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AH RECEPTOR LIGANDS IN CANCER: FRIEND AND FOE

Iain A. Murray, **Andrew D. Patterson**, and **Gary H. Perdew**

Department of Veterinary and Biomedical Sciences and The Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, PA

Preface

The Aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that is best known for mediating the toxicity and tumor promoting properties of the carcinogen 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, commonly referred to as “dioxin”. The AHR influences the major stages of tumorigenesis- initiation, promotion, progression, and metastasis. Physiologically relevant ligands that have been characterized are often formed during disease states or heighten innate and adaptive immune responses. Interestingly, ligand specificity and affinity varies between rodents and humans. Studies of aggressive tumors and tumor cell lines have revealed that AHR levels are elevated and can be found constitutively in the nucleus. This leads to the hypothesis that the AHR is chronically activated in tumors, thus facilitating tumor progression. The potential for therapeutic modulation of AHR activity in tumors will also be discussed.

Introduction

The Aryl hydrocarbon receptor (AHR) is a member of the basic helix–loop–helix–PER–ARNT–SIM (bHLH–PAS) subgroup of the bHLH superfamily of transcription factors and is the only member of this family known to be activated by ligands[1]. The AHR was discovered as the receptor that binds 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) with high affinity and is capable of sustained hyperactivation, resulting in a myriad of toxicologic outcomes. The half-life of TCDD in humans is approximately 10 years, due to its inability to be metabolized to a polar derivative that can be excreted. These properties contribute to the potency of TCDD as a promoter of liver and skin carcinogenesis in rodents [2]. The AHR has been studied extensively for its ability to induce transcription of several cytochrome P-450s that are important in the metabolism and bioactivation of carcinogens, and in particular, polycyclic aromatic hydrocarbons after heterodimerization with the Ah receptor nuclear translocator (ARNT) (Fig. 1). DNA microarray studies have established that the AHR either directly or indirectly regulates a myriad of genes[3]. These studies have shown that the AHR regulates genes involved in a wide variety of biochemical pathways, including energy metabolism, lipid and cholesterol synthesis, xenobiotic metabolism and various transporters. AHR knockout mice have provided insight into the physiological role of the AHR and have been useful for exploring the influence of AHR expression on susceptibility to carcinogens.

Correspondence to: Gary H. Perdew, Department of Veterinary and Biomedical Sciences and The Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, PA 16802, Tel: (814) 865-0400, Fax: (814) 863-1696, ghp2@psu.edu.

The AHR is now known to be involved in a variety of cellular processes, such as the cell cycle, epithelial barrier function, cell migration, and immune function.

A high degree of complexity has emerged regarding the role of AHR in cancer, with clear discrepancies between pro- and anti-tumorigenic activities evident when utilizing cell culture versus in vivo models of malignancy. Furthermore, various classes of AHR ligands and indeed ligands within the same class can differentially modulate AHR to influence tumorigenic outcomes. Nevertheless, the AHR is a potentially important drug target that can be effectively modulated with different classes of ligands. Here we will focus on what has been established about the activity of the AHR using animal models and human tissue, with particular emphasis on the role of the AHR in immune surveillance and cancer. An issue that will be covered in this review that has not received sufficient prior attention is the difference between endogenous ligand specificity for the human versus mouse AHR and how this may impact tumor biology. In addition, a wide variety of tumor types exhibit both high levels of AHR expression relative to the parent cell type, and significant constitutive receptor activity, thus AHR antagonists could potentially be employed in cancer treatment.

Mechanisms of AHR activation

AHR agonists

Numerous chemicals exhibit high affinity binding to the AHR, altering its activity in a ligand dependent manner thus rendering this receptor an attractive small molecule target (Table 1). For many years the focus was on the identification of xenobiotics that exhibit strong agonist activity, such as persistent planar halogenated polycyclic hydrocarbons (e.g. TCDD, TCDF, PCBs) and polycyclic aromatic hydrocarbons (e.g. benzo(a)pyrene, benzanthracene). More recently, a wide variety of lower affinity agonists have been identified from diverse sources[4]. Through the use of various assay systems that detect AHR transcriptional activity, many commercial and consumer products, fruits, vegetables and spices, were determined to have AHR activation potential, which could significantly contribute to our total AHR ligand exposure levels[5, 6]. However, the identity of specific compounds with AHR activity was not established in these studies. Other research revealed several drugs that exhibit off-target activity through binding to the AHR (Table 1)[7]. In addition, a number of reports have identified specific dietary constituents that are AHR ligands. For example, certain flavonoids (e.g. quercetin, apigenin, kaempferol) exhibit AHR agonist and antagonist activity in a cell line specific manner[8, 9]. Moveover, cruciferous vegetables contain significant amounts of indole glucosinolates that, upon consumption, are degraded to indole-3-carbinol. This compound then undergoes condensation reactions in the acidic environment of the stomach, creating several products that are capable of activating the AHR, with the compound of highest affinity for the AHR being indolo[3,2 *b*]carbazole[10].

The presence of AHR ligands at the site of epithelial barriers may indicate that the AHR plays a role in the response to microflora. Metabolism of tryptophan by bacteria in the intestinal tract leads to the formation of AHR ligands such as indole, indole-3-acetate and indole-3-aldehyde[11, 12]. A chemical library derived from a probiotic bacterium was screened for molecules that activate the AHR and the compound 1,4-dihydroxy-2-naphthoic acid was

identified as an AHR agonist[13]. Specific strains of yeast (e.g. *Malassezia* species) isolated from patients with certain skin diseases are capable of synthesizing several potent AHR ligands; including indirubin, indolo[3,2*b*]carbazole and malassezin[14]. It is possible that the host responds to the presence of these chemicals by mediating AHR activation thus enhancing barrier function, although these effects will likely be tissue and context dependent. Consistent with this concept is the observation that coal tar, a rich source of AHR ligands, is capable of enhancing skin barrier function in an AHR-dependent manner[15]. Humans have significant exposure to AHR ligands from a variety of sources and whether these exposure levels could lead to enhanced tumorigenesis warrants further investigation. For example, one question that should be addressed is whether AHR ligand concentrations are elevated in cancer patients as the disease progresses.

Ligand independent activation

Another aspect of AHR behavior to consider is the possibility that there is genuine ligand independent activation. In support of this concept is the apparent ability of elevated cAMP levels to activate the AHR[16]. Furthermore, it is possible that in cells that have relatively high AHR levels, such as many tumor cell lines, the AHR undergoes dynamic nucleocytoplasmic shuttling, which could lead to AHR and ARNT heterodimerization in the absence of ligand[17]. Support for this concept can be found upon comparing the level of retained nuclear AHR in a human head and neck squamous cell carcinoma cell line, NH30, compared to normal human keratinocytes[18]. However, the possibility that AHR ligands are present in these cells cannot be excluded. Shear stress in endothelial cells can also lead to AHR activation, although the mechanism of activation is not known [19, 20]. Co-expression of a mutant AHR unable to bind ligand (AhR-A375I) and ARNT increased AHR-mediated transcriptional activity suggests that the AHR can potentially heterodimerize with ARNT in the absence of ligand, although heterodimerization potential appear to be quite inefficient[21].

