

MICROBIOME-DERIVED TRYPTOPHAN METABOLITES AND THEIR ARYL HYDROCARBON RECEPTOR-DEPENDENT AGONIST AND ANTAGONIST ACTIVITIES

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Abbreviations: AHR, aryl hydrocarbon receptor; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; DREs, dioxin response elements; FBS, fetal bovine serum; FICZ, 6-formylindolo-[3,2-b]-carbazole; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol-12-myristate 13-acetate; SAhRMs, selective aryl hydrocarbon receptor modulators; TBP, TATA binding protein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

ABSTRACT

The tryptophan metabolites indole, indole 3-acetate and tryptamine were identified in mouse cecal extracts and fecal pellets by mass spectrometry. The aryl hydrocarbon receptor (AHR) agonist and antagonist activities of these microbiota-derived compounds were investigated in CaCo-2 intestinal cells as a model for understanding their interactions with colonic tissue which is highly aryl hydrocarbon (AH)-responsive. Activation of AH-responsive genes demonstrated that tryptamine and indole 3-acetate were AHR agonists, whereas indole was an AHR antagonist that inhibited 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced *CYP1A1* expression. In contrast, the tryptophan metabolites exhibited minimal anti-inflammatory activities, whereas TCDD decreased phorbol ester-induced *CXCR4* gene expression, and this response was AHR-dependent. These results demonstrate that the tryptophan metabolites indole, tryptamine and indole 3-acetate modulate AHR-mediated responses in CaCo-2 cells, and concentrations of indole that exhibit AHR antagonist activity (100-250 μ M) are detected in the intestinal microbiome.

INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that forms a nuclear heterodimer with the AHR nuclear translocator protein to activate gene transcription (Denison et al., 2011; Hankinson, 1995). AHR complex-mediated gene expression involves interaction with dioxin responsive elements (DREs) in 5'-promoter regions of target genes, and the overall mechanisms of this response have been intensively investigated using the *CYP1A1* gene and other drug-metabolizing enzymes as models (Whitlock, 1999). More recent studies demonstrate that this genomic pathway for activation of the AHR is more complex than the classical induction of CYP1A1, and there is also evidence for extranuclear activity of the AHR (Huang and Elferink, 2012; Tanos et al., 2012). 2,3,7,8-Tetrachlordibenzo-*p*-dioxin (TCDD) and structurally related halogenated aromatics and polynuclear aromatic hydrocarbons were among the first compounds identified as AHR ligands (Bigelow and Nebert, 1982; Poland et al., 1976). However, subsequent studies have shown that this receptor binds structurally-diverse compounds which include industrial compounds, pharmaceuticals, phytochemicals such as flavonoids and indole derivatives, and endogenous biochemicals including indigoids, kynurenine, 7-ketocholesterol, 6-formylindolo-[3,2-*b*]-carbazole (FICZ), and bilirubin (reviewed in Denison and Nagy, 2003; Safe et al., 2012). AHR ligands exhibit both agonist and antagonist activities and there is evidence that some compounds exhibit tissue/cell-specific agonist or antagonist activities (Jordan, 2007). For example, α -naphthoflavone and 3'-methoxy-4'-nitroflavone were initially characterized as AHR antagonists but subsequent studies showed that they exhibit both agonist and antagonist activity (Lu et al., 1996; Zhou and Gasiewicz, 2003). The AHR-active pharmaceutical mexiletine induces hepatic CYP1A1 *in vivo* and *in vitro* and binds the AHR (Hu et al., 2007); however, in MDA-MB-468 breast cancer cells, mexiletine inhibits induction of

CYP1A1 by TCDD and is an AHR antagonist (Jin et al., 2012) and this is typical of a tissue-specific selective AHR modulator (SAhRM).

There is increasing evidence that the endogenous AHR plays an important functional role in multiple tissues and organs (Barouki et al., 2007; McMillan and Bradfield, 2007) and this has been amply demonstrated in AHR^{-/-} mice and by AHR silencing in non-transformed and transformed cell lines. For example, in gastrointestinal tissues including lymphoid tissues, the receptor and its ligands modulate inflammatory responses including those associated with induced colitis in animal models (Arsenescu et al., 2011; Benson and Shepherd, 2011; Furumatsu et al., 2011; Kawajiri et al., 2009; Kiss et al., 2011; Li et al., 2011; Singh et al., 2011). The AHR agonist β -naphthoflavone significantly suppresses dextran sodium sulfate-induced colitis in C57BL/6 mice (Furumatsu et al., 2011) and in the APC^{min} model of colon cancer, the loss of the AHR enhances colon carcinogenesis and AHR ligands inhibit polyp formation in APC^{min}/AHR^{+/+} mice (Kawajiri et al., 2009).

Previous studies in our laboratory have identified the tryptophan metabolite indole as a major extracellular metabolite produced by gut bacteria such as *Escherichia coli* (Bansal et al., 2010) and, in human feces, concentrations of indole can reach mM levels (Karlin et al., 1985; Zuccato et al., 1993). Indole exhibits AHR agonist activity in a yeast assay but is inactive in an Ah-responsive liver cancer cell line (Heath-Pagliuso et al., 1998; Miller, 1997). However, other tryptophan-derived compounds including tryptamine, indole-3-acetate, and 3-indoxyl sulfate have previously been characterized as AHR agonists (Gillner et al., 1985; Heath-Pagliuso et al., 1998; Miller, 1997; Schroeder et al., 2010; Vikstrom Bergander et al., 2012). It has been well established that the gut microbiome and its metabolites have a direct effect on intestinal homeostasis (DiNatale et al., 2010; Maynard et al., 2012; Tremaroli and Backhed, 2012; Villard

et al., 2007), and the AHR plays an important role in maintaining gut homeostasis (Arsenescu et al., 2011; Benson and Shepherd, 2011; Furumatsu et al., 2011; Kawajiri et al., 2009; Kiss et al., 2011; Li et al., 2011; Singh et al., 2011). Therefore, the major objectives of this research were to investigate the AHR agonist or antagonist activity of tryptophan-derived microbiota metabolites in CaCo-2 human epithelial colon cancer cells and other cell lines, and determine their role in modulating inflammation in CaCo-2 cells. The results clearly demonstrate the production of high levels of tryptophan metabolites in the gut microbiome, and the AHR agonist and antagonist activities of these metabolites are both response-, cell context- and compound-dependent which is typical of a SAhRM.

