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Article

Identification of Mid-Polar and Polar AhR Agonists in Cetaceans from Korean Coastal Waters: Application of Effect-Directed Analysis with Full-Scan Screening

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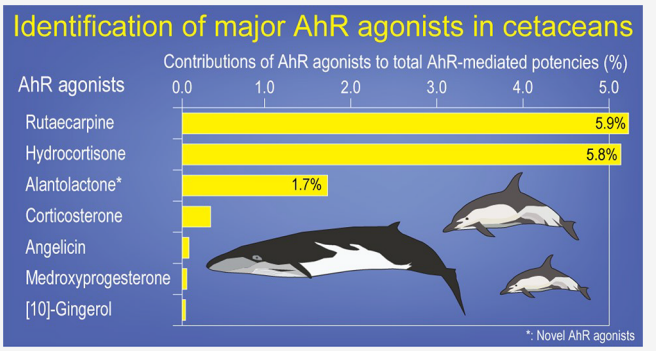
ABSTRACT: Major aryl hydrocarbon receptor (AhR) agonists were identified in extracts of blubber, liver, and muscle from six long-beaked common dolphins (*Delphinus capensis*) and one fin whale (*Balaenoptera physalus*) collected from Korean coastal waters using effect-directed analysis. Results of the H4IIE-*luc* bioassay indicated that the polar fractions of blubber and liver extracts from the fin whale exhibited relatively high AhR-mediated potencies. Based on full-scan screening with high-resolution mass spectrometry, 37 AhR agonist candidates, spanning four use categories: pharmaceuticals, pesticides, cosmetics, and natural products, were selected. Among these, five polar AhR agonists were newly identified through toxicological confirmation. Concentrations of polar AhR agonists in cetaceans were tissue-specific, with extracts of blubber and liver containing greater concentrations than muscle extracts. Polar AhR agonists with great log K_{OA} values (>5) were found to biomagnify in the marine food chain potentially. Polar AhR agonists contributed 8.9% of the observed AhR-mediated potencies in blubber and 49% in liver. Rutaecarpine and alantolactone contributed significantly to the total AhR-mediated potencies of blubber, whereas hydrocortisone was a major AhR contributor in the liver of the fin whale. This study is the first to identify the tissue-specific accumulation of polar AhR agonists in blubber and liver extracts of cetaceans.



KEYWORDS: *cetaceans, aryl hydrocarbon receptor-mediated activity, H4IIE-luc transactivation bioassay, biomagnification, full-scan screening, marine mammals, hierarchical clustering analysis*

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INTRODUCTION

Persistent toxic substances can be transferred to top predators along the food chain and can accumulate in great concentrations in marine mammals. The biomagnification tendencies of dietary chemicals accumulated in aquatic species mainly depend on polarity as represented by the logarithm of the octanol−water (log K_{OW}) and octanol-air partition coefficient (log K_{OA}) of the chemicals.[1] In the marine-mammalian food web, which includes air-breathing organisms, chemicals with lesser log K_{OW} values (2.0−5.0) could be biomagnified due to greater log K_{OA} values (>5.0).[2] This is because compounds with greater K_{OA} values are less volatile and tend to partition into lipid-rich substances instead of being released into the atmosphere.[3] A recent report identified several polar aryl hydrocarbon receptor (AhR) agonists, such as canrenone, mepanipyrim, medroxyprogesterone, rutaecarpine, genistein, tretinoin, etofenprox, [10]-gingerol, and eupatilin, in livers of black-tailed gulls from South Korea.[4] These rater polar compounds demonstrated significant biomagnification potential in the marine food webs.[4]

The AhR is a transcription factor that can be activated by various ligands.[5] It plays a role in regulating diverse biological processes, including xenobiotic metabolism, immune function, developmental toxicity, and cancer induction, by promoting the expression of target genes upon binding to specific DNA sequences in the nucleus.[6] Initially located in the cytoplasm, the AhR forms a complex with heat shock proteins and translocates to the nucleus after binding to a ligand.[6] Traditionally, it has been known that AhR-mediated activity is mainly caused by mid-polar and non-polar compounds, such as dioxins, furans, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). In addition, recent studies have identified novel polar AhR ligands that accumulate

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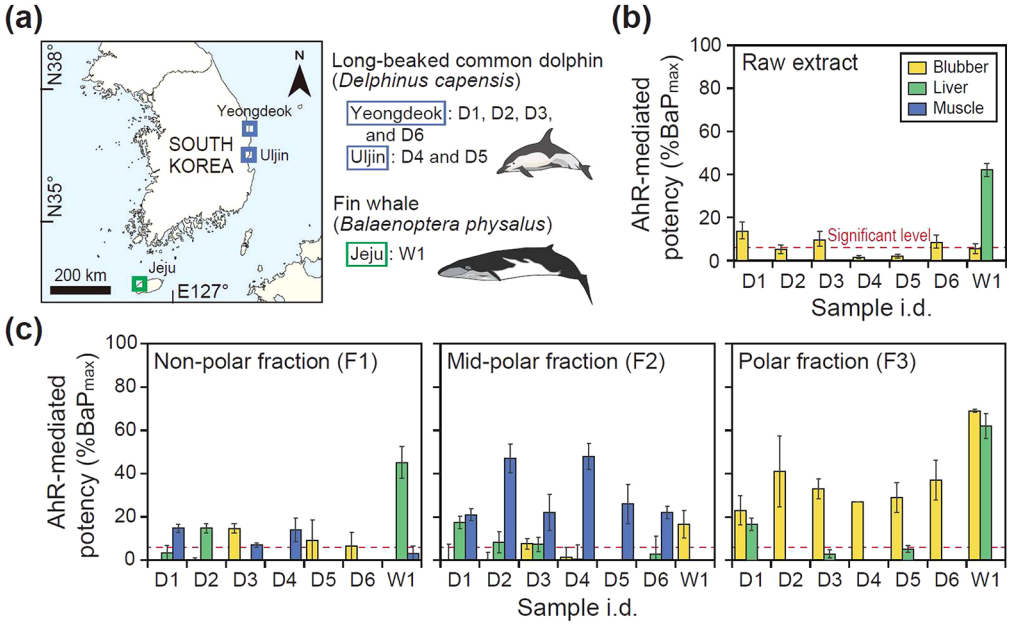

Figure 1. (a) Map showing the sampling sites of six long-beaked common dolphins (*Delphinus capensis*) and one fin whale (*Balaenoptera physalus*) from Korean coastal waters. Long-beaked common dolphins and a fin whale were collected in 2012 and 2019, respectively. Blubber, liver, and muscle tissues were used for the effect-directed analysis. (b) AhR-mediated potencies in raw organic extracts. Red lines represent the significant level (7.1%) of AhR-mediated potencies measured in this study. The significant level (%) was calculated by dividing the standard deviation of the RLU value of the solvent control group by the RLU value of the maximum concentration of the positive control group. (c) AhR-mediated potencies in silica gel fractions of blubber, liver, and muscle extracts from long-beaked common dolphins and a fin whale (error bar: mean ± standard deviation (SD); n = 3).

