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Naturally-Occurring Marine Brominated Indoles are Aryl Hydrocarbon Receptor Ligands/Agonists

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Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that mediates the toxic and biological effects of structurally diverse chemicals, including the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). As part of a larger effort to identify the full spectrum of chemicals that can bind to and activate the AhR, we have examined the ability of several naturally-occurring marine-derived brominated indoles and brominated (methylthio)indoles (collectively referred to as “brominated indoles”) to bind to the AhR and stimulate AhR-dependent gene expression. Incubation of mouse, rat and guinea pig recombinant cell lines containing a stably transfected AhR-responsive luciferase reporter gene with eight brominated indoles revealed that all compounds stimulated luciferase reporter gene activity, although some species-specific differences were observed. All compounds induced significantly more luciferase activity when incubated with cells for 4 h as compared to 24 h, demonstrating that these compounds are transient activators of the AhR signaling pathway. Three of the brominated indoles induced *CYP1A1* mRNA in human HepG2 cells *in vitro* and *Cyp1a* mRNA in zebrafish embryos *in vivo*. The identification of the brominated indoles as direct ligands and activators/agonists of the AhR was confirmed by their ability to compete with [³H]TCDD for binding to the AhR and to stimulate AhR transformation and DNA binding *in vitro*. Taken together, these marine-derived brominated indoles are members of a new class of naturally-occurring AhR agonists.

Keywords

Aryl hydrocarbon receptor; AhR; ligand; brominated indoles; marine natural product

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INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a soluble, ligand-activated transcription factor belonging to the basic-helix-loop-helix-PAS (bHLH-PAS) family of regulatory proteins. The AhR is best known for its role in mediating many of the biological and toxic responses observed following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related dioxin-like chemicals, including a wide variety of other halogenated and polycyclic aromatic hydrocarbons (HAHs and PAHs, respectively). To initiate AhR signaling, ligands diffuse into the cell and bind to the cytosolic AhR which exists as a multiprotein complex containing HSP90,¹ XAP2² and p23.³ Following ligand binding, exposure of the nuclear localization sequence facilitates translocation of the ligand:AhR protein complex into the nucleus where it is released from its associated protein subunits through dimerization with the AhR nuclear translocator (ARNT) protein and converted into its high-affinity DNA binding form.⁴⁻⁷ Binding of the ligand:AhR:ARNT complex to its specific DNA recognition sequence, the dioxin responsive element (DRE), results in increased transcription of downstream genes.^{5,6}

The best-characterized high affinity ligands for the AhR include a variety of synthetic HAHs, such as the polychlorinated/polybrominated dibenzo-p-dioxins, dibenzofurans, and biphenyls, as well as numerous PAHs and PAH-like chemicals, such as benzo(a)pyrene, 3-methylcholanthrene, β -naphthoflavone and others.^{6,8-10} However, recent studies have demonstrated that the AhR can bind and be activated by a diverse range of both synthetic and naturally-occurring chemicals whose structures and chemical and physical characteristics are substantially different from those of HAH and PAH AhR ligands.^{8,11} Although the majority of these ligands are of lower potency and bind to the AhR with lower affinities than those of HAH/PAH ligands, some compounds can bind to the AhR with an affinity comparable to that of TCDD.^{12,13} Recently there has been a significant increase in the number of studies examining activation of the AhR and AhR signaling pathway by endogenous and naturally-occurring compounds, largely due to the identification of physiologic and developmental processes involving the AhR.^{6,8,11,14-16} While these studies have led to the identification of AhR-active natural products representing several chemical classes, a large number of these AhR agonists were either indole-containing or indole-derived,¹¹ and several of these compounds have been suggested to be endogenous physiological ligands for the AhR.¹⁷⁻²¹

While the majority of studies directed toward isolation and identification of naturally-occurring and endogenous AhR ligands have predominantly focused on substances extracted from mammalian species, terrestrial environmental matrices, or food sources, the marine environment represents a potential major source of potent AhR agonists that has been largely overlooked. Not only do marine organisms synthesize a wide range of chemical compounds (it is estimated that there are more than 15,000 marine natural products of diverse structures),^{22,23} but the presence of relatively high concentrations of naturally-occurring halogens in seawater results in 15–20% of all newly discovered marine natural products being organohalogens.²² Since halogenated compounds are some of the most potent AhR agonists currently identified,⁸ these marine-derived organohalogens could represent novel AhR activators. Although a wide variety of marine natural products have

been identified, many with structures that will undoubtedly be determined to be AhR ligands (i.e. naturally-occurring brominated/chlorinated dibenzo-p-dioxins^{24–27} and some with similarity to polychlorinated biphenyls^{28,29}), the majority of these compounds have not been examined for AhR activity. Of the limited functional analysis that has been carried out on naturally-occurring marine products, several novel, structurally-diverse AhR agonists have been identified and characterized.^{30–33} Thus, marine natural products may represent a significant source of AhR ligands, and screening of such compounds may identify new classes of potent AhR agonists. Here we describe the results of studies examining the AhR agonist activity of several novel brominated indoles isolated from a marine alga³⁴ and demonstrate that several of these compounds are relatively potent naturally-occurring AhR agonists.

