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RESEARCH ARTICLE

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The role of receptor-mediated activities of 4- and 5-ring unsubstituted and methylated polycyclic aromatic hydrocarbons (PAHs) in developmental toxicity

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Abstract

The present study evaluated the aryl hydrocarbon receptor (AhR), estrogen receptor-α (ER-α), and retinoic acid receptor (RAR) mediated activities of nine 4- and 5-ring unsubstituted and monomethylated polycyclic aromatic hydrocarbons (PAHs) using a series of Chemical-Activated LUciferase gene eXpression (CALUX) assays. The potential role of these aforementioned receptors in relation to the developmental toxicity of these PAHs was further assessed in the zebrafish embryotoxicity test (ZET). The results show that all nine tested PAHs were AhR agonists, benz[a]anthracene (BaA) and 8-methyl-benz[a]anthracene (8-MeBaA) were ER-α agonists, and none of the tested PAHs induced ER-α antagonistic or RAR (ant)agonistic activities. In the AhR CALUX assay, all the methylated PAHs showed higher potency (lower EC50) in activating the AhR than their respective unsubstituted PAHs, implying that the addition of a methyl substituent on the aromatic ring of PAHs could enhance their AhR-mediated activities. Co-exposure of zebrafish embryos with each individual PAH and an AhR antagonist (CH223191) counteracted the observed developmental retardations and embryo lethality to a certain extent, except for 8-methyl-benzo[a]pyrene (8-MeBaP). Co-exposure of zebrafish embryos with either of the two estrogenic PAHs (i.e., BaA and 8-MeBaA) and an ER-α antagonist (fulvestrant) neutralized embryo lethality induced by 50 μM BaA and the developmental retardations induced by 15 μM 8-MeBaA. Altogether, our findings suggest that the observed developmental retardations in zebrafish embryos by the PAH tested may partially be AhR- and/or ER-α-mediated, whereas the RAR seems not to be relevant for the PAH-induced developmental toxicity in the ZET.

KEYWORDS
aryl hydrocarbon receptor, developmental toxicity, estrogen receptor-α, methylated polycyclic aromatic hydrocarbons, retinoic acid receptor, zebrafish embryotoxicity test

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1 | INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) form a group of diverse organic compounds with at least three fused benzene rings, containing only carbon and hydrogen atoms (EFSA, 2008; Masood et al., 2017). PAHs are generated in both natural-biological and anthropogenic processes (i.e., pyrogenic and petrogenic sources) (Balmer et al., 2019; Skoczyńska & de Boer, 2019). Pyrogenic substances comprise mainly unsubstituted parent PAHs, whereas petrogenic substances, such as crude oil and its refined products, contain predominantly alkylated PAHs (Saha et al., 2012, 2009; Skoczyńska et al., 2013; Yang et al., 2014).

Some PAHs have been associated with negative health impacts on humans such as prenatal developmental toxicity (Choi et al., 2006; Choi et al., 2008; Perera et al., 2003; Perera et al., 2006; Tang et al., 2006). To date, investigations of the developmental toxicity of PAHs mainly focus on unsubstituted/naked PAHs. For example, prenatal exposure to a 5-ring PAH, benzo[a]pyrene (BaP) resulted in compromised fetal survival, decreased fetal weight, and increased incidence of resorptions in the offspring of pregnant rats (Archibong et al., 2002; Bui et al., 1986). However, there is limited knowledge on the developmental toxicity of the substituted (e.g., alkylated) congeners of PAHs. Available studies reported that methylated congeners of the 3-ring phenanthrene (PHE) induced higher embryotoxicity than PHE itself (Mu et al., 2014; Turcotte et al., 2011) and that methylated benz[a]anthracene (BaA) either increased or decreased the embryotoxicity as compared to the unsubstituted parent BaA (Lin et al., 2015).