in aquatic environments.[4,5,7,8] Polar compounds are known to have increased bioavailability as they bind to membrane transport proteins and are transported into cells.[5]

Effect-directed analysis (EDA) is a tool that combines the use of fractionations based on physicochemical properties with bioassays and instrumental analysis to identify bioactive constituents in environmental samples.[9] Effect-based monitoring involves the use of cell-based and whole-organism bioassays to evaluate the toxic potencies and efficacies of samples.[10] To simplify the complexity of environmental samples, fractionations are employed to separate compounds based on their chemical properties.[11] Bioassays are then used to identify those fractions that exhibit the greatest response or the most potency.[4] Bioanalytical equivalent concentrations (BEQ_{chem}) are determined by combining the concentrations and relative potencies (RePs) of individual chemicals in the samples.[4,12] By comparing the BEQ_{chem} and bioassay-derived BEQ_{bio} for the same sample, the contribution of specific chemicals to observed biological effects can be quantified.[10] It should be noted that targeted chemicals often only account for small portions of the overall potency determined in the bioassay, where many more unmeasured chemicals likely contribute to the observed effects.[10]

Advanced EDA, EDA combined with full-scan screening analysis (FSA), has been developed and applied to identify biologically active substances that have not been previously monitored in environmental samples. FSA, utilizing high-resolution mass spectrometry (HRMS), can provide information about all substances in environmental samples.[13] HRMS analysis is conducted on fractions exhibiting greater responses, and candidates are identified by matching compounds using chemical library searching software.[14,15] Despite library matching, environmental samples might still contain numerous compounds that have not been detected or identified.[16,17] Thus, causative agents are identified through a series of selection criteria, and novel toxicants can be confirmed through chemical and toxicological confirmation.[16,17]

The long-beaked common dolphin (*Delphinus capensis*) is primarily found within 180 km of coastal areas and feeds on a variety of fish and squids.[18] The fin whale (*Balaenoptera physalus*) inhabits oceans ranging from polar to tropical regions and primarily feeds on small fish, squid, and crustaceans.[19] Top predators, such as dolphins and whales, possess characteristics including wide mobility, diverse prey sources, low metabolic activity, and great lipid content.[20,21] These unique features make them valuable as sentinels for biomonitoring environmental pollutants in aquatic environments.[20,21] Long-term exposure to various environmental pollutants can have adverse effects on the health of top predators and contribute to increased environmental stress in the marine ecosystem.[4] However, only a limited number of studies have investigated the biological effects of environmental pollutants and the presence of unknown toxicants in cetaceans.

The purpose of this study was to apply EDA to identify major AhR agonists in organic extracts of blubber, liver, and muscle from six long-beaked common dolphins (D1−D6) and one fin whale (W1) collected from Korean coastal waters (Figure 1). In particular, we focused on the mid-polar and polar fractions, which exhibited significant AhR-mediated activities in cetacean tissues. The flowchart showing the experimental design of this study is presented in Figure S1 of the Supporting Information. The specific objectives were to (1) investigate the contamination levels of persistent organic pollutants (POPs) in cetaceans, (2) measure the AhR-mediated potencies using H4IIE-*luc* bioassays, (3) conduct FSA for more toxic fractions and select AhR agonist

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candidates, (4) identify novel AhR agonists, (5) determine the contributions of the identified AhR agonists to the total AhR-mediated potencies, and (6) evaluate additional toxicities of candidate compounds using in silico modeling and the EPA ToxCast database.

MATERIALS AND METHODS

Sample Collection and Preparation. D1−D3 and D6 were collected in Yeongdeok, and D4 and D5 were collected in Uljin in 2012, respectively. Additionally, W1 was collected in Jeju, South Korea in 2019 (Figure 1a). All cetacean carcasses were entangled in fishing nets. After collecting the cetaceans, the specimens were transported to the Cetacean Research Institute in Ulsan City, South Korea. During the dissection process, information on sex, body length, and growth stage was recorded (Table S1). Samples of blubber, liver, and muscle were dissected from the bodies and stored at −20 °C until analysis. Lipid contents, except for blubber, could not be obtained due to limitations of the sample amount. In the present study, lipid content in blubber samples was measured to evaluate the concentrations of POPs in cetaceans and to compare them with previous studies. This is because blubber samples serve as representative tissue for monitoring POPs.[22] Wet samples were homogenized by adding anhydrous sodium sulfate. Two grams of blubber, 5 g of liver, and 10 g of muscle samples [wet mass (wm)] were used for organic extraction. Raw organic extracts (REs) were obtained by Soxhlet extraction using 400 mL of 25% hexane (Honeywell, Charlotte, NC) in methylene chloride (J.T. Baker, Phillipsburg, NJ) for 16 h. The blubber extract was concentrated to 11 mL, and then, a 1 mL sample was collected to measure the lipid content using the gravimetric method. Subsequently, the lipid fraction was removed from the extract using gel permeation chromatography columns packed with Bio-Beads S-X3 (Bio-Rad Laboratories, Hercules, CA).

POPs Analysis. Concentrations of POPs in cetaceans were measured only on blubber samples. This data provides information on the contamination of anthropogenic compounds in cetacean samples used in this EDA study. The analysis of POPs has followed the methods of previous studies,[23,24] and details are presented in the Supporting Information (Tables S2 and S3).

Silica Gel Column Fractionation. Of the 2 mL of REs in the blubber (1.0 g wm mL^{-1}), liver (2.5 g wm mL^{-1}), and muscle samples (5.0 g wm mL^{-1}), 1.5 mL was fractionated using silica gel column chromatography (70−230 mesh; Sigma-Aldrich, Saint Louis, MO). The remaining 0.5 mL of REs was substituted with dimethyl sulfoxide (DMSO; Sigma-Aldrich) for bioassays. More details about the fractionation are presented in the Supporting Information.

