**Regular Article**

**The Differential Selectivity of Aryl Hydrocarbon Receptor (AHR) Agonists towards AHR-Dependent Suppression of Mammosphere Formation and Gene Transcription in Human Breast Cancer Cells**

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We had previously reported that treatment with the aryl hydrocarbon receptor (AHR) agonist β-naphthoflavone (βNF) suppressed mammosphere formation derived from cancer stem cells in human breast cancer MCF-7 cells (Cancer Lett., 317, 2012, Zhao et al.). Here, using several AHR agonists, we have investigated the association of this suppression with the classical ability to induce AHR-mediated gene transcription in the xenobiotic response element (XRE). The mammosphere formation assays were performed using wild-type and AHR-knockout MCF-7 cells in the presence of AHR agonists including 3-methylcholanthrene (3MC), benzo[a]pyrene (BaP), 7,12-dimethylbenz[a]anthracene (DMBA), 6-formylindolo[3,2-b]carbazole (FICZ), indirubin, indole-3-carbinol (I3C), indole-3-acetic acid (IAA), and kynurenine (KYN), followed by the XRE-reporter gene assays of the agonists. We showed that treatments with 3MC, BaP, and DMBA strongly suppressed mammosphere formation of the stem cells in an AHR-dependent manner, while other agonists showed weaker suppression. In reporter gene assays, the strength or duration of AHR/XRE-mediated gene transcription was found to be dependent on the agonist. Although strong transcriptional activation was observed with 3MC, FICZ, indirubin, I3C, IAA, or KYN after 6 h of treatment, only weak activation was seen with BaP or DMBA. While transcriptional activation was sustained or increased at 24 h with 3MC, BaP, or DMBA, appreciable reduction was observed with the other agonists. In conclusions, the results demonstrated that the suppressive effects of AHR agonists on mammosphere formation do not necessarily correlate with their abilities to induce AHR-mediated gene transcription. Hence, different AHR functions may be differentially induced in an agonist-dependent manner.

**Key words**  aryhydrocarbon receptor; cancer stem cell; breast cancer; mammosphere

**INTRODUCTION**

The aryl hydrocarbon receptor (AHR) is a member of the basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) family and acts as a ligand-activated transcription factor. AHR is located in the cytoplasm and upon ligand binding, it translocates to the nucleus and heterodimerizes with the AHR nuclear translocator (ARNT). The heterodimer then binds to the xenobiotic response elements (XREs) of its target genes, in order to regulate their transcription. The target genes include those encoding drug-metabolizing enzymes such as CYP1A1, CYP1A2, and CYP1B1. Hence, AHR is a key regulator of the metabolism of xenocides and endogenous compounds.

AHR was discovered in 1976 as a dioxin-binding protein and has since been widely studied. Several studies have focused on its roles in transmitting the toxic effects of environmental compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAHs). The latter includes known AHR ligands such as 3-methylcholanthrene (3MC), benzo[a]pyrene (BaP), and 7,12-dimethylbenz[a]anthracene (DMBA). On the other hand, structurally diverse endogenous and exogenous compounds have also been identified as AHR ligands. These include tryptophan derivatives such as kynurenine (KYN) and 6-formylindolo[3,2-b]carbazole (FICZ) and indole-3-acetic acid (IAA), and indole derivatives such as indole-3-carbinol (I3C) and indirubin and kynurenine (KYN), followed by the XRE-reporter gene assays of the agonists. We showed that treatments with 3MC, BaP, and DMBA strongly suppressed mammosphere formation of the stem cells in an AHR-dependent manner, while other agonists showed weaker suppression. In reporter gene assays, the strength or duration of AHR/XRE-mediated gene transcription was found to be dependent on the agonist. Although strong transcriptional activation was observed with 3MC, FICZ, indirubin, I3C, IAA, or KYN after 6 h of treatment, only weak activation was seen with BaP or DMBA. While transcriptional activation was sustained or increased at 24 h with 3MC, BaP, or DMBA, appreciable reduction was observed with the other agonists. In conclusions, the results demonstrated that the suppressive effects of AHR agonists on mammosphere formation do not necessarily correlate with their abilities to induce AHR-mediated gene transcription. Hence, different AHR functions may be differentially induced in an agonist-dependent manner.