MATERIALS AND METHODS

Materials

The brominated indoles used in this study (Figure 1) were isolated from the red alga *Laurencia brongniartii* as previously described.³⁴ Stock solutions in DMSO were made to a concentration of 2 mM. TCDD was a kind gift from Dr. Steve Safe (Texas A&M University), 2,3,7,8-tetrachloro[1,6-³H]dibenzo-*p*-dioxin ([³H]TCDD; 35 Ci/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) from Ultra Scientific (Hope, RI). **Caution:** *Since TCDD and TCDF are highly toxic chemicals, their handling required the use of disposable glass and plasticware, absorbent bench top paper, and appropriate personal protective equipment and disposal of contaminated materials followed procedures approved by the UC Davis and WHOI Offices of Environmental Health & Safety.* Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO), [γ -³²P]ATP from PerkinElmer (Waltham, MA) and synthetic oligonucleotides were synthesized by Operon Biotechnologies (Huntsville, AL). All chemical stocks were made in DMSO and either these stock solutions or their dilutions were used for all chemical treatments.

PLHC-1 Culture and Ethoxyresorufin O-Deethylase (EROD) Assay

Teleost fish hepatoma (PLHC-1) cells were grown at 30°C in Eagle's MEM containing 25 mM HEPES, Earle's salts, non-essential amino acids, L-glutamine, and 10% (v/v) calf serum as previously described.³⁵ The EROD assay was performed using a modification of the protocol from Kennedy and coworkers.³⁶ Briefly, PLHC-1 cells were plated in 48-well plates at a density of 400,000 cells/cm² and allowed to recover for 24 h, followed by replacement of the medium with 0.5 ml of fresh medium. DMSO (0.5% (v/v)), TCDD (0.1 nM), or brominated indole (10 μ M) was added to each well to obtain the indicated final chemical concentration. The brominated indole concentration of 10 μ M was essentially the maximum that could be achieved given the limited availability of the compounds, their solubility in DMSO and the concentration of vehicle that could be used in the assays. Following 24 h of incubation, cells were rinsed with PBS followed by the addition of 100 μ l of sodium phosphate buffer (50 mM, pH 8.0), and 100 μ l of 7-ethoxyresorufin (2 mM final concentration) to each well. The reaction was allowed to proceed for 10 min at room temperature and was terminated by the addition of 150 μ l of ice-cold Fluorescamine in

acetonitrile. Fluorescence was read using the CytoFluor™ 2300 Fluorescence Measurement System (Millipore Corporation, Bedford, MA) and product formation was determined by comparison to a resorufin standard curve. The data was normalized to protein content using a fluorescence-based assay as previously described³⁷ and activity expressed as a percentage of the EROD activity induced by TCDD.

Luciferase Gene Induction

Recombinant mouse hepatoma (H1L1.1c2), rat hepatoma (H4L1.1c4), and guinea pig intestinal adenocarcinoma (G16L1.1c8) cell lines containing a stably integrated AhR-responsive luciferase reporter gene were used for the detection of AhR agonist activity as previously described.^{38–40} All cell lines were grown in alpha-Minimum Essential Medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) under conditions of 37°C, 5% CO₂ and greater than 80% humidity. Cells were plated at a density of 75,000 cells per well in clear bottom 96-well assay plates (Corning Incorporated, Corning, NY) and allowed to attach for 20 to 24 h. For the 24 h induction studies with H4L1.1c4 cells, culture medium in each well was replaced with fresh medium (100 µl) containing either the vehicle control (DMSO 1% (v/v)), TCDD (1 nM final concentration), or one of the brominated indoles (10 µM final concentration), and the cells incubated for 24 h at 37°C. For the 4 h induction studies, all cell lines were handled as described above but chemical treatment occurred 40 to 48 h post-plating. Termination of the 4 and 24 h incubations occurred simultaneously, wherein the treatment medium was removed, cells were washed twice with PBS and 50 µl of luciferase cell culture lysis reagent (Promega, Madison, WI) was added to each well. Plates were incubated for 20 min at room temperature with shaking followed by determination of luciferase activity in an Orion Microplate Luminometer (Berthold Detection Systems, Oak Ridge, TN) with automatic injection of Promega stabilized luciferase substrate (50 µl) and a read integration time of 10 s.