MATERIALS AND METHODS

Cell lines, antibodies, and reagents. CaCo-2 human colon cancer cell line and MDA-MB-468 and MDA-MB-231 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). CaCo-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) nutrient mixture supplemented with 20% fetal bovine serum (FBS), 10 ml/L 100X MEM non-essential amino acid solution (Gibco), and 10 ml/L 100X antibiotic/antimycotic solution (Sigma-Aldrich). MDA-MB-468 and MDA-MB-231 cells were maintained in DMEM nutrient mixture supplemented with 10% fetal bovine serum (FBS) and 10 ml/L 100X antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in the presence of 5% CO₂, and the solvent (dimethyl sulfoxide, DMSO) used in the experiments was ≤ 0.2%. Monoamine oxidase-A (MAO-A) siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz). CYP1A1, CYP1B1, AHR, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz) and β-catenin antibody were purchased from Cell Signaling. The CYP1A1 and CYP1B1 antibodies show minimal cross-reactivity. All compounds used in this study were purchased from Sigma-Aldrich.

Sample collection. Female C57BL/6 mice at 5 weeks of age were purchased from Jackson Laboratories (Bar Harbor, Maine) and allowed to acclimate for 1 week. All mice were maintained in a pathogen free animal facility located at Texas A&M Health Science Center. The animals were handled in accordance with the Institutional Animal Care and Use Committee guidelines under an approved animal use protocol. Mice ($n = 7$) were sacrificed at 6 weeks of age. The entire cecum (tissue with luminal contents) and fecal pellets were collected from each animal. The samples were weighed, flash frozen, and stored at -80°C before processing for extraction. Metabolites were extracted from cecal tissue or fecal pellets using a

methanol/chloroform extraction method (Sellick et al., 2010) with minor modifications. Cold methanol/chloroform (2:1, v/v; 1.5 mL) was added to a pre-weighed cecal or fecal sample and homogenized on ice. The sample tube was centrifuged at 15,000 x g for 10 min at 4°C and the supernatant transferred to a new sample tube through a 70 µm cell strainer. Ice-cold water (0.6 mL) was added and the sample tube was vortexed and centrifuged (15,000 x g, 5 min, 4°C) to obtain phase separation. The upper and lower phases were separately collected in fresh sample tubes with a syringe, taking care not to disturb the interface. The polar (upper) phase (500 µL) was evaporated to dryness in a Savant speedvac concentrator (Thermo Scientific, Asheville, NC), and then reconstituted in 50 µL of methanol/water (1:1, v/v). Extracted metabolites were stored at -80°C until analysis.

Metabolite analysis. Prior to sample analysis, MS parameters were optimized for each target metabolite to identify the MRM transition (precursor/product fragment ion pair) with the highest intensity under direction injection at 10 µL/min. The following parameters were optimized operating in positive mode: declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP). The optimized parameter values for the target metabolites analyzed in this study are shown in Supplemental Table 1. The target metabolites in samples were detected and quantified on a triple quadrupole linear ion trap mass spectrometer (3200 QTRAP, AB SCIEX, Foster City, CA) coupled to a binary pump HPLC (1200 Series, Agilent, Santa Clara, CA). Peak identification and integration were performed using Analyst software (version 5, Agilent, Foster City, CA). Samples were maintained at 4°C on an autosampler prior to injection. Chromatographic separation was achieved on a hydrophilic interaction column (Luna 5 µm NH₂ 100 Å 250 mm × 2 mm, Phenomenex, Torrance, CA) using a solvent gradient method (Bajad et al., 2006). Solvent A was an ammonium acetate (20 mM)

solution in water with 5% acetonitrile (v/v). The pH of solvent A was adjusted to 9.5 immediately prior to analysis using ammonium hydroxide. Solvent B was pure acetonitrile.

Cell proliferation (MTT) assay. Cells (5×10^3 per well) were plated in 96-well plates and allowed to attach for 16 hr. The medium was then changed to DMEM medium containing 2.5% FBS and 1X MEM non-essential amino acid, and either vehicle (DMSO) or different concentrations of the compounds were added. After 24 hr, treatment medium was replaced with fresh medium containing 0.05 mg of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) per 100 μ L and incubated for 4 hr. Medium was then removed, and 100 μ L of DMSO was added to wells. The optical density of each sample was read on a microplate reader (FLUOstar OPTIMA) at 570 nm against a blank prepared from cell-free wells. Cell proliferation was expressed as a % of relative absorbance of untreated controls.

Colonic crypt isolation and three-dimensional culture. Colonic crypts were isolated essentially as described (Davidson et al., 2012). Briefly, intact colons were everted on a disposable mouse gavage needle and incubated with 20 mM EDTA in Ca/Mg free HBSS at 37°C for 30 min. Colonic crypts were released by mechanical disruption and purified by a series of PBS washes and centrifugation steps (Davidson et al., 2012). Purified crypts were kept on ice, and resuspended in cold growth factor reduced, phenol red-free Matrigel (BD Bioscience, San Jose, CA) at a density of 15 crypts/ μ L. A total of 750 crypts/50 μ L Matrigel were plated onto the center of wells in a 24 well-plate and incubated at 37 °C for 10 min. After polymerization, complete medium containing Advanced DMEM/F12 (ADF) (Life Technologies, Grand Island, NY), EGF [50 ng/mL] (Life Technologies), Noggin [100 ng/mL] (Peprotech, Rocky Hill, NJ), R-Spondin (500 ng/mL] (Sino Biological, Beijing, China), N2 supplement [1X] (Invitrogen), B27 supplement [1X] (Life Technologies), N-acetylcysteine [1 μ M] (Sigma, St. Louis, MO) and Wnt

conditioned medium was added to the wells. After 7 d of culture to allow development of mature organoids, the culture was then incubated for 6 hr at 37 °C with 1 nM TCDD, 500 μM indole, or a combination of TCDD and indole.