**Key words**  arylydrocarbon receptor; cancer stem cell; breast cancer; mammosphere

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these beneficial effects can be separated from the AHR-mediated toxic effects. Although various AHR ligands have been identified, there is little information about the ligand selectivity for various AHR functions. It is also unclear whether all the ligands agonists have the same set of AHR functions. Therefore, in this study, we have investigated whether AHR agonists other than βNF could show suppressive effects on mammosphere formation. Furthermore, we investigated the association between AHR-induced suppression of mammosphere formation and AHR/XRE-dependent gene transcription.

MATERIALS AND METHODS

Chemicals  βNF, 3MC, and DMBA were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). BaP, I3C, IAA, and KYN were obtained from Tokyo Chemical Industry (Tokyo, Japan). FICZ and indirubin were procured from Enzo Life Sciences (Farmingdale, NY, U.S.A.) and Biomol GmbH (Hamburg, Germany), respectively. Their chemical structures are shown in Fig. 1. Other chemicals used were of the highest grade commercially available and were obtained from commercial sources.

Knockout of AHR Gene The knockout of AHR gene in the MCF-7 human breast cancer cells obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) was performed using the clustered regularly interspaced short palindromic repeats CRISPR-associated proteins 9 (CRISPR/Cas9) system. The guide RNA (gRNA) sequence was designed with the CHOPCHOP online tool (https://chopchop.rc.fas.harvard.edu/).31) The insert oligonucleotides for human AHR gRNA were 5-ccggGTA AAG CCA ATC CCA GCT GA-3/5-aaacTCA GCT GGG ATT GGC TTT AC-3. The gRNA targets the second exon of AHR. The gRNA was annealed and subcloned into a pGuide-it vector (Clontech Laboratories, Mountain View, CA, U.S.A.). MCF-7 cells were transfected with the subcloned pGuide-it/gRNA vector using PEI Max (Polysciences, Warrington, PA, U.S.A.). After transfection, the cells expressing ZsGreen1 fluorescent protein were collected by fluorescence-activated cell sorting using JSAN JR Swift (Bay Bioscience, Kobe, Japan). The collected cells were cloned by limiting dilution to obtain AHR-knockout (AHR-KO) MCF-7 cells.

Cell Culture Wild-type and AHR-KO MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) containing 5% fetal bovine serum and 100 U/mL penicillin, and 100 µg/mL streptomycin (Pen Strep, Gibco, Grand Island, NY, U.S.A.) in a humidified atmosphere of 5% CO2 at 37°C.

Western Blotting Wild-type and AHR-KO MCF-7 cells were harvested and the whole-cell lysates were subjected to Western blotting with anti-AHR antibody as described previously.32)

Mammosphere Formation Assay The mammosphere formation assays were performed as described previously.27) Briefly, single cells were seeded at a density of 500 cells per well in ultra-low adherent 96-well plates using MammoCult Medium (StemCell Technologies, Inc.; Vancouver, BC, Canada), and cultured in a medium containing an AHR agonist at indicated concentrations or solvent (0.1% dimethyl sulfoxide, DMSO) in a 5% CO2 incubator at 37°C for 5d. The mammospheres were viewed under a Zeiss confocal microscope and measured using Zeiss Axiovision software (Carl Zeiss, Oberkochen, Germany).

MTS Assay The MCF-7 cells were seeded in 96-well microplates at a density of 5000 cells per well, cultured for 24h, and treated with AHR agonists at the indicated concentrations. After 48h of incubation, cell viability was evaluated using the MTS assay (Promega, Madison, WI, U.S.A.) according to the manufacturer’s instructions. The number of living cells is directly proportional to the absorbance at 490nm of a formazan product reduced from MTS by living cells.

