Characterization of marine-derived halogenated indoles as ligands of the aryl hydrocarbon receptor

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ABSTRACT

The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor thought to mediate a number of physiological roles in the body, is becoming a target of interest for the development of new therapeutics. However, previous research has demonstrated that the downstream effects of AhR ligands cannot be predicted based simply on whether a ligand acts as an agonist or antagonist and the persistence of AhR signaling is thought to be a key determining feature. The current study investigated the AhR activity of four halogenated indoles isolated from the New Zealand red alga, Rhodophyllis membranacea: 4,7-dibromo-2,3-dichloroindole (4DBDCI), 7-bromo-2,3-dichloro-6-iodoindole (BDCII), 6,7-dibromo-2,3-dichloroindole (6DBDCI) and 2,6,7-tribromo-3-chloroindole (TBCI). Their ability to activate AhR signaling, measured as CYP1A1 activity via the ethoxyresorufin-O-deethylase (EROD) assay, was determined in human HepG2, mouse Hepa1c1c7 and rat H4IIE liver cancer cells. All four compounds induced CYP1A1 activity in HepG2 cells, suggesting they all acted as AhR agonists. 4DBDCI was particularly efficacious, inducing an 11-fold increase. Hepa1c1c7 and H4IIE cells, however, were generally less responsive to the halogenated indoles. All four compounds were persistent AhR agonists, inducing peak CYP1A1 activity after 72 h. Moreover, the 2,3,6,7-substituted BDCII, 6DBDCI and TBCI, but not 4DBDCI, competed with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for AhR binding as observed by the inhibition of TCDD-induced CYP1A1 activity. Overall, the current study has characterized four previously untested AhR ligands, highlighting differences in species sensitivity and persistence of signaling to provide a framework for their potential future use.

1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor of the basic helix-loop-helix-Per-ARNT-Sim family, conserved in vertebrates and expressed ubiquitously in humans [1,2]. Though initially discovered as the mediator of polycyclic and halogenated aromatic hydrocarbon toxicity, research over the last couple of decades has demonstrated a regulatory role of the AhR in inflammatory and immune responses and cell proliferation, and its physiological significance remains to be uncovered [3-5]. Indeed, in addition to the well-studied pollutants that induce toxicity via AhR binding, an ever-increasing number of dietary-derived and endogenously-produced ligands have been reported, supporting the notion of its physiological roles [6-8].

In the canonical mechanism of AhR signaling, cytosolic ligand binding of the AhR triggers translocation of the ligand-AhR-chaperone complex into the nucleus, where the chaperone proteins, heat shock protein 90, hepatitis B virus X-associated protein and p23, dissociate to allow binding of the AhR nuclear translocator (ARNT) protein [9]. The newly-formed heterodimer then binds to specific DNA sequences, known as xenobiotic response elements (XREs), found upstream of a myriad of target genes [10,11]. Those involved in drug metabolism,
such as UDP-glucuronosyltransferase 1A6 (UGT1A6), NAD(P)H quinone oxidoreductase 1 (NQO1) and various cytochrome P450 (CYP) isoenzymes, are commonly associated with AhR activation [12–16]. In particular, CYP1A1, especially in the liver, is so tightly regulated by the AhR it often serves as the basis of environmental contaminant detection [17–19].

Outside of xenobiotic-metabolizing enzymes, however, the link between AhR activation and gene expression is unclear, with different ligands inducing different downstream transcriptional responses. A study comparing two potent AhR agonists, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 6-formylindolo[3,2-b]carbazole (FICZ), found that TCDD and FICZ had opposing effects on Th1 and Th2 cell frequency in influenza A virus-infected mice [20]. Similarly, Wheeler et al. [21] found that TCDD modulated immune responses to influenza A virus infection, while FICZ had no effect, which was attributed to the persistence of AhR stimulation and cell-specific responses. Another study in which total AhR activation over time was matched between ligands confirmed the idea that the downstream immune responses were dependent on both the amount and duration of AhR stimulation [22].