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed using ChIP-IT Express Magnetic Chromatin Immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. CaCo-2 cells (5×10^6 cells) were treated with TCDD and/or compounds for 2 hr. Cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to desired chromatin length (~200-1500 bp). The sonicated chromatin was immunoprecipitated with normal rabbit IgG or AHR antibodies and protein A-conjugated magnetic beads at 4°C for overnight. After the magnetic beads were extensively washed, protein-DNA crosslinks were reversed and eluted. DNA was prepared by proteinase K digestion followed by PCR amplification. The CYP1A1 primers were 5'-TCA GGG CTG GGG TCG CAG CGC TTC T-3' (sense), and 5'-GCT ACA GCC TAC CAG GAC TCG GCA G-3' (antisense), and then we amplified a 122-bp region of human Cyp1A1 promoter which contained the AHR binding sequences. PCR products were resolved on a 2% agarose gel in the presence of ETBR.

Quantitative real-time PCR. cDNA was prepared from the total RNA of cells using amfiRivert cDNA Master Mix Platinum (GenDEPOT, Barker, TX). Each PCR was carried out in triplicate in a 20 μL volume using SYBR Green Mastermix (Applied Biosystems) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min in the Bio-Rad iCycler (MyiQ™2) real-time PCR System. The comparative CT method was used for

relative quantitation of samples. Values for each gene were normalized to expression levels of TATA-binding protein (TBP). The sequences of the primers used for real-time PCR were as follows: CYP1A1 sense 5'- GAC CAC AAC CAC CAA GAA C-3', antisense 5'- AGC GAA GAA TAG GGA TGA AG -3'; CXCR4 sense 5'- TTT TCT TCA CGG AAA CAG GG-3', antisense 5'- GTT ACC ATG GAG GGG ATC AG -3'; ICAM1 sense 5'- TGA TGG GCA GTC AAC AGC TA -3', antisense 5'- AGG GTA AGG TTC TTG CCC AC -3'; IL-1 β sense 5'- GAA GCT GAT GGC CCT AAA CA -3', antisense 5'- AAG CCC TTG CTG TAG TGG TG -3'; MMP-9 sense 5'- TTG GTC CAC CTG GTT CAA CT -3', antisense 5'- ACG ACG TCT TCC AGT ACC GA-3'; MAO-A sense 5'- TGG AGA ATC AAG AGA AGG CG -3', antisense 5'- CAG TCA AGA GTT TGG CAG CA -3'; and TBP sense 5'-TGC ACA GGA GCC AAG AGT GAA-3', antisense 5'-CAC ATC ACA GCT CCC CAC CA -3'.

Western blot analysis. Cells (3×10^5) were plated in six-well plates in DMEM media containing 2.5% FBS (and 1X MEM non-essential amino acid for CaCo-2 cells) for 16 hr and then treated with different concentrations of the compounds. Cellular lysates were prepared in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecylsulfate (SDS), 1 mM NaF, 1 mM Na₃VO₄, 1 mM phenyl methyl sulfonyl fluoride, 10 μ L/ml protease inhibitor cocktail (GenDEPOT, Barker, TX) and 1% NP-40. The cells were disrupted and extracted at 4°C for 30 min. After centrifugation, the supernatant was obtained as the cell lysate. Protein concentrations were measured using the Bio-Rad protein assay. Aliquots of cellular proteins were electrophoresed on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). The membrane was allowed to react with a specific antibody, and

detection of specific proteins was carried out by enhanced chemiluminescence. Loading differences were normalized using a polyclonal β -actin antibody.

Transfection of siRNA. Cells (2×10^5 cells/well) were plated in 6-well plates in DMEM medium containing 20% FBS and 1X MEM non-essential amino acid. After 16 hr, the cells were transfected with 20 nM of each siRNA duplex for 24 hr using RiboCellin siRNA Delivery Reagent (BioCellChallenge, France) following the manufacturer's protocol. The medium was then changed and incubated for 24 hr. After incubation, the cells were treated with either vehicle (DMSO) or different concentrations of the compounds, and cells were collected for western blot analysis and quantitative real-time PCR assay.

Statistics. All of the experiments were repeated a minimum of three times. The data are expressed as the means \pm SE. Statistical significance was analyzed using either Student's t-test or analysis of variance (ANOVA) with Scheffe's test. The results are expressed as means with error bars representing 95% confidence intervals for three experiments for each group unless otherwise indicated, and a *P* value of less than 0.05 was considered statistically significant.

RESULTS

Previous studies show that indole is a major bacterial metabolite and, in this study, we analyzed the cecal material (luminal contents and tissue) and fecal pellets from 6-week old C57BL/6 mice for the presence of indole and other tryptophan-derived microbiota metabolites. The major tryptophan metabolites identified include indole, indole 3-acetate and tryptamine (Fig. 1A). Levels of these metabolites were quantified in both cecal extracts and fecal pellets using MRM mass spectrometry, and the concentration of indole in the cecal material ranged between 200 - 400 μ M while tryptamine and indole 3-acetate were less abundant (concentrations ranging from 10 - 20 and 10 - 40 μ M, respectively). Similar levels of these metabolites were detected in fecal pellets (Fig. 1D).

These compounds were investigated as AHR agonists and antagonists in CaCo-2 colon cancer cells. The effects of the tryptophan metabolites alone and in combination with 10 nM TCDD on *CYP1A1* mRNA and CYP1A1, CYP1B1 and AHR protein levels (markers of altered AH-responsive gene expression) were determined. Treatment of CaCo-2 cells with tryptamine (50 - 1000 μ M) induced *CYP1A1* mRNA and protein and CYP1B1 protein (Fig. 2A) with significant induction at the lowest concentration (50 μ M); AHR levels were unchanged at 50 - 500 μ M concentrations but decreased at the highest concentration (1000 μ M) (Fig. 1A). High concentrations of tryptamine also decreased expression of CYP1A1/CYP1B1 proteins and this may be due to cytotoxicity (Supplemental Fig. 1). TCDD alone decreased AHR protein levels as previously described for other AHR agonists in colon cancer cells (Kawajiri et al., 2009). TCDD also induced CYP1A1 (mRNA and protein) and CYP1B1. In combination with tryptamine, the TCDD-induced responses were slightly attenuated at the two highest concentrations of tryptamine (500 and 1000 μ M), indicating that this compound is primarily an AHR agonist in

CaCo-2 cells as previously described in other cancer cell lines (Heath-Pagliuso et al., 1998; Miller, 1997; Vikstrom Bergander et al., 2012), with possible partial antagonist activity at the higher concentrations. In contrast to a previous report (Vikstrom Bergander et al., 2012), our data show that the AHR agonist activity of tryptamine (i.e. CYP1A1 induction) was not affected by a monoamine oxidase inhibitor (Supplemental Fig. 2).

