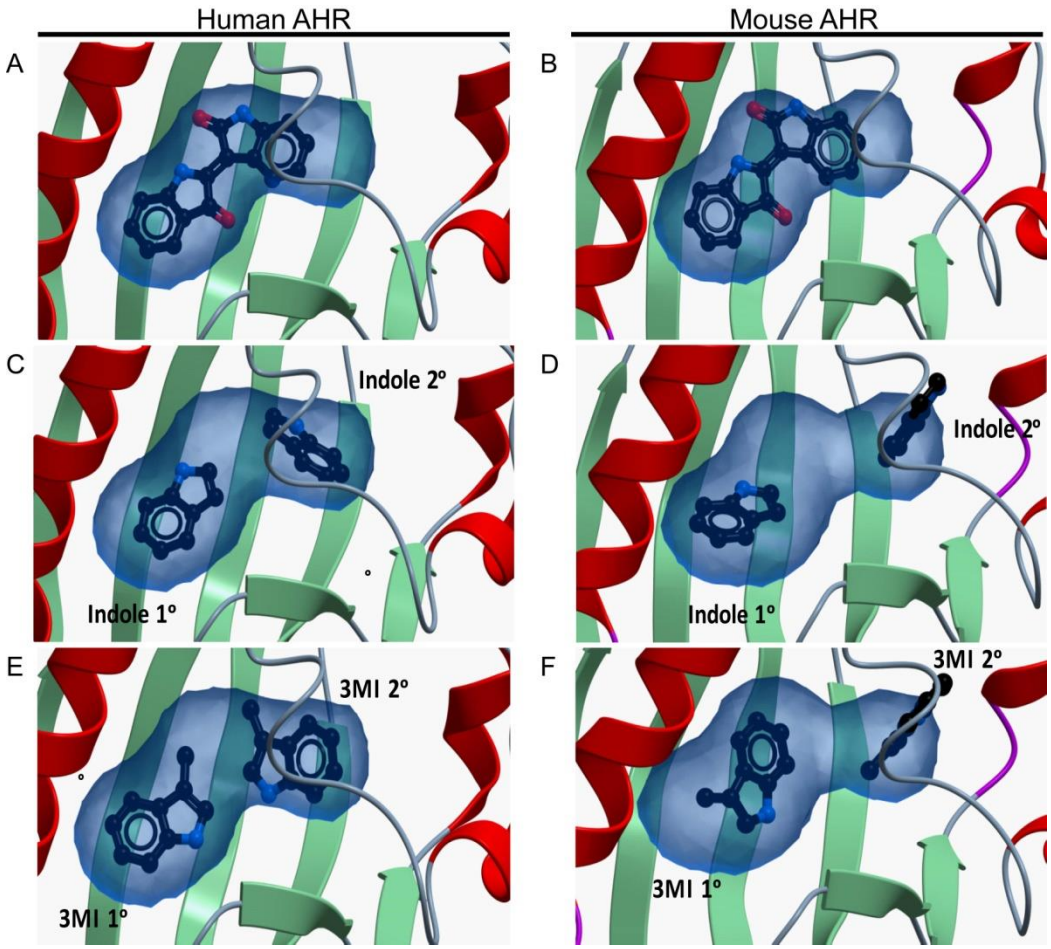
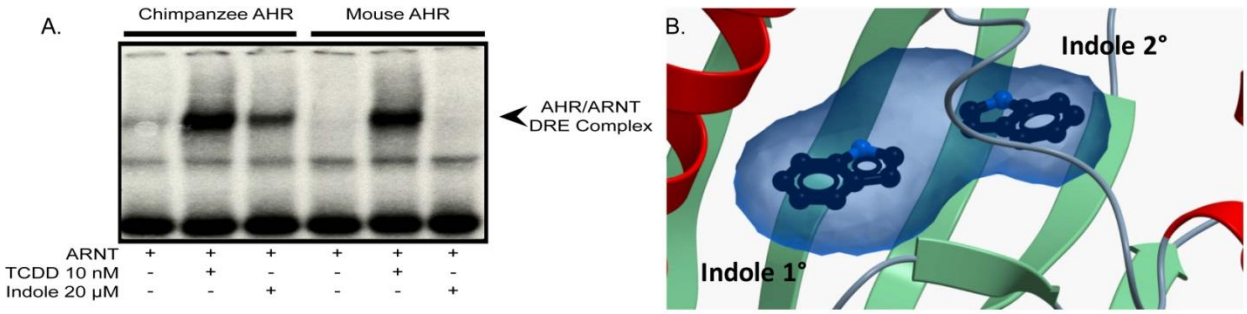


Figure 2.13: In silico modeling of human versus mouse AHR binding of indole derivatives. Homology modeling of indirubin optimized ligand binding within hAHR (A) and mAHR (B). The predicted two indole binding model within hAHR (C) and mAHR (D). The predicted two 3-methyl indole binding model within hAHR (E) and mAHR (F).

Activation of AHR by indole is conserved across hominids

The species specific activation of human AHR by indole suggests that selective adaptation of the *Ahr* locus occurred during the course of mammalian evolution that resulted in a gain of function in the form of microbial indole sensitivity. In order to determine if indole sensitivity is restricted solely to humans or if this function evolved prior to human speciation, we investigated the capacity for indole to activate the AHR of *Pan troglodytes* (chimpanzee). A gel retardation assay was conducted using in *vitro* translated murine or chimpanzee AHR treated with vehicle, TCDD (10 nM) or indole (20 μM) **(Figure 2.14A)**. A robust increase in chimpanzee AHR/ARNT/DNA complex was observed relative to mouse AHR, indicating that chimpanzee AHR is sensitive to activation by indole. The receptivity of the chimpanzee AHR LBD to binding two molecules of indole was evaluated using previously described *in silico* modeling techniques. The ligand-docking simulations suggest that the LBD of chimpanzee AHR would favorably accommodate two indole molecules in a manner comparable to that observed with human AHR **(Figure 2.14B)**. Amino acid sequence alignments that illustrate the LBD of chimpanzee, human, and mouse AHR reveal only a single amino acid difference between chimpanzee and human at position 381. At this position, or its homologous site within murine AHR (375), chimpanzee and mouse both contain an alanine residue, relative to a valine residue in humans **(Figure 2.14C)**. These results suggest that the mutation of residue 381 in man is unlikely to contribute to indole sensitivity and that the determinant for such sensitivity in primates is likely due to other alterations in AHR structure that differentiate primate AHR from that of its mouse homolog.



C.

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Chimpanzee  247-HGQKKKGKDGSILPPQLALFAIATPLQPPSILEIRTKNFIFRTKHKLDF-295
     Human  247-HGQKKKGKDGSILPPQLALFAIATPLQPPSILEIRTKNFIFRTKHKLDF-295
     Mouse  241-HGQNKKGKDGALLPPQLALFAIATPLQPPSILEIRTKNFIFRTKHKLDF-289

Chimpanzee  296-TPIGCDAKGRIVLGYTEAELCTRGSGYQFIHAADMLYCAESHIRMIKT-343
     Human  296-TPIGCDAKGRIVLGYTEAELCTRGSGYQFIHAADMLYCAESHIRMIKT-343
     Mouse  290-TPIGCDAKGQLILGYTEVELCTRGSGYQFIHAADILHCAESHIRMIKT-337

Chimpanzee  344-GESGMIVFRLLTKNNRWTWVQSNARLLYKNGRPDYIIATQRPLTDEE-390
     Human  344-GESGMIVFRLLTKNNRWTWVQSNARLLYKNGRPDYIIVTQRPLTDEE-390
     Mouse  338-GESGMTVFRLLAKHSRWRWVQSNARLIYRNGRPDYIIATQRPLTDEE-384
```

Figure 2.14: Chimpanzee AHR is activated by indole.
(A)*In vitro* translated AHR/ARNT gel shift assay showing indole treatment capacity to transform chimpanzee AHR into AHR/ARNT/DNA complex. (B) In silico modeling of chimpanzee LBD occupancy by two indole molecules. (C) Amino acid sequence alignment comparing chimpanzee, human, and mouse AHR ligand binding domains.

2.5 DISCUSSION

The AHR, consistent with its established function as a xenobiotic sensor, exhibits a marked degree of promiscuity with regard to ligand binding[184, 313]. Our results demonstrate that indole, a product of microbial tryptophan metabolism, is able to bind and activate the human AHR and mediate subsequent target gene transcription. Moreover, the murine AHR homolog was not permissive for activation by indole. This result is atypical relative to previous findings in which the murine AHR possesses increased sensitivity to prototypical AHR agonists, such as TCDD, PAHs, and HAHs[314]. However, recent insights that have characterized novel endogenous molecular entities of increasingly diverse structure that exhibit higher agonist activities within the context of the human AHR[232, 255, 274]. Initial *in silico* modeling of AHR-ligand docking simulations using the presumptive hypothesis of a single ligand entering the AHR LBD failed to account for differences in ligand specific activation potential of methyl indole isomers upon the human AHR. The observation that single molecules of indole, 3-methyl-indole, and 2-methyl-indole all bound similarly within the human AHR is expected due to the ability of the LBD to accommodate large molecules such as B(a)P and TCDD, suggesting the large binding surface would present little steric hindrance for the entry of low molecular weight indoles. Therefore a more complex mechanism of indole-AHR binding interactions was paramount to explain the structure and species specific activation potential of these molecules. Notably, both human and mouse AHR are able to be activated by the pigment molecule indirubin. However, indirubin exhibits a potency equivalent to or greater than TCDD within human cells, whereas it is only a weak agonist when acting upon the mouse AHR[315]. Indeed, the structural composition of indirubin is reminiscent of covalently attached indole moieties. This led us to the hypothesis that the LBD of AHR would possibly accommodate two individual indole molecules, and that

variations in the steric environment of the LBD would present a novel molecular basis for the interspecies sensitivity to indole. In agreement with our hypothesis, *in silico* modeling simulations suggest that the steric environment of the human AHR LBD was more amenable for interactions with two indoles, relative to mouse AHR. Further utilization of this dual-molecule binding model was used to assess the likelihood of other indole derivatives to exhibit a similar species specific binding mechanism. In support of previous experimental evidence, *in silico* modeling suggested that only 3-methyl-indole, but not its constitutional isomers 1-methyl-indole or 2-methyl-indole, were able to favorably bind within the human AHR LBD. Such a bimolecular binding mechanism by the AHR has not been previously characterized or considered. This novel binding mechanism supplants previous assumptions that the AHR is restricted to activation by large polycyclic molecules, typified by structures of prototypical ligands, such as TCDD, and suggests that additional ligands with uncharacteristic structures and low molecular weights may yet to be identified.

Loss of homeostatic symbiosis between the host and commensal microbes have been found to associate with numerous pathological outcomes such as inflammatory bowel disease, Crohn's disease, ulcerative colitis, obesity, alcoholic and non-alcoholic steatosis[316-318]. Recent evidence has implicated the AHR as an important factor in the maintenance of intestinal homeostasis. Previous works have demonstrated that the AHR plays a critical role in the maintenance of type III innate lymphoid cells, and regulation of epithelial barrier function via the IL22 signaling axis. These AHR-dependent functions were thought to be strictly mediated by dietary AHR ligands, such as polyphenolic flavonoids *e.g.* quercetin, or glucobrassin-derived gastric acid condensation products *e.g.* indolo-[2,3*b*]-carbazole. Additional dietary activators of the receptor maybe generated through anthropogenic sources such as the high temperature

cooking of foods in the form of PAHs and heterocyclic amines[202, 212, 319, 320]. However, sole reliance upon dietary-derived AHR ligands for necessary stimulation beneficial intestinal signaling would be restrictive during times of sporadic or limited consumption. Therefore, microbial derived indole may provide a more steady-state level of AHR agonists. Notably, agonist mediated activation of the receptor has been found to be protective in models of intestinal inflammation, inflammation-associated colorectal tumorigenesis, and pathogenic microbial infections[222, 296-300, 321]. However, further studies are needed to assess the propensity of microbial-derived indole to activate the AHR and facilitate a similar therapeutic response. Recent evidence has found that indole supplementation was able to attenuate disease severity within murine models of non-steroidal anti-inflammatory drug (NSAID) enteropathy, however, the dependency of this effect upon AHR activity was not assessed[322]. Additionally, heightened fecal indole levels in combination with levels of indole-producing microbes were found to correlate with decreased susceptibility to *Cryptosporidium* infection[323]. This observed effect may have further implications in human diseases associated with persistent intestinal colonization by pathogenic microbes, such as *Clostridium difficile*. Fecal transfer therapies have been recently recognized as a viable method to combat *C. difficile* associated pathologies. It would be of great interest to assess indole and indole producing bacterial levels in individuals as a biomarker for susceptible to *C. difficile* infections and also to monitor indole levels following successful fecal transplant to see if they correlate with disease remediation or recurrence.