Guinea Pig Hepatic Cytosol and Electrophoretic Mobility shift Assay (EMSA)

Hepatic cytosol was prepared from male Hartley guinea pigs (400 g; Charles River Laboratories, Wilmington, MA) in HEDG buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol) as previously described.⁴¹ Protein concentration was determined using the method of Bradford,⁴² and samples were stored at –80°C until use. The EMSA procedure for the ligand-dependent transformation and DNA binding of guinea pig cytosolic AhR was carried out as described in detail.⁴¹ Briefly, cytosol (8 mg protein/ml) was incubated with DMSO (2% (v/v)), TCDD (20 nM final concentration) or brominated indole (64 µM final concentration) for 2 h at 20°C, followed by incubations with poly(dI•dC) (Roche Applied Science, Indianapolis, IN) and [³²P]-labeled dioxin responsive element (DRE)-containing oligonucleotides. Protein[³²P]DNA complexes were resolved in non-denaturing polyacrylamide gels and visualized by autoradiography.

AhR Ligand Binding

Specific binding of [³H]TCDD to the AhR and competitive binding of brominated indoles was determined by sucrose gradient centrifugation as previously described in detail.^{43,44} Briefly, cytosol (1 mg protein/ml) prepared from mouse hepatoma (Hepa1c1c7) cells was

incubated with [^3H]TCDD (1 nM) in the absence or presence of 0.2 μM TCDF or 5 μM of the indicated brominated indole for 1 h on ice. Unbound ligand was removed by dextran-coated charcoal treatment (1 mg dextran and 10 mg charcoal/ml) for 5 min on ice and 300 μl of each sample applied to 10–30% sucrose gradients along with the internal sedimentation markers ^{14}C -catalase (11.3 S) and ^{14}C -ovalbumin (3.6 S). Samples were centrifuged for 140 min at 65,000 rpm at 4°C in a VTi65.2 rotor, fractions (150 μL) collected and radioactivity in each fraction determined by liquid scintillation.

Cyp1a induction in zebrafish embryos *in vivo*

Zebrafish embryos [TL strain; 48 hours post fertilization (hpf)] were placed in 20-ml scintillation vials containing 15 ml of 0.3x Danieau's solution containing DMSO (0.5%), TCDD (2 nM final concentration), or brominated indole (2 or 10 μM) and incubated for 6 hr at 28°C. There were sixty embryos per vial. Following exposure, three replicate groups of 20 embryos from each vial were frozen in liquid nitrogen. Total RNA was isolated from frozen embryos using the Aurum mini kit (Bio-Rad) and quantified using a NanoDrop spectrophotometer. cDNA was synthesized from 1 μg of total RNA using the iScript kit (Bio-Rad). Real-time RT-PCR for *cyp1a* and *beta-actin* was performed using the iTaq Universal SYBRgreen Supermix (Bio-Rad) in a CFX96 Real Time Thermo Cycler (Bio-Rad). Primer sequences and calculations of fold-induction of *cyp1a* using the 2^{-C_T} method were as described previously.²¹

CYP1A1 induction in human HepG2 cells

HepG2 cells (human liver hepatoma; ATCC) were seeded in two 24-well cell culture plates at a density of 1.6×10^5 cells/well in Eagle's Minimum Essential Medium (ATCC Catalog No. 30-2003) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) (0.5 ml/well). The plates were incubated at 37°C with 5% CO_2 and allowed to grow for 48 hr until confluent. Triplicate wells were dosed with DMSO (0.5% v:v), TCDD (2 nM), or brominated indole (10 μM). Both plates were dosed at the same time; one plate was harvested after 6 hr and the other was harvested after 24 hr. Media was aspirated and the cells were rinsed with 500 μl 1xPBS. STAT-60 (Tel-Test) (1 ml) was added to each well, and the cells were scraped off and transferred to a 1.5 ml Eppendorf tube. Cells in STAT-60 were stored at -80°C until RNA preparation within one week. Total RNA was isolated using the Aurum mini kit (Biorad), and quantified using the Nanodrop. cDNA was synthesized with the iScript kit (Bio-Rad) using 1 μg of total RNA. Real-time RT-PCR was performed using the iTaq Universal SYBRgreen Supermix (Bio-Rad) in a CFX96 Real Time Thermo Cycler (Bio-Rad). Primers used were: *β -actin*: 5'-ATATCGCCGCGCTCGTCGTC-3' and 5'-ATGCCACCATCACGACCTG-3'; *CYP1A*: 5'-CAAGAGACACAAGTTTGAAAGG-3' and 5'-TGGGTTGACCCATAGCTTCTG-3'.

