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IDENTIFICATION OF 2,8-DIHYDROXYQUINOLINE AS A NOVEL AGONIST OF THE HUMAN ARYL HYDROCARBON RECEPTOR

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor known for its ability to metabolize a vast array of xenobiotics, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Recent literature has implicated the role of AHR in intestinal homeostasis and immunity via activation by endogenous and dietary ligands, suggesting that certain ligands may impart a protective effect and increase barrier function of the intestinal epithelium. This study characterizes 2,8-dihydroxyquinoline (2,8-DHQ) as a novel AHR ligand, exhibiting specificity for the human AHR. 2,8-DHQ is a product of gastrointestinal microbial metabolism identified in body fluids. A number of dihydroxyquinoline compounds were investigated for potential AHR activity through a luciferase-based reporter assay, which identified 2,8-DHQ as having the highest affinity for the human receptor. Administration of 2,8-DHQ increased transcription of the AHR target gene *CYP1A1* in a dose-dependent manner in cultured human cells. A photoaffinity ligand (PAL) competition assay identified 2,8-DHQ as a competitive ligand for the human AHR in cell culture, however it is interesting to note that this was not true when the assay was repeated *in vitro* in liver extracts. This may be due to the need for 2,8-DHQ to undergo metabolism, a process that also increases the affinity of indole to bind AHR when it becomes indoxyl sulfate. Finally, a nuclear retention assay was conducted that showed 2,8-DHQ treatment to cause a significant increase in nuclear translocation and retention of AHR. Further research is needed to investigate the ability of 2,8-DHQ to activate AHR *in vivo* and to characterize its potential to impact human health through attenuation of intestinal inflammation.

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LIST OF ABBREVIATIONS

2,8-DHQ	2,8-Dihydroxyquinoline
2-HQ	2-Hydroxyquinoline
8-HQ	8-Hydroxyquinoline
AHR	Aryl Hydrocarbon Receptor
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
bHLH-PAS	Basic Helix-Loop-Helix and Per, Arnt/AHR, Sim
BNF	β-naphthoflavone
Caco-2	Human Colon-Carcinoma Cell Line
DRE	Dioxin Response Element
HAH	Halogenated Aromatic Hydrocarbon
HSP90	Heat Shock Protein 90
I3C	Indole-3-Carbinol
I3S	Indoxyl Sulfate
IAA	Indole-3-Acetic Acid
IBD	Inflammatory Bowel Disease
ICZ	Indolo[3,2-*b*]Carbazole
IR	Indirubin
p23	Heat Shock Protein 90 Co-Chaperone p23
PAH	Polycyclic Aromatic Hydrocarbon
PAL	Photoaffinity Ligand
SOD	Superoxide Dismutase
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TCDD/Dioxin	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
Treg	Regulatory T Cell
XAP2	X-Associated Protein 2

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I would like to thank Dr. Gary Perdew for generously welcoming me into his lab my sophomore year and making it possible for me to balance my school work with lab time. I would also like to acknowledge Dr. Troy Hubbard for mentoring me through my first few years of laboratory work and being such a wonderful teacher and valuable resource to me. Finally, I would like to thank my parents for helping me achieve my dream of attending veterinary school this fall and encouraging me to work hard and stay focused both in and out of the classroom.

INTRODUCTION

The Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor, historically responsible for mediating gene expression in response to exposure to a variety of xenobiotic compounds. [1] The AHR is a nuclear receptor, part of the bHLH-PAS (basic helix-loop-helix and Per, Arnt/AHR, Sim) superfamily along with its heterodimer, the aryl hydrocarbon receptor nuclear translocator (ARNT). [2]

Typical AHR ligands are polycyclic aromatic hydrocarbons (PAH) and halogenated aromatic hydrocarbons (HAH) such as 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent activator of AHR and a highly toxic environmental pollutant. In addition to these synthetic compounds, many endogenous dietary ligands that induce AHR are being identified and are of increasing interest. [3]

Recently, it has been shown that AHR null ($Ahr^{-/-}$) mice develop growth and hepatic defects, indicating that AHR plays a role in normal physiological function. [4] AHR is present in the cytoplasm of most cells of the body, with highest concentrations in barrier organs such as the lung, skin, and gastrointestinal tract as well as metabolically active tissues such as the liver. [5] Chemicals enter cells through the plasma membrane, then bind to unliganded AHR complexes in the cytosol and translocate into the nucleus, where AHR dimerizes with ARNT and stimulates transcription of a specific gene. The majority of genes regulated by AHR encode xenobiotic metabolizing enzymes such as cytochrome P450 isozyme 1A1 (CYP1A1) a monooxygenase that

catalyzes many reactions involved in drug metabolism and is often used as a hallmark of AHR activation. [3]

The AHR has been shown to have additional functions outside of drug metabolism, such as immune function, cell differentiation, and disease attenuation. [6] As the AHR is known to bind a wide variety of ligands, it is of great interest to identify novel AHR ligands that may function in a manner to improve human health or disease status. Sources of AHR ligands include exogenous compounds, endogenous compounds, bacterial-derived metabolites, and compounds ingested via diet, all of which mediate AHR activation in various tissues of the body.

Functional Domains

As a member of the bHLH-PAS family of transcription factors, the AHR contains the following in the N-terminal region: the bHLH domain, two PAS repeats (PAS-A, PAS-B), and the nuclear localization signal (NLS). The C-terminal region of the receptor contains a glutamine rich domain (Q-rich). Ligand binding occurs on the PAS-B repeat, HSP90 binding as well as dimerization occur on the bHLH domain and PAS repeats, DNA binding is restricted to the bHLH domain, and transcriptional activation occurs on the Q-rich region. [7] The domains are illustrated in Figure 1 below.

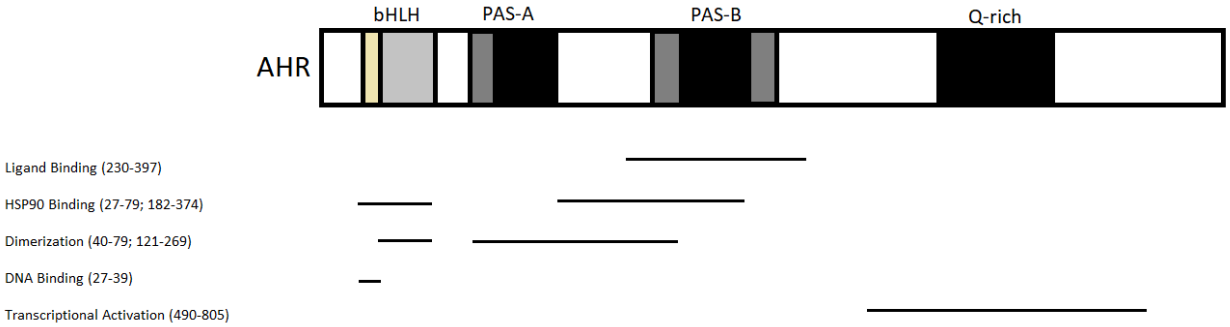


Figure 1: Functional Domains of the AHR

Canonical AHR Signaling Pathway

Unliganded AHR resides within the cytoplasm of most cells in the body, complexed with X-associated protein 2 (XAP2), two molecules of heat shock protein 90 (HSP90), and one molecule of heat shock protein 90 cochaperone p23 (p23). [8], [9], [10] Occupation of the ligand-binding domain by an AHR agonist leads to a conformational change of the receptor and transport of the receptor-ligand complex into the cell's nucleus. [11] Inside the nucleus, AHR dissociates from its cytoplasmic complex (XAP2, HSP90, p23) and associates with ARNT to form a heterodimeric transcription factor capable of binding to DNA. [12] The AHR/ARNT complex then binds to a dioxin response element (DRE), a DNA sequence containing (5'-TNGCGTG-3'). [13] Coactivators are recruited to the transactivation domain of AHR after binding to the DRE sequence, enabling chromatin remodeling and leading to transcription of the target gene. Figure 2 depicts this signaling pathway.