Microbial indole production and its subsequent hepatic bioactivation to the uremic toxin indoxyl sulfate are of increased relevance to investigational studies of renal disease. Indoxyl sulfate is a known agonist of the human AHR, but is also limited in its ability to activate the mouse AHR[232]. Concentrations ranging from 10 to 130 mg of indoxyl sulfate are produced in the

body daily, which is then excreted in the urine of healthy individuals[324] CKD patients are limited in their ability to excrete indoxyl sulfate, leading to elevated circulating levels 20-fold greater than normal[325, 326]. Indoxyl sulfate levels are inversely correlated with kidney function and found to associate with increased incidence of thrombosis, aortic calcification, and arterial stiffness[233, 327]. Such data suggests that levels of indole synthesis by resident microbes may have important implications in the pathogenesis of renal disease. Inhibition of the Tryptophanse enzyme mediated conversion of tryptophan to indole or identification of bacterial communities with a limited tryptophan metabolism potential may have therapeutic applications within CKD patients[328].

Moreover, the hepatic conversion of indole to indoxyl-sulfate by CYP2E1 metabolism and subsequent conjugation by sulfotransferases may have further implications within obesity associated metabolic diseases. Hepatic steatosis, also referred to as fatty liver, is characterized by the accumulation of lipid deposits within liver cells mediated by the increased uptake of free fatty acids or *de novo* synthesis of triglycerides. Steatosis has also been found to be induced by alcohol consumption via multiple mechanisms[329]. Notably, activation of the AHR was found to enhance the induction of steatosis[330]. Therefore we speculate that generation of indole by microbes, in combination with enhanced alcohol induced CYP2E1 metabolism, may facilitate increased hepatic concentration of the potent AHR ligand indoxyl sulfate. Enhanced levels of indoxyl sulfate could play a significant role in the disease progression of alcohol induced steatosis, and may further define the combinatorial contribution of dietary consumption and bacterial metabolism to complex disease phenotypes.

The recent identification of additional dietary/endogenously derived AHR agonists highlight a complex multifaceted network of signaling pathways that dictate AHR activity within

a given host. Additional microbial metabolites, such as 1,4-dihydroxy-2-napthoic acid, indole-3-acetate, and tryptamine, also are agonists of the AHR and are known to confer protective activities in murine models of colitis[132, 133, 208, 210, 211]. This observed promiscuity of the AHR LBD to multiple microbial metabolites may function to allow recognition of various microbial species and monitor microbial communication networks by sensing of quorum signaling molecules. In contrast to the human specificity of indole, multiple microbial ligands have been found to establish beneficial intestinal functions in murine models. This suggests that the murine AHR is permissive to binding low molecular weight compounds containing indole moieties, but doesn't clarify the varying degrees of interspecies sensitivity to these classes of ligands that yet may exist. Identification of molecular activators of AHR within an organism can be further complicated by the consumption or endogenous syntheses of AHR antagonists[331]. These molecules would actively compete for occupation within the AHR LBD and dictate overall AHR-dependent function, and may too exhibit species-dependent activities.

The identification of indole as a human-specific AHR agonist raises the many questions, such as when did this activity arise during mammalian evolution and what important physiological function does it confer? The evolutionary conservation of the AHR across species, ranging from invertebrates to vertebrates, implies that the AHR is a critical mediator of important biological functions. However, interspecies differences in AHR sensitivity to distinct classes of molecules suggests that differential selection upon the *AHR* locus that could have led to species specific alterations within AHR structure. Our findings indicate that the human AHR and the AHR of other hominid species *e.g. P. troglodytes* are able to respond to indole, suggesting that adaptation of the receptor to accommodate indole arose prior to the splitting of *Homo* and *Pan* lineages. Comparative analyses of the ligand binding domain indicate a single

amino difference (chimpanzee A381, human V381), that is unlikely to affect indole binding. However, further dose response studies are needed in order to more quantitatively assess possible differences in indole sensitivity between chimpanzee and human AHR. Notably, the V381 mutation present in humans is known to be the determinant for a reduced capacity to responds to TCDD and other PAHs, similar to the *Ahr*$^{d/d}$ allele[314, 332]. This implies that additional selection upon the *AHR* locus occurred following the separation of the human and chimpanzee lineages. Mutations within the AHR LBD that facilitate indole responsiveness may provide a foundation for the establishment of an inter-kingdom (host-microbe) signaling paradigm that represents an advantageous commensal relationship between bacteria and their host. The nature of the advantage is speculative, but may involve the establishment of signaling that promotes enhanced maintenance of intestinal homeostasis through AHR stimulation by microbiota-derived indole. This benefit would provide greater protection from intestinal insult, and as a result affect host health, life span, and overall fitness. Indeed recent studies in *Drosophila* present evidence that the maintenance of intestinal integrity is a critical bio-marker of organism health and longevity[333, 334].

In summary, this chapter highlights our findings that the human AHR is permissive for activation by the microbial tryptophan metabolite indole through a novel bi-molecular binding mechanism. The identification of indole as a human AHR agonist further adds to the compendium of known endogenously derived AHR agonists. Given the abundance of indole generation within the intestinal tract by resident microbes coupled with the activation of human AHR at physiologically relevant concentrations, it is likely that indole signaling may have implications in normative host intestinal health. Future studies utilizing “humanized” AHR mice

are required to further elucidate the mechanisms by which microbial indole signaling is able to influence host physiology and disease through the AHR.

CHAPTER 3: DIVERGENT AH RECEPTOR LIGAND SELECTIVITY DURING HOMININ EVOLUTION

3.1 ABSTRACT

Here, we have identified a fixed nonsynonymous mutation within the aryl AHR-LBD of humans (Val381; derived variant) that differs from that of our closest extinct relative, Neanderthals (Ala381; ancestral variant). Comparative exome sequence analyses between three Neanderthals, one Denisovan individual, and nine modern humans identify a total of 90 sites in which archaic hominins are fixed for an ancestral nonsynonymous variant and modern humans are fixed for a derived variant. Of these sites, there exist only 27 examples (including Val381 in the AHR) in which there is no reported variability within the genome of modern humans, according to the human dbSNP database. This finding suggests that the derived Val381 AHR variant was a mutation that occurred during the speciation of *Homo sapiens* and exhibits complete penetrance within modern humans. Functional analyses of the ancestral Ala381 AHR variant, carried by Neanderthals and non-human primates, indicate an enhanced responsiveness to PAHs as evidenced by heightened ligand binding, transformative capacity, and AHR-mediated transcriptional activities when compared to the human Val381 variant. Relative to the human AHR, the Neanderthal AHR exhibited 150-1000-times greater sensitivity to induction of *Cyp1a1* and *Cyp1b1* expression following exposure to PAHs. The resulting CYP1A1/CYP1B1 enzymes are a necessary component of PAH first pass metabolism, which lead to the production of toxic intermediates and possible AHR-associated toxicities. Notably, *H. sapiens* AHR responsiveness to endogenous ligands derived from tryptophan and indole remains unperturbed and is equivalent to that of the ancestral variant. These findings indicate that a functionally significant change in AHR ligand selectivity occurred uniquely in humans, relative to other primates, that would attenuate responsiveness to environmental pollutants, such as those present in wood smoke.

3.2 INTRODUCTION

The AHR is a ligand activated transcription factor, of the basic region helix-loop-helix-PER/ARNT/SIM homology super-family, first identified as the mediator of TCDD toxicity[335]. The receptor possesses a promiscuous ligand binding domain that is responsive to an array of xenobiotics[335]. Upon ligand activation and heterodimerization with its binding partner ARNT, the AHR facilitates increased expression of target genes, such as the drug metabolism enzymes *CYP1A1*, *CYP1A2*, and *CYP1B1*[39, 336]. This pathway represents a negative feedback mechanism that evolved to limit exposure to planar hydrophobic chemicals through recognition/activation of the AHR and subsequent induction of phase one metabolism to promote their clearance. PAHs are well characterized AHR agonists formed from the incomplete combustion of organic materials (*e.g.* wood). PAH exposure through inhalation, dermal, or dietary routes can facilitate activation of the AHR. As a result of this, CYP1A1 and CYP1B1 enzymatic targeting of PAHs is enhanced, leading to the formation of hydroxylated and reactive epoxide PAH intermediates[171]. The chemical nature of these metabolites makes them notoriously reactive and genotoxic and at sufficient concentrations can lead to overt cellular toxicity and death in a CYP1A1-activity dependent manner [337].

While the AHR has been previously established as a primary mediator of xenobiotic metabolism, recent insights suggests that the AHR influences numerous endogenous functions, such as cell cycle progression, metabolism, immune function, and epithelial barrier integrity[338, 339]. Further *in vivo* studies utilizing *Ahr*$^{-/-}$ mice and those done in the absence of exogenous ligand treatment prompted the identification of numerous endogenous, dietary, and microbial derived ligands[340, 341]. Many endogenous or pseudo-endogenous AHR ligands originate from various mechanisms of tryptophan and indole metabolism and have diverse structures that

deviate from that of prototypical exogenous AHR agonists. These observations would suggest that activation of AHR by endogenous and dietary ligands would be of physiological benefit and therefore likely evolutionarily conserved.

Differential AHR ligand sensitivity between species has been previously observed in comparisons of human and mouse AHR homologs[315]. The mouse AHR homolog (*e.g. Ahr*$^{b/b}$) is known to bind exogenous ligands such as TCDD and PAHs with ~10-fold higher affinity than the human AHR [314, 332]. The determinant for this difference was mapped to a single amino acid difference within the LBD, in which the human AHR possesses a valine residue at position 381, whereas the mouse AHR contains an alanine residue at the analogous 375 position[314]. This observation led to the assumption that the human AHR possesses a global loss of ligand affinity relative to the mouse *Ahr*$^{b/b}$ allele. However, recent research has found that the human AHR possesses a higher relative affinity for indirubin, indoxyl sulfate, kynurenic acid, and indole, relative to mouse AHR[104, 231, 232, 342]. These latter studies would suggest that ligand responsiveness of the human AHR is not indiscriminately decreased with regard to all ligands, but that evolutionary selection has produced a uniquely responsive AHR variant. The origin of such differential responsiveness may lie rooted in disparate selective pressures among species that acted upon the *Ahr* locus.

Selective pressure from exposure to environmental or manmade toxicants and there resultant detrimental outcomes could be contributing factor to the microevolution or divergence of species. Insecticide resistance of the common house fly (*Musca domestica*) resulted from over-use of the insecticide dichlorodiphenyltrichloroethane (DDT). Resistance to DDT was conferred by a mutation within the promoter of the *Cyp6g1* gene that facilitated over expression of the peptide and rapid metabolism of DDT[343]. Constant exposure to PCBs within contaminated

water was found to promote a selective mutagenesis of the *Ahr2* locus within populations of Atlantic Tomcod (*Microgadus tomcod*). Tomcod populations that inhabited the polluted waters of the Hudson River were found to possess a higher allelic frequency of an *AHR2* polymorphism (6 base deletion in the LBD) relative to those from non-contaminated waters[344]. This deletion mediates decreased affinity of AHR for PAHs, PCBs, and ablates AHR-mediated toxicity of these compounds. A similar loss of AHR transcriptional activity was found to be associated tolerance of PCB-polluted waters in Atlantic killifish populations[345]. A recent evaluation of four separate killifish populations identify multiple genetic variants within the AHR signaling pathway, such as *AHR*, *XAP2*, *ARNT*, or *CYP1A1*, indicating toxin tolerance could be mediated through selective targeting of multiple molecular targets within a common signaling pathway[346]. Notably, it is plausible for PCB-tolerant fish populations to have existed prior to habitat pollution. However, exposure of PCB-sensitive populations to environmental toxins likely led to acute embryo mortality under conditions of sustained AHR activation. This selective pressure would lead to rapid expansion of PCB-tolerant phenotypes that may have initially represented only a small proportion the overall population. Such evidence indicates that environmental toxicant exposure and resultant AHR activation can promote rapid selection in populations that promotes divergence of ligand responsiveness.

Paleogenetics is of heightened interest following the publication of the *H. neanderthalensis* and contemporary *H.sapiens* genomes[347-349]. Variation of ancestral peptide homologs and consequent differences in biological function allow researchers to question how polymorphisms within the genome might have influenced Darwinian selection and speciation of anatomically modern humans. Mutations in genes such as Forkhead box protein 2 (*FOXP2*), a determinant of speech and language development, and alcohol dehydrogenase class IV (*ADH4*),

a gene involved the metabolism of alcohols, have been investigated for possible roles in the evolutionary divergence of *hominids*[350, 351].