Indole 3-acetate was also identified in the cecal extract and feces (Fig. 1) and the activity of this compound was compared to that of the uremic toxicant 3-indoxyl sulfate which has previously been described as an AHR agonist in liver cancer cell lines (Schroeder et al., 2010). Both indole 3-acetate and 3-indoxyl sulfate induced CYP1A1 (mRNA and protein) and CYP1B1 (protein) and, in combination with TCDD, indole 3-acetate did not affect CYP induction by TCDD (Figs. 2B and 2C). Although 3-indoxyl sulfate was an AHR agonist, induction of CYP1A1 and CYP1B1 protein by TCDD was decreased in CaCo-2 cell cotreated with TCDD plus 3-indoxyl sulfate, suggesting some partial AHR antagonist activity. However, we also observed that 50 μ M 3-indoxyl sulfate was cytotoxic (Supplemental Fig. 1), and this may contribute to decreased CYP1A1/CYP1B1 proteins. The two 3-substituted indole derivatives differed with respect to their effects on the AHR; like TCDD, 3-indoxyl sulfate decrease AHR expression, whereas indole 3-acetate did not affect levels of AHR protein, even though the fold induction of *CYP1A1* mRNA by this compound was similar to that observed for TCDD. Treatment of CaCo-2 cells with 50 - 1000 μ M indole significantly induced *CYP1A1* mRNA levels only at the two higher concentrations (500 - 1000 μ M); however, this was not accompanied by induction of CYP1A1 and CYP1B1 proteins and levels of the AHR protein were unchanged (Fig. 2D). In combination experiments, indole (500 - 1000 μ M) inhibited TCDD-mediated induction of CYP1A1 (mRNA and protein) and CYP1B1 protein, and

downregulation of the AHR demonstrated that indole was an AHR antagonist for these responses (Fig. 2D). The EC₅₀ values for induction of CYP1A1 mRNA by tryptamine indole 3-acetate and indole were 0.048, 0.37 and > 1 mM, respectively.

We further investigated the AHR antagonist activities of indole as an inhibitor of TCDD-induced *CYP1A1* gene transcription after treatment of CaCo-2 cells for 2 hr (Fig. 3A). The results showed that at this early time point, indole was a full AHR antagonist, whereas tryptamine and indole 3-acetate did not inhibit TCDD-induced *CYP1A1* RNA (Fig. 3B). Indole also significantly inhibited TCDD-induced *Cyp1a1* and *Cyp1b1* mRNA expression in mouse-derived colonic crypts in 3-dimensional culture (Fig. 3C) and these data complemented results in CaCo-2 cells. We also observed that indole inhibited indole 3-acetate-induced *CYP1A1* mRNA (2 and 24 hr) and protein (24 hr) expression in CaCo2 cells (Fig. 3D), demonstrating potential inhibitory interactions among the indole-derived AHR agonists. Effects of the tryptophan metabolites on TCDD-induced recruitment of the AHR complex to a DRE region on the CYP1A1 promoter were investigated in ChIP assays (Fig. 3E). Treatment of CaCo-2 cells with TCDD for 2 hr resulted in recruitment of the AHR to the CYP1A1 promoter. Similar results were observed for tryptamine and indole 3-acetate and, in cells cotreated with TCDD plus tryptamine or indole 3-acetate, the AHR binding to the DRE was essentially unchanged. Indole alone did not induce AHR-DRE binding and, in cells cotreated with TCDD plus indole, there was a dramatic decrease in TCDD-induced DRE binding which was consistent with the AHR antagonist activity of indole observed for transactivation.

Confirmation of the AHR agonist/antagonist activity of the tryptophan metabolites was determined in MDA-MB-468 and MDA-MB-231 cells which we have previously used to investigate the activities of several AHR-active pharmaceuticals (Jin et al., 2012). Figure 4A

shows that the pattern of CYP1A1 induction by tryptamine was similar in both breast cancer cell lines. Tryptamine alone was an AHR agonist but, in combination with TCDD, decreased expression of *CYP1A1* mRNA and protein and partially blocked TCDD-mediated AHR downregulation at the higher concentrations. Indole-3-acetate was a partial AHR agonist in MDA-MB-468 and MDA-MB-231 cells and, in cells cotreated with this compound plus TCDD, there was no evidence of AHR antagonist activity (Fig. 4B). Indole exhibited weak AHR agonist activity in both breast cancer cell lines and, in combination studies, indole inhibited TCDD-induced CYP1A1 protein and mRNA levels and blocked TCDD-mediated downregulation of the AHR. In MDA-MB-468 cells, a highly AH-responsive cell line, indole exhibited almost complete AHR antagonist activity. Thus, the results for the three tryptophan metabolites as AHR agonists/antagonists were comparable in the colon and breast cancer cell lines.