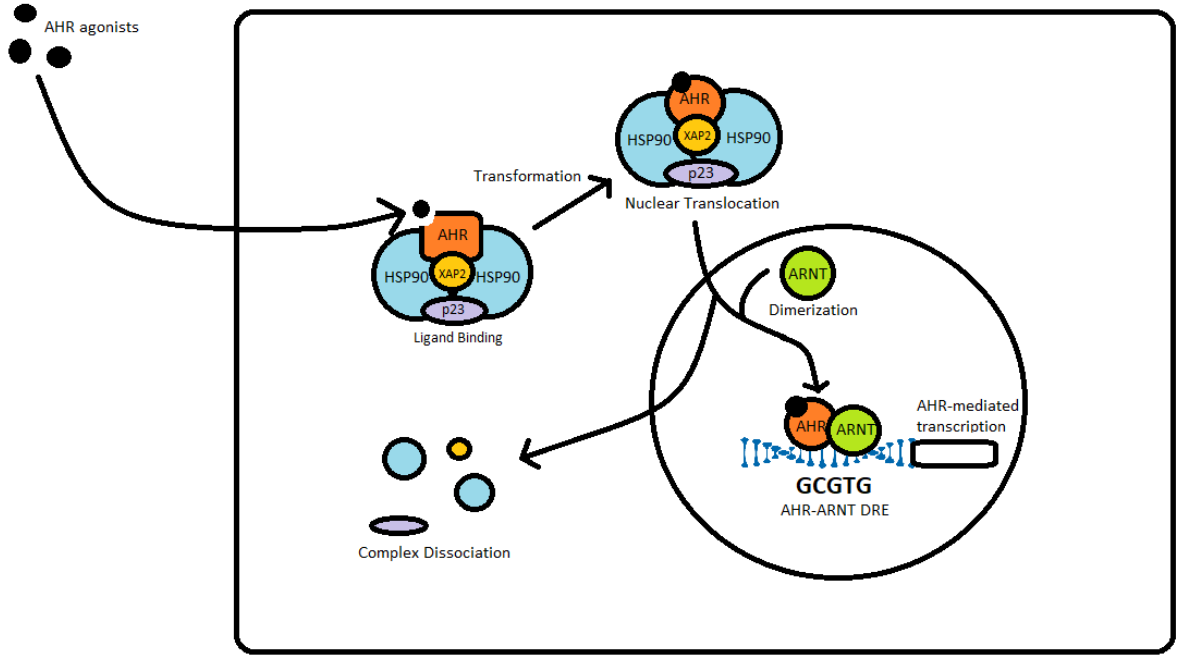


Figure 2: Canonical AHR Signaling Pathway

Role of AHR in Immunity

Proper barrier function of the intestinal epithelium is vital to preventing inflammation and infection in the gut. Activation of the AHR has been shown to impact immune function, particularly by regulating barrier function of gastrointestinal epithelial cells. The intestinal tissue of patients with inflammatory bowel disease (IBD) exhibit downregulation of AHR, suggesting that AHR signaling is important for the regulation of gastrointestinal inflammation via an IL-22 dependent pathway. [14] The induction of effector cytokines such as IL-17 and IL-22 have been linked to AHR activation in T helper cells and innate lymphoid cells. [15] IL-22-dependent mucosal resistance to inflammation and infection has been shown to be mediated by the activation of AHR by bacterial tryptophan metabolites such as indole-3-aldehyde (IAA). [16]

Peripherally-induced T regulatory cells (Tregs) in the gut show higher expression of AHR when compared to thymus-derived Tregs, indicating that AHR is differentially expressed by Tregs in various tissues of the body, and is particularly abundant in the gastrointestinal tract. AHR activation in the gut also promotes homing of Tregs, shown by the reduction of the following proteins in AHR-deficient mice: GPR15 (assists homing of Tregs to the large intestine), CCR6 (chemokine receptor), and CD103 (important for Treg activation and retention at inflammatory sites). [17] Expression of AHR in gut Tregs also played a protective role against gastrointestinal inflammation in an *in vivo* T cell transfer model of colitis, as AHR deficient mice had impaired suppressive function against colitis when compared to mice whose Tregs expressed AHR. [17]

Identification of AHR Ligands

As the AHR ligand-binding pocket is capable of interacting with a variety of chemicals, identification of new ligands is of high priority due to the impact of AHR activation on human health and wellness. Previously identified ligands come from a variety of sources—exogenous, endogenous, dietary intake, and gastrointestinal commensal bacteria metabolism. Examples of relevant AHR ligands and their structures are listed in the Appendix.

Exogenous Ligands

AHR was originally identified due to its role as a mediator of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD/dioxin) toxicity in the human body. [18] Dioxin is a HAH and a potent environmental toxicant produced as a byproduct of multiple industrial processes. Murine exposure to dioxin causes a wide variety of toxicities including carcinogenicity, teratogenicity, and lethality. [19] Human exposure to dioxin is classically known to produce chloracne. [20] Other well-known xenobiotic compounds that induce AHR activity typically fall into the category of PAH's, HAH's, and other similar compounds.

Endogenous Ligands

The creation of an AHR null (*Ahr* $^{-/-}$) mouse model lead to significant discoveries about the physiological role of the receptor outside of xenobiotic metabolism. AHR null mice were found to be immune to the toxic effects of dioxin administration, however they expressed a variety of developmental defects. In mice lacking AHR, the ductus venosus failed to close

properly, organ development was altered, and liver size was decreased in addition to many other abnormalities that exhibited the decreased fitness of null mice. [21] AHR may also support gut immune function, as AHR null mice were less likely to withstand intestinal challenge. [22] The importance of AHR activation throughout development suggests that endogenous AHR ligands must be present in order to induce activity. Many proposed endogenous AHR ligands are derived from the essential amino acid tryptophan and its metabolite indole. [23]

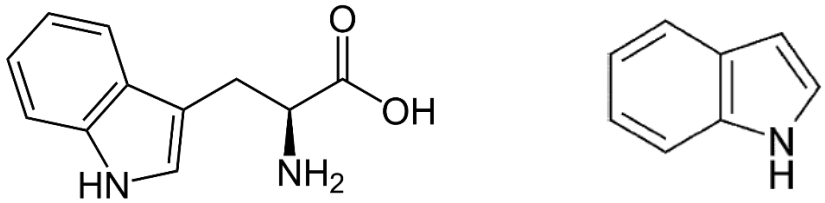


Figure 3: Structure of Tryptophan (Left) and Indole (Right)

Dietary Ligands

A variety of AHR ligands and ligand precursors have been identified from extracts of herbs and some vegetables such as corn and jalapeno pepper. [24] The compound glucobrassicin, whose structure is indole-based, is found in high quantities in cruciferous vegetables, such as broccoli and cabbage. [25] Host mastication promotes the enzymatic degradation of glucobrassicin by myrosinases, which are naturally found in plant cells, into indole-3-carbinol (I3C), a weak activator of AHR. [26], [27] I3C undergoes further metabolism when exposed to gastric acid in the stomach, generating indolo[3,2-*b*]carbazole (ICZ), an additional AHR ligand. [28]

Microbiota-Derived Ligands

Commensal bacteria in the gastrointestinal tract contribute a great deal to the metabolism of dietary compounds ingested by the host. Specifically, many microbes use tryptophan as a nitrogen source and convert the amino acid into mono-substituted indole compounds such as indole, indole-3-acetic acid (IAA), and tryptamine, which have been identified in murine fecal material. [29] In a yeast DRE-dependent reporter assay, using yeast strains expressing AHR and ARNT, AHR was activated by tryptophan, indole, IAA, tryptamine, and especially so by indole-3-carbinol (I3C), which had an EC_{50} of ~10 μM. [30] In human colon-carcinoma (Caco2) cell line, AHR was activated by both tryptamine and IAA at physiologically relevant concentrations present in the gut. [29]