Environmental factors responsible for promoting the positive or negative selection of various traits specific to anatomically modern humans remain to be elucidated. Learned traits, such as the use of fire and cooking of food, would have numerous impacts upon the environment and fitness of upper Paleolithic *H. sapiens* and *H. neanderthalensis*. The controlled use of fire would have provided a source of light, heat, and cooked meals. Cooking rather than raw consumption of diets has many benefits, such as increased efficiency of caloric utilization from foods and decreased risk of bacterial contamination and subsequent infection[352, 353]. It is theorized that increased caloric/nutrient utilization from cooked foods may have been a contributing factor to the rapid expansion of brain volume and digestive tract shortening that occurred in the development of anatomically modern humans between two million and eight hundred thousand years ago[354, 355]. While the benefits of cooking/fire use are numerous, they came at the cost of continuous exposure to a multitude of PAHs, such as benzo(a)pyrene and benzanthracene, present in wood smoke[356].

Here we examine the evolutionary divergence of AHR ligand selectivity in primates, more specifically; we compare the differences in transcriptional activity of Neanderthal and human AHR following exposure to exogenous and endogenous ligands. Phylogenetic analysis of *AHR* nucleotide sequences among primate species revealed that the determinant for reduced PAH sensitivity by a valine substitution at position 381 is unique to modern humans. In addition, our experimental observations demonstrate that reduced PAH sensitivity by human AHR does not affect responsiveness to endogenous ligands relative to other primates. These results demonstrate that evolutionary selection has adapted the AHR to produce a biological sensor that

is distinctive in its responsiveness to environmental toxins and endogenous ligands, the combination of which is unique to *H. sapiens* relative to our closest ancestors.

3.3 MATERIALS AND METHODS

Reagents: TCDF was obtained from AccuStandard. 1,2-benz(*a*)anthracene and benzo(*a*)pyrene were purchased from Sigma. Indoxyl-3-sulfate and indirubin were purchased from Alfa Aesar and Enzo Life Sciences, respectively. Indole was purchased from Sigma and purified as previously described [342]. The photoaffinity ligand 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin was synthesized as described [357]. Cultured supernatant from monoclonal antibody hybridoma clone RPT1 was produced as previously described [29]. COS-1 and BP8 cells were obtained from American Type Culture collection (Manassas, VA) and Martin Göttlicher (Institute of Toxicology and Genetics, Eggenstein, Germany), respectively. BP8 cells are deficient in AHR expression and were generated as described [358].

Plasmids and mutagenesis: The *H. sapiens*, *P. troglodytes*, and *C. sabaeus* AHR cDNA optimized for mammalian codon use and minimal secondary mRNA structure were synthesized by GenScript (Piscataway, NJ) and were inserted into pcDNA3. The nucleotide sequences for these cDNAs are shown in supplemental figure 5. The plasmid pSV-Sport1-ARNT was kindly provided by Oliver Hankinson (University of California, Los Angeles).

Electrophoretic Mobility Shift Assays: Electrophoretic mobility shift assays were performed using *in vitro* translated AHR of a given species as indicated and human ARNT generated via TnT® Coupled Reticulocyte Lysate Systems according to manufacturer's protocol (Promega, Madison, WI). Hominid AHR *in vitro* translations were supplemented with sodium molybdate to a final concentration of 1.25 mM to enhance stability of the receptor. The AHR and ARNT proteins were combined at a 4 μL:4 μL, supplemented with 1.5 μL of HEDG buffer (25 mM HEPES, 1mM EDTA, 10mM sodium molybdate, and 10% glycerol), with the addition of indicated treatments (0.5 μL) and incubated at room temperature for 15 min. Following treatment

incubation, $2x10^6$ cpm of ^{32}P labeled DRE oligonucleotides were added to each reaction and incubated at room temperature for an additional 15 min. Upon addition of 2 μL of 0.25% xylene cyanol in 20% (w/v) Ficoll to the reaction mixture, samples were loaded onto a 6% non-denaturing DNA retardation gel (Invitrogen, Carlsbad, CA) and separated by electrophoresis. The gels were fixed in a 7:1:1:1 water:methanol:acetic acid:glycerol solution for 20 min, dried for 60 min, and then subjected to autoradiography for analysis.

mCcl20_3.1kb DRE Oligonucleotide EMSA Sequence

Ccl20-3.1kb DRE-F-5'-TTG TGT GTG TGC GTG TGT GCG TGT GTT AC-3'

Ccl20-3.1kb DRE-R-5'-TGT AAC ACA CGC ACA CAC GCA CAC ACA C-3'

Cell Culture: COS-1 and BP8 cell lines were maintained in α-modified essential media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan UT). Cells were cultured at 37 °C in a humidified atmosphere composed of 95% air and 5% CO_2 in the presence of 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich).

AHR Ligand Binding Assay: COS-1 cells were grown to 90% confluency in 6-well cell culture plates. Cells were transfected using the Lipofectamine® 2000 transfection reagent and PLUS™ reagent (Life Technologies, Carlsbad, CA) with 1.5 μg of AHR plasmid construct per well, according to the manufacturer's protocol. All binding experiments were performed in the dark prior to photo-crosslinking. These experiments were done essentially as described previously [314, 359]. Media from the transfected cells in six-well plates was removed, and fresh media containing the appropriate amount of photoaffinity ligand was added to each well. Fresh media containing 0, 0.5, 1.0, 1.5, 2.0, or 2.5 pmoles of AHR photoaffinity ligand, 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin was applied to cells for 1 h (in duplicate) at 37°C in 95% air/5%

carbon dioxide. The bound PAL was cross linked to AHR via UV cross linking (302 nM) for 4 min at a distance of 8 cm. Cells were then lysed in 200 μL of lysis buffer [MENG, pH 7.28, 20 mM sodium molybdate, 500 mM sodium chloride, protease inhibitor cocktail and 1%(v/v) NP-40]. Samples incubated on ice and were spun at 13,000 x g for 10 min. A volume of 50 μL of the lysate supernatant per sample was resolved upon an 8% Tricine/SDS polyacrylamide gel. Protein and bound PAL were transferred to polyvinylidene fluoride (PVDF) membrane and visualized by autoradiography. Band intensities were quantified by filmless auto-radiographic analysis using a Cyclone storage phosphor screen instrument (PerkinElmer Life and Analytical Sciences). Band intensities were determined by γ-counting or filmless auto-radiographic analysis, and both methods yield similar results.

BP8 cellular transfection/AHR ligand dose-response Assays: Rat hepatoma BP8 cells that lack AHR expression were plated to 90% confluency in 12-well cell culture plates and transfected using the Lipofectamine® 3000 transfection reagent (Life Technologies, Carlsbad, CA) with a total of 1 μg of plasmid DNA per well. Transient transfections utilized pcDNA3.syn.hAHR or pcDNA3.syn.NeAHR were tittered for equal protein expression between constructs. Upon completion of the transfection at 6 h, Lipofectamine reagents were removed and replaced with Opti-MEM® (Life Technologies, Carlsbad, CA) supplemented with 2% fetal bovine serum. After 17 h post transfection, cells were treated as indicated for 4 h. Total RNA was isolated from cells using TRI Reagent (Sigma-Aldrich), followed by reverse transcription using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD) was used to determine mRNA levels, and analysis was conducted using MyIQ software, in conjunction with a CFX Connect™ Real-Time System (Bio-Rad

Laboratories, Hercules, CA). To account for potential differences in AHR expression or transfection efficiency, quantification of ligand-induced *Cyp1a1*/*1b1* mRNA level was transformed to represent percentage of maximal induction elicited by a saturating dose of TCDD (20 nM) for *H. sapiens* and *H. Neanderthalensis* AHR transfected cells respectively. Data were subjected to non-linear regression using a four parameter variable slope $\log_{10}$[ligand] v response curve-fitting model to yield final dose-response plots and EC_{50} values (GraphPad Prism).

Real-time PCR Primers

Rat *Cyp1a1*-F 5'- CCC TAA CTC TTC CCT GGA TGC-3'

Rat *Cyp1a1*-R 5'- GGA TGT GGC CCT TCT CAA ATG-3'

Rat *Cyp1b1*-F 5'- GAC ATC TTTG GAG CCA GCC A -3'

Rat *Cyp1b1*-R 5'- TCC GGG TAT CTG GTA AAG AGG A -3'

Rat *Rpl13a*-F 5'- AAG CAG CTC TTG AGG CTA AGG -3'

Rat *Rpl13a*-R 5'- TGG GTT CAC ACC AAG AGT CC -3'

Plasmid Mutagenesis: The Neanderthal AHR expression and mutant constructs were synthesized by QuikChange Site-Directed Mutagenesis (Agilent Technologies, Wilmington, DE) of the corresponding synthetic human AHR construct (pcDNA3.syn.hAHR) according to manufacturer's protocols. The V381A and R554K codons were altered to generate the Neanderthal AHR cDNA.

Mutagenesis Primers

hAHR-V381A-F 5'- GAT TAC ATC ATC GCT ACC CAG CGG CCC-3'

hAHR-V381A-R 5'-GGG CCG CTG GGT AGC GAT GAT GTA ATC -3'

hAHR-R554K-F 5'-CCT GGG CAT CGA TTT CGA AGA CAT CAA GCA CAT GCA GAA CG -3'

hAHR-R554K-R 5'-CGT TCT GCA TGT GCT TGA TGT CTT CGA AAT CGA TGC CCA GG -3'

Protein electrophoresis and Western blot analysis: Following Transfection**, c**ells were lysed in RIPA buffer (Tris 100 mM, EDTA 1mM, Sodium Molybdate 20mM, EGTA 0.5 mM, sodium chloride 140 mM, deoxycholate 0.1%, SDS 0.1%, Triton X-100 1%, protease inhibitor cocktail), 100 μg of protein was resolved by Tricine SDS-PAGE, and transferred to PVDF membrane. The relative level of AHR was determined using mAb RPT 1, a biotinylated goat anti-mouse IgG secondary antibody and [^{125}I]streptavidin. B-actin detected with mAb B-actin (C4, Santa Cruz Biotechnology) was utilized as a loading control. Protein bands were visualized using autoradiography and quantified by densometric analyses.

***In silico* molecular modeling:** The homology model of the human AhR-PASB-LBD based on the NMR apo of the HIF-2a-PASB (PDB 1P97) was prepared and optimized as described [360]. Molecular docking was run as previously described [342].

Statistical Analysis: Data in the ligand binding assays were analyzed using one-way ANOVA with Tukey's multiple comparison post-test using GraphPad Prism (v.5.01) software to determine statistical significance between treatments. Data represents the mean change in a given endpoint +/- s.e.m. (n=2/treatment group) and were analyzed to determine significance (**P,0.01; ***P<0.001).

Genomics/Database analysis: We first queried the database of archaic hominin and modern human 'exome' genetic variation produced by Castellano et al. [361]. The nucleotide variants within this database were identified based on paleogenomic sequence data from three Neanderthal individuals (Altai, El Sidron, and Vindija) and one individual from the Denisovan archaic hominin population, plus genome sequence data from nine modern human individuals (three individuals each with sub-Saharan African, European, and Asian ancestry). Genotypes were estimated for only those positions covered by a minimum of six independent sequencing reads in each individual, and ancestral and derived variant states were determined via comparisons to the gorilla and orangutan genome sequences. We used the program PolyPhen-2 [362] to infer whether each nucleotide variant in the database was nonsynonymous (amino acid changing; potentially functional) or synonymous (resulting in no change to the amino acid sequence; typically neutral with respect to fitness). Of the 15,383 nonsynonymous SNPs in the database for which ancestral and derived states could also be determined, only 90 were fixed for the derived allele in the modern human sample but the ancestral allele in the archaic hominin sample (Neanderthals plus the Denisovan individual). We then queried the dbSNP database (Build 144) to discover that there is no record of variation in modern humans for 27 of these positions. We also queried the 1000 genomes database [[363]; 2/19/15] for record of modern human *AHR* gene variation. We further examined the full AHR-binding region of one Neanderthal individual [364] and one Denisovan individual [365], for which high-coverage genome sequence data are available (these data are also represented in Castellano et al.[361] database, but with the omission of some lower-coverage sites for the other two Neanderthal individuals). For the Neanderthal individual, we downloaded the read alignment file from the European Nucleotide Archive (accession ERP002097; L9105.bam) and used the variant detector program called

freebayes to reconstruct the Neanderthal reference AHR protein sequence [366]. For the Denisovan sequence, we used an alignment track from the UCSC genome browser [367]. We also downloaded the genome sequencing read alignment file for a 45 ky modern human individual (European Nucleotide Archive PRJEB6622) [349] and used freebayes for variant calling. Multiple sequence alignments were performed using the program Clustal Omega [368].