H4IIE-*luc* In Vitro Bioassays. The AhR-mediated potencies of REs and silica gel fractions from cetacean samples were determined by using the H4IIE-*luc* transactivation bioassay. The detailed methodology for the in vitro bioassay has been described elsewhere.[25] All dosed samples, including positive control, solvent control, negative control, and test samples, were prepared with a 0.1% concentration of DMSO. To minimize the effect of cytotoxicity, the MTT assay was used to determine the noncytotoxic dose prior to performing the AhR-mediated activity assay (>80% cell viability). Consequently, cell exposure concentrations were set at 10 g of wm mL^{-1} for blubber, 2.5 g of wm mL^{-1} for liver, and 50 g of wm mL^{-1} for muscle. The positive control used was benzo[*a*]pyrene (BaP), which specifically targets mid-polar and polar compounds that are readily metabolized in the H4IIE-*luc* bioassay after a 4 h exposure. The reliability, reproducibility, and sensitivity of the H4IIE-*luc* bioassay were evaluated by confirming the dose−response relationship of the positive control (BaP), which showed consistency with previous studies (Table S4).[4,7] Luminescence activities were measured by using a Victor multilabel plate reader (PerkinElmer, Waltham, MA). The luminescence values obtained from all dosed samples are expressed in relative luminescence units (RLU). The RLU values for the test samples were calculated as a percentage of the RLU values obtained from the maximum concentration of BaP. The concentrations of BaP-equivalent bioactivity (BaP-EQ_{bio}) were obtained from dose−response relationships [based on effective concentrations (ECs) at the 20% level] of samples with BaP maximum (BaP_{max}) values of 20% or greater (Figure S2).

Full-Scan Screening Analysis and Data Processing. FSA was conducted on the polar fractions of blubber and liver extracts from W1 using high-performance liquid chromatography (HPLC, 1290 infinity system, Agilent Technologies, Santa Clara, CA) interfaced to a quadrupole time-of-flight mass spectrometer (QTOFMS 5600 system, AB Sciex, Framingham, MA). The mass scan type of the FSA used the information-dependent acquisition (IDA) mode. The detailed instrumental conditions for LC-QTOFMS are presented in Table S5. AhR agonist candidates were selected through a five-step process.[4,8,26] The information about the selected candidates is presented in Table S6. Among these, standard materials for 11 AhR agonist candidates were commercially available. Hydroxygenkwanin, alantolactone, isoliensinine, norbuprenorphine, norfloxacin, strychnine, and thiothixene were purchased from Sigma-Aldrich. Raloxifene, peimisine, and donepezil were obtained from Toronto Research Chemicals (Toronto, ON, Canada). Fluphenazine was purchased from Alfa Chemistry (Ronkonkoma, NY).

Toxicological and Chemical Confirmation. Detailed methodology of toxicological and chemical confirmation for AhR agonist candidates has been described elsewhere.[4] Standards for individual AhR agonist candidates were prepared at six concentrations (1000, 333, 111, 37, 12, and 4.1 $\mu g\ mL^{-1}$) using a 3-fold serial dilution. The RePs of the AhR agonists were calculated based on ECs at the 20% level observed in comparison to BaP. For the chemical confirmation of the five novel AhR agonists, an HPLC (1290 Infinity II, Agilent Technologies) coupled with a triple quadrupole mass spectrometer (6470 MS/MS system, Agilent Technologies) was used. Five novel AhR agonists were separated using a ZORBAX Eclipse XDB-C18 column (150 mm, 2.1 mm inner diameter, and 5 μm particle size). The mobile phase consisted of (A) 0.1% formic acid and 10 mM ammonium formate in water and (B) 0.1% formic acid in acetonitrile. Detailed instrumental conditions are presented in Table S7. Optimization information for the analysis of the novel polar AhR agonists is provided in Table S8.

Target AhR Agonists Analysis. Target AhR agonists, including 15 traditional-PAHs (t-PAHs), 11 emerging-PAHs (e-PAHs), and 10 styrene oligomers (SOs) were quantified using Agilent 7890B gas chromatograph coupled with 5977 mass selective detector (Agilent Technologies) (Table S9). In addition, 19 polar AhR agonists were quantified using HPLC-MS/MS. Detailed information on these compounds has been described in previous studies.[4,7,9,13,25,27] ReP values for the

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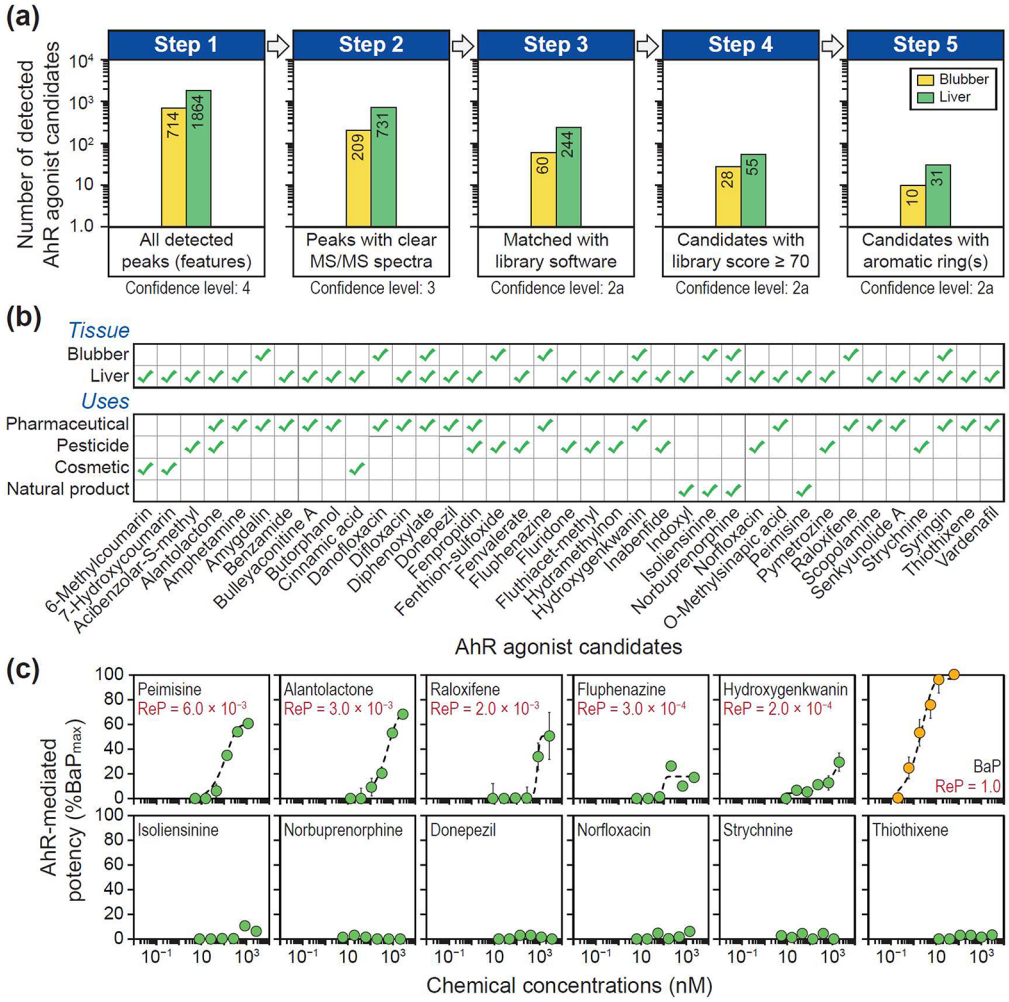

Figure 2. (a) The stepwise approach used for LC-QTOFMS data analysis to identify AhR agonist candidates in polar fractions of blubber and liver extracts from a fin whale. A five-step selection criterion was applied. The confidence level indicates the certainty or probability of identifying a specific chemical as determined by high-resolution mass spectrometry.[33] (b) Detected tissues and known uses of 37 AhR agonist candidates. Detailed information about the uses of the compounds is presented in Table S6. (c) Dose−response curves for the AhR-mediated potencies of 11 AhR agonist candidates with six concentrations (1000, 333, 111, 37, 12, and 4.1 μg mL^{-1}) and benzo[*a*]pyrene in the H4IIE-*luc* bioassays (Error bar: mean ± SD; n = 3).