Tryptamine is derived from tryptophan via direct decarboxylation, while the conversion of tryptophan to IAA occurs via the indole-3-acetamide pathway, mediated by the enzymes tryptophan monooxygenase and indole-3-acetamide hydrolase. [31] The generation of indole by bacterial metabolism of tryptophan occurs via the tryptophanase pathway in Escherichia coli bacteria. [32] Indole has also been identified as a component of human fecal material at high enough concentrations to impact health, and was shown to improve barrier function and have anti-inflammatory properties in a human intestinal epithelial cell line (HCT-8). [33]

Indole can be further metabolized into indoxyl sulfate (I3S), which is a potent and direct activator of the human AHR, and to a lesser extent the mouse AHR. [34] I3S is an atypical AHR ligand, as classic ligands are typically hydrophobic and planar. There is also evidence that the sulfate group is important for AHR activation, especially as I3S induces AHR significantly better than indole, meaning that sulfation should be considered as a positive trait when searching for new ligands and the sulfation of known ligands may increase their activity.

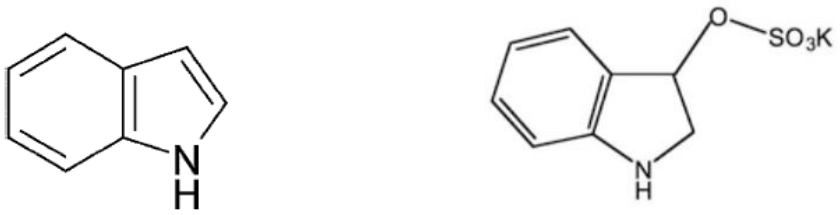


Figure 4: Structures of Indole (Left) and Indoxyl Sulfate (Right)

Ligand Specificity for Human versus Mouse AHR

Variations in the structure of human versus mouse AHR has led to the identification of differences in their functions. The amino-terminal region of the receptor exhibits 85% homology between the two species, while the carboxy-terminal region shows a great deal of variation in the transactivation domain. The ligand binding domain is contained within the amino-terminal region, where the amino acid sequence variations cause mouse and human AHR to exhibit differences in ligand affinity and selectivity. Notably, mouse AHR binds TCDD with 10-fold higher affinity than human AHR due to a single amino acid substitution, while human AHR binds endogenous indolic derivatives with a relative higher affinity than the mouse receptor does. [35], [36] As murine models are often used for *in vivo* study of AHR activation and its impact on overall health, it is important to note the variances between mouse and human AHR before drawing conclusions that the same effects will occur in humans.

Identification of Quinoline Derivatives

Quinoline is a heterocyclic aromatic organic compound, derivatives of which have been identified in body fluids as products of microbial metabolism in the gastrointestinal tract. 2,8-dihydroxyquionline (2,8-DHQ) has been isolated from the urine of rats fed a diet rich in cornmeal. [37] Additionally, in an *in vivo* study using mice that were administered Tempol, a compound similar to superoxide dismutase (SOD) that has antioxidant and anti-inflammatory effects, 2,8-DHQ was identified as a urinary endogenous metabolite. [38] Activation of peroxisome proliferator-activated receptor alpha (PPARα) by administration of the PPARα ligand Wy-14,643 in mice significantly enhances the excretion of both 2,8-DHQ and its glucuronide conjugate. [39]

2,8-DHQ and its derivatives 2-hydroxyquinoline (2-HQ) and 8-hydroxyquinoline (8-HQ) should be investigated in order to determine if the human AHR exhibits specificity for one conformation over another. The structure of these potential ligands are shown below in Figure 5.

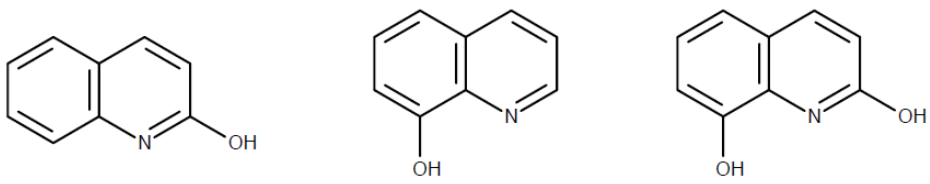


Figure 5: Structure of 2-HQ (Left), 8-HQ (Middle), 2,8-DHQ (Right)

Overview and Significance of Research

The objective of this study is to characterize 2,8-DHQ as a novel human AHR ligand. This will be accomplished through administration of 2,8-DHQ within a variety of in vitro models to assess its capacity to facilitate induction of AHR target gene expression and enzyme activity. Luciferase-based reporter assays, real-time PCR, photoaffinity ligand competition assays, and nuclear translocation assays will be performed. The outcome of this study will add to the expanding compendium of known AHR ligands that have the possibility to impact human health.

As the consumption of cornmeal gives rise to the formation of 2,8-DHQ, dietary cornmeal could impact endogenous functions of the AHR, such as intestinal homeostasis, inflammatory signaling, immune regulation, and carcinogenesis. Previous research suggests that non-toxic AHR agonists act similar to probiotics and promote beneficial effects upon intestinal health, therefore dietary consumption of cornmeal or supplementation of one's diet with 2,8-DHQ could be of increased biomedical interest through activation of AHR in the gastrointestinal tract. In a recent study, it was shown that consumption of a 15% broccoli diet in mice lead to protective effects against chemically induced colitis, dependent on AHR status and associated with AHR activation. [6] AHR activation in the gastrointestinal tract by 2,8-DHQ after consumption of cornmeal in the diet has the potential for physiologically relevant benefits.

MATERIALS AND METHODS

Cell Culture

HepG2 40/6 (human hepatoma stable cell line), Hepa 1.1 (mouse hepatoma cell line), and Caco2 (human colon carcinoma cell line) were maintained in α-modified essential media (Sigma-Aldrich, St. Louis, MO) containing 8% fetal bovine serum (HyClone Laboratories, Logan, UT) and 1% penicillin (100 units/mL)/streptomycin (100 μg/mL) (Sigma-Aldrich, St. Louis, MO). The cell culture plates were kept at 37˚C in an atmosphere containing 5% CO_2.

Luciferase-Based Reporter Assay

Hepa 1.1 and HepG2 40/6 cells were incubated at 37˚C in 6-well plates and were treated as indicated. Cells were lysed with 400 mL of reporter lysis buffer [25mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100] in each well and plates were placed in an -80˚C freezer overnight. 80 μL of Luciferase Reporter Substrate (Promega, Madison, WI) and 20 μL of lysate from each well were combined and luciferase activity was measured using a TD-20e Luminometer (Turner Systems, Sunnyvale, CA).

RNA Isolation and Quantitative Real-Time PCR

Isolation of RNA from cultured cells was performed using Tri Reagent (Sigma-Aldrich, St. Louis, MO). RNA concentration was determined via spectrophotometry at wavelengths of 260/280nm. Preparation of cDNA from the isolated RNA was achieved with a High Capacity

cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qRT-PCR was run on a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercultes, CA) with PerfeCTa SYBR Green Supermix for iQ (Quanta Biosciences, Beverly, MA). Normalization of gene expression was performed using ribosomal protein L13a (*Rpl13a*/RPL13A) to yield relative mRNA level. The primers (Integrated DNA Technologies, Coralville, IA) used are listed in Table 1 below.