3.4 RESULTS

The human AHR contains a V381 residue that is unique among primates

Previous studies from our laboratory have identified the location of a single amino acid residue at position 381 within the human AHR LBD as a determinant of exogenous ligand binding affinity [15, 314]. The human AHR contains a valine at residue 381 (V381), whereas the mouse AHR contains an alanine at the analogous 375 residue. The human AHR valine substitution results in a 10-fold reduction in affinity for the radiolabeled dioxin analog 2-azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin (PAL)[314]. This observation prompted an evaluation of AHR conservation across mammalian species through sequence comparison of mouse, human, chimpanzee, and African green monkey using reference sequences available from the NCBI database **(Figure 3.1)**. Chimpanzees are our closest extant relative with 99% of our genomic DNA conserved between species. Examination of AHR LBD amino acid sequences identified conservation of an alanine at position 381 among non-human primates. The lack of AHR V381 retention among non-human primates suggested that the origin of this mutation occurred after the separation of human and chimpanzee lineages. The recent availability of archaic hominin genomic sequences from Neanderthals (99.5% DNA conserved with human) and the Denisovan man (an archaic hominin population more closely related to Neanderthals than modern humans) prompted further comparative analyses of the AHR[361, 364, 365]. We first considered a database of genome-wide gene coding region ('exome') variants among population samples of humans (n=9 individuals or n=18 diploid chromosomes), Neanderthals (n=3 individuals; n=6 chromosomes), and one Denisovan individual (n=2 chromosomes), based on published paleogenomic sequence data [361, 364, 365]. Interspecies comparison of the *AHR* locus confirmed that the AHR is highly conserved (~99.7%) among evaluated hominins **(Figure 3.2)**. However, humans and archaic

hominins were found to segregate at two distinct amino acid residues. Neanderthals and the Denisovan individual were found to be fixed for a lysine residue within the transactivation domain at codon 554 (K554), whereas modern humans are polymorphic at this location, possessing either an arginine or lysine (R554 or K554). Previous investigations have been unable to ascribe alteration of AHR function dictated by variability at residue 554[369]. Interestingly, paleogenomic AHR sequence analyses also found Neanderthals and the Denisovan man to be fixed for the ancestral variant at codon 381 (A381) in contrast to humans, which were found to be fixed for the derived variant (V381) **(Figure 3.3)**. The ethnic distribution of the V381 was found to be invariant among modern humans through AHR sequence evaluation of the 1000 Genomes Project and the dbSNP databases. Recently, the genomic sequence of a 45,000 year old anatomically modern human became available[349]. This Paleolithic human was also homozygous for the V381 AHR variant, suggesting that the derived variant's origins are not a consequence of modern selective pressures. The occurrence of fixed nonsynonymous exomic variations between humans and archaic hominin genomes is considered rare with only 90 total exomal variants observed[361]. Notably, only 27 of these sites, including the V381 AHR variant, also exhibit no reported variability (according to the dbSNP) and are therefore considered invariant within modern humans **(Table 3.1)**. Furthermore, the AHR was the only protein among those analyzed to have implications in an organism's response to environmental stimuli (e.g. xenobiotic metabolism).

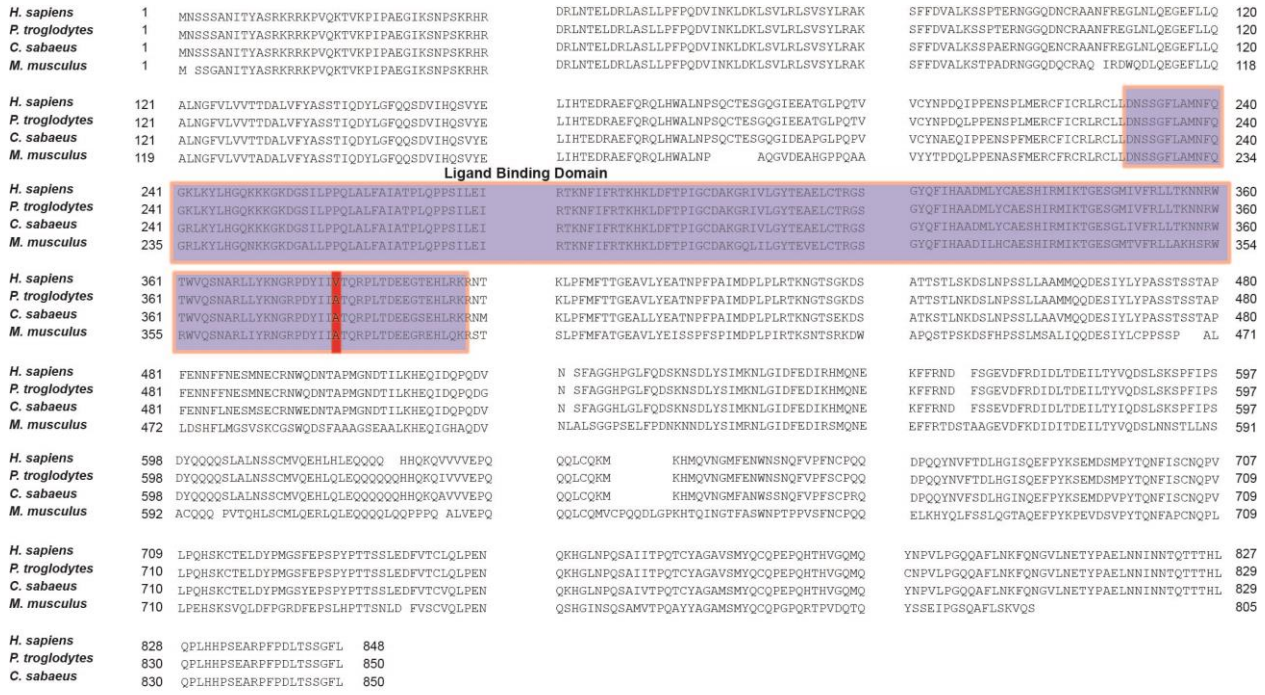


Figure 3.1: Mammalian divergence of AHR structure.
Amino acid sequences for *H. sapiens* (human), *P. troglodytes* (chimpanzee), *C. sabaeus* (African green monkey), and *M. musculus* (mouse) were aligned using NCBI protein-protein blat (blastp) algorithm. AHR ligand binding domain is highlighted in blue, nonsynonymous variant at position 381 (primates) or 375 (mouse) are indicated in red.

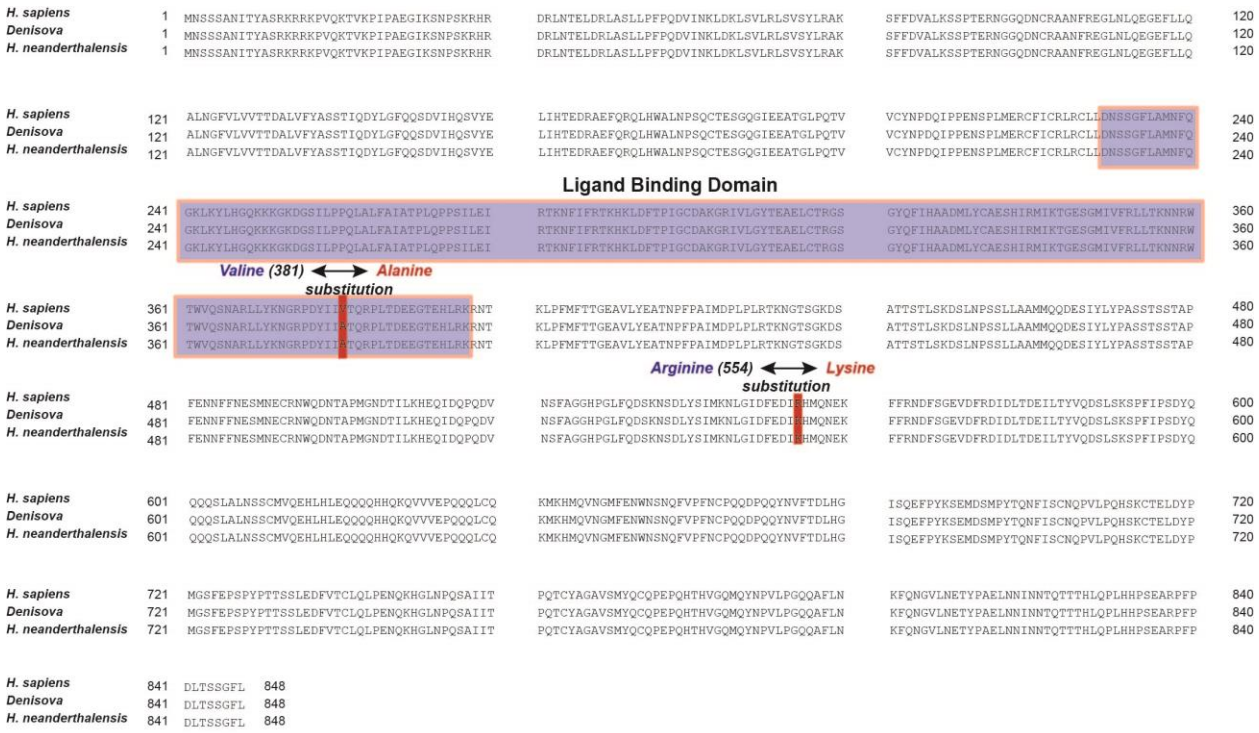


Figure 3.2: Hominin divergence of AHR structure.
Amino acid sequences for *H. sapiens* (human), Denisova, and *H. neanderthalensis* (Neanderthals) were aligned using NCBI protein-protein blat (blastp) algorithm. AHR ligand binding domain is highlighted in blue, nonsynonymous variants at position 381 and 554 are indicated in red.

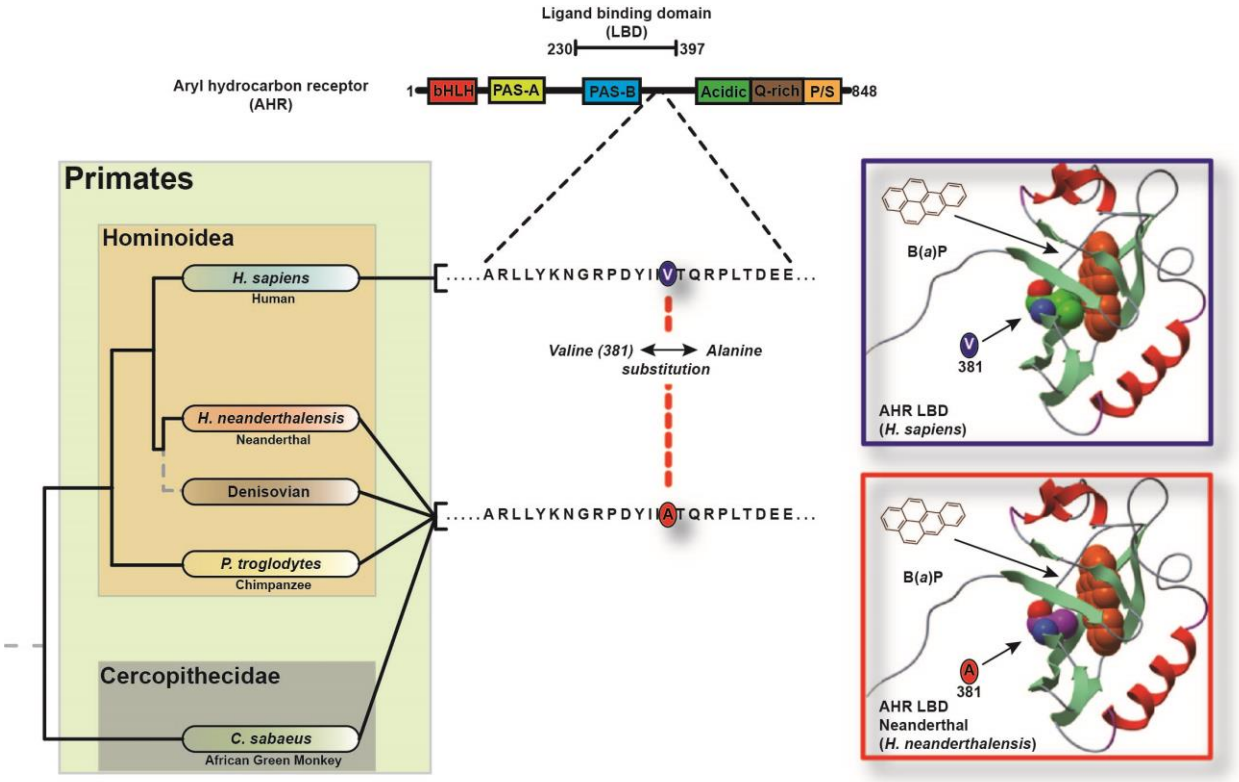


Figure 3.3: The human AHR ligand-binding domain segregates from other primates.