AhR activity of individual target AhR agonists are presented in Table S10. Method detection limits (MDL) were calculated at standard deviation ×3.707 of the lowest calibration standards. MDL of targeted AhR agonists are presented in Table S11.

Statistical Analysis and Potency Balance Analysis. Hierarchical clustering analysis (HCA) was conducted using IBM SPSS Statistics 26 (Armonk, NY) to verify the distribution pattern of the polar AhR agonists. To enable a comparison of the concentrations of polar AhR agonists in the samples, concentrations were converted to logarithmic values. Concentrations of AhR agonists less than the MDL were analyzed statistically using MDL/2. Principal component analysis (PCA) was performed using IBM SPSS Statistics 26 to identify the specific results of the bioassay and chemical analysis in different types of tissue extracts of D1−D6 and W1.

The contribution of individual AhR agonists to the AhR-mediated potencies in biota extracts was evaluated by comparing BaP-EQ$_{chem}$ and BaP-EQ$_{bio}$. The concentrations of BaP-EQ$_{chem}$ were determined through the multiplication of chemical concentrations and their assay-specific ReP values (eq 1)

$$\text{BaP-EQ}_{chem} = \sum [(\text{concentrations of AhR agonist}_i)\text{ReP}_i] \quad (1)$$

Prediction of Additional Toxicities of AhR Agonist Candidates. VirtualToxLab in silico modeling, the EPA ToxCast database, and VEGA quantitative structure−activity relationships (QSARs) were utilized to predict the potential toxicity of 37 AhR agonist candidates. Detailed methods are presented in the Supporting Information.

RESULTS AND DISCUSSION

Concentrations of POPs in the Blubber of Cetaceans. The concentrations of POPs were relatively greater in the blubber of D1−D6 compared to that of W1 (Table S2). This result can be attributed to differences in habitat, diet, and trophic levels of the two species, which is consistent with findings from previous studies.[18,23,28] Due to their persistence, toxicity, and bioaccumulation characteristics, dichlorodiphenyltrichloroethanes and PCBs, which have been regulated for decades, continue to pose environmental threats. Consequently, these compounds are still detected in marine

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mammals. Concentrations of POPs in organic extracts of blubber were compared with those of other cetaceans reported previously (Table S12).[22,23,29−32] Compared with those found in dolphins from other regions, concentrations of POPs in blubber samples from long-beaked common dolphins in this study, excluding polybrominated diphenyl ethers and hexachlorobenzene (HCB), were relatively small. Similarly, concentrations of POPs in the blubber of a fin whale were relatively small or similar to other samples of whales, except for hexachlorocyclohexanes and HCB. These differences can be attributed to variations in the sex and growth stage of cetaceans as well as differences in sources and geographic characteristics of released POPs among countries. Although the concentrations of POPs analyzed in this study and previous studies were not calculated as the sum of the same congeners, the concentrations of POPs in cetaceans in this study were not great (Table S12). However, it is important to consider the possibility that other unmonitored toxic substances accumulate in the cetacean samples.

AhR-Mediated Potencies in Cetaceans. When AhR-mediated potencies were measured using the H4IIE-*luc* bioassay in the REs of D1−D6 and W1, significant AhR-mediated potencies were observed in blubber from D1, D3, and D6, and liver of W1 (BaP_{max} > 7.1%; Figure 1b). It should be noted that the complexity of various compounds present in the REs of organisms can inhibit AhR binding activity.[4] As a result, AhR-mediated potencies might appear slightly less in the REs compared with the potency of fraction samples. In F1 (non-polar fraction), AhR-mediated potencies were relatively less than other fractions, except for the extract of liver of W1 (Figure 1c). AhR-mediated potencies in F2 (mid-polar) were relatively great in extracts of muscle from D1−D6 (mean = 31%). In F3 (polar), AhR-mediated potencies were relatively great in extracts of blubber from D1−D6 and W1 and in the extract of liver from W1. These results suggest that AhR-mediated activities with environmental pollutants occur in various tissues of cetaceans. Meanwhile, this study evaluated the AhR-mediated potencies in cetaceans using H4IIE-*luc*, rat hepatoma cells, and further research is needed on cetacean-specific AhR response profiles and ligand interactions.

Identification of Novel Polar AhR Agonists. The main focus of this study was on the mid-polar and polar fractions, which exhibited relatively great AhR-mediated activities in cetacean tissues. Relatively greater AhR-mediated potencies were observed in the polar fractions of blubber and liver of W1. AhR agonist candidates were identified through selection criteria consisting of five steps (Figure 2a). In the first step, as a result of FSA using IDA mode, a total of 714 and 1864 compounds were detected in extracts of blubber and liver, respectively (confidence level: 4).[4,33] The second step involved selecting compounds with clear MS/MS spectra,[8] resulting in narrowing down to 209 and 731 compounds in extracts of blubber and liver, respectively (confidence level: 3). In the third step, 60 compounds in blubber and 244 compounds in liver were matched with library software (AB Sciex, Framingham, MA).[4] These library-matched compounds meet the minimum data requirements for confidence level 2a, including MS, MSMS, and library MSMS data.[33] However, the library spectrum of the compounds can occasionally be inaccurate. Thus, it is important to exercise caution when comparing spectra captured under differing acquisition parameters, such as resolution, collision energy, ionization, and MS level.[33] In the fourth step, 28 and 55 compounds in blubber and liver, respectively, were selected based on a library score ≥ 70.[4,7] The selection of using a score of 70 or higher was commonly used in this field and has been shown to be effective in accurately aligning library spectra with compounds.[34−36] The fifth step involved identifying 10 aromatics in blubber and 31 aromatics in liver.[26]

As a result, 41 AhR agonist candidates were selected from blubber and liver, with 4 compounds being identified in common (Figure 2b and Table S6). These AhR agonist candidates were classified into four usage categories: pharmaceuticals, pesticides, cosmetics, and natural products, with pharmaceuticals and pesticides being the most abundant. Toxicological confirmation was performed on 11 commercially available compounds (Figure 2c). Based on the results of the H4IIE-*luc* bioassay for AhR binding efficacy, peimisine, alantolactone, raloxifene, fluphenazine, and hydroxygenkwanin were identified as novel AhR agonists. Meanwhile, using the sequential window acquisition of all theoretical fragment-ion spectra (SWATH) mode in FSA for environmental samples could obtain more abundant fragment ions than using IDA mode;[37] thus, it can be considered in the follow-up study.