Our recent study (Fang et al., 2022) evaluated the in vitro developmental toxicity potency of nine unsubstituted and methylated 4- and 5-ring PAHs using the zebrafish embryotoxicity test (ZET). The ZET data showed that addition of a monomethyl substituent could either increase or decrease the in vitro developmental toxicity of PAHs, depending on the location of the methyl substituent on the aromatic ring of PAH tested. For example, 8-methyl-BaP (8-MeBaP) was more potent than its parent BaP to induce developmental retardation (at nonlethal concentrations) in zebrafish embryos (according to the calculated BMC20s, see Table 1 of Fang et al., 2022), whereas 7-methyl-BaP (7-MeBaP) tested negative for developmental toxicity in the ZET.

To date, the mode of action underlying the developmental toxicity of PAHs remains unclear, although some studies have suggested the involvement of the aryl hydrocarbon receptor (AhR) in mediating such effects (Goodale et al., 2013; Lammer et al., 2009). The AhR is a cytosolic ligand-activated transcription factor (Denison & Nagy, 2003). AhR activation can further activate cytochrome P450 monooxygenases (CYP450), which could subsequently lead to the biotransformation of PAHs into their reactive metabolites (Hawliczek et al., 2012; Puga et al., 2005). The PAH-induced AhR activation has been suggested to contribute to the in vitro developmental toxicity of some individual PAHs (Hawliczek et al., 2012; Huang et al., 2012; Incardona et al., 2006) and PAH-containing petroleum substances (Kamelia et al., 2018; Kamelia et al., 2021; Kamelia, Brugman, et al., 2019; Kamelia, de Haan, et al., 2019). Besides the AhR, the developmental toxicity observed with some PAHs may also relate to their interaction with other nuclear hormone receptors (NHRs), such as the estrogen receptor-α (ER-α) and the retinoic acid receptor (RAR) (Barlow et al., 1999; Maqbool et al., 2016; Piersma et al., 2017; Van Lipzig et al., 2005). Both ER-α and RAR play an important role in vertebrate embryonic development, and disturbance of these receptors may lead to abnormalities in fetus (Bondesson et al., 2015; Lammer et al., 1985; Mark et al., 2009). Several unsubstituted PAHs, for

TABLE 1 Overview of the AhR and ER-α-mediated activities of the nine unsubstituted and methylated PAHs in the CALUX reporter gene assays under study and their in vitro developmental toxicity in the ZET (Fang et al., 2022)

Test compound	AhR CALUX agonist assay (6-h exposure)		AhR CALUX agonist assay (24-h exposure)		ER -α agonist CALUX assay		ZET[a]
	EC50 (μM)	95% confidence intervals	EC50 (μM)	95% confidence intervals	EC50 (μM)	95% confidence intervals	BMC20 (μM)
BaA	0.013 [+++]	0.0037–0.047	1.27 [++]	0.98–1.63	27.00 [+]	21.52–33.88	5.29 [++]
4-MeBaA	0.00081 [+++]	0.00041–0.0016	0.14 [+++]	0.096–0.21	NA [–]		5.34 [++]
8-MeBaA	0.00080 [+++]	0.00038–0.0017	0.17 [+++]	0.12–0.26	11.99 [+]	8.080–17.85	4.81 [++]
9-MeBaA	0.00051 [+++]	0.00025–0.0010	0.0036 [+++]	0.0017–0.0076	NA [–]		15.7 [+]
BaP	0.0046 [+++]	0.0020–0.010	0.78 [+++]	0.53–1.14	NA [–]		4.31 [++]
3-MeBaP	0.0030 [+++]	0.00085–0.011	0.20 [+++]	0.076–0.50	NA [–]		15.1 [+]
7-MeBaP	0.0010 [+++]	0.000523–0.0020	0.15 [+++]	0.11–0.19	NA [–]		NA [–]
8-MeBaP	0.00035 [+++]	0.00016–0.00075	0.048 [+++]	0.032–0.071	NA [–]		0.38 [+++]
DB[a,h]A	0.00070 [+++]	0.00030–0.0016	0.035 [+++]	0.020–0.060	NA [–]		6.71 [++]

Note: NA (not applicable) means the compound induced less than 50% luciferase induction in the AhR or ER-α agonist CALUX assays, or less than 20% reduction of the extended-GMS in the ZET. The EC50 of the AhR and ER-α agonist CALUX assays, and the in vitro developmental toxicity in the ZET (expressed as BMC20) were grouped as follows: + (10 μM < EC50/BMC20 < 50 μM), ++ (1 μM < EC50/BMC20 < 10 μM), +++ (EC50/BMC20 < 1 μM); the negative results were presented by the "-" symbol.