Table 1: Primer Sequences used for qRT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Rpl13a	TTCGGCTGAAGCCTACCAGAAAGT	GCATCTTGGCCTTTTTCCGTT
RPL13A	CCTGGAGGCGAAGCGGAAAGAGA	GAGGACCTCTGTGTATTTGTCAA
Cyp1a1	CAGAAGCGGAGGCTTACCAT	CTCTGTATTGAGGCGGTCCC
CYP1A1	TCTTCCTTCGTCCCCTTCAC	TGGTTGATCTGCCACTGGTT

Photoaffinity Competitive Ligand Binding Assay

The protocol used for the PAL assay followed the method laid out by Ramadoss and Perdew. [40]

Nuclear Retention Assay

The method to determine nuclear retention of ligands was carried out using the protocol from Singh, Hord, and Perdew. [41]

Statistical Analysis

GraphPad Prism 5 Software was utilized for statistical analysis of data sets, through student's t-test and one-way ANOVA, and generation of histograms. Statistical significance is demonstrated by p-value ≤ 0.05 (*), p-value ≤ 0.01 (**), p-value ≤ 0.001 (***).

RESULTS

Identification of 2,8-DHQ as an AHR Agonist

A reporter assay was conducted in both Hepa 1.1 and Hep G2 (40/6) cells in order to compare the ability of different DHQ compounds to induce transcription of either the mouse or human AHR. Compounds selected were: 2,4-DHQ, 2,8-DHQ, 2,6-DHQ, and 4,8-DHQ. Relative luciferase units (RLU) were compared to both vehicle and TCDD treatment. In Hepa 1.1 cells, 4,8-DHQ was the only compound of interest found to induce AHR at significant levels. In Hep G2 (40/6) cells, 2,8-DHQ induced AHR at levels compared to TCDD, a potent AHR ligand. Therefore, 2,8-DHQ was selected for further investigation as a potential human AHR ligand. The data is shown in Figure 6 below.

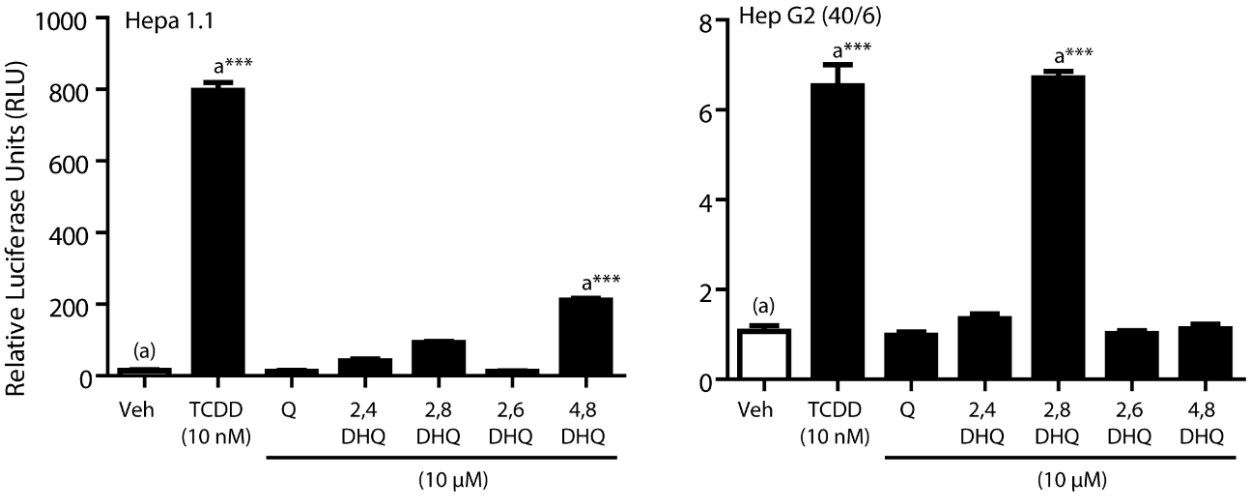


Figure 6: Ability of DHQ Compounds to Bind AHR

A dose response experiment was conducted to compare the ability of 2-HQ, 8-HQ, and 2,8-DHQ induce mouse and human AHR. In Figure 7 below, each compound was compared to TCDD and indirubin (IR), another known potent AHR ligand. In mouse cells, 2-HQ was able to induce transcription of AHR significantly at a dose of 10μM, while neither 8-HQ or 2,8-DHQ were significant at any dose. In human cells, 2-HQ induced AHR at 10 μM, while 2,8-DHQ was able to induce transcription at both 10 μM and 1μM doses. This reporter data shows that 2,8-DHQ is an agonist of the human AHR.

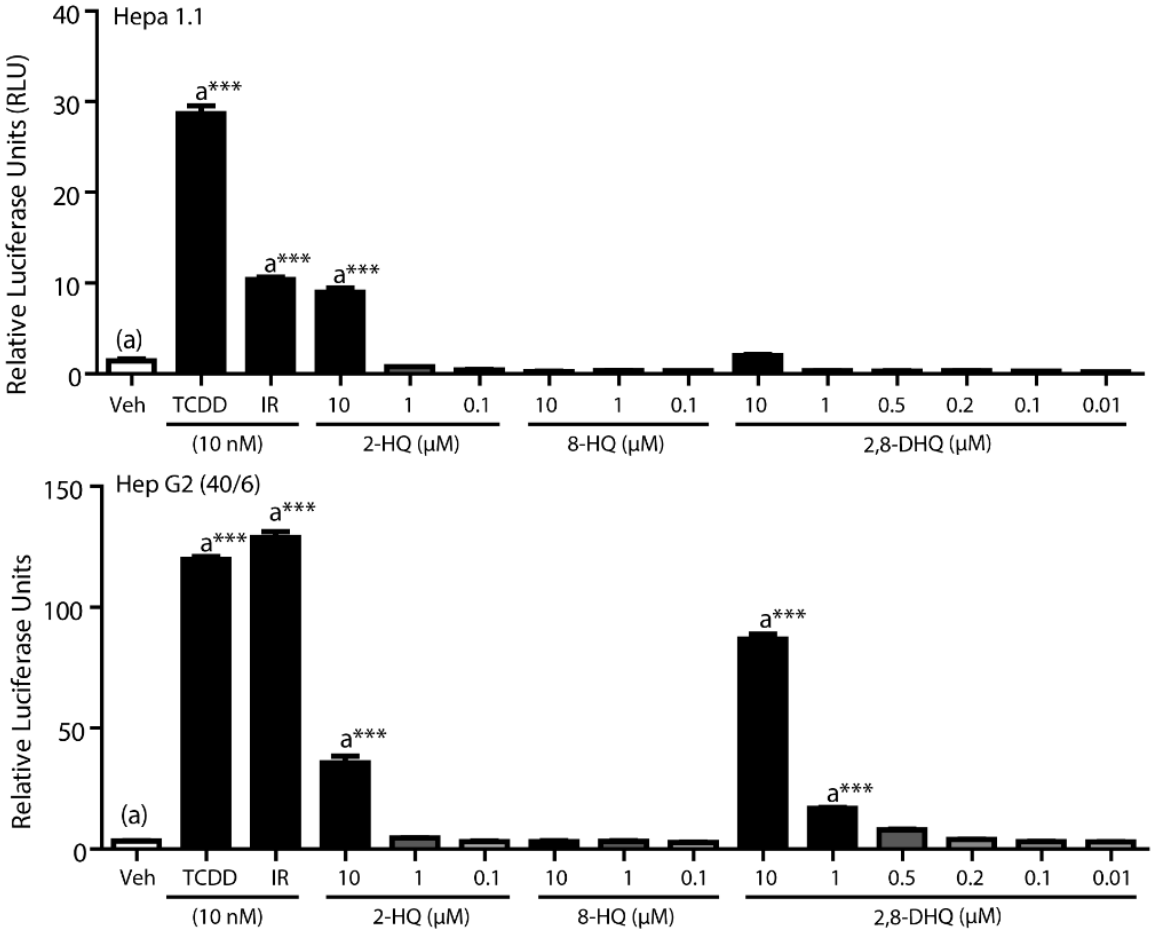


Figure 7: Dose Response of 2,8-DHQ and Derivatives

2,8-DHQ Induces Transcription of AHR Target Genes

Hepa 1.1 and Caco2 cells were cultured and treated with varying doses of 2,8-DHQ as well as TCDD as a control. After RNA isolation and cDNA reaction, qRT-PCR was conducted to measure the expression of *Cyp1a1*/CYP1A1, a known target of AHR.