TCDD and hepato-carcinogenesis: exemplar of AHR agonist-mediated cancer

TCDD is considered both a complete epigenetic carcinogen and a potent tumor promoter through sustained activation of the AHR and this topic has been extensively reviewed[22, 23]. High affinity AHR ligands can mediate significant AHR transcriptional activity even in the presence of modest levels of AHR expression, suggesting that hyper-activation of the AHR would affect a wide range of cell types. Many of these studies have focused on the role of the AHR in liver cancer in rodents. Recent progress has been made on the mechanisms of liver tumor promotion with the recognition of inflammatory signaling as a major component of hepatocellular carcinoma[24]. Using a two-stage carcinogenesis model, it has been shown that the receptors for TNFA, TNFB, IL1A, and Il1B play a major role in the ability of the carcinogen diethylnitrosamine to mediate tumor incidence in mice[25]. Furthermore, the absence of these receptors greatly attenuated the ability of TCDD to promote the number and size of liver tumors. One possible explanation for these results is the key role that IL6 signaling plays in malignant progression of HCC[26]. Indeed, the AHR can synergistically induce IL6 in the presence of an inflammatory signal through both dioxin and NFkB response elements in the Il6 promoter[27]. These observations would support testing whether

TCDD can promote carcinogenesis in an Il6 null mouse. Thus, the disruption of excessive AHR activation by the pool of endogenous and exogenous AHR agonist would appear to be a logical goal when considering the AHR as a therapeutic target.

Presence of endogenous AHR ligands that may influence tumorigenesis

Perhaps one of the most fundamental questions concerning the role of the AHR in carcinogenesis is whether endogenous ligands can mediate sufficient receptor activity to influence tumor progression. A number of studies have examined the role of the AHR in tumorigenesis and a complex story has emerged with reports that detail the ability of AHR activation to enhance or repress tumorigenesis. Whether AHR ligands are present in the tumor microenvironment needs to be addressed, as well as how the type of ligand (e.g. endogenous or exogenous) influences AHR activity. Early in the tumorigenesis process a complex multi-cellular inflammatory microenvironment develops. In this situation the level of AHR expression and activity probably resembles that of the tissue of origin and whether AHR ligands present are produced locally or systemically is not known. However, environmental ligands such as halogenated planar polycyclic hydrocarbons, as well as dietary ligands previously described, could facilitate a significant level of activation through systemic circulation. Indeed, AHR ligands have been detected in serum from cancer-free individuals through the use of reporter assay systems[28, 29]. Several indole-derived potent human AHR ligands, indirubin, indigo and metabolites of 6-formylindolo[3,2-*b*]carbazole, have been identified in human urine[30, 31]. Whether these compounds are dietary, generated by the host, or through bacterial metabolism has not been firmly established. Nevertheless, these compounds appear to add to the number of AHR agonists, found in healthy humans (Box 1).

As a tumor grows and a complex inflammatory microenvironment develops, comprised of a number of cell types, endogenous ligands appear to be produced that could set up autocrine/paracrine pathways, leading to sustained AHR activation (Fig. 2). Perhaps the most significant are the tryptophan dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) pathways that produce kynurenine, a relatively weak AHR ligand, from tryptophan[32]. In the absence of a disease state, TDO is predominantly expressed in the liver and is the major degradation pathway that controls circulating tryptophan levels, while IDO is expressed in several immune cell types, particularly in an inflammatory environment. TDO and/or IDO, encoded by the *TDO2* and *IDO1* genes, can be expressed in a variety of tumors, leading to significant production of kynurenine[33]. Immune cells such as macrophages and dendritic cells within the tumor microenvironment are likely to have elevated levels of IDO1, contributing to the local depletion of tryptophan, along with increased tryptophan metabolites (AHR ligands), which contribute to increased formation of T_{Reg} cells and immune tolerance[34]. There is a positive correlation between,the expression of TDO and the AHR regulated gene *CYP1B1* in human glioblastoma tissues. In addition, glioma cell lines can produce high levels of kynurenine capable of activating the AHR [35]. These studies also demonstrated that increased *CYP1B1* mRNA expression positively correlated with poor survival in patients with glioblastomas. A survey of glioma cell lines revealed that many are capable of producing kynurenine up to a concentration of 60 μM in cell culture[35]. Whether the level of AHR activity observed in these tumors is predominantly due to kynurenine or

the presence of other AHR ligands was not established. Indeed, other products of the IDO degradation pathway, such as kynurenic and xanthurenic acid, are more potent AHR ligands than kynurenine[36]. In fact, kynurenic acid levels have been observed to positively correlate with the size of pancreatic adenocarcinomas[37]. Utilizing a glioma line with AHR expression ablated in a xenograph model, results suggested that many of the effects of kynurenine on tumor cell growth appear to be mediated by the AHR. Thus, the high normal circulating levels of tryptophan coupled with increased TDO/IDO expression in late stage tumors, could lead to significant levels of AHR ligand production and subsequent AHR activation.

Indole is a weak human AHR agonist that is generated from tryptophan in significant quantities by gut bacteria in the colon[11]. Indole is then absorbed by the host, circulates to the liver where it is hydroxylated by CYP2E1 followed by sulfation, forming indoxyl sulfate. The kidney then efficiently excretes this metabolite. Indoxyl sulfate is a potent endogenous ligand for the human AHR but exhibits a 40–fold lower activation potential for the mouse AHR[38]. In healthy humans the serum concentration of indoxyl sulfate is 2 μM, with almost 100% bound to serum proteins. In contrast, the free concentration of indoxyl sulfate in dialysis patients has been estimated to be 12-30 μM, and this level would likely lead to increased AHR activity[39, 40]. In support of this concept is the observation that in a nephrectomized rat model elevated indoxyl sulfate levels resulted in a significant increase in the AHR target gene *Cyp1a2* in kidney and liver [41]. Interestingly, the longer a patient is on kidney dialysis the greater the risk of cancer after kidney transplantation [42]. Compromised kidney function often occurs in cancer patients. Thus, in the late stage of tumor progression, a wide spectrum of AHR agonists exists that may drive AHR transcriptional activity. This work firmly suggests that AHR activation could play an important role in human tumor progression[43].