Mid-Polar AhR Agonists in Cetaceans. Mid-polar AhR agonists, such as t-PAHs, e-PAHs, and SOs, were detected in all extracts of D1−D6 and W1 (Tables S11, S13, and S14). In cetacean samples, low molecular mass PAHs (2−3 rings) were found to be more prevalent compared with higher molecular mass PAHs (4−6 rings). Concentrations of e-PAHs were greater in extracts of blubber and liver from D1−D6 and W1 compared to those from muscle. Among the detected e-PAHs, the mean concentration of 11H-benzo[*a*]fluorene (11BaF) was the greatest. The concentration of SOs was approximately 20−50 times greater in extracts of blubber, 3−8 times greater in liver, and 4−13 times greater in muscle compared to AhR-active PAHs. The liver of W1 exhibited relatively high concentrations of SOs (880 ng g^{-1} wm) compared to other samples. Although SOs are widely distributed in the coastal environment of South Korea,[38,39] few studies have reported their bioaccumulation in marine organisms. Further studies are imperative to understand the exposure pathway, bioaccumulation, biomagnification, and potential risk of SOs in cetaceans. Concentrations of mid-polar AhR agonists did not exhibit a significant correlation with the sampling sites, growth stage, and lipid content in blubbers, but they exhibited a significant negative correlation with body length (Figure S3). The pattern of accumulation of PAHs in long-beaked common dolphins observed in this study, where concentrations decreased with increasing body length, is similar to that found in Indo-Pacific humpback dolphins from the Pearl River Estuary.[40] Concentrations of mid-polar AhR agonists in long-beaked common dolphins were inversely proportional to body length, which indicated processes such as biodilution and biotransformation.[18,40] Concentrations of mid-polar AhR agonists in the fin whale were comparable to or greater than those in long-beaked common dolphins, showing a slightly different trend compared to the pattern of distributions of POPs observed in this study. This difference might be attributed to interspecific and sexual differences in metabolic activity and the effects of biodilution on POPs and PAHs.[18] Additionally, variations in feeding habits and environmental conditions could also contribute to these differences.[18,19]

Polar AhR Agonists in Cetaceans. Nineteen polar AhR agonists were detected in all extracts from D1−D6 and W1 except for protopine (Tables S11, S13, and S14). Concen-

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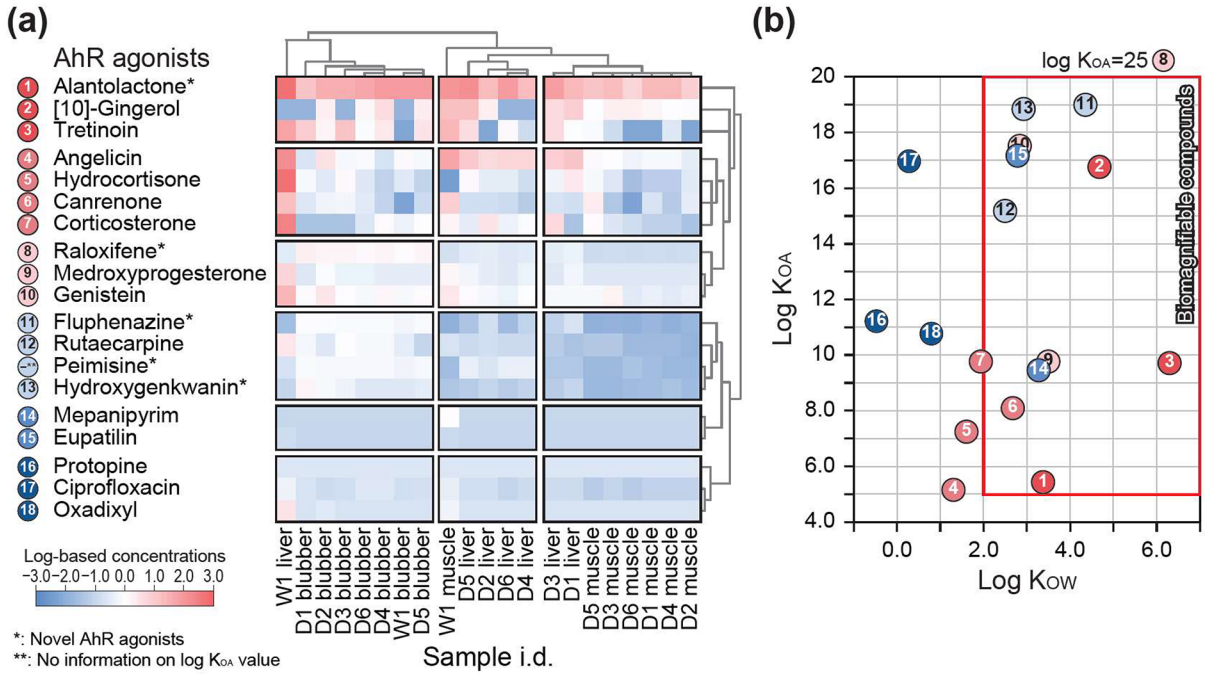

Figure 3. (a) Hierarchical clustering analysis of polar AhR agonists in the polar fraction of blubber, liver, and muscle extracts from long-beaked common dolphins and a fin whale. Concentrations of detected compounds, represented on a logarithmic scale, were visualized as heat map. (b) Evaluation of biomagnification potentials for detected polar AhR agonists in the samples. Compounds with a log K_{OA} greater than 5.0 and a log K_{OW} greater than 2.0 are considered to have a biomagnification potential (red box). Chemical space map for polar AhR agonists based on partition coefficients (log K_{OA}, log K_{AW}, and log K_{OW} values were obtained from ChemSpider).[44]

trations of polar AhR agonists varied among the six long-beaked common dolphins, with different concentrations for each individual. However, no significant associations were observed between concentrations of these compounds and environmental and biological factors, such as sampling sites, body length, growth stage, or lipid content in blubber (Figure S3). Samples were classified into three clusters based on their tissue-specific characteristics (Figure 3a). For instance, blubber functions as a storage site for lipids,[22] while the liver is involved in lipid metabolism and the maintenance of lipid homeostasis.[41] In contrast, muscle tissue is primarily composed of protein fibers and contains relatively lesser lipid contents.[42] Notably, it is acknowledged that the accumulation of compounds in marine mammals is influenced by the lipid content.[43] The evident grouping of tissue samples seems to align with the concentration of polar AhR agonists, suggesting an association with the tissue lipid content. The first cluster consisted of blubber from all cetaceans and the liver of W1. The second cluster included muscle from W1 and liver from D2, D4, D5, and D6. The third cluster contained liver from D1 and D3 and muscle from D1−D6.