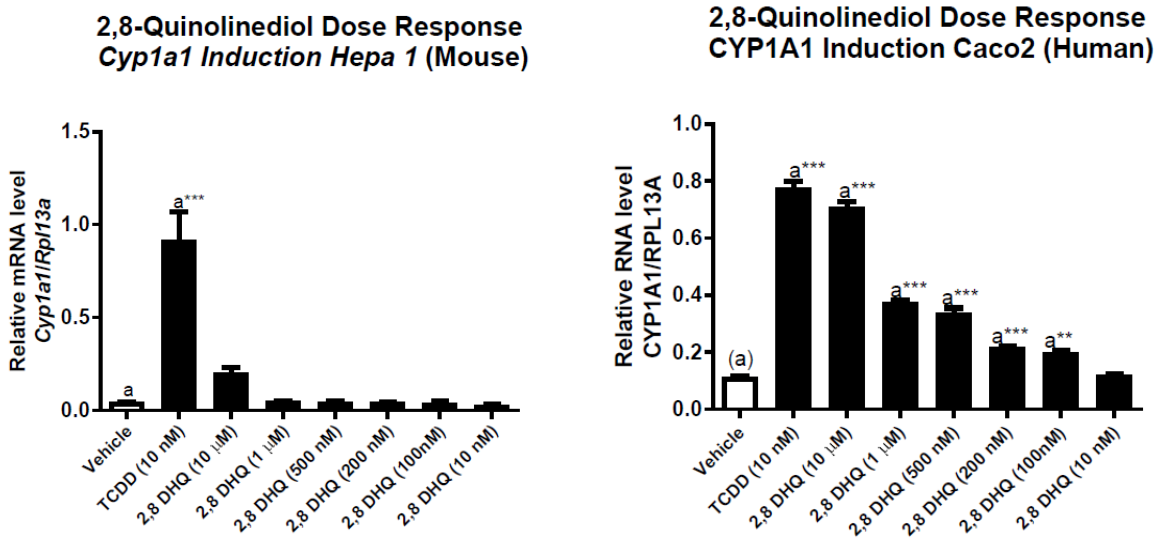


Figure 8: Dose Response of Cyp1a1/CYP1A1 Induction

In Figure 8 above, murine AHR induction by 2,8-DHQ was not significant at any dose, however they appear to show a dose-response pattern of weak induction. Human AHR was induced by treatment of 2,8-DHQ at all doses except the smallest dose, 10 nM. Therefore, 2,8-DHQ is able to induce the transcription of CYP1A1 in Caco2 cell in a dose-dependent manner, relevant to AHR activation in the gastrointestinal tract.

Treatment of primary human hepatocytes with 10 μM of 2,8-DHQ resulted in the induction of CYP1A1, shown in Figure 9 below. Showing that 2,8-DHQ is also an agonist for AHR in primary human hepatocytes makes the case that this is more physiologically relevant, as it was able to induce CYP1A1 in multiple human cell types.

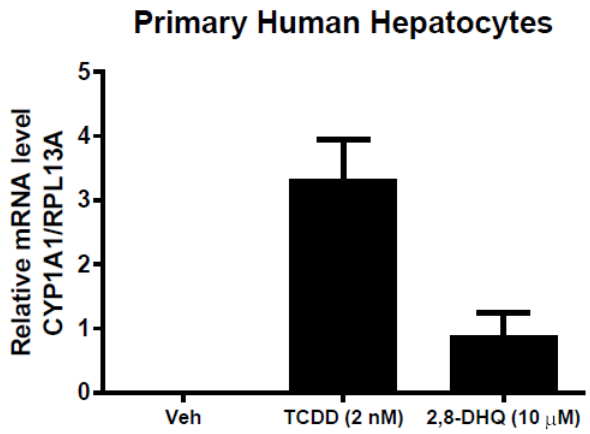


Figure 9: CYP1A1 Induction by 2,8-DHQ in Primary Human Hepatocytes

2,8-DHQ is a Competitive AHR Ligand in Cell Culture

2,8-DHQ was compared against β-naphthoflavone (BNF), a potent AHR ligand, to determine if it could compete with a photoaffinity ligand (PAL) for AHR binding sites. In mouse Hepa 1.1 cells, BNF binds to AHR with high affinity, as shown by the largely outcompeted level of bound PAL. 2,8-DHQ did not compete significantly for binding, as was expected with its low affinity for murine AHR. In Hep G2 (40/6) cells, BNF showed a lower affinity as it shows specificity toward mouse AHR. However, 2,8-DHQ significantly outcompeted PAL for binding to human AHR, meaning that 2,8-DHQ can compete for the human AHR ligand binding domain in cell culture. This data is shown in Figure 10 below, demonstrated by percent PAL binding.

A second study was conducted in order to determine if 2,8-DHQ had the ability to bind AHR competitively *in vitro*. When a PAL competition assay was run in human liver extracts, 2,8-DHQ was unable to compete with PAL for binding (data not shown). This leads us to believe that 2,8-DHQ may require further metabolism to bind AHR outside of cell culture.

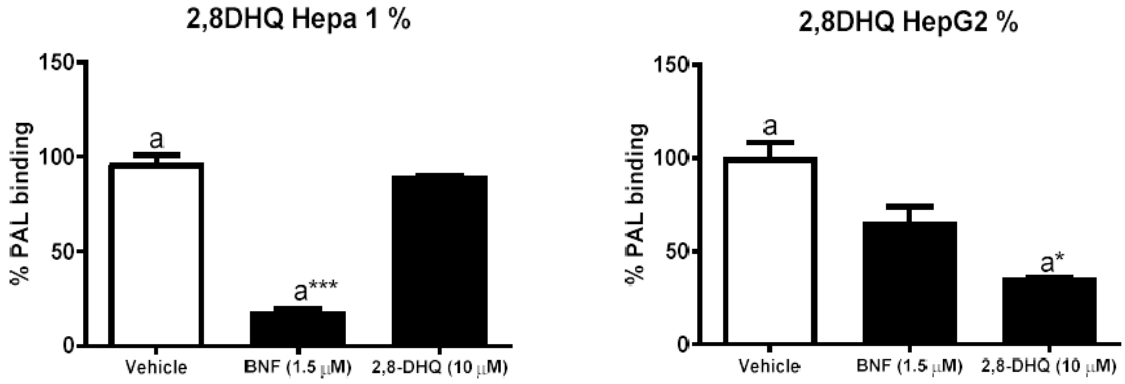


Figure 10: PAL Competition Assay

2,8-DHQ Causes Increased Nuclear Retention of AHR

It is important to determine whether 2,8-DHQ is able to not only bind AHR, but also cause AHR to translocate into the nucleus of cells, heterodimerize with ARNT, and initiate transcription. A nuclear retention assay was conducted comparing the ability of 2,8-DHQ and TCDD (a potent AHR activator) to cause AHR to translocate and remain in the nucleus of Hep G2 40/6 cells in culture. As expected, TCDD caused a significant increase in the amount of nuclear AHR content. A dose of 10 μM 2,8-DHQ was also able to cause nuclear translocation of AHR significantly as compared to vehicle nuclear extracts. The data is represented in Figure 11 below.