The involvement of the AhR in numerous physiological processes makes it an interesting therapeutic target. However, the categorization of AhR ligands into agonist or antagonist is clearly insufficient to describe the effect these ligands will have on downstream pathways and the persistence of AhR stimulation needs to be considered. In this study, the AhR activity of four halogenated marine indoles (Fig. 1) isolated from the endemic New Zealand red alga, Rhodophyllis membranacea [23], was assessed. The aim was not just to identify whether these compounds would act as AhR agonists or antagonists, but to also identify any differences in persistence of AhR stimulation and competition with TCDD for AhR binding that could provide a more useful profile of these ligands for future experiments.

2. Materials and methods

2.1. Materials

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, 50 μg/mL in DMSO, >99.9%) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA) and GNF-351 was purchased from Santa Cruz Biotechnology (Dallas, TX). Trichloroacetic acid was purchased from Merck (Darmstadt, Germany). Resorufin ethyl ether, 3,3'-methylene-bis(4-hydroxy-<br>ocoumarin), sulforhodamine B and DMEM/F12 powder were purchased from Sigma (St Louis, MO). 100x L-glutamine and penicillin-streptomycin were purchased from Gibco (Dublin, Ireland) while fetal bovine serum was purchased from Cytiva Life Sciences (Marlborough, MA). HepG2, Hepa1c1c7 and H4IIE cell lines were purchased from ATCC (Manassas, VA).

2.2. Indole extraction

Indoles, 4,7-dibromo-2,3-dichloroindole (4DBDCI), 7-bromo-2,3-dichloro-6-iodoindole (BDCII), 6,7-dibromo-2,3-dichloroindole (6DBDCI) and 2,6,7-tribromo-3-chloroindole (TBCI) were extracted and purified from Rhodophyllis membranacea as previously described [23].

2.3. Cell culture

Human hepatocellular carcinoma (HepG2) and rat hepatoma (H4IIE) cells were grown in DMEM/F12 supplemented with 5% fetal bovine serum, 1% penicillin streptomycin and 1% L-glutamine. Mouse hepatoma (Hepa1c1c7) cells were grown in MEM supplemented with 5% FBS and 1% penicillin streptomycin. All cells were incubated at 37 °C, 5% CO₂ and passaged at ~80% confluence.

2.4. Cell viability

HepG2, Hepa1c1c7 and H4IIE cells were plated in 96-well plates at 1 × 10⁴, 7.5 × 10³ and 7.5 × 10³ cells per well, respectively. Cells were treated the following day with the specified concentration of indole or vehicle control (0.5% DMSO) and incubated for 72 h. The sulforhodamine B (SRB) assay was used to determine the effect of the individual indoles on cell viability as previously described [24]. The resulting absorbance was measured at 510 nm using the Bio-Rad Benchmark Plus microplate spectrophotometer. Absorances were compared to a cell density standard curve and the results are expressed as a percent of the vehicle control.

2.5. CYP1A1 catalytic activity

CYP1A1 activity was determined via the ethoxyresorufin O-deethylase (EROD) assay, as previously described [25]. For assessing EROD activity after 6 or 24 h, HepG2 cells were plated in black, clear-bottom 96-well plates at 7 × 10⁴ cells per well, while Hepa1c1c7 and H4IIE cells were plated at 5 × 10⁴ cells per well. For EROD assays conducted over 72 h of treatment, HepG2 cells were plated at 1.5 × 10⁵ cells per well. In all cases, cells were allowed to attach to the wells for 24 h and were then treated with the indicated concentration of indole or vehicle control and incubated at 37 °C. EROD assays were then conducted by incubating the cells with ethoxyresorufin (5 μM) and dicoumarol (5 μM) in PBS for 30 min at 37 °C. The reaction was terminated by the addition of ice-cold methanol and the resulting fluorescence was measured on the SpectraMax 13x Multi-Mode microplate reader with an excitation/emission wavelength of 550/585 nm. Fluorescence output was expressed as a function of assay duration (30 min) and protein content, as determined by the bicinchoninic acid assay (P. K. [26]).