Luciferase Reporter Gene Assay The MCF-7 cells were transfected with both the pGL3-XRE luciferase reporter plasmid (XRE-luc)33) as well as Renilla pGL4.74 (hRluc/TK; Promega) as an internal standard, using the reverse-transfection method with PEI Max (Polysciences). After overnight incubation at 37°C, the cells were treated with AHR agonists for 6 or 24h. This was followed by the measurement of the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). The activity of the firefly luciferase was normalized to that of Renilla luciferase.
RESULTS

βNF Suppressed Mammosphere Formation in MCF-7 Cells in an AHR-Dependent Manner  We had previously reported that treatment with the AHR agonist, βNF, suppressed mammosphere formation by CSCs in human breast cancer-derived MCF-7 cells. As a first step, we investigated whether the observation was AHR-dependent or not. To this end, we established AHR-KO cell lines using the CRISPR/Cas9 system-based method. Mutation of two clones was analyzed by direct sequencing (Fig. 2A; named KO#1 and KO#2). To confirm the knockout of the AHR gene, Western blotting was performed with the cell lysates. In the two cell lines established, no AHR proteins were detected (Fig. 2B). Consistent with the expectations, treatment with the typical AHR agonist 3MC induced the expression of the XRE-driven luciferase reporter gene in wild-type MCF-7 cells, but not in the AHR-KO cells (Fig. 2C). Moreover, treatment with 3MC increased CYP1A1 mRNA levels in the wild-type cells, but not in the AHR-KO cells (data not shown). These results clearly demonstrated appropriate knockout of AHR in both the clones. Thereafter, we used the KO#2 clone as AHR-KO MCF-7 cells in this study.

Using the established AHR-KO cells, we first investigated whether the βNF-induced suppression of mammosphere formation was AHR-dependent. The wild-type and AHR-KO cells were cultured under non-adherent conditions in the presence of various concentrations of βNF for 5 d, following which the mammosphere formation was evaluated. As expected, the treatment with βNF reduced the number of mammospheres formed in wild-type MCF-7 cells, but not in AHR-KO cells (Fig. 3).

Extent of AHR-Dependent Suppression of Mammosphere Formation Differed among the AHR Agonists Used  We then investigated other AHR agonists, including 3MC, BaP, DMBA, FICZ, indirubin, I3C, IAA, and KYN, for their abilities to suppress mammosphere formation. The ranges of test compound concentrations were selected based on previous reports. The treatments with 3MC, BaP, and DMBA strongly suppressed mammosphere formation in a concentration-dependent fashion, as previously observed with βNF, while the suppression by FICZ, indirubin, I3C, IAA, and KYN was weaker in comparison. In the AHR-KO cells, no significant effects on mammosphere formation of the AHR agonists used, except for I3C and IAA at the highest concentration, were observed. These results suggest that various AHR agonists suppress mammosphere formation in an AHR-dependent manner, but the extent of suppression varies among AHR agonists.

Since treatment with I3C or IAA at 100 µM suppressed mammosphere formation in both wild-type as well as AHR-KO cells, the cytotoxicities of AHR agonists used were investigated. After 48 h of treatment under adherent culture conditions, cell viability was not affected by these compounds at the concentrations examined (Fig. 4).