Consequences of AHR activation in cancer

Genetic manipulation of AHR expression and cancer

One approach to gain insight into the role of the AHR in tumorigenesis would be to utilize $Ahr^{-/-}$ mice. After diethylnitrosamine treatment $Ahr^{-/-}$ mice developed significantly more liver adenomas and exhibited elevated proliferative and inflammatory marker gene expression compared to $Ahr^{+/+}$ mice[44]. Cecal tumorigenesis was also enhanced in $Ahr^{-/-}$ mice and heightened inflammatory signaling was observed[45]. In addition, loss of *Ahr* expression increased the incidence of prostate tumor formation in a TRAMP mouse model, further supporting the theory that expression of the AHR attenuates tumorigenesis[46]. In order to examine a model that would exhibit high constitutive AHR transcriptional activity, a transgenic mouse was bred that expresses the AHR with the hsp90-binding domain deleted (a.a. residues 288-421). This mouse line was termed CA-AhR and revealed enhanced levels of liver tumors after diethylnitrosamine exposure[47]. Elevated levels of gastric tumors were also observed to form spontaneously in CA-AhR mice[48]. The drawback of using genetic models is that one does not know whether the effects observed are due to developmental issues or are early or late in the carcinogenesis process. In addition, it is difficult to totally assess whether these mouse models truly yield insight as to what effect targeting AHR has on the tumorigenesis process. Nevertheless, these observations support the overall

conclusion that AHR expression attenuates carcinogenesis and AHR activation enhances carcinogenesis. However, the lack of AHR expression should not be equated with repression of AHR activity, especially when repression occurs late in the tumorigenesis process, such as the treatment of an existing tumor with an antagonist.

Proliferation

The AHR function with regard to tumor cell proliferation appears to depend on which cell line or tumor model is used and the mechanism of action under investigation. An integrated and comprehensive view of the role of AHR in determining tumor growth is currently lacking due to the fact that most studies have been restricted to cell culture systems using clonal cancer lines with different oncogenic backgrounds. These cloned cell lines are usually derived from different tissues and cultured under optimal conditions, which lack the complex milieu of immune, stromal and tumor-associated signaling that occurs with *in vivo* malignancy. Nonetheless, it is clear from numerous lines of evidence that AHR does impact tumor cell proliferation through diverse and contradictory mechanisms, thus making AHR a theoretical target for suppression of tumor growth[49].

Several growth factors are known to be important in tumor cell proliferation and elevated expression levels are associated with a range of cancers. Importantly, epiregulin and amphiregulin, fibroblast growth factor-9, osteopontin, and vascular endothelial growth factor all exhibit a degree of AHR dependency with regard to their expression[50-55]. In fact, the promoters of many of these genes (e.g. epiregulin) contain AHR DNA response elements and exhibit enhanced expression following exposure to AHR agonists[50]. As potent mitogens, enhanced expression of these factors is likely to contribute to tumor cell proliferation [54, 56-58]. Indeed, these factors are present at increased levels within tumors of different origins. It has thus far not been established whether the elevated levels can be attributed to heightened AHR activity, although increased AHR expression is often observed in tumors (Box 2), and that, combined with the omnipresence of AHR agonists (environmental, dietary, endogenous and tumor-derived), renders some level of AHR involvement likely. Furthermore, such mitogens have been demonstrated to respond positively to tumor-associated inflammatory stimuli (*e.g.* Il6 and IL1B) due to the presence of the NFkB response element in their promoters, which in cell culture models provide a synergistic induction in combination with AHR agonist [18, 50]. These effects of AHR upon growth factor signaling may account for the tumor promoting activity of TCDD observed in rodents [2]. In addition to modulating expression of upstream mitogenic factors, multiple components of the core cell cycle machinery are directly influenced by AHR activity in cell culture (Fig. 3). Several excellent reviews have been published on this subject [59, 60].

AHR/ER crosstalk and cancer

The endocrine disrupting effects of compounds subsequently identified as AHR ligands, point to the AHR as a modulator of hormone receptor function. Subsequent studies have revealed that both the androgen receptor and the estrogen receptor (ER), in particular, are sensitive targets of AHR inhibition in a ligand-dependent manner[61]. Consequently, there has been an extensive analysis of the role of AHR in estrogen-dependent breast cancer. A complex picture is emerging with numerous mechanisms proposed to account for the

generally anti-estrogenic nature of AHR in breast and other cancers [62-64]. For example, AHR agonists stimulate CYP1A1/B1-mediated estrogen depletion [65]. Proteosomal degradation of ER is enhanced by AHR acting as a component of the ubiquitin ligase complex. However, its exact role in these complexes has not been established [66, 67]. In addition, estrogen-dependent transcription of numerous proliferation/apoptosis target genes is suppressed in an AHR-dependent fashion [68]. These mechanisms of AHR activity are largely mediated through direct interaction with ER, resulting in mutual inhibition of DNA binding and disruption of coactivator/repressor recruitment [66, 69, 70]. These suppressive mechanisms are believed to account for some of the anti-proliferative/pro-apoptotic effects of AHR agonists upon estrogen-dependent tumor growth models.

Metastasis

The signaling events that initiate metastatic progression of tumors have yet to be fully elucidated, but increasing evidence points to a role for the AHR in the modulation of cell adhesion and migratory potential (Fig. 4)[16]. Studies have identified increased transient AHR nuclear translocation and activity during loss of cell-cell contact through a mechanism involving Jun N-terminal kinase (JNK) activation [71, 72], as well as augmented migration in multiple cell culture models[73, 74]. Thus, AHR activation, whether arising from a loss of cell adhesion or agonist stimulation, may promote metastasis. A number of pro-migratory factors appear to be impacted by AHR, including adhesion components [75], proteases[74], cytokines [76], signal transduction adaptors [77, 78] and transcription factors [79, 80]. In contrast, expression of the chemokine CXCR4 is down regulated by AHR agonist mediated activity in breast cancer cells, suggesting an anti-metastatic effect of AHR activation [81, 82]. Clearly, in vivo studies are needed to take into account the effects of AHR activation on metastatic potential within the complex tumor microenvironment.

Extrinsic factors that modulate AHR activity in tumors

An important consideration concerning AHR expression in tumor cells is that elevated expression may or may not correlate with increased AHR activity, such that any AHR-mediated response is likely dictated by numerous factors, both intrinsic and extrinsic to AHR signaling. Localized generation and availability of AHR ligands clearly will influence AHR activity at the resolution of a single cell. However, the combination of AHR and its ligands may not be permissive for any given AHR activity in the context of competing signals. For example, estrogen receptor status in breast cancer is likely to influence AHR activity [83, 84]. Both receptors exhibit mutual antagonism with regard to the other, indeed this is the basis for the extensive studies purporting the beneficial aspects of AHR agonists in limiting the proliferative effects of the estrogen receptor in models of hormone-dependent breast cancer [70, 85]. Estrogen receptor-dependent suppression of AHR activity may attenuate the anti-proliferative action of AHR ligands. Conversely, hormone-dependent inhibition may mitigate AHR-mediated growth factor expression, leading to tumor suppression. Therefore, AHR activity in estrogen receptor positive breast cancer cells likely differs from that in estrogen receptor negative cells. Indeed, the ability of AHR to crosstalk with numerous signaling components introduces a confounding level of complexity when trying to assess the responsiveness of AHR in any given tumor cell. Additional factors may limit AHR responsiveness such as, the metabolic and transporter activities of tumor cells, which may

limit exposure to AHR ligands. AHR activity may also vary within a given tumor due to nutrient availability, cell cycle status, redox status, hypoxia, cell-cell contact, or cytokine levels, all of which are reported to influence AHR-mediated signaling [86-89]. Thus, it seems likely that the response of AHR to ligands in cancer is probably highly contextual, which may account for the dichotomous observations that AHR exhibits both tumorigenic and suppressor activity. Such contextual complexity is inherently difficult to dissect and cannot be effectively addressed with in vitro cell culture studies. Even in vivo models examining the role of AHR in cancer are highly likely to impart model-dependent contextual bias.