2.6. Statistical analysis

Data were analyzed using either a t-test, or one- or two-way ANOVA coupled with a Bonferroni post-hoc test following a significant ANOVA, as indicated in the figure legends. Statistical significance was set at p < 0.05. Data were normalized to an appropriate control, and variability in the control group was accounted for in the statistical analysis.
3. Results

3.1. Cell viability in response to halogenated indoles

To ensure that changes in enzymatic activity were not caused by cell death, the SRB assay was used to assess cell viability in response to the indoles. The results showed that indoles BDCII, 6DBDCI and TBCI were not significantly cytotoxic to any of the cell lines at concentrations up to 20 µM (Supplementary Table 1). At concentrations of 20 µM, BDCII and TBCI did decrease Hepa1c1c7 cell viability to 52% and 64% of control, respectively, but neither was statistically significant (Supplementary Table 1). 4DBDCI, however, was significantly more cytotoxic to each cell line, reducing cell viability after 72 h to 47.8 ± 13.5% (HepG2), 12.5 ± 13.3% (Hepa1c1c7) and 33.1 ± 13.5% (H4IIE) of control at 20 µM (p < 0.05, one-way ANOVA with Bonferroni post-hoc test) (Supplementary Table 1). Therefore, experiments that examined structure-activity relationships between the four indoles used the same range of concentrations at a maximum concentration of 10 µM.

3.2. Short-term induction of EROD activity

To determine whether these compounds act as AhR agonists, their ability to induce CYP1A1 catalytic activity was determined after at 6 and 24 h. In human HepG2 cells, all four compounds caused a significant increase in EROD activity, albeit to varying extents (Figs. 2). At its peak, the most potent, 4DBDCI, increased EROD activity 11-fold relative to control. BDCII and 6DBDCI increased EROD activity 2-3-fold at 24 h, while TBCI elicited a 7-fold increase, though with considerable variability. The trends observed in HepG2 cells did not translate to either mouse hepatoma (Hepa1c1c7) or rat hepatoma (H4IIE) cells. For example, in Hepa1c1c7 cells, 4DBDCI was particularly potent with near maximal EROD induction at 0.05 µM, while BDCII, 6DBDCI and TBCI were relatively ineffective. Furthermore, H4IIE cells were the least sensitive to the halogenated indoles with only 4DBDCI increasing EROD activity ~4-fold after 24 h. Given the relative insensitivity of the mouse and rat cells to these indoles, no further investigation was conducted with these cell lines.

3.3. EROD activity in competition with GNF

While the EROD assay is generally considered adequate for determining AhR activation given the tight regulation of CYP1A1 by the AhR, the possibility remains that CYP1A1 could have been induced independent of the AhR [27,28]. Thus, to ensure the observed effects were mediated by the AhR, EROD assays were conducted in combination with the AhR antagonist, GNF-351 (0.25 µM) [29]. The results showed that GNF-351 completely abrogated the indole-induced CYP1A1 activity, suggesting that all four compounds induced CYP1A1 via AhR binding (Fig. 3).

3.4. Long-term induction of EROD activity

Having observed differences in short-term (6–24 h) CYP1A1 activity between the indoles, their effect on CYP1A1 activity over a 72-h period was determined in HepG2 cells. Although 1 µM or less of the halogenated indoles generally did not induce significant CYP1A1 activity at 24 h, 0.1, 1 and 10 µM of each indole was examined to see if the lower concentrations could persist and induce CYP1A1 activity. Within 12 h, the CYP1A1 activity induced by 4DBDCI (10 µM) was elevated 2-fold over control and remained elevated at 72 h (Fig. 4). Both BDCII and TBCI (10 µM) induced peak CYP1A1 activity at 72 h, reaching approximately 2.5- and 5-fold, respectively, above control. In contrast, 6DBDCI did not induce significant changes in CYP1A1 activity throughout the 72-h period, though it followed a similar trend to that observed for BDCII and TBCI (Fig. 4).