RESULTS

Brominated Indoles are Weak Agonists of AhR-Dependent CYP1A Expression in PLHC-1 Cells

Initial studies examining the ability of selected naturally-occurring marine brominated indoles (Figure 1) to activate the AhR and stimulate AhR-dependent gene expression were

carried out using PLHC-1 cells. This hepatocellular carcinoma cell line, derived from *Poeciliopsis lucida* (desert topminnow), contains the AhR and ARNT proteins and responds to AhR agonists with the induction of cytochrome P450 1A (CYP1A) gene expression.³⁵ PLHC-1 cells were incubated with DMSO, 0.1 nM TCDD or 10 μ M of each brominated indole and EROD activity determined after 24 h. The results of these studies demonstrated that although most of the compounds induced EROD activity (Figure 2), the overall magnitude of the induction response was relatively low compared to TCDD, indicating that the brominated indoles are very weak AhR agonists in this system under these exposure conditions. At 10 μ M, the most efficacious inducers, JN2-13-1 and JN2-39-2, stimulated EROD activity to only 5.5% and 3.9% of the maximal activity induced by TCDD respectively, while other compounds such as JN2-10-21, JN2-11-1 and JN2-36-12 showed no agonist activity.

Brominated Indoles are Moderately Potent Transient Activators of the Mammalian AhR Signaling Pathway

While the brominated indoles are relatively weak agonists of the fish AhR, significant species differences have been observed in AhR ligand specificity and selectivity.⁸ We therefore examined the ability of these compounds to stimulate AhR-dependent gene expression in cell lines derived from several mammalian species. For these experiments, we used recombinant mouse and rat hepatoma (H1L1.1c2 and H4L1.1c4, respectively) and guinea pig intestinal adenocarcinoma (G16L1.1c8) cells. These cell lines contain a stably integrated AhR-responsive firefly luciferase reporter gene that responds to AhR agonists with the induction of luciferase gene expression.^{38,39} Each cell line was incubated with DMSO, TCDD (1 nM) or the indicated brominated indole (10 μ M final concentration) for 4 h, after which luciferase activity was determined (Figure 3). This incubation time was selected to minimize metabolism of the test chemicals. In contrast to the results obtained with PLHC-1 cells after a 24-hr exposure, all brominated indoles were able to induce AhR-dependent luciferase activity in each of the mammalian cell lines examined. While relatively high levels of luciferase activity (>50% of that of 1 nM TCDD) were induced by all compounds in G16L1.1c8 and H4L1.1c4 cells lines, all brominated indoles tested produced significantly lower levels of luciferase induction in mouse H1L1.1c2 cells. Of the brominated indoles examined, JN2-11-2 produced the greatest induction response in all three cell lines (60 – 100%), while the level of luciferase induction was relatively similar for the remaining compounds in both G16L1.1c8 and H4L1.1c4 cell lines. In the H1L1.1c2 cells, the rank order of luciferase induction was JN2-11-2 followed by JN2-10-21 and JN2-18-4 in decreasing order (Figure 3), with the remaining compounds inducing luciferase activity between 20–30% of TCDD.

In contrast to dioxin-like HAHs, which are metabolically stable due to the presence of halogen substituents (which are typically resistant to removal by metabolic enzymes like cytochrome P450), most AhR agonists are metabolically labile and thus are only transient activators of the AhR and AhR signaling pathway.^{45–47} While initial examination of the brominated indoles suggested that they might be transient AhR inducers, the presence of bromine substituents could reduce their rate of degradation, resulting in activation of the AhR pathway for an extended period of time. To examine the relative persistence of AhR

activation by these compounds, H4L1.1c4 cells were incubated with DMSO, TCDD (1 nM) and the indicated brominated indole (10 μ M) for 4 or 24 h followed by determination of luciferase activity (Figure 4). While TCDD showed a sustained luciferase induction response over the 24 h period, the induction of luciferase activity by all of the brominated indoles was substantially lower at 24 h. Most compounds exhibited a decrease in luciferase activity of 80–90% compared to the 4 h time point (Figure 4). Two of the more active compounds (JN2-11-2 and JN2-18-4) showed a less dramatic decrease in luciferase activity (to ~50%) indicating that some brominated indoles appear to be more resistant to cellular degradation than others. Overall, however, these compounds are transient, rather than persistent, inducers of the AhR signal transduction pathway.

Brominated Indoles Stimulate AhR Transformation and DNA Binding *In Vitro*

While the ability of the brominated indoles to strongly stimulate luciferase gene induction suggests that they activate the AhR and AhR signaling pathway, this method does not confirm whether the compounds directly bind to and activate the AhR or whether AhR activation is indirect (i.e. through metabolism of the parent compound into an AhR agonist or stimulation of AhR activation through another signaling mechanism like phosphorylation by kinases). To determine whether these compounds directly activate the AhR, we first examined their ability to stimulate AhR transformation and DNA binding *in vitro* by EMSA using guinea pig hepatic cytosol. Given that the relative level of gene induction was greatest in the recombinant guinea pig cell line (Figure 3), combined with our reported ability to achieve almost complete ligand-dependent transformation and DNA binding of guinea pig hepatic cytosolic AhR,^{48,49} we used guinea pig cytosol to examine the DNA-binding activity of these compounds. Similar to the luciferase gene induction results, the EMSA results (Figure 5) demonstrate that all eight brominated indoles were able to stimulate AhR transformation and DNA binding to relatively high levels. The relative amount of brominated indole-inducible guinea pig cytosolic AhR:DNA complex was comparable to the relative magnitude of induction of luciferase activity by each compound in guinea pig G16L1.1c8 cells (compare Figure 5 to Figure 3), indicating that the induction of gene expression by each compound was mediated by their ability to directly stimulate the AhR.