AHR and innate immunity

The central role of innate immune signaling in the development and progression of tumorigenesis is becoming increasingly apparent. Multiple cell types within the tumor contribute to the formation of an anti-apoptotic, increased angiogenic, invasive, and metastatic phenotype[90]. Within the tumor microenvironment the neoplastic, immune and stromal cells can all participate in the expression of cytokines, chemokines and other inflammatory mediators, leading to positive paracrine feedback loops, which ultimately result in sustained or chronic inflammation. Recent reviews detail the altered expression of a wide variety of inflammatory signaling molecules after AHR ligand exposure[63, 91, 92]. In *Ahr* $^{-/-}$ mice, a challenge with lipopolysaccharide (LPS) leads to a heightened induction of a number of cytokines, suggesting that the AHR may play a role in constitutively attenuating inflammation[93]. Whether AHR activation regulates cytokine/chemokine expression directly or indirectly is important to consider when assessing the role of the AHR in inflammation. Evidence for direct transcriptional regulation of cytokines by the AHR is somewhat limited, but has been established for *Il1b*, *Il6*, and *Il21* through promoter analysis[27, 94, 95]. In type 1 regulatory T cells both *Il10* and *Il21* are directly regulated by c-maf and the AHR in a synergistic manner. Treatment of MCF-7 or ECC-1 cancer cell lines with an AHR ligand in the presence of an inflammatory signal synergistically induces IL6[96]. The mechanism of this synergism is driven by AHR/ARNT occupancy of DRE elements ~3 kB upstream from the transcriptional start site, which in turn mediates displacement of histone deacetylase 1 (HDAC1) from the *Il6* promoter and subsequent acetylation of NF-kB (Fig. 4)[27]. This mode of regulation was also observed in head and neck squamous cell carcinoma lines[76]. The proximal promoters of a number of cytokine/chemokine genes have DRE elements, raising the possibility that the AHR directly regulates a much larger subset of genes, especially in combination with activation of a transcription factor(s) (e.g. NFkB) associated with inflammation[97]. Further support for synergistic regulation of cytokine/chemokine expression (e.g. *Il6*, *Il10*, *Il22*, *Cxcl3*) was observed through AHR activation in the presence of LPS in bone marrow-derived dendritic cells[98]. Cyclooxygenase 2 gene expression is also directly regulated by activation of both NFkB and AHR in a variety of cell types[99, 100]. The logic for the AHR mediating inflammation in this manner is most likely based on the role of the AHR in barrier function and as a sensor for ligands generated by flora or the host at the site of an infection. Furthermore, in an inflammatory environment such as a carcinoma line of epithelial origin, the AHR can participate directly in the regulation of several growth factors, consistent with observations in a barrier tissue injury[50]. Along this line of reasoning, the inflammatory microenvironment within a tumor of epithelial origin likely leads to a broader repertoire of genes altered by the AHR through combinatorial gene regulation.

The AHR appears to regulate innate inflammatory signaling, not only through binding to its cognate response element in association with ARNT, but also through direct binding to RelA and RelB, members of the NFkB family of transcription factors [101, 102]. Interaction of the AHR with RelA has been shown to either repress or enhance RelA transcriptional activity in a context specific manner[103]. The AHR has been shown to bind to RelB and this complex can interact with p52/RelB within the promoter of a number of RelB target genes[102, 104]. AhR/RelB complexes have also been shown to bind to DREs, further increasing the complexity of target genes influenced by this heterodimer. In addition, interaction of RelB and AHR in the breast cancer cell lines MDA-MB 436 and MCF-7 appears to mediate IL-8 expression[105]. These studies further support the concept that AHR activation contributes to inflammatory signaling in tumors through multiple mechanisms.

AHR and adaptive immunity

Historically, the importance of adaptive immunity and immune tumor surveillance in tumorigenesis has been controversial. More recent studies have now firmly established that tumors escape immune surveillance through deletion and inactivation of self-reactive lymphocytes and that this is an important early event in tumor development[106-108]. Locally induced T-regulatory 1 (T_R1) and thymus-derived natural T_{Reg} cells are believed to play a central role in mediating a suppressed immune environment within a tumor. The AHR has been shown to be an important regulator of T cell differentiation, with AHR levels markedly induced during this process[95, 109, 110]. The AHR functions in concert with c-Maf to enhance *Il10* and *Il21* expression, leading to the formation of T_R1 cells. TCDD can also induce T_{Reg} cell production, suggesting that the presence of endogenous AHR ligands would enhance T_{Reg} cell production in the tumor microenvironment[111]. However, the ability of the AHR to influence chronic disease progression from an adaptive immunity perspective has been examined largely in autoimmune disease models. The AHR was initially shown to favor the formation of T_{Reg} cells, following treatment with TCDD[112]. TCDD has been shown to attenuate autoimmune type1 diabetes in a NOD mouse model and colitis in mice, which correlated with the enhanced production of T_{Reg} cells[113-115]. The AHR can induce both T_{Reg} and T_H17 cells, with TCDD favoring the production of T_{Reg} cells concomitant with a decrease in experimental autoimmune encephalomyelitis (EAE) progression[116]. In contrast, the AHR agonist FICZ favors T_H17 production and a subsequent increase in the severity of EAE. This would clearly suggest that the AHR agonist utilized in a given study could influence the outcome in an autoimmune disease model. A new class of AHR ligands, benzimidazoisoquinolines, has been identified that is capable of mediating enhanced T_{Reg} production in a mouse graft-versus-host autoimmune model, leading to suppression of clinical symptoms[117]. This study supports the concept that an AHR agonist could be developed that would be useful in organ transplants (e.g. bone marrow transplants) if the potential for possible long-term adverse effects of agonist treatment are resolved. One possible contributor to the in vivo production of T_{Reg} cells is AHR-mediated induction of IDO and kynurenine [32]. In addition, naringenin a dietary flavonoid that is an AHR agonist, is also capable of inducing T_{Reg} cell production[118]. These results suggest that AHR likely participates in the regulation of T_{Reg} differentiation due to the presence of endogenous, exogenous, and dietary ligands. Considering the studies examining the effects of AHR ligands on suppression of autoimmunity, it is reasonable to hypothesize that AHR activity

within the tumor microenvironment, such as increased production of T_{Reg} cells, may in part explain the tumor promoting properties of TCDD.