The polar AhR agonists were divided into six clusters based on their concentrations in samples. The first cluster included alantolactone, [10]-gingerol, and tretinoin, which showed relatively greater concentrations than the other compounds. Alantolactone, an anti-inflammatory agent and nematicide,[45−47] was detected at the greatest concentrations in samples. [10]-Gingerol and tretinoin are used as anti-inflammatory agents[48] and a neoplastic agent,[49] respectively. These compounds were identified as biomagnifiable compounds in air-breathing organisms (Figure 3b).[44] The second cluster included angelicin, hydrocortisone, canrenone, and corticosterone. Angelicin is used as an anticonvulsant.[50] Hydrocortisone is used as an anti-inflammatory agent and is also a glucocorticoid hormone.[51] Canrenone, used as a diuretic,[52] was widely detected in cetacean samples. Corticosterone is a representative glucocorticoid hormone in vertebrates.[53] Glucocorticoid hormones, such as hydrocortisone and corticosterone, are crucial for the stress response and osmotic pressure regulation in vertebrates.[54,55] These are released by the adrenal gland in response to these reactions and accumulate in various tissues and organs.[54,55] Hydrocortisone can partition into organic phases, such as sediments and suspended soils due to its hydrophobic properties,[7] potentially leading to its accumulation in the organisms. Although hydrocortisone and corticosterone had greater log K_{OA} values (>5.0), they did not meet the criteria for biomagnification potential due to their log K_{OW} values being lower than 2.0. Angelicin had comparatively low log K_{OA} and log K_{OW} values and did not have a biomagnification potential. The third cluster consisted of raloxifene, medroxyprogesterone, and genistein. Raloxifene is used as a bone density conservation agent,[56] and the concentration of blubber extract was relatively great compared to other samples, which is likely influenced by its great log K_{OW} value of 6.1. Medroxyprogesterone and genistein are used as uterine cancer agent[57] and anticancer agent,[58] respectively. The fourth cluster included fluphenazine, rutaecarpine, peimisine, and hydroxygenkwanin. Fluphenazine, an antipsychotic agent,[59] was frequently detected in aquatic environments across Europe.[60] Peimisine is produced by Fritillaria inhabiting the temperate region of the Northern Hemisphere.[61] Rutaecarpine is known as a herbal medicine.[62] Hydroxygenkwanin is used as an anti-inflammatory agent that can treat edema, ascites, cough, asthma, and cancer.[63] These compounds were found to exhibit greater levels of concentration in blubber extracts compared with other

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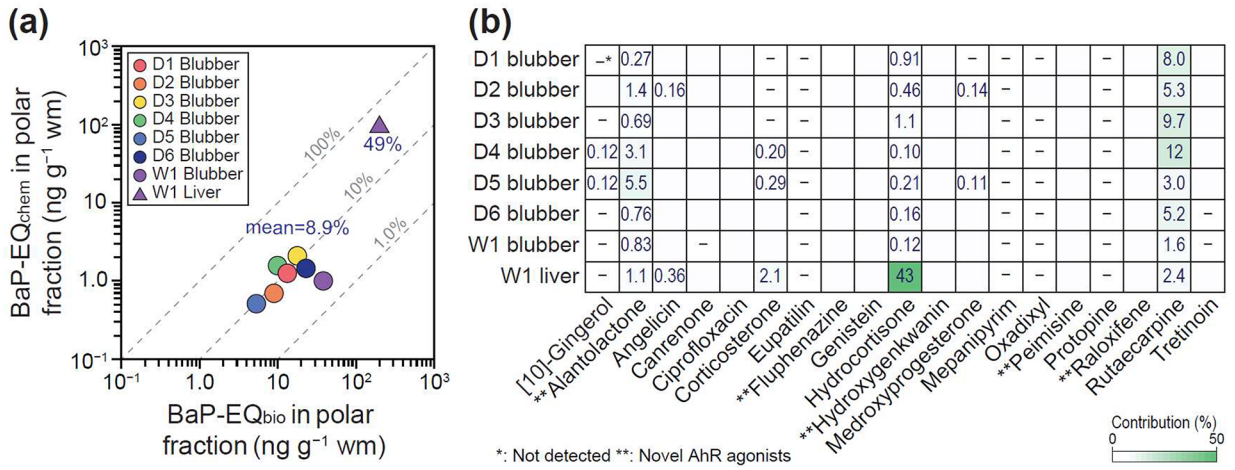

Figure 4. (a) Comparison of instrument-derived BaP-EQ$_{chem}$ and bioassay-derived BaP-EQ$_{bio}$ concentrations in polar fractions of organic extracts of long-beaked common dolphins and fin whale. Potency balance analysis in polar fractions was performed only for samples with a BaP$_{max}$ value of 20% or more. (b) The numbers represent the contribution percentage of AhR agonists to total induced AhR-mediated potencies (-: below detection limits; blank: < 0.1%).

Table 1. Potency Balance between BaP-EQ$_{chem}$ and BaP-EQ$_{bio}$ Concentrations in Polar Fractions of Blubber and Liver Extracts from Long-Beaked Common Dolphins and a Fin Whale in Korean Coastal Waters