The diagram displays a map of AHR functional domains and the single amino acid variation in the human AHR relative to other *Hominoidea*. The homology modeling depicts the differential orientation of the compound benzo(a)pyrene within the LBD of human versus Neanderthal AHR.

Data Table 3.1: Exomes that are fixed for a derived nonsynonymous variant in humans compared to Neanderthal/Denisovan that also exhibit no reported dbSNP variability.

Gene	Protein	Gene	Protein
SPAG 17	Sperm Associated Antigen 17	***CCDC15***	Coiled-Coil Domain Containing 15
SPAG17	Sperm Associated Antigen 17	***PRDM10***	PR Domain Containing 10
SLC27A3	Solute Carrier Family 27 Member 3	***NOVA1***	Neuro-Oncological Ventral Antigen 1
ZBTB24	Zinc Finger And BTB Domain Containing 24	***GPR132***	G Protein-Coupled Receptor 132
AHR	Aryl Hydrocarbon Receptor	***KIAA1199***	Cell Migration Inducing Protein, Hyaluronan Binding
DNAH11	Dynein, Axonemal, Heavy Chain 11	***CDH16***	Cadherin 16, KSP-Cadherin
ADAM18	ADAM Metallopeptidase Domain 18	***SPAG5***	Sperm Associated Antigen 5
RB1CC1	RB1-Inducible Coiled-Coil 1	***SSH2***	Slingshot Protein Phosphatase 2
NEK6	NIMA-Related Kinase 6	***RFNG***	RFNG O-Fucosylpeptide 3-Beta-N-Acetylglucosaminyltransferase
NR6A1	Nuclear Receptor Subfamily 6, Group A, Member 1	***KIAA1772***	Growth Regulation By Estrogen In Breast Cancer-Like
C9orf86	RAB, Member RAS Oncogene Family-Like 6	***C19orf28***	Major Facilitator Superfamily Domain Containing 12
ARRDC1	Arrestin Domain Containing 1	***RSPH1***	Radial Spoke Head 1 Homolog (Chlamydomonas)
FAM178A	SMC5-SMC6 Complex Localization Factor 2	***ADSL***	Adenylosuccinate Lyase
DCHS1	Dachsous Cadherin-Related 1		

The human AHR exhibits reduced binding of TCDD analogs relative to Neanderthal AHR

The archaic hominin A381 AHR variant is conserved among other non-human primates and rodents, and is known to mediate an enhanced affinity for binding of TCDD and PAHs relative to the human V381 variant [314, 332, 370]. *In silico* modeling of B(a)P docking within the human or Neanderthal AHR LBD suggest that the amino acid residue present at position 381 may directly affect occupancy. The model suggests that the smaller alanine at position 381 mediates a more sterically accommodating ligand-binding cavity, than the larger valine residue **(Figure 3.3)**. To empirically assess differential ligand binding capacities of Neanderthal and human AHR, cell-based ligand binding assays were utilized **(Figure 3.4)**. In these assays AHR-deficient COS-1 cells were transiently transfected with AHR expression constructs in such a manner to facilitate equal expression of human or Neanderthal AHR protein **(Figure 3.4A)**. Following transfection, cells were incubated with increasing doses of the radiolabeled dioxin analog PAL **(Figure 3.4B)**. The PAL compound is structurally similar to TCDD and mimics binding mechanics of other AHR ligands, such as dibenzofurans and PAHs. Quantitation of AHR-PAL binding interactions revealed enhanced covalent interactions of the PAL with Neanderthal AHR, relative to human **(Figure 3.4C)**. Reciprocal mutation of the V381 to A381 or vice versa within the context of human/Neanderthal AHR proteins confirmed the 381 residue as the sole determinant for differential capacity to bind dioxin-like compounds. Therefore form this data we presume that a substitution of an arginine/lysine at position 554 does not significantly contribute to ligand binding selectivity of the human or Neanderthal AHR. Due to the high radio-specific activity of the PAL, the amount of ligand used was significantly below the threshold required to saturate the ligand-binding domain.

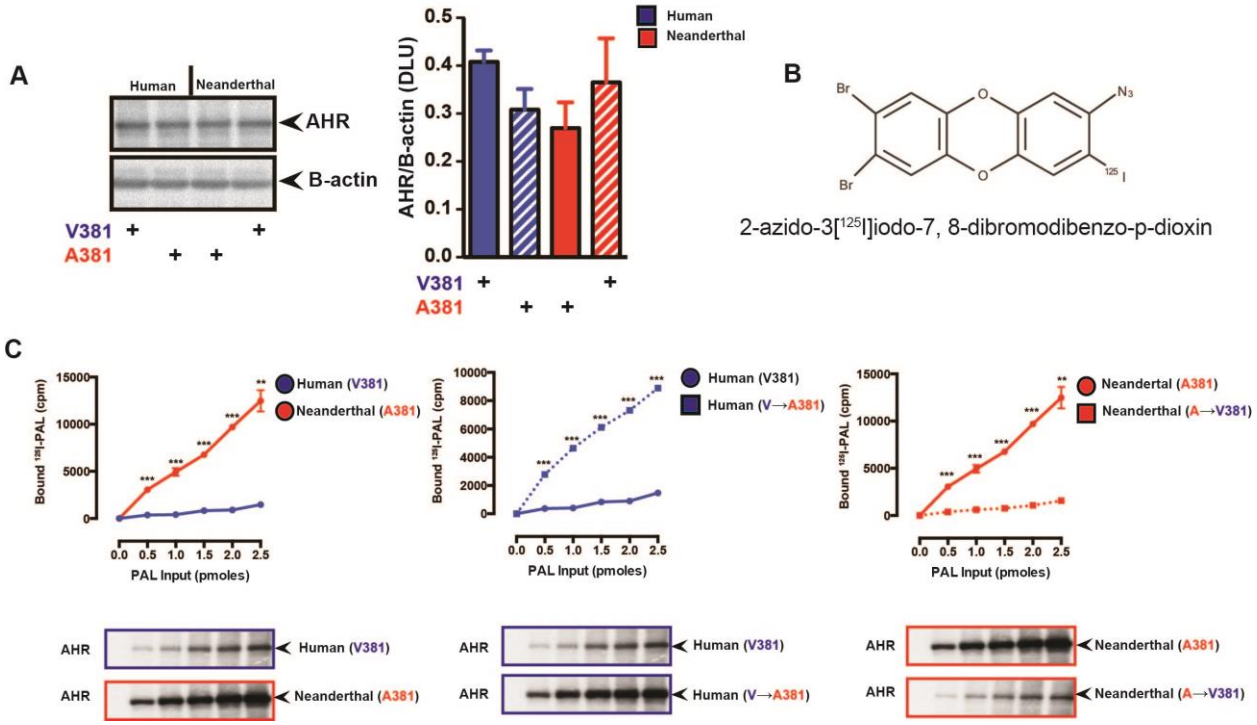


Figure 3.4: Neanderthal AHR exhibits enhanced binding of dioxin analogs relative to the human AHR.

(A) Equivalent expression of AHR in COS-1 cells was assessed by western blot analysis. **(B)** Structure of AHR photo-affinity-ligand (PAL). **(C)** COS-1 cells transiently expressing human, Neanderthal, or indicated mutants were incubated with increasing concentrations of PAL, cells were exposed to UV-light, and cellular extracts containing covalently cross-linked AHR/PAL were analyzed by SDS-PAGE and quantified.

The human AHR exhibits reduced transformation activity following exposure to PAHs, relative to the Neanderthal receptor

Following ligand stimulation, the AHR initiates a process of receptor transformation characterized by a change in structure that facilitates translocation into the nucleus, heterodimerization with ARNT, and binding to cognate DNA response elements within the promoter of target genes. The capacity for various PAH ligands (TCDF, B(*a*)P, and BA) to induce differential rates of AHR transformation were assessed using electrophoretic mobility shift assays with *in vitro* translated AHR protein **(Figure 3.5)**. Efficiency of translations was assessed by western blot to ensure equivalent AHR protein levels between all expression constructs **(Figure 3.5 B)**. Comparison of human, Neanderthal, and their reciprocal A↔V831 mutant AHR forms revealed enhanced PAH-induced transformation and resultant DNA-binding dependent in AHR isoforms containing an alanine residue at position 381. Conversely, the presence of valine at position 381 mediated a decrease in transformation potential as evidenced by a reduction in DNA-binding **(Figure 3.5C)**. This effect was further substantiated through investigation of B(*a*)P mediated transformation of other primate AHR homologs (chimpanzee and African green monkey) **(Figure 3.5C)**. In support of our previous results, the enhanced capacity for PAHs to mediate receptor transformation segregated among species by the amino acid encoded at residue 381 (alanine or valine).

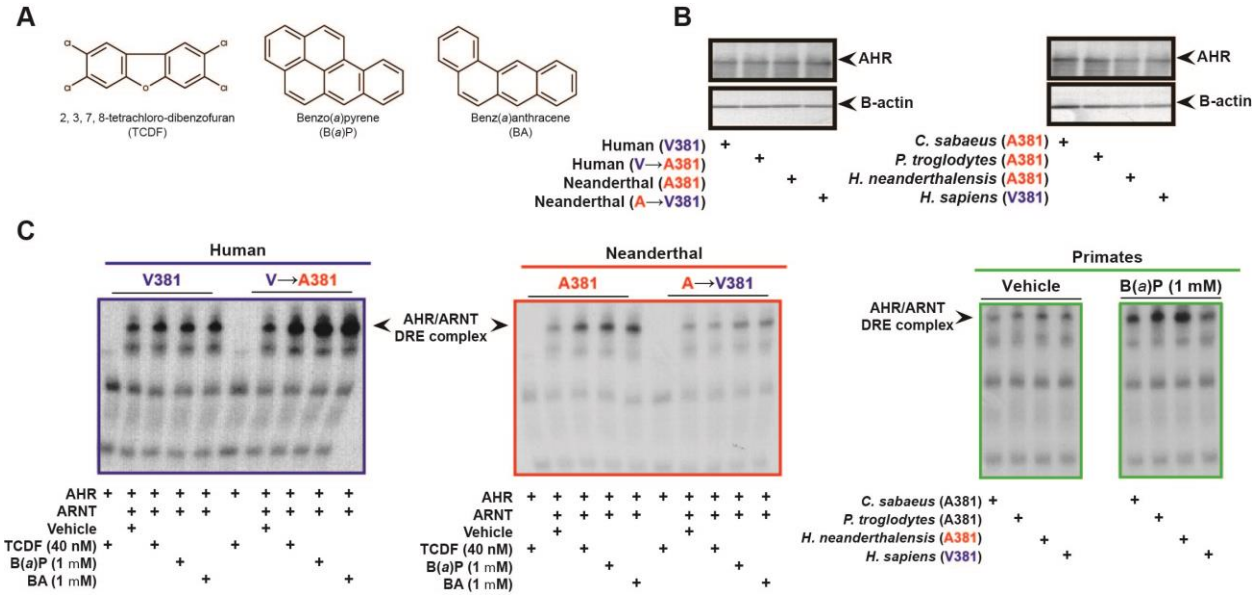


Figure 3.5: Residue 381 is the determinant for enhanced AHR transformation and DNA-binding capacity following exposure to exogenous ligands.
(A) Structures of exogenous AHR ligands tested. **(B)** AHR expression within *in vitro* translations as measured by western blot. **(C)** Interspecies analyses of exogenous ligand responsiveness through use of electrophoretic mobility shift assays using in vitro translated AHR/ARNT protein.