Previous studies in colon cancer cell lines show that AHR agonists have variable effects on endogenous and induced inflammatory responses including ICAM-1, IL-1 β , MMP9 and β -catenin (Furumatsu et al., 2011; Kawajiri et al., 2009; Villard et al., 2007) and CXCR4 is suppressed by AHR ligands in breast cancer cells (Hsu et al., 2007). Treatment of CaCo-2 cells with tryptamine or indole slightly increased *ICAM-1* mRNA; indole 3-acetate and TCDD decreased (Fig. 5A) and indoxyl 3-sulfate had no effect on expression of this gene (Supplemental Fig. 3). Phorbol-12-myristate 13-acetate (PMA) only slightly induced ICAM1 in CaCo-2 cells and, in combination with the tryptophan metabolites, expression was increased (tryptamine, indole), decreased (indole-3-acetate) or unchanged (3-indoxyl sulfate), and similar results were observed for TCDD. Expression of *MMP-9* in CaCo-2 cells was low and unaffected by the indole derivatives or TCDD (Fig. 5B); *MMP-9* was induced 10-fold by PMA and, in the cotreatment experiment, only tryptamine (slight decrease) affected the PMA-induced response.

IL- β expression was slightly increased (indole and indole 3-acetate), decreased (TCDD) or unaffected (indole and 3-indoxyl sulfate) by the AHR ligands and PMA only slightly induced (1.5 - 2.5-fold) IL-1 β mRNA levels (Fig. 5C). Cotreatment with PMA plus the indole derivatives resulted in dose-dependent effects for some compounds; 100 and 500 μ M tryptamine enhanced and inhibited *IL-1 β* mRNA levels, respectively; indole and indole 3-acetate enhanced and 3-indoxyl sulfate decreased *IL-1 β* mRNA and TCDD slightly decreased the PMA-induced response. Tryptamine, indole-3-acetate, TCDD and 3-indoxyl sulfate decreased and indole had no effect on endogenous *CXCR4* gene expression. PMA significantly induced *CXCR4* mRNA levels and cotreatment with the highest concentration of all four tryptophan metabolites and TCDD decreased PMA-induced *CXCR4* mRNA levels (note: 100 μ M indole increased this response). The most consistent effect of TCDD and the tryptophan metabolites was the decreased expression of *CXCR4* and this response has previously been observed for other AHR agonists in breast cancer cells (Hsu et al., 2007).

Results in Figure 6A show that the AHR antagonist CH223191 alone did not affect basal or PMA-induced *CXCR4* mRNA levels but significantly blocked the effects (downregulation) of TCDD, and CH223191 also inhibited TCDD (\pm PMA)-induced *CYP1A1* mRNA levels in CaCo-2 cells. CH223191 partially reversed tryptamine-mediated downregulation of (induced) *CXCR4* but did not significantly alter the effects of indole 3-acetate or 3-indoxyl sulfate (Fig. 6B). It was also evident that CH223191 was less effective as an inhibitor of tryptamine-/indole 3-acetate-/3-indoxyl sulfate-induced *CYP1A1* mRNA (Fig. 6C) than observed for TCDD (Fig. 6A) and these results are consistent with the reported activity of CH223191 as specific inhibitor of TCDD and related halogenated aryl hydrocarbons (Zhao et al., 2010). However, AHR knockdown by RNA interference shows *CYP1A1* inducibility is decreased for both TCDD and the tryptophan

metabolites (Fig. 6D). It should also be noted that CH223191 inhibits induction of Ah-responsive luciferase activity by the natural ligand 6-formylindo[3,2b]carbazole and methyl 2-(1-H'-indolo-3'-carbonyl)-thiazole-4-carboxylate in HepG2 liver cells suggesting a broader spectrum of AHR antagonist activity for this compound (Choi et al., 2012).

A previous study reported that several AHR agonists decreased β -catenin expression in DLD-1, SW480 and HCT116 colon cancer cells (Kawajiri et al., 2009); however, TCDD did not affect β -catenin levels in CaCo-2 cells and, among the tryptophan metabolites, only the higher concentrations of tryptamine decreased β -catenin protein levels (Supplemental Fig. 4A) and this may be due to cytotoxicity (Supplemental Fig. 1). However, the AHR antagonist CH223191 did not modulate downregulation of β -catenin by tryptamine but clearly inhibited induction of CYP1A1 protein by tryptamine (500 μ M) (Supplemental Fig. 4B). The effects of 100 μ M tryptamine on CYP1A1 downregulation were not affected by CH223191. These results demonstrate that the AHR-dependent effects of TCDD and the indole derivatives are response- and cell context-dependent which is consistent with their activity as SAhRMs.

DISCUSSION

The AHR and its ligands play an increasingly important role in gut homeostasis and in various intestinal diseases including colitis and colon cancer (Arsenescu et al., 2011; Benson and Shepherd, 2011; Furumatsu et al., 2011; Kawajiri et al., 2009; Kiss et al., 2011; Li et al., 2011; Singh et al., 2011). For example, the AHR is required for the postnatal expansion of CD4-ROR γ T⁺ innate lymphoid cells and dietary AHR ligands such as indole-3-carbinol (I3C) promote the postnatal expansion of these cells which protect against intestinal infections (Bansal et al., 2010). β -Naphthoflavone, a relatively non-toxic AHR agonist, protects against dextran sodium sulfate-induced colitis in a mouse model and decreased lipopolysaccharide-induced inflammatory responses in SW480 colon cancer cells (Furumatsu et al., 2011). APC^{min/+} mice are extensively used as a model for intestinal carcinogenesis, and loss of the AHR enhanced cecal tumor incidence and, in wild type mice, dietary administration of I3C or diindolylmethane (AHR agonists) protected against intestinal carcinogenesis (Kawajiri et al., 2009). These results clearly demonstrate an important role for the AHR and AHR agonists in maintaining gut health and protecting against intestinal diseases.

Tryptophan metabolites such as the indole derivatives have previously been investigated as AHR ligands, and a recent study showed that the AHR agonist kynurenine (Davarinos and Pollenz, 1999) plays an important pro-oncogenic role in glioma (Opitz et al., 2011). Therefore, the expression of tryptophan metabolites by gut microflora and their AHR agonist or antagonist activities could significantly impact Ah-responsive intestinal functions. Previous studies in our laboratory have identified indole as a major microbial metabolite (Bansal et al., 2010) and indole concentrations in human feces can be in the low mM range (Karlin et al., 1985; Zuccato et al., 1993). In this study, we used LC-MRM-MS and identified the tryptophan metabolites indole,

tryptamine and indole-3-acetate in the cecum and feces of 6-week old C57BL/6 mice, at concentrations comparable to those reported in human feces (Karlin et al., 1985; Zuccato et al., 1993). The concentration of indole was almost an order of magnitude greater than tryptamine or indole-3-acetate in both cecal extracts and fecal samples and concentrations of tryptamine and indole in the cecum and fecal pellets were comparable to those needed for AHR agonist and antagonist activity, respectively. Concentrations of these tryptophan metabolites (Fig. 1) are probably underestimated due to the overall efficiencies of extraction which are < 100%.