Potential therapeutic manipulation of AHR activity

Antagonism of AHR activity

AHR antagonists are increasingly being examined as possible therapeutic agents. Perhaps the first AHR antagonist described was a derivative of TCDD, 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin[119]. Historically, the most utilized AHR antagonist is α-naphthoflavone, which actually exhibits weak agonist activity at high concentrations[120, 121]. Other flavone-based antagonists that have been described include 3′methoxy-4′-nitroflavone and 6,2′,4′-trimethoxyflavone, with the latter exhibiting no agonist activity[122, 123]. Interestingly, some of the beneficial activity of resveratrol has been attributed to its antagonism of the AHR[124]. More recently, higher affinity AHR antagonists have been identified that appear to have no agonist potential[125-127]. For example, the compound CH-223191 has been successfully used in rodents[128]. However, it fails to block agonist activity of β-naphthoflavone, highlighting the possibility that a given AHR antagonist may not block all agonist activity in a given setting[129]. A high affinity AHR antagonist, StemRegenin 1, was identified by a high throughput screen for compounds that enhance the expansion of hematopoietic stem cells ex vivo[126]. Interestingly, this compound has much higher affinity for the human versus mouse AHR, illustrating the difference in ligand specificity between species. GNF-351 is an AHR antagonist that was generated during medicinal chemistry optimization of StemReginin 1[127]. GNF351 does not exhibit any agonist activity and efficiently inhibits both the human and mouse AHR. These recently identified AHR antagonists should serve as useful pharmacologic tools to study the role of the AHR in tumorigenesis.

Development of Selective AHR modulators

Selective nuclear receptor ligands were first described for the estrogen receptor (ER) and were named selective ER modulators (SERMs). These compounds (e.g. tamoxifen, raloxifene) have been shown to alter the spectrum of genes that are activated by the liganded ER complex through differential co-activator/co-repressor recruitment[130]. This mechanism is based on the concept that the AF-2 transactivation domain in the ER is localized near the ligand-binding domain and SERMs alter the coactivator recruitment interface. The ligand binding and transactivation domains of the AHR appear to be distinct and it is not clear whether different ligands can mediate differential coactivator recruitment. However, selective Ah receptor modulators (SAhRM), such as 6-methyl-1,3,8-trichlorodibenzofuran and 1,1′-dimethyl diindolylmethane, have been identified that exhibit weak AHR agonist activity, yet still fully repress estrogen receptor activity through ER/AHR crosstalk in a manner similar to a full agonist[131]. Whether or not this anti-nuclear receptor activity is the primary or only therapeutic activity elicited by these compounds warrants further investigation. Another class of selective ER ligands has been generated that dissociates agonist or DNA binding activity from transrepression of NFkB and AP-1 transcriptional activity [132, 133]. [134]Selective AHR ligands capable of repressing acute phase gene expression, yet exhibiting no AHR agonist activity have been developed (e.g. SGA360 and

3′,4′-dimethoxy-α-naphthoflavone) [134, 135, 136]. Results from these studies clearly indicate that the AHR can exhibit robust anti-inflammatory potential in the absence of DRE-mediated activity, and most likely through protein-protein interactions. The identification of this class of ligands also suggests that the AHR can exhibit constitutive anti-inflammatory activity in the apparent absence of DRE-mediated activity.

SAhRM and modulation of tumorigenesis

Numerous studies have been performed to investigate the role of the AHR upon ligand treatment in cultured tumor cells examining specific endpoints. However, there are far fewer studies that have tested the effect of various functionally distinct AHR ligand classes on tumorigenesis, especially from a therapeutic standpoint in vivo. The laboratory of Dr. Stephen Safe has pioneered the development of SAhRMs that show therapeutic potential [64]. His work has focused on the development of weak AHR agonists that would not exhibit the toxicity of a full agonist, such as TCDD, using *CYP1A1* induction as a measure of transcriptional activity. In the presence of a full agonist these compounds would exhibit AHR antagonistic activity. The SAhRM that was most utilized in in vivo studies is 6-methyl-1,3,8-trichlorodibenzo-*p*-dioxin (MCDF), which has moderate affinity for the AHR but only exhibits weak agonist activity[137]. However, this weak agonist is still able to mediate antiestrogenic activity and repress development of rat mammary tumors after exposure to a carcinogen[138, 139]. MCDF also inhibited growth of ER negative breast cancer and lung metastasis in a xenograph mouse models, suggesting that MCDF exhibited anti-tumorigenic activity through suppresion of tumor cell proliferation and metastasis[140, 141]. These studies indicate that there is considerable potential for the use of SAhRM in cancer treatments.

Concluding remarks

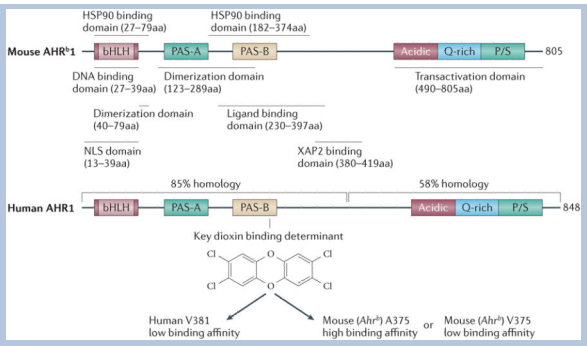
The study of the effects of AHR agonists together with the development of potent antagonists, and SAhRMs/weak agonists, has highlighted many important, if conflicting and counterintuitive, aspects regarding the role of AHR in tumorigenesis. Evaluation of the therapeutic efficacy of AHR ligands with regard to suppression of tumorigenic outcomes awaits the identification and development of specific high affinity AHR ligands that exhibit improved ADME profiles and their subsequent use in multiple in vivo models of cancer. The differences between mouse and human AHR activities suggest that ‘humanized’ AHR in vivo models would represent the preferred route. Additionally, given the contradictory observations that AHR is both pro-tumorigenic and a tumor suppressor suggests that an efficacious AHR ligand may not exhibit a “one-size-fits-all” role for limiting different types of cancer. Indeed, within a single model any AHR ligand may display temporal effectiveness, being beneficial in a chemopreventative regime but deleterious if a tumor becomes established, or *vice versa*. AHR agonists appear effective in attenuating cell proliferation/migration in numerous in vitro settings and limited in vivo models. However, their therapeutic application in humans has yet to be examined and is likely to be tainted by the established roles of AHR activation in biotransformation, mutagenesis, tumor promotion and immune suppression. The multitude of activities elicited by agonists evidently cannot be attributed entirely to direct AHR/ARNT heterodimer/DRE-mediated transcription and may