[a]The BMC20 value of each test compound in the ZET was taken from Fang et al. (2022).

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example, BaP and BaA, were reported to induce estrogenic and/or anti-estrogenic activities (Boonen et al., 2020; Fertuck, Matthews, & Zacharewski, 2001; Gozgit et al., 2004; Tran et al., 1996; Van Lipzig et al., 2005). However, data on ER-α- and RAR-mediated activity of especially monomethylated PAHs are limited at present.

Thus, the present study aims to evaluate the AhR-, ER-α-, and RAR-mediated activities of a series of unsubstituted and monomethylated PAHs, using reporter gene assays, all in relation to the developmental toxicity potency of these PAHs in the ZET. To that purpose, nine 4- and 5-ring PAHs (Figure 1; including BaA and its methylated congeners: 4-methyl-BaA (4-MeBaA), 8-methyl-BaA (8-MeBaA), 9-methyl-BaA (9-MeBaA); BaP and its methylated congeners: 3-methyl-BaP (3-MeBaP), 7-MeBaP, 8-MeBaP; and dibenz[a,h]anthracene (DB[a,h]A)) were tested in the ER-α, RAR, and AhR Chemical-Activated LUciferase gene eXpression (CALUX) reporter gene assays. Moreover, some PAHs were selected and tested in the ZET in the absence or presence of specific receptor antagonists, to further investigate the role of these receptors in mediating PAH-induced developmental toxicity.

2 | MATERIALS AND METHODS

2.1 | Test compound

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, CAS No. 1746-01-6, analytical standard grade), 17β-estradiol (E2, CAS No. 50-28-2, purity ≥98%), fulvestrant (CAS No. 129453-61-8, purity ≥98%), retinoic acid (RA, CAS No. 302-79-4, purity ≥98%), AGN 193109 (CAS No. 171746-21-7, purity ≥98%), CH223191 (CAS No. 301326-22-7, purity ≥98%), 3,4-dichloroaniline (CAS No. 95–76-1, purity 98%), benzo[a]pyrene (BaP, CAS No. 50-32-8, purity ≥96%), benz[a]anthracene (BaA, CAS No. 56-55-3, purity 99%), dibenz[a,h]anthracene (DB[a,h]A, CAS No. 53-70-3, analytical standard grade), 7-methyl-benzo[a]pyrene (7-MeBaP, CAS No. 63041-77-0, purity 98%), 8-methyl-benzo[a]pyrene (8-MeBaP, CAS No. 63041-76-9, purity ≥98%), 4-methyl-benz[a]anthracene (4-MeBaA, CAS No. 316-49-4, purity ≥98%), 8-methyl-benz[a]anthracene (8-MeBaA, CAS No. 2381-31-9, purity ≥ 98%), and 9-methyl-benz[a]anthracene (9-MeBaA, CAS No. 2381-16-0, purity ≥98%) were purchased from (Merck, The Netherlands). 3-Methyl-benzo[a]pyrene (CAS No. 16757-81-6, purity >99%) was synthesized by the Biochemical Institute for Environmental Carcinogens (Grosshansdorf, Germany). All stocks and dilutions of test compounds were prepared in dimethyl sulfoxide (DMSO) (Merck, The Netherlands).