The Neanderthal AHR exhibits heightened PAH-induced transcriptional activity, when compared with the human receptor

The contribution of the V↔A381 fixed difference between human and Neanderthal AHR to PAH-induced transcriptional activity was assessed by dose-responsive quantitative PCR analyses of *Cyp1a1/1b1* expression in transiently transfected AHR-deficient BP8 cells. Again, transient transfections were optimized to ensure equal AHR expression levels **(Figure 3.6A)**. Transcriptional induction of *Cyp1a1/1b1* proved dose responsive with each PAH examined in the cells expressing equal levels of human or Neanderthal AHR. However, exposures to increasing concentrations of PAHs were found to produce differing transcriptional responses among cells expressing human or Neanderthal AHR **(Figure 3.6B)**. The Neanderthal AHR exhibited at least two orders of magnitude heightened sensitivity to ligand-mediated induction of *Cyp1a1*/*1b1* over human AHR, as evidenced by a decrease in fifty percent maximal effective concentration (EC_{50}) values **(Figure 3.6C)**. BP8 cells were ideal for these studies due to their inherent lack of functional AHR protein, which results in no observable ligand mediated transcription of AHR target genes without the input of functional AHR protein **(Figure 3.7A)**. Therefore, all increases in ligand-mediated target gene expression (*Cyp1a1/Cyp1b1*) are solely mediated by the AHR homolog transiently expressed within the cells. Also the relative levels of *Cyp1a1* and *Cyp1b1* induced by the Neanderthal and human AHR were similar following treatment with a saturating dose of TCDD (20 nM) **(Figure 3.7B)**.

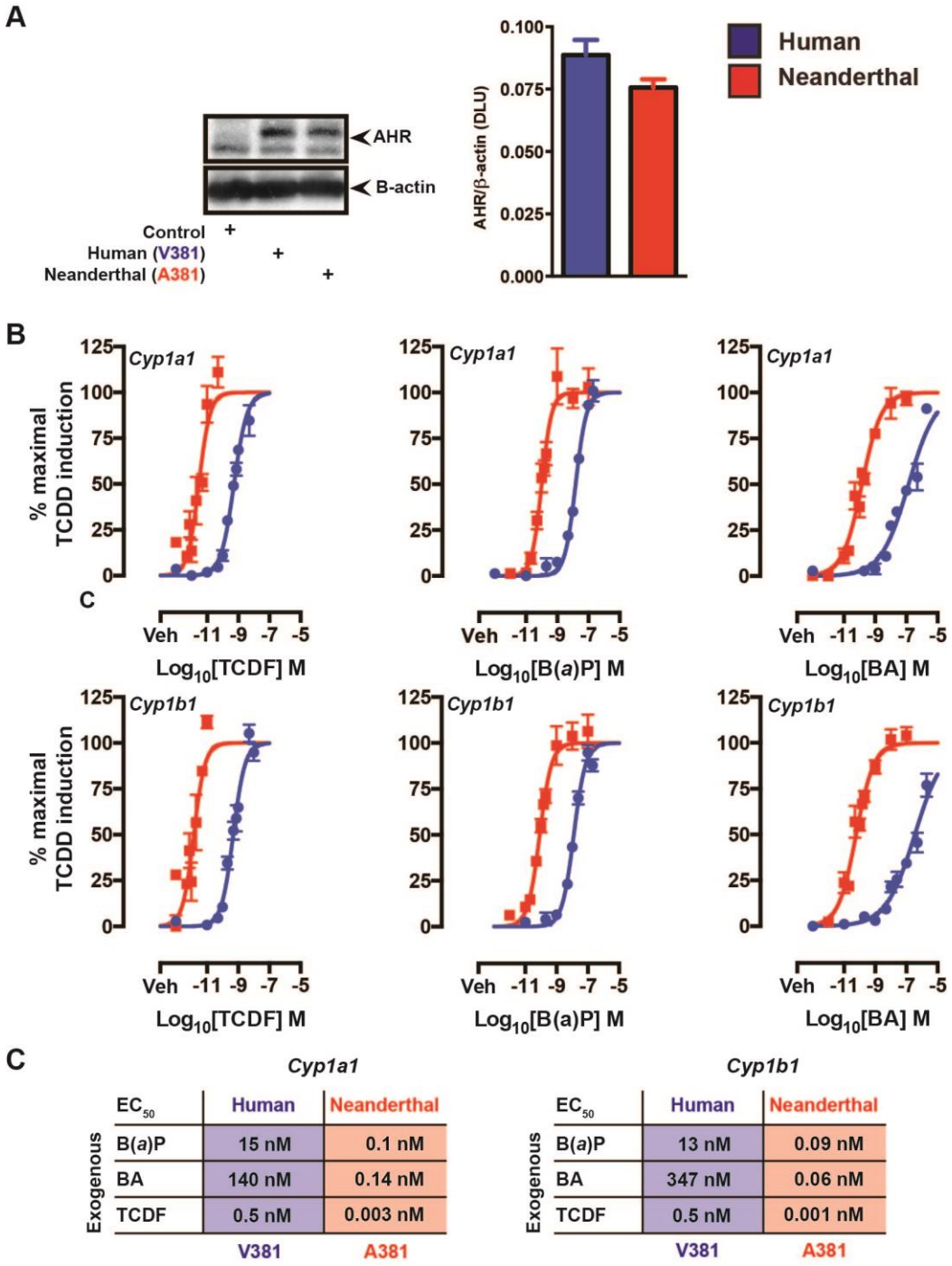


Figure 3.6: Comparative analysis of PAH mediated transcriptional activity of the Neanderthal and human AHR.

(A) AHR expression in transiently transfected BP8 cells as measured by western blot analysis. **(B)** Dose-dependent induction of *Cyp1a1* and *Cyp1b1* expression mediated by Neanderthal or human AHR upon PAH treatment in transfected BP8 cells. Target gene induction was normalized to Rpl13a expression. **(C)** Summary of EC50 values for the dose response experiments in panel B.

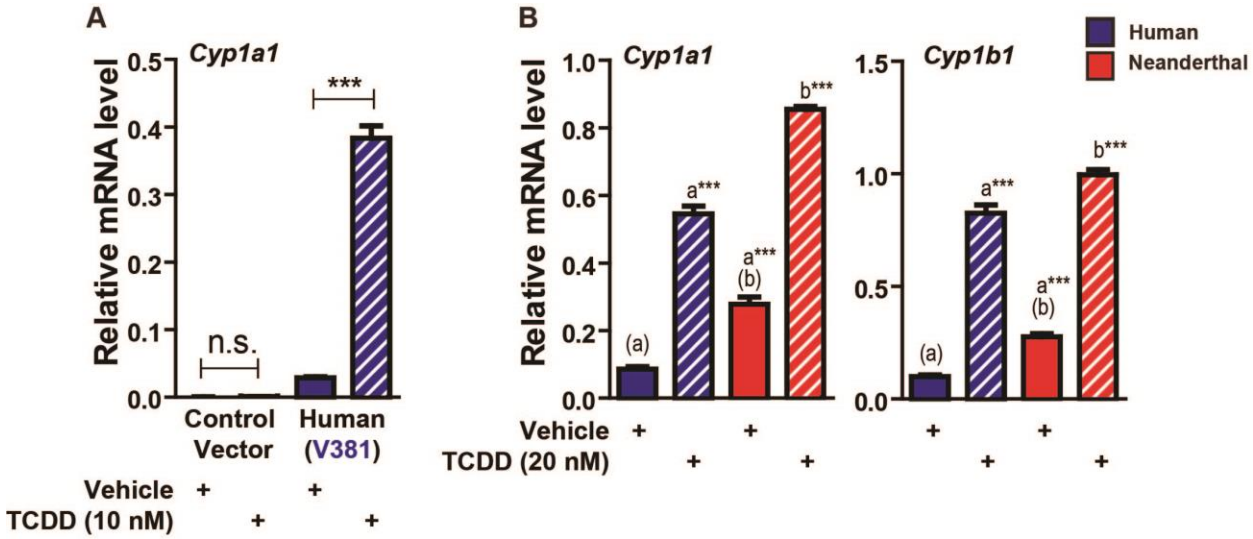


Figure 3.7: BP8 cells are conducive for comparison of transfected AHR expression constructs.

(A) BP8 cells in the absence of ectopic AHR expression are incapable of inducing *Cyp1a1* expression following TCDD treatment. **(B)** Transient transfection of equivalent levels of human and Neanderthal AHR induce similar relative expression of *Cyp1a1/1b1* following treatment with TCDD. Target gene induction was normalized to Rpl13a expression.

The human and Neanderthal AHR exhibit similar levels of activation by endogenous ligands

The previous data suggests that the xenobiotic sensing function of the AHR would differ drastically between human and Neanderthal species. However, expanding physiological roles of the AHR propose further mechanisms to modulate receptor activity through interactions with structurally diverse endogenous or pseudo-endogenous ligands, such as indirubin, indoxyl sulfate, and indole. Due to the apparent contrast in structure it is conceivable that endogenous ligands may interact with the AHR LBD through a mechanism that is distinct from prototypical exogenous ligands. Therefore, we examined if the derived A→V381 desensitization to PAH ligands, observed in the human AHR, would also impact AHR-dependent responsiveness to endogenous ligands. Previous studies have indicated that human AHR (V381) is more sensitive to endogenous ligand treatments then mouse AHR (A375)[231]. However, electrophoretic mobility shift assay studies investigating the transformative capacity of indirubin, indoxyl sulfate, and indole upon human and Neanderthal AHR failed to demonstrate a difference in AHR/ARNT DNA binding activities between species **(Figure 3.8A/B)**. Reciprocal mutations of V↔A381 within human or Neanderthal AHR also reported no observable alteration of DNA binding capacity in response to endogenous ligand treatments **(Figure 3.8B)**. Additionally, endogenous ligand-induced DNA binding activities showed no variability among other primate species (chimpanzee and African green monkey), irrespective of the A/V381 presence within the AHR LBD **(Figure 3.8B)**. These studies indicate that the AHR A/V381 status is not required for establishment of endogenous ligand sensitivity. To further confirm this principle we assessed the contribution of the V↔A381 substitutions upon the AHR dependent transcriptional response following exposure to endogenous ligands. In this study AHR-deficient BP8 cells were made to

express equivalent levels of human or Neanderthal AHR protein and treated with increasing concentrations of indirubin, indoxyl sulfate, or indole. Following treatment AHR-dependent transcriptional activity was assessed by quantitative PCR analyses of *Cyp1a1/1b1* expression. Expression of *Cyp1a1/1b1* proved dose responsive with each endogenous ligand tested in the context of human and Neanderthal AHR **(Figure 3.9A)**. Both human and Neanderthal AHR were found to exhibit equivalent endogenous ligand-mediated transcriptional induction of *Cyp1a1/1b1*, as evidenced by near equivalent EC_{50} data **(Figure 3.9B)**.

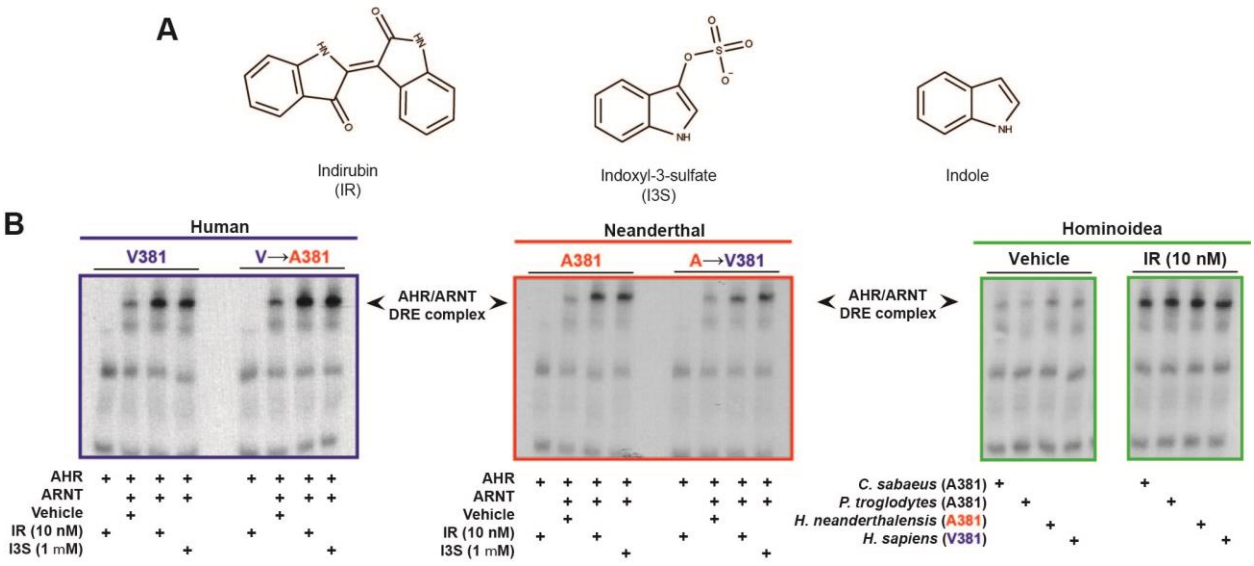
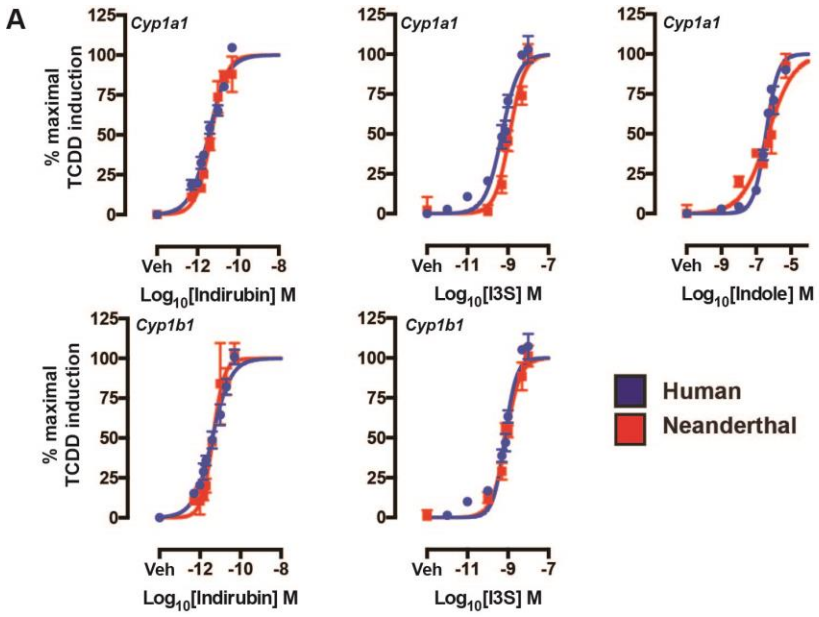


Figure 3.8: Residue 381 does not affect AHR transformation and DNA-binding capacity following exposure to endogenous ligands.