3.5. EROD activity in competition with TCDD

To assess the potential for these halogenated indoles to antagonize AhR binding of potent halogenated aromatic hydrocarbons, EROD assays were conducted in combination with TCDD (1 nM). The results showed that at concentrations of 1 µM BDCII, 6DBDCI and TBCI began to antagonize the TCDD-induced response (Fig. 5). At the highest concentration (10 µM) each compound effectively blocked TCDD binding to the AhR, as evidenced by the reduced EROD activity following co-treatment with the compounds and TCDD. 4DBDCI, however, was unable to antagonize the action of TCDD and appeared to increase TCDD-induced EROD activity, though this was not statistically significant.

4. Discussion

The current study has identified four seaweed-derived indoles as AhR
ligands, with some key differences in their AhR activity. The EROD activity induced by each compound in human HepG2 cells was abrogated when tested in combination with the AhR antagonist, GNF-351, confirming the AhR-dependency of the response. Moreover, though their potencies varied, these ligands appeared to act persistently, inducing peak (2–5-fold increase over control) EROD activity at 72 h. This is in contrast to the findings of DeGroot et al. [30], who investigated eight marine-derived halogenated indoles and found them to act quite transiently, with greater CYP1A1 mRNA expressed at 6 h compared to 24 h.

The distinction between persistent and transient AhR ligands is considered an important determinant of the overall downstream response [31] and transient AhR ligands are thought to have a lower risk of toxicity. Although these indoles were tested for cytotoxicity in liver cell lines, potential toxicity towards other organs is an important consideration. The type and pattern of halogen substitution on an indole core likely determines the persistent or transient nature of these compounds and further investigation into this could be useful in the rational design of AhR ligands. For example, BDCII, 6DBDCI and TBCI all follow the pattern of halogen substitution at the C-2, 3, 6 and 7 positions, differing only by the type of halogens substituted and, as such, had very similar responses across all experiments in this study. Notably these three ligands were effective at inhibiting TCDD-induced EROD activity. This suggests that they can compete with TCDD for AhR binding, effectively antagonizing the effect of TCDD, while still exhibiting partial agonism. Therapeutically, this could be explored further to reduce the body burden of dioxins and dioxin-like compounds. Moreover, this is a therapeutic use in which a slightly persistent effect (to match that induced by dioxins) may be more beneficial than a transient one.

An interesting comparison can be made between 4DBDCI and 6DBDCI, differing only by the position of a single bromine (C-4 vs. C-6).
The EROD activity induced by 4BDDCI appeared to be relatively stable over the course of 72 h, while that induced by 6BDDCI seemed to increase over time. A particularly interesting finding was that, unlike the other indoles which reduced TCDD-induced EROD activity, 4BDDCI appeared to increase the effect of TCDD. It is fair to assume that the mechanism through which 4BDDCI induces CYP1A1 activity is mediated by the AhR, given that GNF-351 antagonized CYP1A1 induction by 4BDDCI. This leaves two possible conclusions: 1) 4BDDCI exhibits weak affinity for the AhR that cannot outcompete TCDD, but still maintains reasonable efficacy and acts relatively persistently, or 2) 4BDDCI and TCDD are capable of binding simultaneously to the AhR to potentiate the response. Although the average EROD activities obtained for the combination treatment of TCDD and 4BDDCI were greater than that observed for TCDD alone, the large variability in the data makes it difficult to draw conclusions regarding the significance of this effect.