Sucrose Density Gradient Analysis of AhR Ligand Binding

The above results demonstrate the ability of these brominated indoles to stimulate AhR transformation and DNA binding, but they do not confirm that these compounds do so by binding directly to the AhR. To confirm the interaction of these compounds with the AhR ligand binding pocket, we examined their ability to competitively inhibit the specific binding of [³H]TCDD to mouse hepatic cytosolic AhR using sucrose density gradient ligand binding analysis. The results of these analyses (Figure 6) reveal that four of the compounds (JN2-10-21, JN2-11-2, JN2-13-1 and JN2-18-4) were able to competitively inhibit >94% of [³H]TCDD and one (JN2-9-1) competitively eliminated ~87% of [³H]TCDD binding. JN2-36-12 and JN2-11-1 showed only moderate competitive inhibition of [³H]TCDD specific binding (52% and 39%, respectively) and JN2-39-2 was a relatively weak competitive ligand, inhibiting only 10% of [³H]TCDD specific binding to the AhR. Taken together, the results of the above analysis demonstrate that these brominated indoles are

AhR agonists that activate the AhR through their ability to directly bind and stimulate AhR transformation and DNA binding.

Induction of Cyp1a mRNA by Brominated Indoles in Zebrafish Embryos *in vivo*

To further investigate the ability of brominated indoles to induce AhR-dependent gene expression and to extend it to induction of an endogenous gene *in vivo*, we exposed zebrafish embryos to three brominated indoles, JN2-11-2, JN2-13-1, and JN2-10-21 and measured the expression of Cyp1a mRNA. These three compounds were selected because they tended to elicit the strongest response in the cell culture assays. In an initial experiment using 10 μ M concentrations of the three compounds, there was substantial mortality of embryos after the 6-hr exposure. In a subsequent experiment using 2 μ M exposure concentrations, survival was high and there was ~300- to 700-fold induction of Cyp1a mRNA (Figure 7). This level of induction, although high, was only 3–6% of that achieved with a 2 nM exposure to TCDD. Thus, these brominated indoles are capable of inducing the classical AHR-dependent marker gene Cyp1a *in vivo*, but with reduced efficacy as compared to TCDD.

Induction of CYP1A1 mRNA by Brominated Indoles in Human HepG2 Cells

To investigate the ability of these compounds to activate AhR and induce CYP1A1 in humans, we used the human liver-derived cell line HepG2, which has been shown previously to have a functional AhR signaling pathway.⁵⁰ We exposed HepG2 cells to brominated indoles JN2-11-2, JN2-13-1, and JN2-10-21 and measured the expression of CYP1A1 mRNA after either 6 hr or 24 hr of exposure. There was strong induction of CYP1A1 after 6 hr exposure (~300- to 2000-fold) (Figure 8). JN2-11-2 gave the strongest response, which was 42% of that produced by exposure to a maximally inducing concentration of TCDD (2 nM). After 24 hr of exposure, the induction response had decreased dramatically for the brominated indoles, whereas the response to TCDD had increased. Thus, these brominated indoles induce CYP1A1 with moderate to strong efficacy in a human cell line, but the induction is transient as compared to the persistent inducer TCDD.

DISCUSSION

The majority of studies examining ligand activation of the AhR and AhR signaling pathway as well as AhR-dependent toxic and biological effects have primarily focused on the activity of synthetic dioxin-like HAHs and PAHs.⁸ However, numerous naturally-occurring chemicals have been identified that can bind to the AhR and activate AhR signal transduction (reviewed in Denison and Nagy;¹¹ Nguyen and Bradfield⁵¹) and the majority of these ligands are either flavonoids or indole-containing/indole-derived compounds. The large number of endogenous halogenated compounds present in marine organisms suggests that these organisms may represent a significant source of unidentified naturally-occurring AhR ligands, and a few marine natural compounds with AhR agonist activity have already been identified.^{30–33} In the studies described here, we examined the biological activity of eight brominated indoles isolated from a marine alga and confirmed that they are members of a new class of AhR ligand by demonstrating their ability to directly bind to and activate

the AhR and AhR signaling pathway both *in vitro*, in cells from a variety of species, and *in vivo*.