2.2 | CALUX reporter gene assays

2.2.1 | Cell line and cell culture conditions

Stably transfected human osteosarcoma cell lines (U2OS), expressing either ER-α (U2OS ER-α CALUX) or RAR (U2OS RAR CALUX) and a firefly luciferase reporter gene under the control of receptor-specific

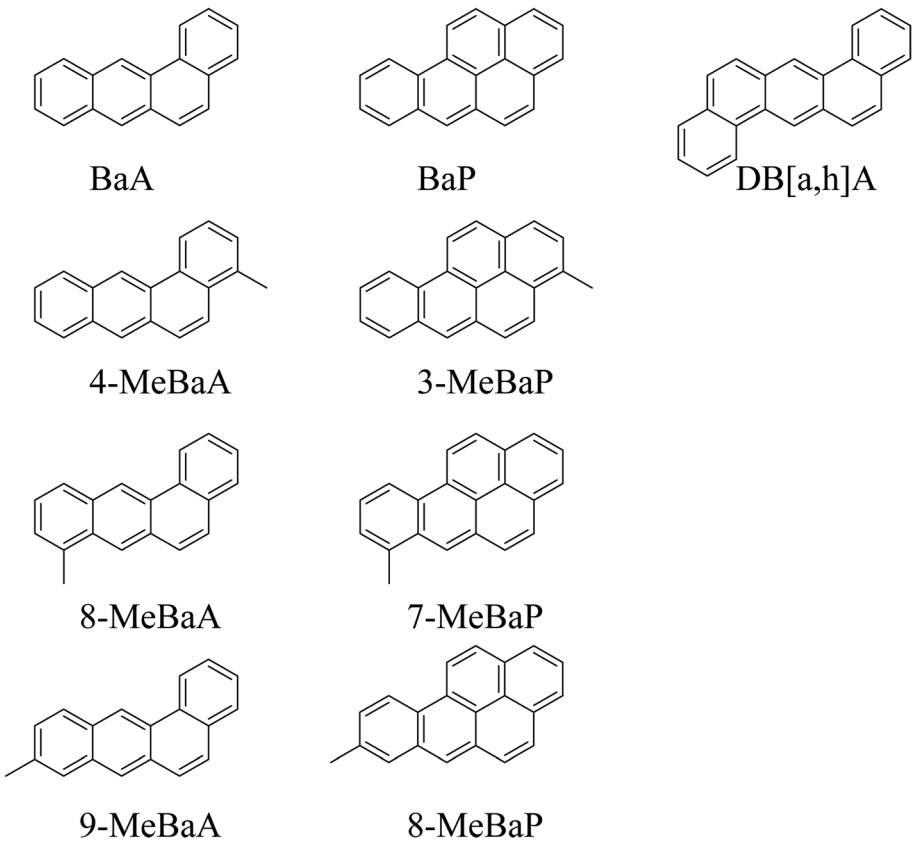

FIGURE 1 Structure of the nine unsubstituted and methylated PAHs tested in the study.

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responsive elements were kindly provided by BioDetection Systems (Amsterdam, The Netherlands). U2OS cytotox cell line (BioDetection Systems), stably expressing only a firefly luciferase reporter gene without any receptor-specific responsive element, was used as a control for luciferase expression in the U2OS-based CALUX reporter gene assay. All the U2OS cell lines were maintained in T75 polystyrene cell culture flasks (Corning, The Netherlands) in Dulbecco's Modified Eagle Medium F-12 nutrient mixture (DMEM/F12; Gibco, Paisley, United Kingdom; catalog no. 31330–038). The DMEM/F12 medium was supplemented with 10% (v/v) fetal bovine serum (FBS; Capricorn, Germany; catalog no. FBS 12A), 0.5% nonessential amino acids (NEEA; Gibco, catalog no. 11140-035), and 200 μg/ml of geneticin G418 (Invitrogen, Breda).

The stably transfected rat hepatoma cell line (H4IIE.luc) was used for the AhR CALUX assay (Aarts et al., 1995). H4IIE.luc cells were cultured in T75 polystyrene cell culture flasks in Minimum Essential Medium (MEM) alpha medium (Gibco; catalog no. 2256-021), supplemented with 10% (v/v) FBS.

All cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere and were routinely subcultured two to three times per week using 0.05% trypsin–EDTA (Gibco; catalog no. 15400-054) to detach the cells.