Compounds	Blubber							Liver
	D1	D2	D3	D4	D5	D6	W1	W1
Known polar AhR agonists (BaP-EQ$_{chem}$, ng g^{-1} wm)								
[10]-Gingerol	ND[a]	1.0×10^{-2}	ND	1.0×10^{-2}	1.0×10^{-2}	ND	ND	ND
Angelicin	1.0×10^{-3}	1.0×10^{-2}	2.0×10^{-3}	1.0×10^{-3}	2.0×10^{-3}	3.0×10^{-3}	1.0×10^{-2}	7.3×10^{-1}
Canrenone	1.0×10^{-3}	2.0×10^{-3}	3.0×10^{-3}	4.0×10^{-4}	1.0×10^{-3}	1.0×10^{-3}	ND	8.0×10^{-2}
Ciprofloxacin	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	2.0×10^{-3}
Corticosterone	ND	ND	ND	2.0×10^{-2}	2.0×10^{-2}	4.0×10^{-3}	1.0×10^{-2}	4.4
Etofenprox	ND	ND	ND	ND	ND	ND	ND	ND
Eupatilin	ND	ND	ND	ND	ND	ND	ND	ND
Genistein	1.0×10^{-3}	3.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	2.0×10^{-2}
Hydrocortisone	1.2×10^{-1}	4.0×10^{-2}	1.9×10^{-1}	1.0×10^{-2}	1.0×10^{-2}	4.0×10^{-2}	5.0×10^{-2}	88
Medroxyprogesterone	ND	1.0×10^{-2}	1.0×10^{-2}	1.0×10^{-2}	1.0×10^{-2}	1.0×10^{-2}	1.0×10^{-2}	9.0×10^{-2}
Mepanipyrim	ND	ND	ND	ND	ND	ND	ND	ND
Oxadixyl	ND	1.0×10^{-4}	ND	ND	ND	ND	ND	ND
Protopine	ND	ND	ND	ND	ND	ND	ND	ND
Rutaecarpine	1.1	4.8×10^{-1}	1.8	1.2	1.6×10^{-2}	1.2	0.60	4.8
Tretinoin	4.0×10^{-3}	1.0×10^{-3}	1.0×10^{-2}	1.0×10^{-3}	1.0×10^{-3}	ND	ND	3.0×10^{-2}
Novel polar AhR agonists (BaP-EQ$_{chem}$, ng g^{-1} wm)								
Alantolactone	4.0×10^{-2}	1.3×10^{-1}	1.2×10^{-1}	3.1×10^{-1}	3.0×10^{-1}	1.8×10^{-1}	3.2×10^{-1}	2.2
Fluphenazine	2.0×10^{-4}	2.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	4.0×10^{-6}
Hydroxygenkwanin	3.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	4.0×10^{-5}	1.0×10^{-4}	5.0×10^{-5}	5.0×10^{-5}	2.0×10^{-5}
Peimisine	3.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	1.0×10^{-3}
Raloxifene	2.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	4.0×10^{-4}
BaP-EQ$_{chem}$ (ng g^{-1} wm)[b]	1.3	0.69	2.1	1.6	0.51	1.4	1.0	100
BaP-EQ$_{bio}$ (ng g^{-1} wm)[c]	13	9.1	18	10	5.4	23	39	200
Contribution (%)	9.3	7.6	12	16	9.4	6.2	2.6	49

[a]ND: Not detected. [b]Calculated by multiplying the concentrations of known and novel polar AhR agonists by their ReP values. [c]Obtained from dose−response tests of samples (based on EC$_{20}$).

tissues. Except for peimisine, which lacked information on its log K_{OA} value, all of these compounds were identified as biomagnifiable compounds. Compounds in the fifth and sixth clusters were detected below the MDL or at relatively low concentrations in samples. In the fifth cluster, mepanipyrim known as a fungicide,[64] and eupatilin, known as a natural product,[65] were confirmed to have biomagnification potential. All compounds in the sixth cluster did not meet the criterion of the biomagnification potential. Meanwhile, verification of compound recovery in the polar fraction through spike and recovery tests using standards was not conducted in this study. Further studies might consider evaluating the recovery rates and stabilities of these compounds within the polar fraction. Such validation processes could enhance the reliability of the findings in the present study.

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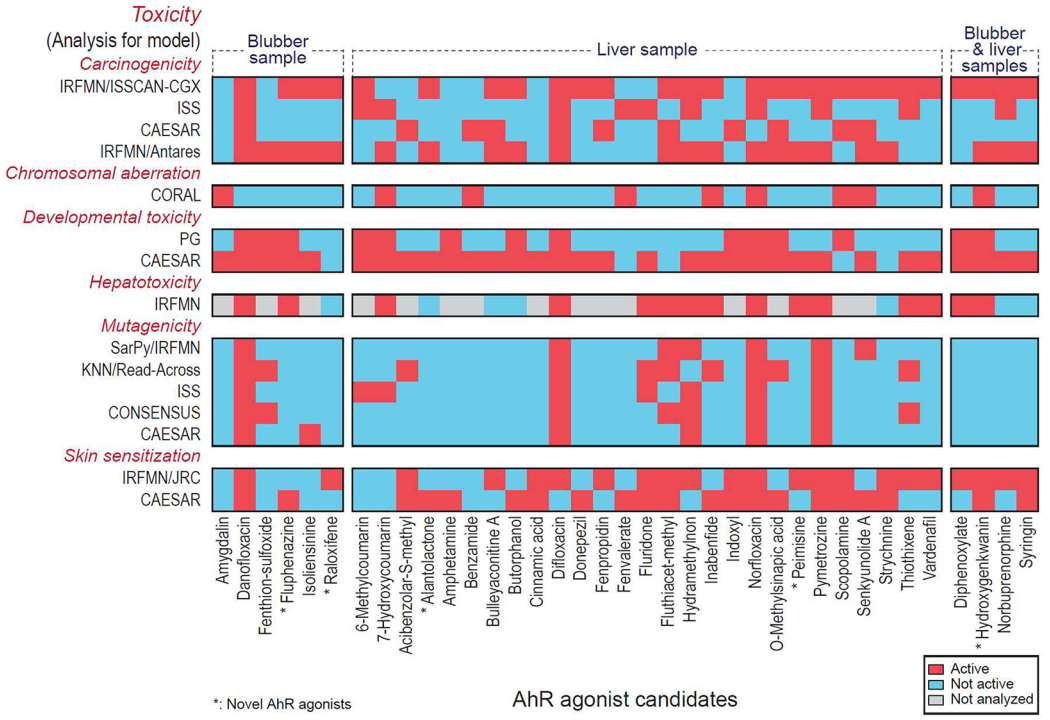

Figure 5. Prediction of additional toxicity of 37 AhR agonist candidates in blubber and liver extracts of a fin whale using VEGA QSARs. A total of 15 models were used for the prediction of toxicities.

Tissue-Specific Distribution of AhR Agonists in Cetaceans. Concentrations of AhR agonists in D1−D6 and W1 were greater in blubber and liver compared to muscle, except for muscle from W1 (Figure S4 and Tables S11, S13, and S14). Blubber is a tissue in which various lipophilic substances accumulate.[66] Livers of marine mammals are known to accumulate and metabolize both endogenous and exogenous ligands.[67] The greater concentrations of AhR agonists in blubber and liver might be attributed to the ability of these tissues to accumulate and store various environmental pollutants that can have a relatively long half-life in the body and are resistant to metabolism and elimination.[68] When PCA was performed based on concentrations of AhR agonists and AhR-mediated potencies in the silica gel fractions of D1−D6 and W1 (Figure S5), it was demonstrated that extracts of tissue could be classified into three distinct groups. The first group mainly included muscle and was associated with AhR activity in F2. This is explained by the relatively great AhR-mediated potencies in F2 of extracts of muscle from D1−D6. The second group mainly included the liver, where polar AhR agonists exhibited a relatively strong correlation, while AhR-active PAHs showed a relatively weak correlation. This pattern can be explained by the greater concentration of environmental pollutants in the livers of cetaceans. The third group mainly consisted of blubber and was correlated to AhR-active SOs and AhR activity in F3. The results of PCA revealed three distinct groups of tissue extracts, each displaying a unique pattern of AhR agonists and AhR-mediated potencies in the samples.