We used the CaCo-2 intestinal colon cell line as a model to investigate the AHR agonist and antagonist activities of indole, indole 3-acetate and tryptamine, and their effects on *CYP1A1* mRNA and protein and *CYP1B1* protein expression were determined as a measure of their AHR-responsiveness. Moreover, in CaCo-2 cells, we also examined the effects of 3-indoxyl sulfate which has previously been characterized as an AHR agonist (Schroeder et al., 2010). TCDD served as a prototypical agonist, and we also compared the effects of TCDD vs. the tryptophan metabolites on proteasome-dependent downregulation of the AHR protein which is observed for some (e.g. TCDD) but not all AHR ligands (14, 17, 39). All the tryptophan metabolites exhibit full or partial (indole) AHR agonist activity in CaCo-2 cells and induced *CYP1A1* mRNA and protein, whereas induction of *CYP1B1* was highly variable. However, after treatment of CaCo-2 cells with tryptamine for 24 hr, there was some indication that this compound may be a partial AHR antagonist (i.e. inhibition of TCDD-induced *CYP1A1* and AHR downregulation) and in short term experiments, neither tryptamine or indole 3-acetate inhibited TCDD-induced *CYP1A1* gene expression (Fig. 3B). In contrast, indole exhibited AHR antagonist activity at both the 24 and 2 hr treatment times in CaCo-2 cells using TCDD (Figs. 2D and 3A) and indole 3-acetate (Fig. 3D) as agonists, confirming the AHR antagonist activity of indole and indicating potential

interactions among the tryptophan/microbiome AHR ligands. Moreover, we also observed that indole inhibited TCDD-induced *Cyp1a1* and *Cyp1b1* expression in mouse colonic crypts in 3-D culture. Thus, at least for the classical AHR-mediated induction of CYP1A1, 100 - 250 μ M indole exhibited weak agonist/partial antagonist in colon cancer cells, suggesting that the relative levels of the tryptophan-derived microbiota metabolites could affect the overall intestinal balance of AHR ligands from dietary or contaminant sources. Previous studies show that dietary AHR agonists mitigate induced colitis in mouse models (Li et al., 2011), and we are currently further investigating their interactive effects with microbiota tryptophan metabolites.

Previous reports suggest that AHR agonists modulate inflammatory response genes in colon epithelial and in colon cancer cells (Furumatsu et al., 2011; Kawajiri et al., 2009; Villard et al., 2007) and this included AHR-dependent-inhibition of IL-1 β in SW480 cells by β -naphthoflavone (Furumatsu et al., 2011). In contrast IL-1 β and MMP-9 were induced by 3-methylcholanthrene in CaCo-2 cells (Villard et al., 2007); however, the role of the AHR in mediating these responses was not confirmed. Results for the tryptophan metabolites in CaCo-2 cells showed highly variable effects of these compounds on endogenous and PMA-induced stress response genes (Fig. 5). Moreover, TCDD did not affect endogenous or PMA-induced *ICAM1*, *MMP-9* or *IL-1 β* gene expression and the only significant response observed was inhibition of *CXCR4* (Fig. 6A), and TCDD-induced downregulation of *CXCR4* was blocked by the AHR antagonist CH223191. Interestingly, tryptamine, indole 3-acetate and 3-indoxyl sulfate also decreased endogenous and PMA-induced *CXCR4* mRNA levels and exhibited the expected TCDD-like AHR agonist activity; however, cotreatment with the AHR antagonist did not reverse *CXCR4* downregulation (Figs. 6B and 6C), suggesting that the effects of these compounds were AHR-independent. An AHR-dependent response previously observed in SW480, DLD-1 and

HCT116 colon cancer cells was the downregulation of β -catenin by several AHR agonists (not TCDD) (Kawajiri et al., 2009), whereas in CaCo-2 cells, TCDD, indole, indole 3-acetate and 3-indoxyl sulphate did not affect β -catenin levels and the tryptamine response was AHR-independent (Supplemental Fig. 4). Tissue- and response-specific agonist or antagonist activity of receptor ligands is due to several factors including different ligand-induced conformational changes in the receptor and differential expression of cofactor in various tissues/cells. We previously observed the SAhRM-like activity for a series of AHR-active pharmaceuticals in breast cancer cells (Jin et al., 2012).

In summary, results of this study demonstrate that the mouse microbiome produces relatively high concentrations of tryptophan metabolites tryptamine, indole 3-acetate and indole, and these compounds differentially activate markers of AH-responsiveness including induction of *CYP1A1* and *CYP1B1*. Previous studies demonstrate that the AHR plays a critical role in intestinal homeostasis and disease, and dietary AHR agonists such as I3C and DIM can protect against induced colitis and colon cancer in mouse models (Arsenescu et al., 2011; Benson and Shepherd, 2011; Furumatsu et al., 2011; Kawajiri et al., 2009; Kiss et al., 2011; Li et al., 2011; Singh et al., 2011). Our results demonstrate that microbiota-generated tryptophan metabolites exhibit both AHR agonist and antagonist activities, and these may also be response- and cell context-specific as observed for other SAhRMS (Jin et al., 2012). Thus, the AHR-active tryptophan metabolites along with other dietary and potential exogenous (i.e. contaminants) AHR ligands can potentially influence host intestinal responses and changes, and levels and ratios of microbiota-derived tryptophan metabolites could affect overall AHR ligand-induced impacts on intestinal functions.