2.2.2 | CALUX reporter gene assays

The principle of the CALUX assay relies on the ability of a test compound to bind and consequently activate or inhibit the transcription of the receptor target genes, which eventually results in different luciferase activities (Van der Linden et al., 2014; Windal et al., 2018).

The U2OS CALUX assays (ER-α, RAR, and cytotox) were performed essentially as described before by Kamelia et al. (2018). In brief, the U2OS cells were seeded in the 60-inner wells of 96-well white plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 1×10^4 cells per well in 100 μl assay medium (DMEM/F-12 without phenol red [Gibco; catalog no. 21041–025], supplemented with 5% fetal bovine serum treated with dextran coated charcoal [DCC-FBS, Gibco; catalog no. 12676029] and 0.5% NEAA). The 36-outer wells of the same white 96-well plate were filled with 200 μl PBS to create an optimal humidity and to limit evaporation from the inner 60-wells. After 24 h incubation in a humidified atmosphere at 37°C with 5% CO_2, assay medium was removed using a vacuum pump and replaced with 100 μl assay medium per well. Then cells were incubated at the same condition for another 24 h. Afterward, cells were exposed to exposure medium containing different concentrations of test compounds in triplicate for 24 h by adding 100 μl of exposure medium to each well. The exposure medium for the U2OS CALUX agonist assay was prepared by addition of 400-times concentrated stock solutions of the test compounds (dissolved in DMSO) to the assay medium. The exposure medium for the U2OS CALUX antagonist assays was prepared by addition to assay medium of 800-times concentrated DMSO-stock solutions of reference-standard agonist compounds to reach a final concentration equal to their EC50 concentration (1×10^{-5} μM E2 for the ER-α antagonist assay, 2×10^{-1} μM RA for the RAR antagonist assay) and adding test compounds from 800-times concentrated DMSO-stock solutions.

For the AhR CALUX agonist assays, 100 μl of H4IIE.luc cells at a density of 3×10^4 cells per well were seeded into the 60-inner wells of the 96-wells white plates, and the 36-outer wells of the same white 96-well plate were filled with 200 μl PBS. After 24 h incubation, cells were exposed to exposure medium containing different concentrations of test compounds in triplicate for either 6 or 24 h. The exposure medium for the AhR CALUX assay was prepared by diluting 400-times concentrated DMSO-stock solutions of the test compounds with preconditioned medium, which is the growth medium in which cells were previously grown for 16 to 24 h. The use of the preconditioned medium for cell exposure is to avoid a high background luciferase signal induced by tryptophan products present in the fresh medium, which can induce AhR activity and thereby cause false-positive results (Hamers et al., 2000; Vrabie et al., 2009).

After 6 (AhR CALUX assay) or 24 h (AhR CALUX and U2OS CALUX assays) exposure, cells were washed with 100 μl ½ PBS (PBS: nano-pure water = 1: 1) and lysed with 30 μl low salt buffer (10 mM Tris (Merck), 2 mM dithiothreitol (DTT, Merck), and 2 mM 1, 2-diaminocyclohexanete triacetic acid monohydrate (Merck); pH 7.8). Afterward, plates were placed on ice for at least 15 min and frozen at −80°C for at least 2 h before measurement.

For the luminescence measurement, plates were thawed at room temperature for 1 h and then shaken for 5 min on a plate shaker. Luciferase activity was measured using a luminometer (Glomax-Multi Detection System, Promega, California) after the addition to each well of 100 μl flash-mix solution. The flash-mix solution consisted of an aqueous solution of (final concentrations) 20 mM tricine (Merck), 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$ (Merck), 2.67 mM magnesium sulfate ($MgSO_4$, Merck), 0.1 mM ethylenedinitrilotetraacetic acid disodium salt dihydrate (Titriplex III; Merck), 2 mM DTT, 0.47 mM D-luciferin (Synchem UG & Co. KG, Felsberg, Germany), and 5 mM adenosine-5-triphosphate (Duchefa Biochemie bv, Haarlem, the Netherlands).