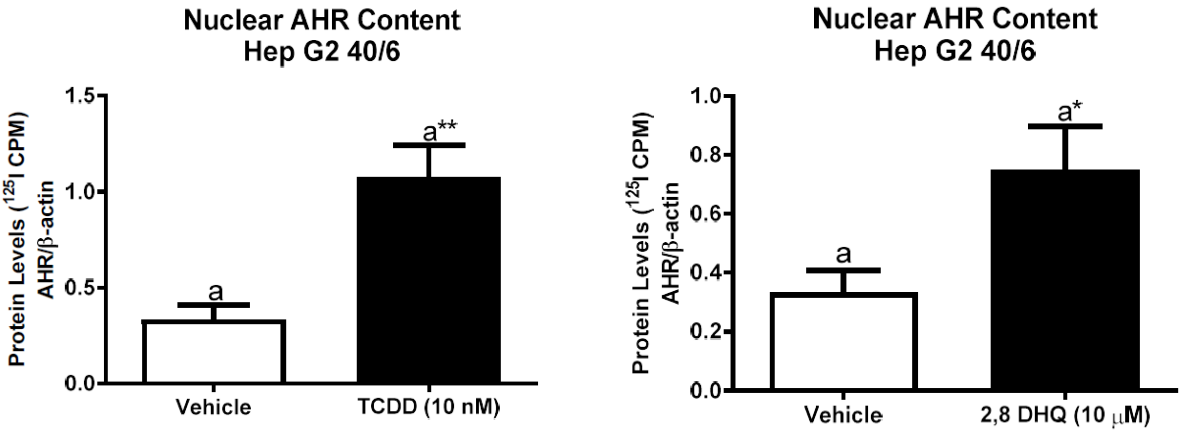


Figure 11: Nuclear AHR Content

DISCUSSION

This study was conducted in order to identify derivatives of quinoline as AHR ligands and then characterize their properties in terms of receptor specificity and potency. Quinoline and its derivatives have been identified as products of metabolism after administration of certain foods and drugs, meaning that they could play an important role in human health and wellness. Compounds that activate AHR in the gut have been shown to have anti-inflammatory effects and help to maintain barrier function of the epithelial lining, an important aspect in the prevention of disease. Activation of AHR also impacts immune cell differentiation and maturation, and induction of effector cytokines such as IL-22. Therefore, the identification of novel AHR ligands, especially those impacted by diet, is of high interest due to the potential human health benefits.

Quinoline and its derivatives are unusual AHR ligand candidates as typical ligands are large, hydrophobic, and planar. Three dihydroxyquinoline compounds were investigated for their ability to bind and activate AHR: 2,4-DHQ, 2,8-DHQ, 2,6-DHQ, and 4,8-DHQ. 2,8-DHQ exhibited specificity for the human AHR over the mouse AHR in a luciferase-based reporter assay. An additional dose-response reporter assay was conducted in order to determine if a derivative of 2,8-DHQ exhibited higher potency than the original compound, revealing that neither 2-HQ or 8-HQ were able to bind AHR as well as 2,8-DHQ. This result allowed for further studies to focus primarily on 2,8-DHQ as a potential human AHR agonist.

As AHR is involved in the metabolism of xenobiotics, its primary target gene is *CYP1A1*, an important enzyme in Phase I metabolism. Typical AHR ligands such as TCDD are known to induce transcription of *CYP1A1* after they activate the AHR. A dose response study treating cells with 2,8-DHQ showed dose-dependent transcription of *CYP1A1* in Caco2 cells, which

additionally proposes the relevance of AHR activation in the gastrointestinal tract. Treatment of primary human hepatocytes with 2,8-DHQ was also able to induce *CYP1A1* transcription, which suggests that 2,8-DHQ is an agonist for all human cell types containing AHR. Again, the compound was unable to significantly activate AHR activity in murine Hepa 1.1 cells, as shown by the lack of significant *Cyp1a1* transcription.

It is important to determine the ability of agonists to compete with each other for ligand binding domains as this will impact physiological relevance. If a compound cannot compete with other more potent AHR ligands, there is little chance it will impart any significant impact on health, either positively or negatively. A photoaffinity ligand competition assay compares the ability of a putative ligand to compete with the PAL, which has similar activity to TCDD. 2,8-DHQ was able to significantly compete with the PAL in human Hep G2 40/6 cells, but not in mouse cells. This suggests that 2,8-DHQ may be able to compete with other AHR ligands for binding in the human body and have a true impact on the health status of an individual.

That being said, a second PAL competition assay was conducted in human liver extracts. This assay did not show that 2,8-DHQ has the ability to compete with PAL *in vitro*. A possible explanation for this phenomenon is that 2,8-DHQ may require host metabolism prior to becoming a potent AHR ligand. This can be compared to the relative potencies of indole and indoxyl sulfate; indole binds AHR with significantly more affinity after it undergoes sulfation. This mechanism may play a role in the affinity of 2,8-DHQ to bind AHR as well. The addition of a sulfate group nearly doubles the size of the compound, meaning that it has a significant impact on the ability of a ligand to fit in the ligand binding domain of a receptor. As most typical AHR ligands are much larger than 2,8-DHQ and indole, sulfation may increase the affinity of these smaller ligands by increasing their size. This result is interesting due to the

involvement of Phase II metabolism in AHR activation, which leads to the activation of Phase I enzymes, suggesting another manner in which the gut maintains homeostasis. Further studies are needed to investigate this proposal including the creation of the sulfated 2,8-DHQ compound and investigating its ability to bind and activate AHR efficiently.

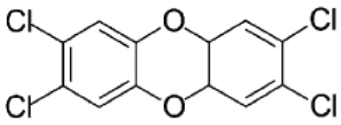
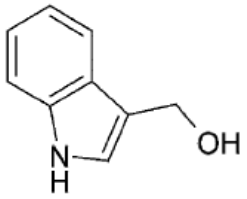
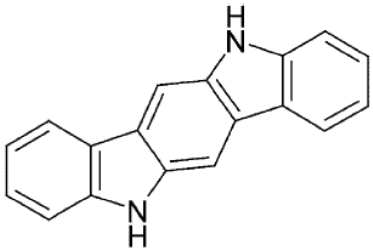
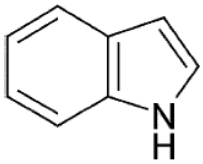
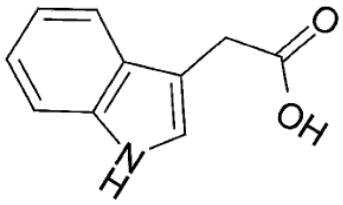
A final assay was completed to investigate the ability of 2,8-DHQ to cause nuclear translocation and retention of AHR. This process is vital to the canonical AHR signaling pathway as the receptor binds ligands in the cytoplasm and then must be transported into the nucleus where it heterodimerizes with ARNT, binds to DNA, and initiates transcription of target genes. Therefore, a ligand that causes AHR to be transported into and remain inside the nucleus of a cell is much more effective than one that fails to do so. Treatment with 2,8-DHQ was able to cause a significant increase in nuclear AHR content as compared to vehicle in human Hep G2 40/6 cells. As a result, it can be gathered that 2,8-DHQ causes effective nuclear translocation and retention of the human AHR.