The ability of the brominated indoles to induce EROD activity in PLHC-1 fish cells and AhR-dependent luciferase reporter gene activity in recombinant mammalian cells revealed some significant species differences in the response to these compounds. Although a relatively high level of brominated indole-dependent induction of luciferase activity was observed in the mammalian cells, the overall level of EROD induction in PLHC-1 cells was extremely low and had a different profile of chemical specificity (compare Figures 2 and 3). While the factors responsible for these quantitative and qualitative differences in responsiveness remain to be determined, there are several possibilities. First, the low induction levels in PLHC-1 cells may result from the fact that these studies were carried out for 24 h as compared to only a 4 h induction study in the mammalian cells. Considering the dramatic reduction in the level of luciferase induction we observed in the mammalian cells by extending the time of incubation/induction to 24 h (compared to 4 h (Figure 4)), a shorter induction period (i.e. 4 h) would be expected to result in a significantly greater level of brominated indole induction response in the PLHC-1 cells. Second, species- or cell-specific differences in the sensitivity and/or ligand specificity of the AhR between the fish and mammalian cell lines have been documented and these could also contribute to the significant differences in the induction response.^{6,8,52-54} For example, when the induction of EROD activity in PLHC-1 cells was compared to that in rat hepatoma (H4IIE) cells following exposure to TCDD or a series of AhR-active chlorinated naphthalenes, the PLHC-1 cells were consistently less sensitive than H4IIE cells.⁵² Other studies in PLHC-1 cells have shown different structure-activity relationships for AhR agonism (e.g. reduced potency of mono-*ortho*-substituted polychlorinated biphenyls) as compared to mammals.⁵³ Together, these aspects could contribute to the relatively low induction response and different chemical profile observed with the brominated indoles in the *Poeciliopsis lucida* cell line.

The ability of these brominated indoles to activate AhR was explored further by using an *in vivo* system, the zebrafish embryo. The Cyp1a induction response after a 6-hr exposure was strong (several hundred fold), even with a compound (JN2-10-21) that appeared inactive in the initial experiments with PLHC-1 cells. However, the efficacy of these brominated indoles was still a fraction of that shown by TCDD. Nevertheless, these studies demonstrate the ability of these compounds to activate AhR and induce an endogenous AhR target gene in an *in vivo* situation.

Interestingly, the brominated indoles were not equally efficacious at inducing luciferase activity in the different mammalian cell lines. While the guinea pig intestinal carcinoma (G16L1.1c8) and the rat hepatoma (H4L1.1c4) cells lines demonstrated relatively similar levels of luciferase induction for all eight compounds, the overall induction response in the mouse hepatoma cell line (H1L1.1c2) was consistently much lower. These results are similar to previous studies that reported increased levels of luciferase induction by AhR agonists in recombinant G16L1.1c8 guinea pig cells as compared to that in H1L1.1c2 mouse cells.^{54,55} Analysis of the brominated indole induction results also revealed that some compounds exhibit species-specific differences in their AhR agonist activity. For example,

JN2-39-2 exhibits a species difference in its ability to activate the guinea pig and mouse AhRs. Not only did JN2-39-2 induce luciferase activity to ~70% of that of TCDD in guinea pig G16L1.1c8 cells, but it stimulated guinea pig AhR transformation and DNA binding to a level comparable to that produced by TCDD. These results are consistent with JN2-39-2 being a relatively efficacious ligand/agonist of the guinea pig cytosolic AhR. In contrast, in mouse H1L1.1c2 cells, JN2-39-2 induced luciferase to only ~20% of that of TCDD in mouse cells and competitively inhibited only ~10% of the binding of [³H]TCDD to the mouse cytosolic AhR. These results are consistent with JN2-39-2 being a relatively poor ligand/agonist for the mouse AhR. The existence of species- and ligand-specific differences in the AhR and AhR signal transduction have been previously reported (reviewed in DeGroot et al.;⁸ Denison et al.⁶) and likely result from amino acid differences within the AhR ligand binding domain and elsewhere that contribute to ligand specificity/selectivity of different AhRs. While the specific amino acids responsible for these differences remain to be identified, recent homology modeling, docking and site-directed mutagenesis studies are providing insights into the mechanisms of ligand binding and ligand-dependent AhR activation and may aid in our understanding of these differences in species- and ligand-specificity.⁵⁶⁻⁵⁹