Three independent experiments were conducted for each test compound in each CALUX assay. A full concentration-response curve of the reference compound for both agonist and antagonist assays was included in each independent experiment. The final concentration of DMSO was kept at 0.25% (v/v). All test compounds were tested at concentrations up to 50 μM in the U2OS CALUX agonist assays and up to 25 μM in the U2OS CALUX antagonist assay, except for DB[a,h]A. DB[a,h]A was tested at concentrations up to 25 μM in the agonist assay and 15 μM in the antagonist assay due to its solubility limitation. In the AhR CALUX assay, the highest final concentration of all test compounds was 5 μM because the highest AhR induction was already obtained upon exposure to test compounds at this concentration. The following final concentration ranges were used for the reference compounds of the various CALUX assays, TCDD (2.5×10^{-7}–2.5×10^{-4} μM) for the AhR agonist assay, E2 (1×10^{-7}-5×10^{-4} μM) for the ER-α agonist assay, fulvestrant (5×10^{-9}-5×10^{-4} μM) for the ER-α antagonist assay, retinoic acid (5×10^{-4} –

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1.5×10^{2} μM) for the RAR agonist assay, and AGN 193109 (1×10^{-4}-3×10^{1} μM) for the RAR antagonist assay.

2.2.3 | Cell viability test

In addition to use of the U2OS-cytotox CALUX assay, the cell viability following exposure to test compounds in all CALUX assays was also determined using the WST-1 assay. Briefly, after the same cell seeding and exposure steps outlined in Section 2.2.2, 5% (v/v) WST-1 reagent (Roche, Mannheim, Germany) was added in each well of the inner 60-wells of the plates. The plates were further incubated for 3 h at 37°C under a 5% CO_2 atmosphere. After this incubation, the absorbances were measured at 440 and 620 nm using a SpectraMax iD3 (Molecular Devices, San Jose, USA). Three independent experiments were performed for each test compound.

2.3 | Co-exposure of zebrafish embryos in the ZET to the selected PAHs and an AhR antagonist or an ER-α antagonist

To further investigate the role of the AhR or ER-α in mediating the PAH-induced developmental toxicity, zebrafish embryos were exposed to the selected PAHs in the presence or absence of an ER-α antagonist (fulvestrant) or an AhR antagonist (CH223191), respectively. The role of RAR in PAH-induced developmental toxicity was not evaluated as none of the PAHs tested induced any agonist or antagonist activity in the RAR CALUX assay (see Section 3).

The concentration of the AhR antagonist CH223191 used in the ZET was 1 μM, which was the highest concentration of CH223191 that did not affect the normal development of zebrafish embryos until 96 hpf (see Supporting Information 1). CH223191 was reported to antagonize the AhR without detectable AhR agonist-like activity or estrogenic activity (Kim et al., 2006). Other studies also report that 0.5 μM CH223191 could sufficiently antagonize the AhR-mediated developmental toxicity in 5D zebrafish embryos (Gerlach et al., 2014; McGee et al., 2013).

The concentration of the ER-α antagonist fulvestrant used in the present study was 3 μM, which is considerably higher than the IC50 (0.8 nM) of fulvestrant to antagonize the in vitro ER-α-mediated activity reported previously (Weir et al., 2016). Moreover, a recent study (Adam et al., 2021) showed that 3 μM fulvestrant could sufficiently antagonize the ER-α-mediated developmental toxicity of the estrogenic compound diethylstilboestrol in the ZET.