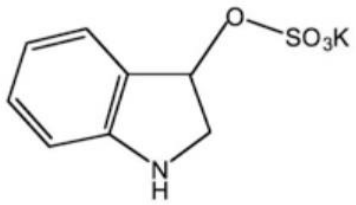
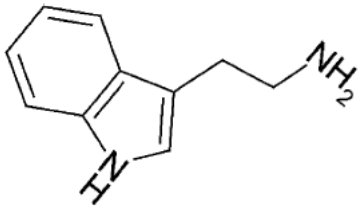
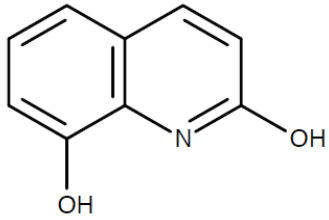
Conclusions

Overall, this research suggests that 2,8-DHQ is an AHR agonist, specific for the human receptor phenotype. Further studies are needed to characterize the structure and activity of the sulfated compound, as this may exhibit higher affinity for the ligand binding domain of the receptor in a similar manner as indoxyl sulfate (data not shown). Additionally, feeding studies utilizing a mouse model with a humanized AHR will be vital to characterizing the ability of 2,8-DHQ to activate AHR *in vivo*. As AHR activation has been shown to impact intestinal health and immunity, 2,8-DHQ should be investigated as a potential supplement, either directly or through administration of foods containing 2,8-DHQ precursors. In conclusion, 2,8-DHQ should be a candidate compound for the treatment of chronic intestinal inflammation and disease due to its high potential for AHR activation in the gut.

Appendix

List of Relevant AHR Ligands

Compound	Origin	Structure	Reference
TCDD	Exogenous		[18]
I3C	Dietary		[28]
ICZ	Dietary		[28]
Indole	Microbial		[30]
IAA	Microbial		[30]

I3S	Microbial		[34]
Tryptamine	Yeast		[40]
2,8-DHQ	Microbial		

REFERENCES

[1] J. Mimura and Y. Fujii-Kuriyama, "Functional role of AhR in the expression of toxic effects by TCDD," *Biochimica et Biophysica Acta,* vol. 1619, no. 3, pp. 263-268, 2003.

[2] S. T. Crews, "Control of cell lineage-specific development and transcription by bHLH–PAS proteins," *Genes & Development,* vol. 12, pp. 607-620, 1998.

[3] M. Nagy, S. Denison and R. Scott, "Activation of the Aryl Hydrocarbon Receptor by Structurally Diverse Exogenous and Endogenous Chemicals," *Annual Review of Pharmacology and Toxicology,* vol. 43, pp. 309-334, 2003.

[4] J. V. Schmidt, G. H. Su, J. K. Reddy, M. C. Simon and C. A. Bradfield, "Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development," *National Academy of Sciences,* vol. 93, no. 13, pp. 6731-6736, 1996.

[5] W. Li, S. Donat, O. Dohr, K. Unfried and J. Abel, "Ah Receptor in Different Tissues of C57BL/6J and DBA/2J Mice: Use of Competitive Polymerase Chain Reaction to Measure Ah-Receptor mRNA Expression," *Archives of Biochemistry and Biophysics,* vol. 315, no. 2, pp. 279-284, 1994.

[6] T. Hubbard, "Dietary broccoli impacts microbial community structure and attenuates chemically induced colitis in mice in an Ah receptor dependent manner," *Journal of Functional Foods,* vol. 37, pp. 685-698, 2017.

[7] B. Fukunaga, M. Probst, S. Reisz-Porszasz and O. Hankinson, "Identification of Functional Domains of the Aryl Hydrocarbon Receptor," *Journal of Biological Chemistry,* vol. 270, pp. 29270-29278, 1995.

[8] G. Perdew, "Association of the Ah receptor with the 90-kDA heat shcok protein," *Journal of Biological Chemistry,* vol. 263, pp. 13802-13805, 1988.

[9] B. Meyer, M. Pray-Grant, J. V. Heuvel and G. Perdew, "Hepatits B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity," *Molecular and Cellular Biology,* vol. 18, pp. 978-988, 1998.

[10] A. Kazlauskas, L. Poellinger and I. Pongratz, "Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (Aryl hydrocarbon) receptor," *Journal of Biological Chemistry,* vol. 274, pp. 13519-13524, 1999.

[11] T. Ikuta, H. Eguchi, T. Tachibana, Y. Yoneda and K. Kawajiri, "Nuclear localization and export signals of the human aryl hydrocarbon receptor," *Journal of Biological Chemistry,* vol. 273, pp. 2895-2904, 1998.

[12] H. Reyes, S. Reisz-Porszasz and O. Hankinson, "Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the AH receptor," *Science,* vol. 256, pp. 1193-1195, 1992.

[13] E. Shen and J. Whitlock, "Protein-DNA interactions at a dioxin-responsive enahncer. Mutational analysis of the DNA-binding site for the liganded Ah receptor," *Journal of Biological Chemistry,* vol. 267, pp. 6815-6819, 1992.

[14] I. Monteleone, A. Rizzo, M. Sarra, G. Sica, P. Sileri, L. Biancone, M. T, F. Pallone and G. Monteleone, "Aryl hydrocarbon receptor-induced signals up-regulate IL-22

production and inhibit inflammation in the gastronintestinal tract," *Gastroenterology,* vol. 248, no. 1, pp. 237-248, 2011.

[15] C. Esser, A. Rannug and B. Stockinger, "The aryl hydrocarbon receptor in immunity," *Trends Immunol,* vol. 30, pp. 447-454, 2009.

[16] T. Zelante, R. Iannitti, C. Cunha, A. DeLuca, G. Giovannini, G. Pieraccini and R. Zecchi, "Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22," *Immunity,* vol. 39, no. 2, pp. 372-385, 2013.

[17] J. Ye, J. Qiu, J. Bostick, C. Jobin, Z. Chen and L. Zhou, "The Aryl Hydrocarbon Receptor Preferentially Marks and Promotes Gut Regulatory T Cells," *Cell Reports,* vol. 21, pp. 2277-2290, 2017.

[18] A. Poland and J. Knutson, "2,3,7,8-Tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity," *Annual Review of Pharmacology and Toxicology,* vol. 22, pp. 517-554, 1982.

[19] M. J. DeVito and L. S. Birnbaum, "Toxicology of Dioxins and Related Chemicals," *Dioxins and Health,* pp. 139-162, 1994.

[20] R. R. Suskind, "Chloracne, "the hallmark of dioxin intoxication"," *Scandinavian Journal of Work, Environment & Health,* vol. 11, no. 3, pp. 165-171, 1985.

[21] J. Schmidt, G. Su, J. Reddy, M. Simon and C. Bradfield, "Characterization of a murine Ahr null allel: involvement of the Ah receptor in hepatic growth and development," *Proc Natl Acad Sci USA,* vol. 93, pp. 6731-6736, 1996.

[22] C. Sutter, S. Bodreddigari, C. Campion, R. Wible and T. Sutter, "2,3,7,8-Tetrachlorodibenzo-p-dioxin increases the expression of genes in the human epidermal differentiation complex and accelerates epidermial barrier function," *Toxicol Sci,* vol. 124, pp. 128-137, 2011.

[23] M. A. Bittinger, L. P. Nguyen and C. A. Bradfield, "Aspartate Aminotransferase Generates Proagonists of the Aryl Hydrocarbon Receptor," *Molecular Pharmacology,* vol. 64, no. 3, pp. 550-556, 2003.

[24] A. Jeuken, B. Keser, E. Khan, A. Brouwer, J. Koeman and M. Denison, "Activation of the Ah receptor by extracts of dietary herbal supplements, vegetables, and fruits," *J Agric Food Chem,* vol. 51, no. 18, pp. 5478-5487, 2003.

[25] W. Loub, L. Wattenberg and D. Davis, "Aryl hydrocarbon hydroxylase induction in rat tissues by naturally occuring indoles of cruciferous plants," *J Natl Cancer Inst,* vol. 54, pp. 985-988, 1975.

[26] T. Shapiro, J. Fahey, K. S. K. Wade and P. Talalay, "Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans," *Cancer Epidemiol Biomarkers Prev,* vol. 10, pp. 501-508, 2001.