(A) Structures of AHR endogenous ligands tested. **(B)** Interspecies analyses of endogenous ligand responsiveness through use of electrophoretic mobility shift assays using in vitro translated AHR/ARNT protein.



B

Endogenous	EC50	Human	Neanderthal
	Indirubin	0.004 nM	0.005 nM
	I3S	0.4 nM	1.2 nM
	Indole	362 nM	473 nM
		V381	A381

Endogenous	EC50	Human	Neanderthal
	Indirubin	0.004 nM	0.004 nM
	I3S	0.7 nM	0.8 nM
	Indole	nd	nd
		V381	A381

Figure 3.9: Comparative analysis of endogenous ligand-induced transcriptional activity of the Neanderthal and human AHR.
(A) Dose-dependent induction of *Cyp1a1* and *Cyp1b1* expression mediated by Neanderthal or human AHR upon endogenous ligand treatment in transfected BP8 cells. Target gene induction was normalized to Rpl13a expression. **(B)** Summary of EC50 values for the dose response experiments in panel A.

3.5 DISCUSSION

The Neanderthal AHR was found to have increased sensitivity to PAH ligand treatments as evidenced by induction of *Cyp1a1* transcription between ~150-1000 fold lower EC_{50} than that observed with its human homolog. This marked difference in ligand responsiveness between species is dictated by the presence of an alanine or valine at residue 381 of the AHR LBD. However, the difference in transcriptional activity is unlikely to be solely attributed to differences in ligand affinity, which through quantitative ligand binding assays were found to only be ~6-10 fold lower for the dioxin analog (PAL) in humans relative to Neanderthals. Therefore it is possible that additional factors that dictate receptor activities may contribute to the observed differences between species, such as transformation potential, nuclear translocation efficiency, heterodimerization efficiency, protein stability, and transactivation potential. Regardless, it is clear that increased PAH binding, DNA binding, and elevated induction of *Cyp1a1/1b1* occurs through the A381 AHR variant. This marked difference in AHR activity provides evidence that Neanderthals, expressing the A381 variant, may have been more susceptible to exogenous PAH mediated toxicities relative to the human V381 variant. Therefore, modern humans were able to retain a specific loss of sensitivity to exogenous PAHs through a mutation of their *AHR* during the course of hominin evolution. However, it appears that this mutation was highly selective in that it did not result in loss of AHR sensitivity to endogenous ligands.

The loss of human sensitivity to exogenous ligands would likely influence AHR-dependent induction of metabolic processes associated with the targeted removal of xenobiotics. However, even with decreased PAH responsiveness it is evident that humans are capable of efficiently metabolizing and excreting PAHs, such as is the case for smokers. A greater

sensitivity to PAH mediated induction of CYP1A1/1B1 activity in non-human primates could lead to decoupling of phase I and phase II drug metabolism. This decoupling is caused by increased production of reactive intermediates through phase I metabolism (CYP1A1/1B1) and a corresponding lack of substrate detoxification via phase II metabolism[182]. A lack in compensation between these components would mediate increased bioaccumulation of toxic intermediates, while not necessarily achieving enhanced rates of substrate clearance. Thus, we propose that environmental exposure to potentially toxic AHR ligands may have provided selection upon the *AHR* locus to promote the expansion of variants that are less sensitive to exogenous substrates.

Nevertheless, evident species differences in AHR ligand selectivity indicate evolutionary adaptation within the LBD occurred. Notably, mutations within the human AHR LBD that may have facilitated an enhanced resistance to exogenous ligand exposures did not result in a global loss of ligand sensitivity. In contrast to exogenous ligands, sensitivity to endogenous ligands is conserved among humans, Neanderthals, and non-human primates. Lack of murine AHR sensitivity to endogenous ligands suggests that sensitivity to such ligands was acquired prior to divergence of the primate lineage[341, 342]. The persistence of this acquired function would suggest responsiveness to endogenous ligands is paramount in the establishment of beneficial physiological functions among primates. For example, AHR responsiveness to such ligands may be critical in the establishment of a host-microbe interkingdom signaling axis[342].

The conservation of the A381 AHR variant among Neanderthals and non-human primates would suggest that the V381 mutation arose recently in the timeline of human speciation. There exists the possibility that this mutation arose within human predecessors and subsisted within the human lineage. However, a lack of reliable anatomically modern human

sequence data (>45,000 years old) exists to effectively establish a timeline for the origin of the AHR V381 mutation. Additionally, the existence of archaic human populations polymorphic for the A/V381 variant is highly plausible. The introduction of the A381 AHR variant into the genome of anatomically modern humans likely emerged through human-Neanderthal admixture. Interbreeding between contemporary human and Neanderthals populations is well supported and suggests introgression of Neanderthal DNA into the gene pool of non-African modern humans that accounts for 1-4% of the total genome[371]. However, the V381 AHR variant is fixed among all modern humans within the available genome sequence data. This suggests that a selective advantage associated with the V381 AHR variant, or a corresponding disadvantage associated with A381 may have contributed to complete penetrance of the V381 allele in modern humans.

The genetic, environmental, and socio-cultural factors that provided sources of selective pressure to promote the evolutionary divergence of the *Homo sapiens* lineage have not been firmly established. The emergence of the AHR V381 mutation may represent evolutionary adaptation in response to environmental stress (e.g. exogenous ligand exposure) in humans, similar to what has been previously observed in Atlantic killifish populations[346]. There exists many potential sources of environmental AHR ligands such as dietary intake, and those derived from the incomplete combustion of organic material. Exposure to combustion byproducts could have occurred through natural events such as wildfires or volcanic eruptions. However, a more likely source of exposure in Paleolithic hominids would be through anthropogenic sources such as fire use.

The controlled use of fire is considered one of the seminal innovations in the prehistory of man. Evidence for the controlled use of fire originates with *Homo erectus* (sensulato) in Eurasia and Africa, and date to at least 250 ka or possibly as old as 760 ka or more[372-374].

Archeological evidence suggests routine controlled use of fire by *Homo heidelbergensis*, the common ancestor of humans, Neanderthals, and Denisovan man, as far back as 790 ka[374]. The controlled use of fire provided many critical functions to archaic hominids such as warmth, cooking, and technological advances through treatment of tools and generation of pigments[375-377]. These innovations were likely necessary for expansion of the *Homo* genus to settle within increasingly northern geographical locations[378]. The process of cooking food was of great benefit to limit the possibility of enteric infection from food borne pathogens and increase nutrient bioavailability[352, 353, 379]. Thus it is likely that fire use in archaic hominids would improve overall fitness[379]. However, the routine use of controlled fires would have come at the cost of increased exposure to smoke and particulate from the combustion of wood and other organic matter, as well as high concentrations of toxic PAHs. Archeological evidence, such as charred bones, heat treated tools, or ash sedimentation, suggests that fires were often situated within caves[378, 380]. As such, it is likely that poor ventilation within such structures would mediate elevated exposure to PAHs and other fire derived toxins. Evidence of micro-fragments of charcoal in Lower Paleolithic hominin dental calculus is consistent with smoke inhalation, and supports the hypothesis of poor smoke remediation in caves[380, 381].

As stated previously, the controlled use of fire by Neanderthals and humans would likely have led to increased PAH exposures, which at high concentrations can result in the acute cellular toxicity or chronic toxicities, leading to detrimental health and exacerbation of co-morbidities[382]. Chronic smoke inhalation is known to associate with various morbidities, such as increased respiratory infection risk, low birth weight, and increased infant mortality following maternal exposure[383, 384]. Additionally, exposure to AHR ligands is known to mediate adaptive immune suppression via induction of T_{Reg} differentiation pathways, which may increase

susceptibility to viral or bacterial infections[198, 385, 386]. Exposure to PAHs has also been shown to increase to time to pregnancy, reduce spermatogenesis, and increase apoptosis of oocytes, all of which have a direct impact upon overall reproductive fitness[387-390]. Detrimental physiological effects of PAHs are likely elicited by formation of reactive electrophilic intermediates as a consequence of metabolism. For example, the smoke substituent B(a)P mediates its own bioactivation to reactive B(a)P epoxides, quinones, and phenols by induction of CYP1A1/1B1 metabolism to supra-physiological levels through activation of the AHR. Excessive production of reactive B(a)P metabolites can result in cellular necrosis and death in rodents[337]. However, lack of cytochrome P450 mediated B(a)P metabolism can also be lethal[169]. This lethality likely occurs through accumulation of hydrophobic PAHs, and resultant toxicity associated with PAH intercalation and disruption of lipid membrane integrity. This result would support that metabolic removal of smoke-derived toxins is necessary, but should be accomplished through highly coordinated phase I/II drug metabolism pathways to ensure limited accumulation of reactive intermediates.

Middle/Upper Paleolithic hominins, who were exposed to high levels of PAHs as a result of controlled fire use, were at increased risk of acquiring PAH-mediated toxicities. It is important to note that both humans and Neanderthals were capable of controlled fire use, and therefore were exposed to similar environmental conditions. However, the V381 AHR mutation may have conferred an evolutionary advantage to anatomically modern humans with regard to decreased responsiveness to PAH exposure and associated co-morbidities. Definitive evidence that PAH-mediated toxicity provided positive selection upon the V381 variant to facilitate complete penetrance in modern humans will be difficult to determine. Nonetheless, our results demonstrate that the human AHR acquired a mutation (V381) that diminished sensitivity to PAHs and other

exogenous ligands and that this mutation is fixed within the modern human lineage. Our hypothesis is further supported by the conservation of the A381 AHR variant among non-human primates due to the absence of our proposed mode of selection through fire-control and resultant smoke exposure in these populations.

Evidence would suggest that Neanderthals were also capable of utilizing controlled fires and may have done so for thousands of years prior to the emergence of anatomically modern humans. However, the V381 AHR mutation did not emerge within Neanderthals. It is important to make the distinction between mutation and evolutionary selection, in that selection enhances proliferation of positive traits and diminishes expansion of negative traits within populations, but it not the source of the selected upon trait (mutation). This principle is clearly evident in the case of industrial melanism of peppered moths. Industrial pollution during the 19th century led to increased soot deposition upon trees, which provided increased camouflage for dark-colored moths (*Biston betularia f. carbonaria)* relative to light colored moths (*Biston betularia f. typica*). The resultant increased predation of lighter-pigmented moths and mediated rapid expansion of the dark pigmentation trait. However, prior to the industrial revolution lighter pigmentation provided enhanced camouflage when resting upon soot-free trees. Notably, both sub-species existed prior to selection. Therefore, the V381 AHR variant was likely derived from a random mutation event within the human lineage and not a consequence of fire-use. However, endogenous metabolism of PAHs, such as B(a)P, leads to the formation of DNA adducts and mutations. Exposure to PAHs may facilitate a unique circumstance in that the proposed source of selection could have mediated the derived mutation.