AUTHORSHIP CONTRIBUTION:

Participated in research design: Jin, Chapkin, Alanz and Safe

Conducted experiments: Jin, S.O. Lee, Sridharan, K. Lee, Davidson, Jayaraman, Alaniz and Safe

Contributed new reagents or analytic tools: Jayaraman, Chapkin and Safe

Performed data analysis: Jin, S.O. Lee, Sridharan, K. Lee, Davidson, Jayaraman, Alaniz and Safe

Wrote or contributed to the writing of the manuscript: Jin, Sridharan, K. Lee, Jayaraman, Chapkin, Alaniz and Safe

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FOOTNOTE:

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FIGURE CAPTIONS

Figure 1. MRM-MS quantification of tryptophan-derived metabolites in murine cecal contents and feces. (A) and (B). Pure standards of tryptophan metabolites were used to generate standard curves for determining the concentration of metabolites in the samples using LC-MRM-MS as described in the Experimental Methods. Detection and quantitation of tryptamine is illustrated. (C) and (D) show the different metabolites in cecal extracts (μM) and feces (μM) in 6-week old mice ($n=5$) quantified by LC-MRM-MS as indicated above.

Figure 2. AHR agonist and antagonist activities of tryptophan metabolites. CaCo-2 cells were treated with tryptamine (A), indole 3-acetate (B), indolyl-3 sulfate (C) or indole (D) alone or in combination with TCDD, and after 24 hr, induction of *CYP1A1* mRNA and AHR, CYP1A1 and CYP1B1 proteins were determined by real time PCR and western blots, respectively, as outlined in the Experimental Methods. Results for mRNA levels are expressed as means \pm SE for three separate experiments and significant ($P < 0.05$) induction (*) or inhibition of TCDD-induced responses (**) are indicated.

Figure 3. AHR activity in transactivation and ChIP assays. CaCo-2 cells were treated with indole (A), indole 3-acetate and tryptamine (B) alone or in combination with TCDD, and after 2 hr, *CYP1A1* mRNA and protein levels were determined by real time PCR. Mouse colonic crypts in culture (C) were treated with indole, TCDD and their combinations for 6 hr, and *Cyp1a1* and *Cyp1b1* mRNA levels were determined as outlined in the Experimental Methods. The 3 bars within each treatment group represent 3 different cultures of colonic crypts. Interactions between indole and indole 3-acetate (D) were determined in CaCo2 cells treated with these

compounds (2 and 24 hr), and *CYP1A1* mRNA and protein were determined by real time PCR and western blots as outlined in the Experimental Methods. Binding to a CYP1A1 DRE (E) was determined in a ChIP assay as outlined in the Experimental Methods. Studies were carried out in triplicate, results are means \pm SE, and significant ($P < 0.05$) increases (*) (compared to DMSO) or inhibition (**) of TCDD-induced responses are indicated.

Figure 4. AHR agonist and antagonist activities of tryptophan metabolites in MDA-MB-468 and MDA-MB-231 breast cancer cells. Cells were treated with tryptamine (A), indole 3-acetate (B) or indole (C) alone or in combination with TCDD for 24 hr, and effects on *CYP1A1* mRNA and AHR and CYP1A1 proteins were determined by real time PCR or western blots, respectively, as outlined in the Experimental Methods. mRNA data are means \pm SE for three replicate determinations, and significant ($P < 0.05$) induction (*) or inhibition (**) of TCDD-induced responses are indicated.

Figure 5. Anti-inflammatory activities of tryptophan metabolites and TCDD in CaCo-2 cells. Cells were treated with the tryptophan metabolites or TCDD alone or in combination with PMA for 24 hr, and expression of *ICAM1* (A), *MMP-9* (B), *IL-1 β* (C) and *CXCR4* (D) mRNA was determined by real time PCR as outlined in the Experiment Methods. Results are expressed as means \pm SE for three replicate determinations, and significant ($P < 0.05$) modulation of basal (DMSO) (*) or PMA-induced (**) responses are indicated.

Figure 6. Role of the AHR in inhibition of *CXCR4* expression. (A) Effects of TCDD on *CXCR4* and *CYP1A1* mRNA expression. CaCo-2 cells were treated with TCDD, PMA or

CH223191 alone and in combination, and expression of *CXCR4* and *CYP1A1* mRNA levels were determined by real time PCR as outlined in the Experimental Methods. CaCo-2 cells were treated with DMSO, PMA, CH223191 or tryptamine (100 μ M), indole 3-acetate (100 μ M), and indole (1000 μ M) alone or in combination, and *CXCR4* (B) and *CYP1A1* (C) mRNA levels were determined by real time PCR. (D) RNA interference. CaCo2 cells were transfected with an oligonucleotide that targets the AHR and after 48 hr, cells were treated with TCDD or the tryptophan metabolites for 24 hr and *CYP1A1*, AHR mRNA levels were determined by real time PCR. Results are expressed as means \pm SE for at least three separate experiments, and significantly ($p < 0.05$) induced *CYP1A1* or decreased *CXCR4* in cells treated with PMA plus the AHR ligand (*) and reversal of these responses by CH223191 (**) are indicated, and a significant ($p < 0.05$) decrease in *CYP1A1* and AHR mRNA (D) is also indicated (*).

Figure 1

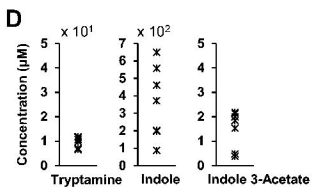
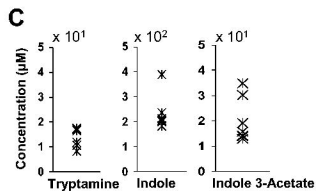
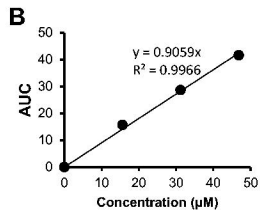
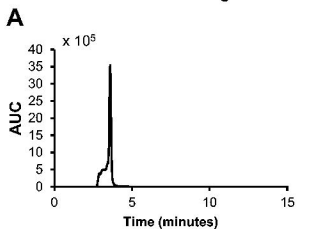