In addition to differences in relative potency and efficacy of different compounds between AHR bioassays from different species, apparent inconsistencies between results obtained with AHR assays from the same species were also observed. For example, although numerous brominated indoles (i.e. JN2-9-1, JN2-11-1, JN2-13-1 and JN2-36-12) were much more effective at competitively inhibiting the binding of [³H]TCDD to the mouse cytosolic AhR than that of JN2-39-2 (Figure 6), these compounds surprisingly only induced luciferase activity in mouse hepatoma cells to ~20%, a level comparable to that induced by JN2-39-2 (Figure 3). There are numerous reasons that could account for the apparent ability of a chemical that can bind well to the AHR in an in vitro ligand binding assay to stimulate AHR-dependent gene expression poorly.^{39,55} These reasons, include, but are not limited to: 1) relatively rapid metabolism/degradation of the compound in the intact cells (which does not occur in the in vitro ligand binding assay), 2) sequestration of the chemical by serum proteins and cell membranes, 3) variation in the ability (inability) of a chemical to cross cellular membranes resulting in differences in intracellular concentrations, 4) ligand binding does not differentiate agonists from antagonists and if the chemical was a partial antagonist, it could bind to the AHR strongly and induce poorly, 5) variations in the ability of the compounds to bind to and stimulate transformation of the mouse AHR in intact cells.^{39,55}

Potential for human exposure and toxicity

Given the source of these brominated indoles as natural products isolated from a marine alga,³⁴ it is reasonable to ask whether humans would be exposed to them, and at what levels. Nothing is known about the presence of these particular compounds in seafood or other routes of human exposure. However, other brominated indoles have been isolated from marine animals and plants⁶⁰⁻⁶² or sediment⁶³ and some of these have been detected in seafood or other marine products consumed by humans, including oysters⁶⁴ and fish oil supplements,^{65,66} at concentrations that can approach those of anthropogenic contaminants

such as mono-*ortho* PCBs.⁶⁵ Additional studies will be needed to better understand the potential human exposure to the suite of brominated indoles including those evaluated here.

Despite the identification of these brominated indoles as AhR agonists, the *in vitro* bioassays used in this study do not provide information relative to the potential toxicity of these compounds. The relatively transient nature of the induction response suggests that these compounds will be similar to other metabolically labile high affinity and efficacious AhR agonists (i.e. beta naphthoflavone and indolo[3,2b]carbazole) that produce little or no AhR-dependent toxicity, even when administered daily at relatively high concentrations.^{67,68} However, while it remains to be determined whether repeated or continuous exposure to brominated indoles is capable of leading to AhR-dependent toxicity in fish and other potentially exposed aquatic organisms, these and other studies suggest that persistent activation of the AHR signaling pathway by labile AHR agonists may not be sufficient on its own to produce the established spectrum of major dioxin-like toxic effects in animals.^{6,8,11,67–69}

The brominated indoles studied here join the ever-expanding category of nonclassical AhR ligands. While exposure to this class of compounds will likely not produce the prototypical spectrum of toxic effects typically observed after exposure to HAHs, it is important to note that adverse effects may still be mediated by other signaling pathways or interactions with cellular components. For example, simple brominated indoles (i.e. those with a single bromine substituent) have been reported to cause developmental toxicity and lethality in zebrafish embryos⁷⁰ and more complex brominated indoles isolated from marine molluscs have activity against human cancer cells.⁷¹ Additionally, the importance of species-specific effects should not be dismissed in any toxicity evaluation of brominated indoles or any other halogenated natural product found to be an AhR ligand, because receptor binding affinity, AhR transcriptional activity, and metabolic degradation are known to vary among species and even strains.⁸ For example, recent studies reported that the indole derivatives, 3-indoxyl sulfate and indirubin were more potent activators of the human AhR as compared to the mouse AhR.^{72,73} Whether a similar situation exists for the brominated indoles remain to be determined, but the results presented here demonstrate that exposure of a human cell line to three of the brominated indoles in this study can cause strong, albeit transient, induction of CYP1A1. The identification of eight naturally-occurring brominated indoles as AhR agonists not only contributes to the development of structure-activity relationships for nonclassical AhR ligands, but it also furthers our understanding of the differences and similarities between naturally occurring halogenated compounds and the man-made HAHs.

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ABBREVIATIONS

AhR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
DMSO	dimethyl sulfoxide
DRE	dioxin responsive element
EMSA	electrophoretic mobility shift assay
EROD	ethoxyresorufin O-deethylase
MEM	Minimum Essential Medium
HAH	halogenated aromatic hydrocarbon
PAH	polycyclic aromatic hydrocarbon
RLU	relative light unit
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TCDF	2,3,7,8-tetrachlorodibenzofuran

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Figure 1.

Structures of the eight naturally-occurring, marine-derived brominated indoles.

Abbreviations used:

JN2-9-1: 2,4,6-tribromo-3-(methylthio)indole

JN2-10-21: 2,3,4,6-tetrabromoindole

JN2-11-1: 6-bromo-2,3-bis(methylthio)indole

JN2-11-2: 2,4,5,6-tetrabromo-3-(methylthio)indole

JN2-13-1: 4,5,6-tribromo-2-(methylthio)indole

JN2-18-4: 4,5,6-tribromo-2,3-bis(methylthio)indole

JN2-36-12: 4,6-dibromo-2-(methylsulfinyl)-3-(methylthio)indole (Itomanindole A)

JN2-39-2: 3-acetyl-4,6-dibromo-2,3-dihydro-1H-indole-3-carboxylic acid.