Contribution of AhR Agonists to Total AhR-Mediated Potencies. The target mid-polar AhR agonists accounted for an average of 70% of the total AhR ligand equivalents determined in the bioassay for extracts of muscle from D1−D6 (Figure S6 and Table S15). Among the mid-polar AhR agonists, indeno[*1,2,3-cd*]pyrene, dibenzo[*a,h*]anthracene, and 11BaF contributed relatively great proportions of the total AhR-mediated potencies. In extracts of blubber from D1−D6 and W1, polar AhR agonists accounted for 2.6−16% (mean = 8.9%), while in the liver extract of W1, they accounted for 49% (Figure 4a and Table 1).

The contribution of rutaecarpine and alantolactone to the total AhR-mediated potencies in blubber from D1−D6 and W1 was relatively great (Figure 4b). In the blubber sample of W1, which exhibited the highest AhR-mediated potency, the contributions of rutaecarpine (1.6%), alantolactone (0.83%), and hydrocortisone (0.12%) were relatively high. This is likely due to the accumulation and storage of anthropogenic substances in blubber, which contribute to their potencies. In the liver of W1, hydrocortisone was a major AhR contributor. The contributions of rutaecarpine (2.4%) and corticosterone (2.1%) were confirmed to be relatively high compared to other AhR agonists. Hydrocortisone and corticosterone are known as endogenous substances,[51,53] and their contributions to AhR activity were high. These results emphasize the importance of identifying both exogenous and endogenous AhR ligands in organisms as the role of endogenous AhR ligands in organisms is still not fully understood.

The mid-polar and polar AhR agonists explained approximately half or more of the observed biological effects in the muscles and liver, respectively. However, polar AhR agonists accounted for only a portion of the AhR-mediated potencies in blubber extracts from D1−D6 and W1. This suggests that there might be tissue-specific differences in the accumulation and potency of AhR agonists, possibly due to the unique characteristics of lipid-rich tissues.[69] Additionally, there might be polar AhR agonist candidates that have not been identified in terms of their chemical structure and toxicological confirmation due to the limited availability of standards, which could accumulate in cetaceans. Biomagnification potential was investigated for AhR agonist candidates that were not found to be active and were not analyzed (Figure S7).

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As a result, some polar AhR agonist candidates were identified to have biomagnification potential, indicating their transfer to cetacean species through the food chain.

In this study, AhR-mediated potency was higher in W1 with lower concentrations of POPs compared to D1−D6. In general, differences in POPs concentrations between species could be attributed to differences in habitat, diet, trophic level, body size, and blubber thickness.[70−73] In addition, the concentrations of POPs and AhR agonists in cetaceans showed different accumulation trends. These accumulation patterns are likely influenced by the chemical properties of these compounds, exposure routes, food sources, and metabolic capacity. The higher AhR activity observed in W1 despite lower POPs concentrations compared to those in D1−D6 likely reflects these species-specific bioaccumulation characteristics.

Additional Toxicity Prediction. For the 37 AhR agonist candidates identified in the polar fractions of blubber and liver extracts from W1, an additional toxicity prediction was conducted. Androgenic receptor, AhR, estrogenic receptor, glucocorticoid receptor, and thyroid receptor binding affinity were predicted using VirtualToxLab in silico modeling and the EPA ToxCast database (Figure S8). Fenthion-sulfoxide and fluthiacet-methyl were not available for analysis in VirtualToxLab. Results of the VirtualToxLab analysis indicated that all AhR agonist candidates were predicted to bind to at least one of the hormone receptors except for danofloxacin, benzamide, bulleyaconitine A, strychnine, and vardenafil (Table S16).

While toxicity prediction using EPA ToxCast provided information for only some compounds, fluphenazine, raloxifene, acibenzolar-S-methyl, fluridone, hydramethylnon, and thiothixene could bind to one or more hormone receptors. Furthermore, the VEGA QSAR analysis predicted that all 37 AhR agonist candidates had at least one potential mechanism of toxicity (Figure 5). Among these, inabenfide and norfloxacin were predicted to exhibit activity against all six potential toxicities. Inabenfide is known as an agricultural chemical.[74] Norfloxacin, an anti-infective agent, has been detected in seawater in fish farms in the South Sea of Korea.[75] Aquatic species exposed to norfloxacin have shown responses, such as oxidative stress and impaired digestive function.[76] These AhR agonist candidates were predicted to have various toxicities, suggesting that they could adversely affect the life cycle of marine organisms.

Environmental Implications. This study effectively demonstrates the utility of EDA to identify unmonitored toxicants in marine organisms. While anthropogenic compounds have beneficial uses that improve human quality of life, they can accumulate in various environmental matrices. The accumulation of toxicants in the marine environment raises concerns about their potential ecological hazards. Nonetheless, studies on the presence of these anthropogenic toxic substances in marine mammals are limited. This study provides evidence of novel toxicants in cetaceans, indicating their potential integration into the marine food chain.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c04311.

Supporting methods; sample information (Table S1); concentrations of POPs in blubbers (Table S2); surrogate recovery for POPs analysis (Table S3); quality control of bioassays (Table S4); instrumental conditions for LC-QTOFMS (Table S5); list of aromatic compounds (Table S6); instrumental conditions for HPLC-MS/MS (Table S7); optimization of HPLC-MS/MS analysis (Table S8); instrumental conditions for GC-MSD (Table S9); ReP values for AhR agonists (Table S10); concentrations of target compounds in blubbers (Table S11); comparison of POPs concentrations in cetaceans from the present study and previous studies (Table S12); concentrations of target compounds in livers and muscles (Tables S13 and S14); potency balance in mid-polar fractions of the muscles (Table S15); results of VirtualToxLab analysis (Table S16); flowchart for study design (Figure S1); dose−response relationships for silica gel fractions (Figure S2); comparison of concentrations of AhR agonists in long-beaked common dolphins (Figure S3); concentrations of AhR-active PAHs and SOs and polar AhR agonists (Figure S4); results of the PCA (Figure S5); contribution of mid-polar AhR agonists to total bioassay results in mid-polar fractions (Figure S6); biomagnification potential for AhR agonist candidates (Figure S7); and toxicity predictions of VirtualToxLab and EPA ToxCast (Figure S8) (PDF)

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Notes

The authors declare no competing financial interest.

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