reflect the ability of agonists to induce selective AHR activity. Continued examination of these agonist-mediated effects will be informative to the development of potent SAhRMs that can harness and specifically target the beneficial selective component of agonists without the associated toxicities. With the recent development of potent AHR antagonists, it will be important to test their effects on tumorigenesis and, perhaps most importantly, as a treatment for existing tumors. Indeed, the fact that antagonists and agonists are both capable of inhibiting tumor cell phenotype in vitro may suggest that they work through distinctly different mechanisms, yet yield the same end result. Considering the emerging role of the AHR in immune tolerance, testing of AHR antagonists as an adjunct to immunotherapy seems to be warranted. The role of endogenous ligands, in particular those produced in the gut and the tumor microenvironment, needs to be explored in terms of whether they participate in tumor progression in a variety of tumor types. The abundance of evidence demonstrating the importance of AHR in dictating tumorigenic outcomes suggests that therapeutic manipulation of AHR in human cancer is on the horizon. Whether this is through agonist or antagonist/SAhRM will be dictated by data obtained from more extensive in vivo studies using these different classes of AHR ligands as a treatment of existing tumors.

Box 1

The human AHR exhibits differential activity relative to the rodent AHR: An emerging issue

In vivo rodent studies have clearly pointed to a role for the AHR in tumorigenesis. However, there are several significant structural differences between the human and mouse AHR that lead to changes in AHR function. The first difference observed was a decrease in stability of the hAHR/hsp90 interaction compared to the mAHR[142]. An amino acid sequence comparison between the mouse and human AHR revealed ~85% sequence homology within the N-terminal half of the receptor, while the C-terminal half exhibits much lower sequence homology, the majority of the non-conserved changes occurring in the transactivation domain. Studies using primary hepatocytes from humans, mice, and humanized mice have revealed that the human and mouse AHR differentially mediate gene expression in hepatocytes[143-145]. One report has shown that the transactivation domain of the hAHR differs relative to the mAHR in terms of its ability to interact with coactivator motifs[146]. These studies would suggest that there are significant species differences in gene transactivation selectivity and potential. Perhaps the most dramatic difference between the human and mouse AHR is the distinct ligand selectivity. The mAHR binds TCDD with a 10-fold higher affinity compared to the hAHR, due to the difference in a single amino acid residue in the middle of the ligand binding pocket[147]. Conversely, the hAHR binds endogenous indolic derivatives such as indirubin and indoxyl sulfate with much higher affinity compared to the mAHR[38, 148]. This in turn may suggest that the human AHR could differ in its “constitutive” activation status in a tumor, which would then imply that studies in mice might underestimate the role of the AHR in human tumorigenesis.



BOX 2

Ah receptor expression during tumorigenesis

The AHR is expressed in most tissues, except skeletal muscle, and expression levels vary widely, with the highest levels observed in liver, lung, spleen, and kidney. Within a given tissue, generally, the greatest level of expression is seen in cells of epithelial origin. Immunohistochemical analysis of breast, prostate, gastric, small cell lung, and liver tumors exhibit increased levels of AHR expression relative to surrounding tissue [64, 149-153]. One possible explanation for the enhanced level of expression in human tumors is the ability of activated STAT6 and NFkB to enhance AHR expression, which correlates with inflammatory status[154, 155]. A specific functional p65/p50 response element was identified in the proximal promoter of the human *Ahr* gene. Another aspect of receptor expression to address is whether the AHR is actually transcriptionally active. One indirect approach is to examine cytoplasmic versus nuclear localization using immunohistochemical visualization of tissue sections. Grading of prostate tissue sections revealed that increased nuclear localization of the AHR correlated positively with the level of poorly differentiated cells[150]. Increased levels of nuclear AHR were also associated with poor prognosis for patients with lung squamous cell carcinoma[152]. In contrast, the levels of AHR in breast cancer were inversely correlated with the histological grade of the tumor[149]. This may be due to the ability of the AHR to antagonize estrogen receptor activity. Another approach to examine the role of AHR activity in human tumor samples is to determine the level of expression of an AHR target gene (e.g. *CYP1B1*). For example, a comparison of *CYP1B1* expression levels with survival rates of patients with glioma revealed that increased *CYP1B1* expression correlated with lower patient survival[35]. Thus, recent studies clearly support that increased AHR expression or activity correlates with promotion of late stage tumorigenesis in most human tumors that have been examined and may confer a selective advantage.

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Biographies

Andrew D. Patterson received his Ph.D. from the Graduate Partnerships Program with George Washington University and the National Institutes of Health with Dr. Albert J. Fornace, Jr. and did his post-doctoral work with Dr. Frank J. Gonzalez. At the Pennsylvania State University, the Patterson lab is focused on understanding the host-microbiome metabolic axis with particular emphasis on the Ah receptor as a major regulator of this complex interaction. The lab employs NMR- and mass spectrometry-based metabolomics, metagenomics, and conventional and gnotobiotic genetically-modified mice to facilitate the study of these pathways and understand their impact on human health and disease.

Iain A. Murray obtained his Ph.D from the University of Manchester in the Endocrine Sciences Research Group under Professors Anne White & Julian Davis. Post-doctoral and subsequent research associate positions with Dr. Gary H. Perdew within the Center for Molecular Toxicology at the Pennsylvania State University have focused on investigating the biology and function of the Aryl hydrocarbon receptor using a range of in vitro and in vivo methodologies. Current research is aimed at the development of novel AHR ligands to dissociate beneficial/therapeutic aspects of AHR activation from established toxicological end-points with their potential application as anti-inflammatory agents.

Gary H. Perdew received his Ph.D from Oregon State University and completed post-doctoral training at McArdle Laboratory for Cancer Research in Dr. Alan Poland's laboratory. First faculty appointment was in the Department of Foods and Nutrition at Purdue University and after eight years moved to Penn State University where he is currently an endowed professor in the Department of Veterinary and Biomedical Sciences. The Perdew laboratory is interested in the biological function of the Ah receptor and mechanisms of dioxin toxicity mediated by hyperactivation of this receptor. His laboratory also focuses on possible therapeutic potential of selective activation of the Ah receptor.