The ZET was performed as described previously (Kamelia, Brugman, et al., 2019). In short, fertilized eggs of the wild-type zebrafish (*Danio rerio*) AB line were purchased from the research facility Carus, Wageningen University and Research, The Netherlands. The ZET was initiated at 4–5 h postfertilization (hpf) and terminated at 96 hpf. The exposure was performed in 24-well plates (Greiner Bio-One). Twenty wells of the 24-well plates were used for exposure to two concentrations of the test compounds (10-wells/concentration), and the other four-wells were used for the internal plate control. The egg water was prepared by adding 10 ml 100-times egg water stock solution (3 g sea salt [Tropic marine, Wartenberg, Germany] in 500 ml demineralized water) to 990 ml demineralized water. The ZET was performed in the absence or presence of the AhR antagonist CH223191 (1 μM) or the ER-α antagonist fulvestrant (3 μM). Exposure medium in the absence of CH223191 or fulvestrant was prepared by adding to egg water the 400-times concentrated DMSO-stock solution of the selected PAHs. Exposure medium for the presence of fulvestrant or CH223191 was prepared by adding to egg water an 800-times concentrated DMSO-stock solution of the selected PAHs and an 800-times concentrated DMSO-stock solution of CH223191 or fulvestrant. The final solvent (DMSO) concentration was kept at 0.25% (v/v). The exposure medium was transferred (2 ml/well) into 10-wells of the 24-well plate, and for the internal plate control, 2 ml egg water was added into each of the four remaining wells. Then, one zebrafish embryo was transferred to each well of the 24-well plate. The plates were sealed with self-adhesive film covers (Greiner Bio-One) to prevent evaporation and were incubated at 26°C with a photo period of 14 h light:10 h dark. For the co-exposure studies in the ZET, the selected PAHs were tested at a concentration of 15 and 50 μM, except for 3-MeBaP and DB[a,h]A, which were tested at only 15 μM due to solubility limitations. Three types of controls were included in each independent experiment: negative control (egg water), solvent control (0.25% (v/v) DMSO in egg water), and positive control (4 μg/ml 3,4-dichloroaniline). Embryos were scored daily until 96 hpf for developmental abnormalities and embryo lethality using an inverted microscope, based on the extended general morphological scoring (extended-GMS) system described by Beekhuijzen et al. (2015) (See Supporting Information 2). Any deviation from normal development of zebrafish embryos until 96 hpf results in a decreased extended-GMS score, which corresponds to a certain extent of developmental retardation. Each independent ZET was considered valid only if the following criteria were met (at 96 hpf): (1) ≤ 1 dead embryos (out of 4) in the internal plate control of the exposed-plate, (2) ≤ 1 dead embryos (out of 10) in the negative control plate, (3) ≤ 1 dead embryos (out of 10) in the solvent control plate, and (4) ≤ 7 alive embryos (out of 10) in the positive control plate. Three independent experiments were performed for each test compound.

2.4 | Co-exposure of H4IIE.luc cells to the selected PAHs and an AhR antagonist in the AhR CALUX assay

The effect of the AhR antagonist CH223191 to antagonize the PAH-induced AhR activation in the AhR CALUX assay was also evaluated. To that purpose, in the AhR CALUX assay, AhR H4IIE.luc cells were exposed to each test compound at its EC50s for either 6 or 24 h in the absence or presence of 100 μM CH223191. The luciferase induction was subsequently quantified. The EC50 reflects the concentration of test compounds that induce 50% AhR-mediated activity in the AhR CALUX assay and was derived from the obtained concentration-response curve of each PAH tested in the AhR CALUX assay (see

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Table 1). In addition, cell viability after 24 h exposure to each test compound in the co-exposure experiments (with or without CH223191) was evaluated using the WST-1 assay. Three types of controls were included in each independent experiment: solvent control (0.25% v/v DMSO), CH223191 only (100 μM), and positive control TCDD (0.25 nM) Three independent experiments were conducted for each test compound in this co-exposure experiment.

2.5 | Estimating free concentrations of the nine PAHs tested in the CALUX assays and ZET

Free (unbound) exposure medium concentrations of PAHs in the CALUX assays (AhR, ER-α and RAR) and the ZET were estimated using an in vitro chemical distribution model (Comenges et al., 2017; Groothuis et al., 2015; Kramer, 2010; Kramer et al., 2022 (manuscript in preparation), 2012). This model was previously used to estimate the free in vitro concentration of the 3-ring PAH PHE (Kramer et al., 2012). In this model, physiochemical properties of the chemical substances, that is, the octanol–water partition coefficient (K_{ow}) and Henrys law constant (K_H), in combination with test system-specific information (e.g., well plates dimensions, exposure medium volume, cell density, cell lipid content, serum concentration, and exposure temperature) were used as the input parameters to predict the in vitro free concentrations (Supporting Information 8). The output of this in vitro chemical distribution model is: (1) a modeled free fraction of the compound in the assay medium, (2) a modeled fraction bound to serum constituents in the assay medium, (3) a modeled fraction of the compound in the cellular compartment (CALUX cells or zebrafish embryos in our case), (4) a modeled fraction of the compound bound to plastic, and (5) a modeled fraction in the headspace (due to evaporation). The modeled results are presented in Supporting Information 9–12. Moreover, concentration-response curves based on the predicted free fraction in medium were made for the AhR, ER-α, and RAR CALUX assays (Supporting Information 13–15).