Fig. 2. The Establishment of MCF-7-Derived AHR-KO Cell Lines

A A model for targeting the AHR by CRISPR/Cas9. The modified bases are highlighted in gray. The sequence used as the target is underlined and the protospacer adjacent motif (PAM) sequences are shown in bold. B Whole-cell lysates of wild-type MCF-7 cells (WT) and two clones of AHR-KO cells (KO#1, KO#2) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were detected by immunoblotting using anti-bodies against AHR and tubulin (as a loading control). C Wild-type MCF-7 cells and two AHR-KO cells (KO#1 and KO#2) were transfected with the reporter plasmid XRE-luc. and pGL4.74. On the following day, the cells were treated with 3MC (2.5 µM) as a positive control or solvent (0.1% DMSO) for 24 h, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System. The results are shown as relative values to the solvent-treated MCF-7 cells after Renilla normalization. Each column represents the mean ± standard deviation (S.D.) (n = 3).
study suppressed mammosphere formation in an AHR-dependent manner, the magnitude of suppression differed among them. Hence, we investigated whether the suppressive abilities of the agonists were correlated with their abilities to induce the AHR/XRE-dependent gene transcription, a classical function of AHR. To this end, the reporter gene assays with the XRE-luc reporter plasmid were performed (Fig. 5). Strong transcriptional activation was observed after 6 h of treatment.
with 3MC, FICZ, indirubin, I3C, IAA, or KYN; however, only weak activation was observed after treatment with BaP or DMBA 6 h. The treatment with 3MC, BaP, I3C, IAA, or KYN for 6 h also transactivated the reporter gene expression in a dose-dependent manner. In the case of 3MC, BaP, and DMBA, the induction of luciferase reporter activity was sustained or increased at 24 h, but appreciable reduction of the activity was observed in the cells after 24 h of the treatment with FICZ, indirubin, I3C, IAA, or KYN. In addition, βNF could not be tested in this assay since it is known to directly inhibit luciferase enzyme activity. 38) These results indicate that the strength or duration of transcriptional activation of AHR varies, depending on the type of AHR agonist.

**DISCUSSION**

In our previous studies, we demonstrated that treatment with βNF, a well-known AHR agonist, resulted in the suppression of mammosphere formation derived from CSCs in human breast cancer MCF-7 cells. 27,28) In this study, AHR-KO cells were established from MCF-7 cells using the CRISPR/Cas9 system, and by use of the established cell line, the suppressive effect of βNF was demonstrated to be AHR-dependent (Figs. 2, 3). We also utilized several structurally diverse AHR agonists, other than βNF, to investigate whether the suppressive effects on mammosphere formation was a general characteristic of AHR agonists. It was indicated that all the 9 agonists examined showed the abilities to suppress mammosphere formation in an AHR-dependent manner. These results suggest that the suppression of mammosphere formation is one of the functions of AHR.

Although the suppression of mammosphere formation was observed with all the AHR agonists tested, the extent of suppression varied among the agonists (Fig. 3). The environment-driven PAH-type agonists, including 3MC, BaP, and DMBA, showed strong suppressive effects even at low concentrations, whereas FICZ, indirubin, and KYN did only weak even at higher concentrations. The effect of βNF was moderate in comparison to these two groups.

Based on these differences, we investigated their association with the potency of AHR agonists for AHR/XRE-dependent gene transcription and found that the magnitude of suppression of mammosphere formation did not necessarily correlate with that of AHR/XRE-dependent gene transcription. For
instance, FICZ and indirubin induced reporter gene expression as much as 3MC at 6h, although FICZ and indirubin showed much weaker suppressive effects on mammosphere formation compared to 3MC. These results suggest that the two different functions of AHR, the suppression of mammosphere formation and AHR/XRE-dependent gene transcription, are independently induced depending on the type of AHR agonists.