Glossary

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, Dioxin)	A polycyclic halogenated hydrocarbon that is highly toxic to rodents and exhibits high affinity for the AHR
Full agonist	An AHR ligand that maximally elicits canonical DRE-mediated transcriptional responses
Weak agonist	An AHR ligand that displays partial agonist activity, eliciting a sub-maximal DRE-mediated transcriptional response. In addition, in the presence of a strong agonist will exhibit antagonist activity
SAhRM	An AHR ligand that displays functional selectivity, exhibiting negligible DRE-mediated transcriptional responses while maximally stimulating non-DRE mediated AHR activity

Antagonist	An AHR ligand that inhibits canonical DRE-mediated and non-DRE mediated AHR activity
Polycyclic aromatic hydrocarbons	A group of over 100 different stable organic molecules comprised of only carbon and hydrogen. They are large, planar molecules assembled from a collection of fused benzene-like rings. They are formed during the incomplete burning of coal, oil and gas, garbage, or other organic substances like tobacco or charbroiled meat
Barrier function	This term refers to the integrity of a protective epithelial layer that serves as a barrier and allows selective absorption
Epithelial-mesenchymal transition (EMT)	The process by which cells convert from an epithelial to a mesenchymal phenotype. This process, which is enacted during normal embryonic development, can be abnormally activated in carcinomas, resulting in altered cell morphology, the expression of mesenchymal proteins and increased invasiveness

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Key Points

The Ah receptor is a ligand activated transcription factor that is best known for mediating the toxicity and tumor promotional properties of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, commonly referred to as “dioxin”.

Three distinct classes of ligands bind to the Ah receptor, agonist, antagonist and selective Ah receptor modulators.

The human Ah receptor compared to the mouse Ah receptor exhibits significant differences in ligand specificity, which may influence the progression of cancer and thus complicates the validity of the mouse model for human carcinogenesis.

Numerous studies demonstrate the ability of the Ah receptor to modulate proliferative and migratory potential.

The Ah receptor is activated by endogenous ligands such as kynurenine, kynurenic acid and indoxyl sulfate.

Physiologically relevant flora can produce potent Ah receptor ligands from tryptophan.

Ah receptor directly modulates inflammatory signaling.

Ah receptor agonist-mediated activity can play a key role in the production of T_{Reg} cells and thus could play a role in immune tolerance in cancer.

Ah receptor levels are often increased in tumors, most likely through inflammatory signaling.

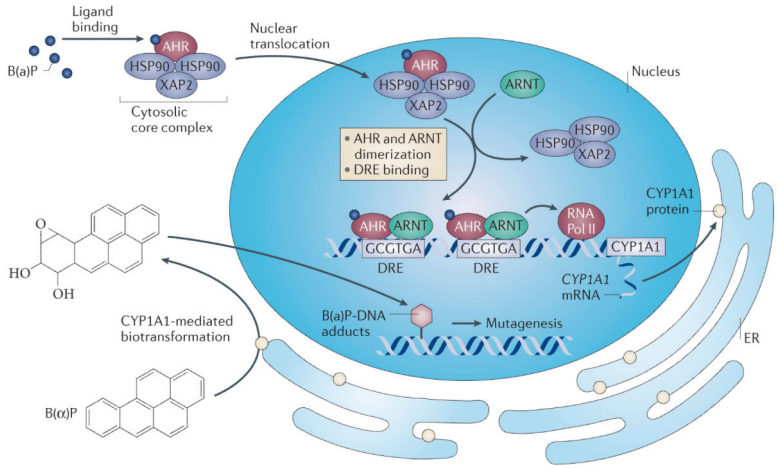

Figure 1. Agonist-mediated activation of the AHR

The unliganded AHR resides in the cytoplasm of a cell, complexed with a dimer of HSP90 and the co-chaperone protein X-associated protein 2[156]. The AHR contains both a nuclear localization and a nuclear export signal sequence and undergoes nucleocytoplasmic shuttling. Upon binding an agonist, the AHR complex translocates into the nucleus and ARNT mediates HSP90 displacement, leading to AHR/ARNT heterodimer formation. This heterodimer is capable of binding to a dioxin responsive element (DRE) with the sequence 5′-T/G/TCGTGA/CG/TA/T-3′. Both the AHR and ARNT can recruit coactivators, leading to transcription of a wide variety of genes. The AHR target gene *CYP1A1* is almost totally dependent on AHR activity for expression and is highly induced by AHR activation through multiple DREs. CYP1A1 metabolizes a number of pro-carcinogens, such as benzo(a)pyrene, to intermediates that can react with DNA to form adducts, resulting in subsequent mutagenesis.

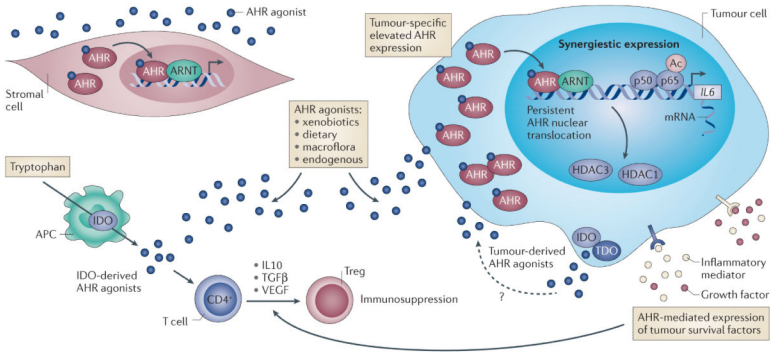

Figure 2. AHR activity within the tumor micro-environment

Tumor-associated AHR activity is elevated when compared to surrounding tissue, suggesting that AHR may influence tumor development. The mechanisms that govern enhanced tumor-associated AHR expression are unclear; notwithstanding, increased AHR expression is likely to elevate basal AHR activity within tumors, especially given the systemic omnipresence of AHR agonist ligands derived from xenobiotic, dietary and microbial sources. Indeed, malignant tissue exhibits enhanced nuclear localization of AHR together with increased expression of the prototypical AHR target gene *CYP1A1*, indicative of higher AHR transcriptional activity. The presence of immune cells (e.g. antigen-presenting cells, APC) within the tumor microenvironment, in conjunction with malignant cells, often exhibit enhanced expression of IDO and TDO [157, 158]. These enzymes generate agonistic AHR ligands from tryptophan within the tumor micro-environment, thus adding to the AHR activation potential within tumors. The consequences of enhanced AHR expression and activities arising from systemic and tumor-derived AHR agonists have not been thoroughly investigated; however, such activation is likely to promote tumor growth. AHR activation and inflammatory cytokine signaling, a common feature of tumors, results in synergistic induction of pro-inflammatory factors, including IL6, exacerbating inflammation while simultaneously promoting diffentiation of immune-suppressive Treg cells through increased IL10, TGF, and VEGF expression. Systemic and tumor-localized generation of AHR ligands, heightened AHR expression/activity may establish a pro-inflammatory yet immune-suppressive tumor micro-environment, favoring tumor survival and escape from immune surveillance, which results in tumor progression.