Figure 2.

Brominated indoles are weak activators of AhR-dependent CYP1A expression in PLHC-1 cells. Teleost fish hepatoma (PLHC-1) cells were incubated with DMSO (0.5%), TCDD (0.1 nM) or each brominated indole (10 μ M) for 24 h after which ethoxyresorufin O-deethylase (EROD) activity was measured and the results normalized to protein content. Results are presented as the percent of the maximum EROD induction by TCDD, and are the mean $\pm$ SE of at least triplicate determinations.

Figure 3.

Brominated indoles stimulate AhR-dependent luciferase reporter gene expression in mammalian cells. Recombinant mouse and rat hepatoma (H1L1.1c2 and H4L1.1c4, respectively) and guinea pig intestinal adenocarcinoma (G16L1.1c8) cells were incubated with DMSO (1%), TCDD (1 nM) or the indicated brominated indole (10 μ M) for 4 h after which luciferase activity was determined. Values are expressed as a mean percent $\pm$ SD of the maximal induction resulting from 1 nM TCDD in triplicate incubations. A decrease in luciferase activity in the H1L1.1c2 cell line compared to both the G16L1.1c8 and H4L1.1c4 cell lines is indicated (* $p < 0.005$ (for both comparisons); Student's t -test). Results are representative of duplicate independent experiments.

Figure 4.

Brominated indoles are transient activators of the AhR signaling pathway. Recombinant rat hepatoma cells (H4L1.1c4) were incubated with DMSO (1%), TCDD (1 nM) or the indicated brominated indole (10 μ M) for 4 and 24 h after which luciferase activity was determined. Values are expressed as an average of relative light units (RLU) $\pm$ SD of triplicate incubations. Results are representative of duplicate independent experiments.

Figure 5.

Brominated indoles stimulate mammalian AhR transformation and DNA binding *in vitro*. Guinea pig hepatic cytosol (8 mg protein/ml) was incubated with DMSO, TCDD (20 nM) and each brominated indole (64 μ M) for 2 h at 20°C followed by incubation with [32 P]DRE oligonucleotide. Protein-DNA complexes were resolved by EMSA and visualized by autoradiography. The arrow indicates the position of the inducible AhR-DNA complex.

Figure 6.

Brominated indoles competitively inhibit the specific binding of [^3H]TCDD to mouse hepatic cytosolic AhR. Cytosolic extracts (1 mg protein/ml) prepared from mouse hepatoma cells (Hepa1c1c7) were incubated with 1 nM [^3H]TCDD, in the absence (●) or presence (▼) of 0.2 μM TCDF, or 5 μM of each brominated indole for 1 h on ice. Aliquots (300 μl) of each incubation were analyzed for ligand binding by sucrose density gradient centrifugation.

Treatment	Relative expression (mean $\pm$ SD)	%TCDD
DMSO	1.01 $\pm$ 0.19	
TCDD	11848 $\pm$ 3183	100%
JN2-11-2	317 $\pm$ 116	3%
JN2-13-1	658 $\pm$ 67	6%
JN2-10-21	699 $\pm$ 70	6%

Figure 7.

Brominated indoles induce Cyp1a mRNA in zebrafish embryos in vivo. Zebrafish embryos (48 hpf) were exposed to DMSO (0.5%), TCDD (2 nM), or each of three indicated brominated indoles (2 μ M) for 6 hr, after which RNA was isolated and Cyp1a mRNA was measured by real time RT-PCR. Values represent the average relative expression of Cyp1a mRNA $\pm$ SD of triplicate samples. Each sample was a pool of 20 embryos. Cyp1A expression was normalized to that of beta-actin.

	6 hr		24 hr	
Treatment	mean ± SD	%TCDD	mean ± SD	%TCDD
DMSO	1.04 ± 0.33		1.30 ± 0.41	
TCDD	4645 ± 1124	100%	8821 ± 3320	100%
JN2-11-2	1958 ± 649	42%	526 ± 126	6%
JN2-13-1	614 ± 148	13%	29 ± 4	0%.
JN2-10-21	293 ± 103	6%	43 ± 13	0%.

Figure 8.

Brominated indoles are transient inducers of CYP1A1 mRNA in human cells. HepG2 cells were incubated at 37°C with DMSO (0.5%), TCDD (2 nM), or each of three indicated brominated indoles (10 µM) for 6 or 24 hr, after which RNA was isolated and CYP1A1 mRNA was measured by real time RT-PCR. Values represent the average relative expression of CYP1A1 mRNA ± SD of triplicate samples. CYP1A1 expression was normalized to that of beta-actin.