An assessment of risks of dioxins for aryl hydrocarbon receptor-mediated effects in polar bear (*Ursus maritimus*) by *in vitro* and *in silico* approaches

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Aim and Procedure
Polar bear (*Ursus maritimus*) populations accumulate dioxins and related compounds (DRCs) at levels that are of health concern. The toxicities of DRCs are primarily mediated via aryl hydrocarbon receptor (AHR) signaling pathway. To estimate the risk of DRCs in polar bears, we initially cloned and sequenced pbAHR complementary DNA (cDNA). We then constructed an *in vitro* reporter gene assay system using a pbAHR expression plasmid and measured the transactivation potencies of DRCs via pbAHR, of which the protein was transiently expressed in U2OS cells. In addition, we built chimeric AHR constructs from pbAHR and C3H/*lpr* mouse AHR (mAHR) and further chimeric AHR constructs with site-directed mutations, and investigated the functional roles of the Per-Arnt-Sim (PAS)-B domain and several amino acid residues in the ligand binding domain (LBD) of pbAHR. To probe the role of specific amino acid residues in pbAHR, protein ligand interaction fingerprint (PLIF) analyses based on *in silico* ligand-receptor docking simulations were performed. Finally, we assessed the risks of DRCs for pbAHR-mediated toxicity from the results of *in vitro* and *in silico* analyses.

Result
In vitro assays showed that the pbAHR was as sensitive to DRCs as C3H/lpr mouse AHR, which is known to be highly sensitive to DRCs. Comparison of pbAHR transactivation potencies indicated that TCDF, 2,3,4,7,8-PeCDF, and BaP exhibited high induction equivalency factors (IEFs). Considering the concentrations of DRCs in polar bears, PCB126 was found to be the most active inducer of pbAHR. The *in vitro* transactivation potencies of ligands of pbAHR showed a significant relationship with *in silico* ligand docking energies in a pbAHR homology model. The protein ligand interaction fingerprint (PLIF) analysis showed different interaction patterns according to ligands. Several amino acids which are highly conserved among mammals, may be involved in species-specific responses via backbone interactions with neighboring amino acid residues which are specific to pbAHR.

Publication/conference presentation
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Perspectives in future
A high correlation between the results of *in silico* pbAHR docking simulations and *in vitro* pbAHR transactivation potencies offers a prospect of high-throughput screening of AHR active compounds in polar bears. Our established approaches thus enable the risk assessment of environmental contaminants in endangered species like polar bears for which *in vivo* toxicity test are ethically, logistically and technically difficult.

In vitro and *in silico* AHR assays for assessing the risk of heavy oil-derived polycyclic aromatic hydrocarbons in fish

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Aim and Procedure
In the aftermath of the Great East Japan Earthquake of March 11, 2011, marine fish in Kesennuma Bay, Japan, have been contaminated with heavy oil containing polycyclic aromatic hydrocarbons (PAHs). The objective of this study was thus to estimate the risk of heavy oil-derived PAHs in fish from Kesennuma Bay. We initially measured PAH concentrations and hepatic EROD and MROD activities as markers of AHR activation in greenlings (*Hexagrammos otakii*) collected from Kesennuma Bay in 2014. We then investigated the AHR transactivation potencies of six PAHs which were detected in the tissue of greenlings from Kesennuma Bay, using the *in vitro* rsAHR-driven reporter gene assay established in our previous study (Bak et al., 2013). The relative effective concentrations of these PAHs that induce 20% of the maximum benzo[α]pyrene (BaP) response ($REC_{20\text{-}BaP}$) or induce 20% of the maximum 2,3,7,8-TCDD response ($REC_{20\text{-}TCDD}$) and their respective REPs, which were compared to the potency of BaP or 2,3,7,8-TCDD, were obtained from the *in vitro* rsAHR responses to the PAHs. We then calculated the total BaP-based induction equivalents (IEQs) of the PAHs in the tissues

of greenlings and compared the geographical trends of the IEQs with those of EROD and MROD activities in the greenlings. In addition, the binding potencies of the six PAHs to each rsAHR were estimated by *in silico* docking simulations. The relationship between the *in vitro* rsAHR transactivation and *in silico* binding potencies was examined and the applicability of an *in silico* approach for screening PAHs as rsAHR ligands was validated.

Result

The results showed that alkylated PAH concentrations and EROD/MROD activities were higher in sites close to the oil-spilled sites than in the control site, suggesting AHR activation by spilled alkylated PAHs. We then investigated AHR-mediated responses to these PAHs in the *in vitro* reporter gene assay system where red seabream (*Pagrus major*) AHR1 (rsAHR1) or rsAHR2 expression plasmids were transiently transfected into COS-7 cells. The *in vitro* assay showed rsAHR isoform-, PAH-, and dose-dependent transactivation potencies. The relative effective concentrations of benzo[α]pyrene, dibenzothiophene, phenanthrene, 2,3,5-trimethylnaphthalene, acenaphthene, and 1-methylphenanthrene that induce 20% of the maximum benzo[α]pyrene response ($REC_{20\text{-}BaP}$) for rsAHR1 activation were 0.052, 38, 70, 88, 270 nM, and no response, respectively, and those for rsAHR2 activation were 0.0049, 32, 53, 88, 60 nM, and no response, respectively. The results showed that the $REC_{20\text{-}BaP}$ values of benzo[α]pyrene for both the rsAHR1 and rsAHR2 isoforms were lower than the concentrations (0.041–0.20 nM) detected in the muscle tissue of fish from Kesennuma Bay, while the $REC_{20\text{-}BaP}$ values of other PAHs were higher than their tissue concentrations. *In silico* rsAHR homology modeling and subsequent ligand docking simulation analyses indicated that the rsAHR activation potencies of PAHs could be predicted from a rsAHR2 model. This study shows that *in vitro* and *in silico* rsAHR analyses may be a useful tool for assessing the risks to fish contaminated with PAHs.

Publication/conference presentation

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Perspectives in future

Our *in vitro* rsAHR transactivation assay supported that PAHs were able to activate fish AHR in a chemical- and concentration-dependent manner. This study also suggests that AHR isoform- and species-specific transactivation potencies could be a critical toxicological issue for the further refinement of the risk assessment of PAHs in fish. This study provides an application of *in vitro* and *in silico* fish AHR ligand screening systems, in particular for PAHs. Further evaluation of the contribution of alkylated PAHs to AHR activation is necessary to better assess the risk of heavy oil contamination.