The persistence of AHR activation might be a relevant factor which influences the agonist-dependent differences in the induction of the AHR functions. In this study, the cells were treated with the agonists for 6 or 24h in reporter assays. As observed in the results shown in Fig. 5, the AHR agonists used in reporter assays are categorized not only on the basis of their activation potency (as discussed above), but their persistence as well. The AHR/XRE-dependent transcription activities induced by 3MC, BaP, and DMBA at 6h were sustained or increased at 24h, while those by FICZ, indirubin, I3C, IAA, and KYN appreciably reduced at 24h. In the former group, 3MC, BaP, and DMBA strongly suppressed mammosphere formation. In contrast, the agonists of the latter group had much weaker effects on mammosphere formation. Although βNF could not assess AHR/XRE-dependent transcription activities, βNF treatment could observe CYP1A1 mRNA induction throughout 24h (data not shown). Thus, βNF may categorize into the former group. Taking into account that the cells were cultured with agonists for 5d in mammosphere formation assays, these results suggest that the persistence of AHR activation, during the mammosphere formation process is important for the inhibition of mammosphere formation by AHR agonists.

Another possible mechanism is related to the characteristics of 3MC, BaP, and DMBA, which showed strong suppressive effects on mammosphere formation in this study. They are well known as carcinogenic polycyclic aromatic hydrocarbons that are metabolically activated by CYP1A1 and/or CYP1B1, and thus treatment of cells with these compounds might induce DNA adduct formation and/or oxidative DNA damage. In addition, BaP induces p53 activation, cell cycle arrest, and cell death in MCF-7 cells. It has also been reported that oxidative stress by reactive oxygen species is involved in the suppression of mammosphere formation in MCF-7 cells. Since the mechanism of the AHR agonist-mediated suppression of mammosphere formation remains unclear, further studies are needed in order to understand the agonist selectivity for the different types of AHR functions.

Treatment with I3C or IAA weakly suppressed mammosphere formation at lower concentrations; however, the compounds showed drastic suppressive effects at the highest concentration in both wild-type as well as AHR-KO cells (Fig. 3). In adherent culture systems, however, treatment with I3C or IAA did not affect cell viability (Fig. 4). These results suggest that these compounds have not only AHR-dependent but also AHR-independent suppressive effects on mammosphere formation. Although further studies are needed for a better understanding of this phenomenon, this information might be useful in mechanistic analyses for the AHR agonist-dependent suppression of mammosphere formation.

A previous study had demonstrated that the repression of AHR by its antagonist or AHR gene knockdown inhibited the proliferation, invasion, and migration of breast cancer cells, suggesting that AHR is associated with cancer malignancy. Furthermore, we previously reported that the activation of inflammatory signaling, induced by the overexpression of HER2, enhanced CSC-derived mammosphere formation, which was observed to be AHR-dependent. However, treatment of the HER2-overexpressing breast cancer cells with βNF suppressed their mammosphere formation. Consistent with these observations, the present study showed that AHR has anti-CSC activity. These results indicate that AHR has diverse roles in cancer cells, and its activation may even show almost opposite effects, depending on the cellular context. Since the identification of AHR as a receptor that mediates the toxic effects of dioxin-like compounds, little research has been done on its role in the suppression of tumor formation and malignant transformation of cancer. However, together with the previous results, the present study suggests that further research on the role of AHR in cancer will provide new insights into the suitability of AHR as a target of anti-cancer drugs. To that end, it is necessary to discover the AHR modulators showing beneficial effects including the suppressive effects of mammosphere formation, but not AHR-mediated toxic effects. The results of this study support the possibility that AHR may be a novel therapeutic target for cancer, if the functions of AHR can be properly modulated by its ligands.

In fact, Safe and colleagues had proposed selective AHR modulators (SAHRMs) that exhibit various AHR functions in a tissue/organ-specific manner, and reported a compound that inhibited estrogen-dependent growth of MCF-7 cells and suppressed the growth of DMBA-induced mammary tumors in rats without CYP1A1 induction. We have shown in this study that the activation of AHR by known agonists (AHR ligands) results in the inhibition of mammosphere formation, and its efficacy is ligand-dependent. Further studies are needed for understanding the mechanism of AHR-induced suppression of CSC-derived mammosphere formation and for discovering new types of the ligands suitable for the control of various AHR functions.

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Conflict of Interest The authors declare no conflict of interest.

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