Figure 2

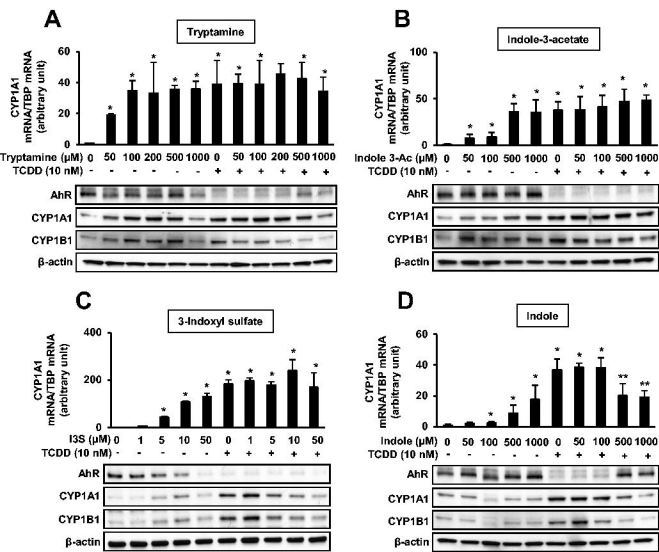


Figure 3

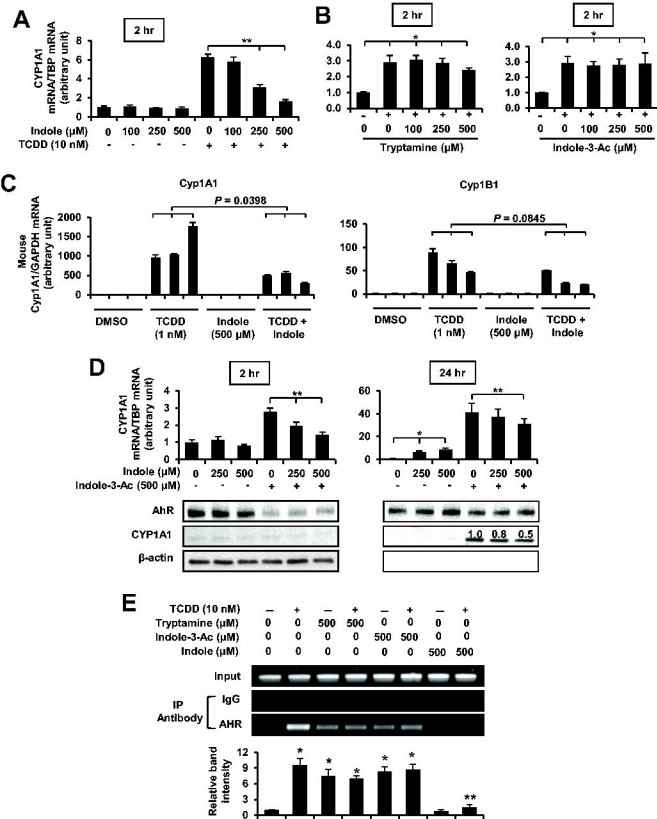


Figure 4

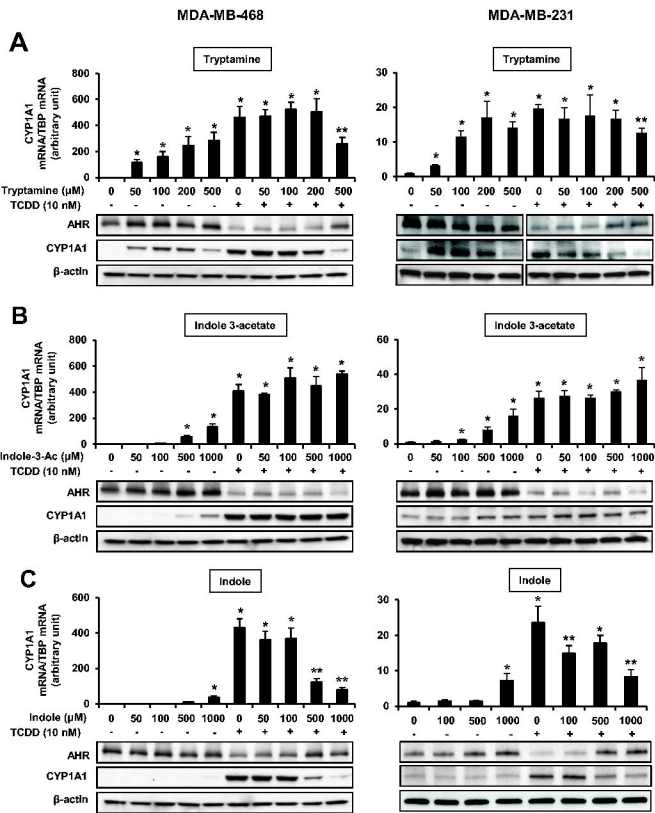


Figure 5

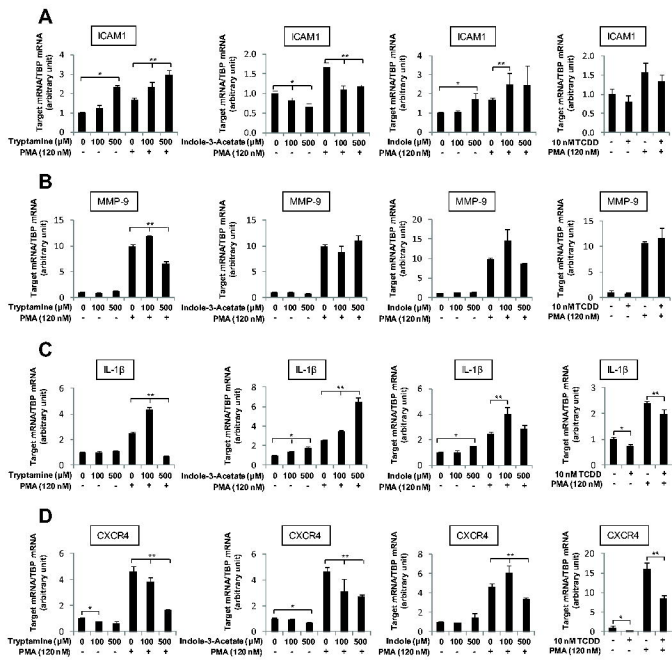


Figure 6