CONCLUSIONS AND FUTURE DIRECTIONS

The AHR was initially characterized as a primary mediator of xenobiotic metabolism through its sensing of large planar PAHs/HAHs and subsequent coordination of transcriptional activity to enhance clearance of chemical toxins. The physiological roles of the AHR have expanded vastly in parallel with the number of chemical entities identified as putative ligands. In the absence of exogenous chemical exposure, AHR activities are induced through recognition of endogenous metabolites[183]. The identification of AHR substrates derived from endogenous metabolism, dietary intake, and microbial synthesis suggest a multi-factorial signaling network that can mediate sustained, transient, or anatomically restricted AHR functions[341]. Further characterization of endogenous ligands, including agonists, antagonists, and selective modulators are necessary to define the myriad of factors that interface to dictate AHR-dependent biological functions. It is important to note that biological functions associated with AHR activity and the ligands that mediate them have been primarily investigated using *in vivo* and *in vitro* murine models. Historically, in the field of AHR biology and toxicology, the human AHR has decreased affinity for dioxin-like compounds and therefore perceived to possess an all encompassing lower affinity for ligands relative to its dioxin-sensitive murine (*Ahr*^{b}) counterpart. However, recent studies from our laboratory have shown the human AHR to be highly inducible by endogenous ligands derived from tryptophan and indole metabolism, such as kynurenic acid, indoxyl sulfate, and indirubin[231, 232, 255]. Therefore, it is likely that the interspecies variations in AHR structure that establish ligand specificity may ultimately mediate differential biological activities. This highlights the importance of further research efforts needed to investigate functions of the human AHR and the ligands that dictate such functions.

In chapter 2, I present data that establishes the microbial tryptophan metabolite, indole, as a ligand permissive for selective activation of the human AHR. The recognition of indole by the AHR was found to be evolutionary conserved in *Pan troglodytes* (Chimpanzee), our nearest extant phylogenetic relative. Murine AHR responsiveness to indole or numerous other agonists containing indole moieties is significantly diminished. Therefore, these results suggest that mouse models do not accurately recapitulate human receptor responsiveness to neither endogenous indoleic derivatives nor toxic polycyclic compounds. These results underscore the critical importance in the utilization of a humanized AHR mouse model to better evaluate the toxicological and biological outcomes of AHR activation by various classes of compounds.

Responsiveness to relatively low molecular weight compounds is atypical when compared to the dogma that the AHR binds to large planar polycyclic compounds. This paradigm shift may yield further insight into small molecules being endogenous regulators of this enigmatic receptor. Through the use of *in silico* modeling techniques, we establish a novel, bi-molecular indole binding hypothesis. The model suggests a unique binding mechanism in which two indole molecules reside in the LBD of the human AHR. However, the mouse LBD is not permissive in the accommodation of two molecules due to steric hindrance. The bi-molecular binding hypothesis was further supported by discrepancies in human AHR responsiveness to differential isomers of methyl-indole. Such bimolecular ligand interactions with the AHR ligand binding pocket has not previously been observed or considered and may vastly broaden the opportunities for targeted modulation of AHR function. However, corroboration of this hypothesis would require determination of a AHR crystal structure, which remains undefined to this date.

Chapter 2 Future Studies: Recently, the microbiome has been shown to play an important role in overall health, consequently the integration of AHR with bacterially derived ligands suggest this mode of AHR activation may have important implications for physiology and warrants further investigation. The recent procurement of a mouse that expresses the human AHR homolog under the control of its analogous murine promoter, will allow assessment of AHR activity as a consequence of indole signaling using a physiologically relevant *in vivo* model.

In my first study, I would hope to determine if bacterial indole production by microbial colonization of the intestinal tract can modulate host expression of AHR target genes. Through collaboration with the lab of Dr. Edward Dudley, at The Pennsylvania State University, three strains of *E.coli* were generated that differ in their capacity to produce indole through tryptophanase enzymatic activity (*tnaA*), producing no indole ($tnaA^{-/-}$), wild-type levels ($tnaA^{+/+}$), or supra-physiological levels ($tnaA^{OE}$). Humanized AHR mice would be administered broad spectrum antibiotics, such as doxycycline, in drinking water to remove resident microbes. This will be followed by oral gavage using one of the three aforementioned strains. Mice will be maintained on drinking water supplemented with kanamycin to select for expansion of administered bacteria that possess the resistance marker, as well as a high protein diet containing elevated levels of tryptophan for 24 days. Following the time course, expression of prototypical AHR target genes (*Cyp1a1/1b1*) will be assessed within the large intestines and liver. I predict that induction of *Cyp1a1/1b1* by the human AHR will positively correlate with enhanced metabolic potential to produce indole.

As discussed previously, indole is capable of being further metabolized by host hepatic enzymes to produce indoxyl sulfate, which is a vastly more potent ligand for the human AHR. Metagenomic and metabolomic analyses of resident microbe populations establish that microbial

tryptophanase activity is necessary for increased generation of indoxyl sulfate[328]. This model also found that dietary restriction of tryptophan levels could significantly impact serum indoxyl sulfate levels. However, the lack of a human AHR within these *in vivo* models limits the potential for ascertaining the AHR-dependent effects of increased indoxyl sulfate load. In further studies, I hope to again utilize the AHR humanized mouse model to further investigate endogenous signaling pathways that regulate indoxyl sulfate levels and their resultant impact upon the host. Hepatic bioactivation of indole to indoxyl sulfate is mediated by CYP2E1 metabolism and subsequent conjugation reactions[226]. Alcohol is a known inducer of CYP2E1 activity[391]. Therefore, I would administer humanized AHR mice diets of increasing protein concentrations and water with or without supplementation of ethanol. I predict that metabolic analyses will reveal that the combination of high protein and alcohol consumption will significantly increase circulating indoxyl sulfate levels and produce a corresponding increase in hepatic AHR activation as evidenced by the increase in expression of prototypical target genes. Such studies would establish that diet and microbial metabolic potential are critical determinants of endogenous indoxyl sulfate levels and AHR activity. These studies could be further expanded to assess the *in vivo* contribution of AHR signaling in the propagation of co-morbidities associated with elevated indoxyl sulfate levels or heightened AHR activation, such as CKD-associated cardiovascular disease or hepatic steatosis[233, 392].

The human AHR exhibits increased binding capacity of endogenous ligands derived from tryptophan and indole metabolism and decreased responsiveness to prototypical PAH agonists when compared to rodent homologues. Such differences suggest selective adaptations may have arisen during the course of mammalian evolution. However, due to limitations in the availability

of genomic sequence data from archaic hominin species, the origin of reduced AHR sensitivity to PAHs and its biological implications remain to be established.

In Chapter 3, I present evidence supporting the hypothesis that the selective desensitization of the human AHR to PAHs arose during or after speciation of *H. sapiens* and discuss its implications with regard to Paleolithic fire use and resultant exposure to toxic constituents within smoke. Interspecies AHR analyses revealed a single amino acid substitution (*A381V*) as the determinant in reduced PAH affinity exhibited by *H. sapiens* relative to *H. neanderthalensis* and other non-human primates. Analyses of the *AHR* gene locus across different ethnic demographics via the thousand genomes database confirm complete penetrance of the *A381V* mutation within anatomically modern humans. Neanderthal AHR was found to exhibit 100-1000 fold increased sensitivity to various smoke derived toxicants. The use of fire and cooking of food provided many healthful benefits, however, heightened exposure to toxic constituents in smoke have been found to associate with increased susceptibility to respiratory infection and decreased reproductive success. Therefore, differential AHR responsiveness observed in humans may have been a selective advantage and could have been a contributing factor to the supplantation of Neanderthals by anatomically modern humans. Notably, *H. sapiens* AHR responsiveness to endogenous ligands derived from tryptophan and indole metabolism remains unperturbed during hominid evolution. This suggests that the *A381V* mutation was highly selective by conserving critical AHR functions dependent upon the responsiveness to endogenous ligands, while providing a beneficial loss in PAH sensitivity.

Chapter 3 Future Studies: The differential sensitivity to PAHs discussed in chapter 3 was identified using *in vitro* cell culture models. To better address the physiological implications of differential PAH sensitivities, a more relevant *in vivo* model must first be developed. This model

would consist of mice expressing either the human or Neanderthal isoforms of the AHR at similar levels. Following development, congenic human/Neanderthal AHR expressing mice can be compared in their responsiveness to PAH exposure. Inhalation studies using increasing concentrations of B(a)P would be used to assess differential toxicological outcomes dependent upon organism *Ahr* status. I predict mice expressing the Neanderthal AHR would display increased sensitivity to B(a)P as evidenced by increased formation of DNA adducts and a lower LC_{50}. Susceptibility to infection was likely a strong source of selection upon Paleolithic hominins. Following B(a)P inhalation, mice could also be subjected to bacterial or viral respiratory infection models to assess combinatorial affects of PAH exposure and infection[393, 394]. Exposure to smoke and activation of the AHR have been previously identified as factors that can influence reproductive fitness[384, 387, 390]. Therefore it would be of interest to establish whether inhaled B(a)P or a combination of smoke constituents can differentially hinder reproductive fitness in human/Neanderthal AHR mice. In this study mice will be exposed to inhaled PAHs and reproductive fitness will be assessed by monitoring time to pregnancy, litter size, and average pup weight.

The data presented in chapter 3 demonstrate that decreased sensitivity to PAHs arose during the speciation of man. However, responsiveness to endogenous ligands appears to be conserved among primates, but the determinant for indole binding and the origin of such activity in mammals remains to be established. The key AHR residues required to bind indole may be determined through comprehensive mutagenesis experiments. However, in-depth phylogenetic analyses of indole sensitivity could identify when this activity arose in mammals. Subsequent comparison of AHR LBD structure could identify the residues necessary for AHR-binding of indole. This more structured approach can be accomplished through BP8 transfection

experiments and EMSA assays comparing indole sensitivity among additional species, such as guinea pig, hamster, or wild boar with increasing structural similarity to that of humans. Once key residues for indole binding are identified they can be further assessed by targeted mutagenesis of human or mouse AHR to mediate gain or loss of indole sensitivity.

In conclusion, my research identifies how evolutionary selection has adapted the AHR to produce a biological sensor that is distinctive in its responsiveness to environmental toxins and endogenous ligands, the combination of which is unique to *H. sapiens* relative to our closest ancestors. By enhancing our understanding of human AHR molecular signaling, I hope to further increase the potential for therapeutic targeting of the AHR and promote the use of transgenic animal models to better assess its implications within various disease states.

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APPENDIX

Portions of this thesis, including texts, figures, and tables were used with permissions from the following peer reviewed articles:

Divergent Ah Receptor Ligand Selectivity during Hominin Evolution. **Hubbard, TD.**, Murray, IA., Bisson, WH., Sullivan, AP., Sebastian, A., Perry, GH., Jablonski, NG., Perdew, GH. *Molecular Biology and Evolution*, **33**, 2648-58 (2016).

Adaptation of the human aryl hydrocarbon receptor to sense microbiota-derived indoles. **Hubbard TD**, Murray IA, Bisson WH, Lahoti TS, Gowda K., Amin SG, Patterson AD, Perdew GH. *Scientific Reports*, **5**, 12689 (2015).

Indole and tryptophan metabolism: endogenous and dietary routes to Ah receptor activation. **Hubbard TD**, Murray IA, Perdew GH. *Drug Metabolism Disposition*, **43**, 1522-35 (2015).

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2. A novel AhR ligand, 2AI, protects the retina from environmental stress. Gutierrez MA, Davis SS, Rosko A, Nguyen SM, Mitchell KP, Mateen S, Neves J, Garcia TY, Mooney S, Perdew GH, **Hubbard TD**, Lamba DA, Ramanathan A. *Scientific Reports*, **6**, 29025 (2016).

3. Adaptation of the human aryl hydrocarbon receptor to sense microbiota-derived indoles. **Hubbard TD**, Murray IA, Bisson WH, Lahoti TS, Gowda K., Amin SG, Patterson AD, Perdew GH. *Scientific Reports*, **5**, 12689 (2015).

4. Indole and tryptophan metabolism: endogenous and dietary routes to Ah receptor activation. **Hubbard TD**, Murray IA, Perdew GH. *Drug Metabolism Disposition*, **43**, 1522-35 (2015).

5. Persistent organic pollutants modify gut microbiota–most metabolic homeostasis in mice through aryl hydrocarbon receptor activation. Zhang L, Nichols RG, Correll J, Murray IA, Tanaka N, Smith P, **Hubbard TD**, Sebastian A, Albert I, Hatzakis E, Gonzalez FJ, Perdew GH, Patterson AD. *Environmental Health Perspectives*, **123**, 679-88 (2015).

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