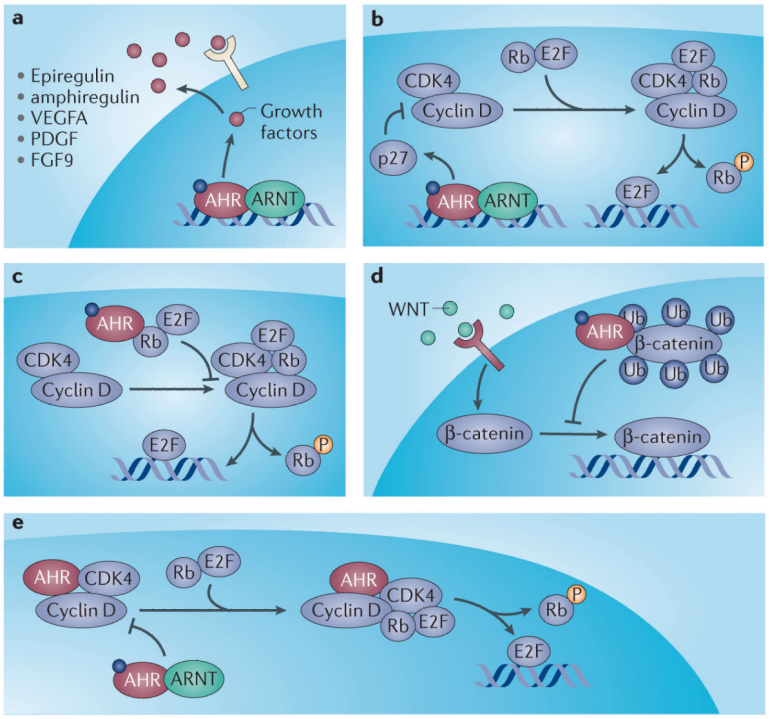

Figure 3. Proposed mechanisms of cell cycle modulation by the AHR

Multiple mechanisms are proposed to account for the pro-/anti-proliferative action of AHR agonists observed with tumor cells in vitro. 1) Binding to AHR response elements in promoters of their respective genes, AHR acts as a direct transcriptional activator stimulating expression of growth factors epiregulin and amphiregulin. As mitogens, these contribute to the proliferation of tumor cells and may account for AHR agonist-mediated tumor cell expansion. 2) Agonist-activated AHR binds to the promoter of p27 (*CDKN1B*) enhancing its expression [159, 160]. As an inhibitor of cyclin-dependent kinase activity, increased p27 limits phosphorylation of retinoblastoma protein (Rb) thus restricting E2F-dependent gene expression and progression through the cell cycle. 3) Association of AHR with Rb in an agonist-dependent manner attenuates both phosphorylation of Rb and liberation of E2F, resulting in cell cycle arrest and inhibition of proliferation [161]. 4) Agonist activation of AHR promotes association with β-catenin and stimulation of a previously unrecognized ubiquitin ligase function of AHR [45, 67]. Ubiquitination of β-catenin in an AHR-dependent fashion promotes proteosomal degradation, restricting cell cycle-dependent gene expression and proliferation. 5) In the absence of ligand, AHR forms a complex with cyclin D and the cyclin-dependent kinases CDK4/6, suppressing phosphorylation of Rb and subsequent E2F-mediated gene expression to promote cell cycle arrest [162]. Exposure to AHR agonist favors the dissociation of the AHR/cyclinD/CDK complex to permit cell cycle progression and tumor cell proliferation. The contradictory nature of these mechanisms may reflect cell-type/culture-dependent differences and emphasize the need to investigate the effect of AHR ligands in the context of in vivo proliferation models.

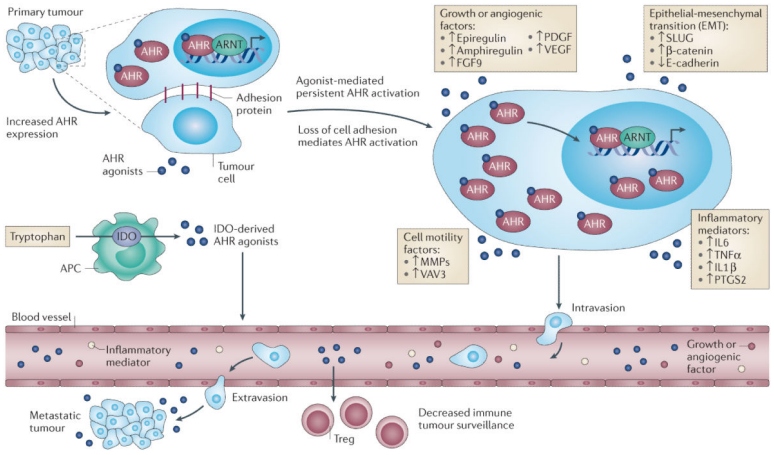

Figure 4. Proposed role of the AHR in tumor metastasis

The dual effect of elevated AHR expression and localized AHR agonist generation by tumor and tumor-associated immune cells increases AHR activity, leading to initiation of epithelial-mesenchymal transition (EMT) and facilitating tumor cell migration, invasiveness, and metastasis. Heightened AHR activity promotes the expression of SLUG, which then inhibits E-cadherin expression thus decreasing cell adhesion. AHR-dependent expression of matrix metalloproteases (MMPs) and intra-cellular signaling factors, which promote cytoskeletal rearrangement (e.g. VAV3) render tumor cells increasingly motile [78]. The presence of tumor-associated inflammatory cytokine signaling results in a self-sustaining synergistic loop in combination with AHR activation, which enhances cell motility [18]. Elevated growth and pro-angiogenic factor gene expression elicited in an AHR-dependent manner provides an escape and proliferative route for motile tumor cells. The generation of AHR agonists by tumor-associated immune cells facilitates the differentiation of immune-suppressive Treg cells, which dampens the immune response to isolated motile tumor cells, thus allowing metastasis and the establishment of distant secondary tumors.

Compounds that exhibit agonist/antagonist/selective AHR activity

Activity	Source	Examples
Agonist	**Xenobiotics**	**Halogenated aromatic hydrocarbons** 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin[163, 164] dibenzofurans[165] biphenyls[166] _ENREF_166
		Polyaromatic hydrocarbons 3-methylchlolanthrene[165] benzo(a)pyrene[148] benzanthracenes[167] benzoflavones[168]
		Pharmaceuticals tranilast[169] leflutamide[170] omeprazole[169, 171]
	Dietary	**Flavonoids** quercetin[148, 172] galangin[9]
		Indoles indole-3-carbino[110, 168] 3, 3-diindoylmethane[10, 168] indolo[3, 2b]carbazole[10]
	Endogenous	**Tryptophan metabolites** kynurenic acid[36] kynurenine[32] 6-formylindolo[3,2b]carbazole[173] indoxyl sulfate[38]
		Others indirubin[30] 7-ketocholesterol[174]
	Microflora	3-methylindole[11] trypthantrin[14] 1, 4-dihydroxy-2-naphtoic acid[13] malassezin[14]
Antagonist	**Xenobiotic Dietary**	6, 2, 4,-trimethoxyflavone[123] GNF351[127] CH-223191[125] resveratrol[124]
Selective AHR modulator	**Xenobiotic**	SGA360[135] 3, 4-dimethoxy-a-naphthoflavone[136] 6-methoxy-1, 3, 8-trichlorodibenzofuran[165]