[27] S. Ito, C. Chen, J. Satoh, S. Yim and F. Gonzalez, "Dietary phytochemicals regulate whole-body CYP1A1 expression through an aryl hydrocrabon receptor nulcear translocator-dependent system in gut," *J Clin Invest,* vol. 117, pp. 1940-1950, 2007.

[28] L. Bjeldanes, J. Kim, K. Grose, J. Bartholomew and C. Bradfield, "Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol in vitro and in vivo: comparisons with 2,3,7,8-tetrachlorodibenzo-p-dioxin.," *Proc Natl Acad Sci USA,* vol. 88, pp. 9543-9547, 1991.

[29] U. Jin, S. Lee, G. Sridharan, K. Lee, L. Davidson, A. Jayaraman, R. Chapkin, R. Alaniz and S. Safe, "Microbiome-Derived Tryptophan Metabolites and their Aryl Hydrocarbon Receptor-Dependent Agonist and Antagonist Activites," *Molecular Pharmacology,* vol. 85, no. 5, pp. 777-788, 2014.

[30] C. Miller, "Expression of the Human Aryl Hydrocarbon Receptor Complex in Yeast," *Journal of Biological Chemistry*, vol. 272, pp. 32824-32829, 1997.

[31] E. Tsavkelova, B. Oeser, L. Oren-Young, M. Israeli, Y. Sasson, B. Tudzynski and A. Sharon, "Identification and functional characterization of indole-3-acetamide-mediated IAA biosynthesis in plant-associated Fusarium species," *Fungal Genet Biol*, vol. 49, no. 1, pp. 48-57, 2012.

[32] C. Yanofsky, V. Horn and P. Gollnick, "Physiological studies of tryptophan transport and tryptophanase operon induction in Escherichia coli," *J Bacteriol*, vol. 173, no. 19, pp. 6009-6017, 1991.

[33] T. Bansal, R. Alaniz, T. Wood and A. Jayaraman, "The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation," *Proc Natl Acad Sci USA*, vol. 107, no. 1, pp. 228-233, 2010.

[34] J. Schroeder, B. DiNatale, I. Murray, C. Flaveny, Q. Liu, E. Laurenzana, J. Lin, S. Strom, C. Omiecinski, S. Amin and G. Perdew, "The uremic toxin 3-indoxyl sulfate is a potent endogenous agonist for the human aryl hydrocarbon receptor," *Biochemistry*, vol. 49, no. 2, pp. 393-400, 2010.

[35] P. Ramadoss and G. Perdew, "Use of 2-azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin as a probe to determine the relative ligand affinity of human versus mouse aryl hydrocarbon receptor in cultured cells.," *Mol Pharmacol*, vol. 66, no. 1, pp. 129-136, 2004.

[36] C. Flaveny, I. Murray, C. Chiaro and G. Perdew, "Ligand selectivity and gene regulation by the human aryl hydrocarbon receptor in transgenic mice.," *Mol Pharmacol*, vol. 75, no. 6, pp. 1412-1420, 2009.

[37] K. Inagmi, M. Kaihara and J. Price, "The Identification of 2,8-Quinolinediol in the Urine of Rats Fed a Diet Containing Corn," *The Journal of Biological Chemistry*, vol. 240, no. 9, pp. 3828-3684, 1965.

[38] F. Li, X. Pang, K. Krausz, C. Jiang, C. Chen, J. Cook, M. Krishna, J. Mitchell, F. Gonzalez and A. Patterson, "Stable Isotope- and Mass Spectrometry-based Metabolomics as Tools in Drug Metabolism: A Study Expanding Tempol Pharmacology," *Journal of Proteome Research,* vol. 12, pp. 1369-1376, 2013.

[39] Y. Zhen, K. Krausz, C. Chen, J. Idle and F. Gonzalez, "Metabolomic and Genetic Analysis of Biomarkers for Persoxisome Proliferator-Activated Receptor α Expression and Actviation," *Moleculuar Endocrinology,* vol. 21, no. 9, pp. 2136-2151, 2007.

[40] P. Ramadoss and G. Perdew, "Use of 2-Azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin as a Probe to Determine the Relative Ligand Affinity of Human versus Mouse Aryl Hydrocarbon Receptor in Cultured Cells," *Molecular Pharmacology,* vol. 66, no. 1, pp. 129-136, 2004.

[41] S. Singh, N. Hord and G. Perdew, "Characterization of the Activated Form of the Aryl Hydrocarbon Receptor in the Nucleus of HeLa Cells in the Absence of Exogenous Ligand," *Archives of Biochemistry and Biophysics,* vol. 329, no. 1, pp. 47-55, 1996.

[42] D. Schrenk, D. Riebniger, M. Till, S. Vetter and H. Fiedler, "Tryptanthrins and other tryptophan-derived agonists of the dioxin receptor.," *Adv Exp Med Biol,* vol. 467, pp. 403-408, 1999.

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EDUCATION

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AWARDS

Dean's List (all semesters)
Schreyer Honors College Academic Excellence Scholarship (2014-18)
Theola F. Thevaos Scholarship (2015-16)
Oswald Scholarship (2014-18)
Veterinary Founders Academic Scholarship (2016-17)
College of Agricultural Sciences Undergraduate Research Grant (Spring, Fall 2017)
Gamma Sigma Delta Honor Society Member (2017-18)
John Danhouse Martz, Jr. Scholarship (2017-18)
Kenton D. Rexford Scholarship (2017-18)

EXPERIENCE

Undergraduate Researcher, Gary H. Perdew, Ph.D. Group — *University Park, PA;* August 2015-May 2018
Conducted research on the impact of dietary ligands on activation of the Ah receptor
Co-authored paper published in the Journal of Functional Foods and featured in news articles
Current and future projects will lead to the completion of my honors thesis in 2018

Veterinary Technician/Student Observer
Veterinary Oncology Services and Research Center — *West Chester, PA*; May 2017-August 2017
Windcrest Animal Hospital — *Wilmington, DE*; June 2016-August 2016
Centreville Veterinary Hospital — *Centreville, DE*; January 2014-June 2014
Cat Vet of South Street — *Philadelphia, PA*; June 2012-August 2016

SKILLS

Laboratory:
Mastered a variety of reactions such as PCR and RNA extraction; ability to maintain cell lines
IACUC certified to handle lab animals and experimental experience with rodents
Ability to record and interpret data in a meaningful way through use of statistical analysis

Veterinary Technician:
Ability to communicate effectively with clients and handle animals
Knowledge of common medications and dosage calculations
Venipuncture and administration of treatments, including chemotherapy
Ability to assist with surgery and utilize sterile technique
Use of radiograph equipment and assisting with ultrasounds
Cared for animals in both emergency situations and general examinations

ACTIVITIES

Rules & Regulations Committee Penn State THON, Fundraising Specialist — October 2015-February 2017
Organized and lead a variety of fundraisers benefitting the Four Diamonds Fund
Communicated effectively with THON directors, captains, and the committee

Volé Ballet, Member — January 2017-May 2018
Penn State's Ballet Club, a student-run organization of over 200 students with a passion for dance
Participated in weekly technique classes, annual performances, social and charitable events

Penn State Learning Edge Academic Program (LEAP), Mentor — June 2015-August 2015
Mentored a group of 24 students during their first semester at Penn State
Planned multiple events every week of summer session for students to acclimate to campus
Worked closely with faculty and coordinators to ensure a smooth transition for students

Penn State Veterinary and Biomedical Sciences Camp, Counselor — June 2015
Organized and lead one week of activities for high school students interested in veterinary medicine

Discover Penn State Stay-Over Program, Public Relations Chair — September 2014-March 2015
Planned a three-day educational program for high school seniors accepted into PSU Pre-Vet program