Previous studies have also reported synergistic AhR activity from TCDD and other phytochemicals. Van der Heiden et al. [32] found that genistein concentration-dependently increased the effect of TCDD on the reporter luciferase response in stably transfected H4IIE, HepG2 and T47D cells. Jin et al. [33] also found that quercetin (10 μM) and TCDD (10 nM) together increased CYP1A1 mRNA more than each individual compound alone in Caco2 cells. Another study investigating a range of methyl- and methoxy-indoles identified 4-methylindole and 7-methoxyindole as two of the most efficacious AhR ligands in human cells, both of which slightly increased CYP1A1 mRNA in combination with TCDD [34]. Interestingly, a synergistic response was obtained by combination treatment of both ligands to AZ-AHR (stable HepG2 luciferase reporter) cells, compared to each indole alone, but the response to an indole combining both groups, 7-methoxy-4-methylindole, was reduced ~10-fold compared to each individual compound. A more thorough investigation into the binding dynamics of AhR ligands, particularly those possessing C4 and C7 substitution, both alone and in combination with TCDD, is clearly necessary to determine the specific mechanism behind these instances of enhanced CYP1A1 induction.

Importantly, EROD induction in response to these seaweed-derived indoles varied considerably among human, mouse and rat cell lines. AhR ligands are often tested in animal models of disease, despite the growing body of literature emphasizing differences between human and other mammalian AhR [35–37]. Hepa1c1c7 cells were particularly sensitive to 4BDDCI, with 0.05 μM inducing an 11-fold increase in EROD activity after 24 h. In contrast, Hepa1c1c7 cells were relatively insensitive to the indoles BDCII, 6DBDCII and TBCI, as were the H4IIE cells. Similarly, H4IIE cells were relatively insensitive to the effects of the halogenated indoles. β-naphthoflavone, a positive control for AhR signaling, induced CYP1A1 activity 9–19-fold in the three cell lines (Supplementary Fig. 1), suggesting that the lack of CYP1A1 induction in response to the halogenated indoles in Hepa1c1c7 and H4IIE cells was due to varying sensitivities of the cell lines to AhR ligands. Humans are thought to have evolved an AhR with greater sensitivity towards indoles, likely due to their presence in the diet, both as phytochemicals and tryptophan metabolites [38]. Similarly, DeGroot et al. [30], found that cells from various species, including fish, mouse, rat, guinea pig and human, differed in their sensitivities to a range of brominated indoles. Future studies investigating the therapeutic effects of an AhR ligand need to consider the relative sensitivity of different species in order to improve their translatability. Furthermore, it should be noted that the effect of these indoles may differ between tissues. A range of phytochemicals discussed in this study induce different responses induced not just between species (mouse vs. human) but also tissue (human breast MCF7 cells vs. human liver HepG2 cells) [39]. The tissue-specific effect of AhR ligands has led to the concept of selective AhR modulators (SAhRMs), which have enormous therapeutic potential, but require thorough testing to best understand their effect in the human body as a whole [40,41].

A limitation of this study is that AhR activation was only assessed via EROD activity, which can be variable between experimental runs. This could potentially be improved by expressing EROD activity values as a ratio of a known inducer, such as TCDD, or normalizing the EROD activity to metabolic activity rather than protein content. However, the emphasis of this study as an initial test of these halogenated indoles was on the qualitative findings. Further validation of these results can be carried out to probe these findings further. Additionally, the pharmacokinetics of these indoles and their direct binding to the AhR have not been determined, so whether the observed CYP1A1 induction was induced by the ligand itself or a metabolite is unconfirmed. However, given that other halogenated indoles have been reported to act as AhR agonists [30] it would be fair to assume that the observed effects are mediated by the halogenated indoles themselves.

Overall, the four previously untested AhR ligands presented here further the understanding of the potential structure-activity relationships governing AhR ligand binding and CYP1A1 induction. Given the differences observed in TCDD-competition binding and timing of CYP1A1 induction, these ligands may differ both in their AhR binding and in their induced downstream effects and thus could serve different therapeutic purposes. There is particular interest in the use of AhR ligands to modulate immune and inflammatory diseases [42], as well as cancer [43] and metabolic diseases [44]. However, species- and tissue-specific AhR effects are common and thus careful investigation into how these ligands are likely to act in humans as a whole is still required.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author statement

All authors agree to the revisions made in this manuscript and have read and approved the final version.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.05.016.

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