2.6 | Data analysis

The results of luminescence measurements were expressed as relative light units (RLUs). First of all, the fold induction was calculated by dividing the mean value of RLUs from the exposed wells by the mean value of RLUs from the solvent control (0.25% v/v DMSO). For the agonist CALUX assays (AhR agonist, ER-α agonist, and RAR agonist CALUX assays), the percentage of luciferase induction was further calculated by setting the maximum fold induction of the corresponding positive controls (TCDD, E2, and RA, respectively) at 100%. For the antagonist CALUX assay (ER-α antagonist and RAR antagonist CALUX assays), the percentage of luciferase induction was calculated by setting the fold induction at the EC50 concentration of the corresponding positive controls (E2 and RA, respectively) at 50%. Results are expressed as mean ± standard error of mean (SEM), obtained from three independent experiments. Figures of concentration-response curves upon exposure to the test compounds were made using GraphPad Prism 5.0 (California, USA). Data were analyzed using non-linear regression and fitted to a sigmoid concentration-response curve with three parameters. EC50 values (effective concentration inducing 50% response) with 95% confidence intervals calculated using GraphPad.

The ZET results were expressed as % of the total extended-GMS score and embryo survival at 96 hpf. Results are presented as mean ± SEM, obtained from three independent experiments. Data were fitted to bar graphs using GraphPad Prism 5.0.

An unpaired student's *t*-test was performed using GraphPad Prism 5.0 to evaluate the significance ($p < 0.05$) between the % of response in the absence or presence of CH223191 (AhR antagonist) or fulvestrant (ER-α antagonist) in the ZET and between the response of AhR-mediated activity in the absence or presence of CH223191 in the AhR CALUX assay.

Data from the WST-1 assay were processed by first subtracting the absorbance values measured at 620 nm (background) from the values measured at 440 nm. Subtracted data were then expressed as the percentage of cell viability relative to the solvent control (0.25% v/v DMSO, cell viability set as 100%). Results are presented as mean ± SEM, obtained from three independent experiments.

3 | RESULTS

It should be noted that the concentrations of test compounds indicated in the present manuscript, for example, in graphs and tables, are the nominal concentrations. Results of the modeled free concentration in medium for the AhR, ER-α, and RAR CALUX assays, and the ZET are presented in Supporting Information 9–12. Concentration-response curves based on the predicted free fraction in medium for the abovementioned CALUX assays can be found in the Supporting Information 13–15.

3.1 | Effects of test compounds on the AhR

In the AhR CALUX agonist assay, exposure time windows of 6 and 24 h were applied to assess the transient or persistent nature of the AhR induction by the test compounds, respectively. The full concentration-response curves of the AhR-mediated activity for all compounds are shown in Figure 2. Table 1 presents the EC50 values derived from the obtained concentration-response curves, and a qualitative overview of the AhR CALUX results (presented by the "+/−" symbols). As shown in Figure 2, all PAHs tested were able to induce concentration-dependent agonist activities in the AhR CALUX assay following both 6 and 24 h exposure. For all PAHs, the EC50 values tested at 6-h exposure were lower than those at 24-h exposure, which indicates a transient nature of the AhR activation. Based on the EC50 values, upon both 6 and 24 h exposure, 9-MeBaA and 8-MeBaP were the most potent 4- and 5-ring PAH tested, respectively. Moreover, all the methylated congeners appear to be more potent than

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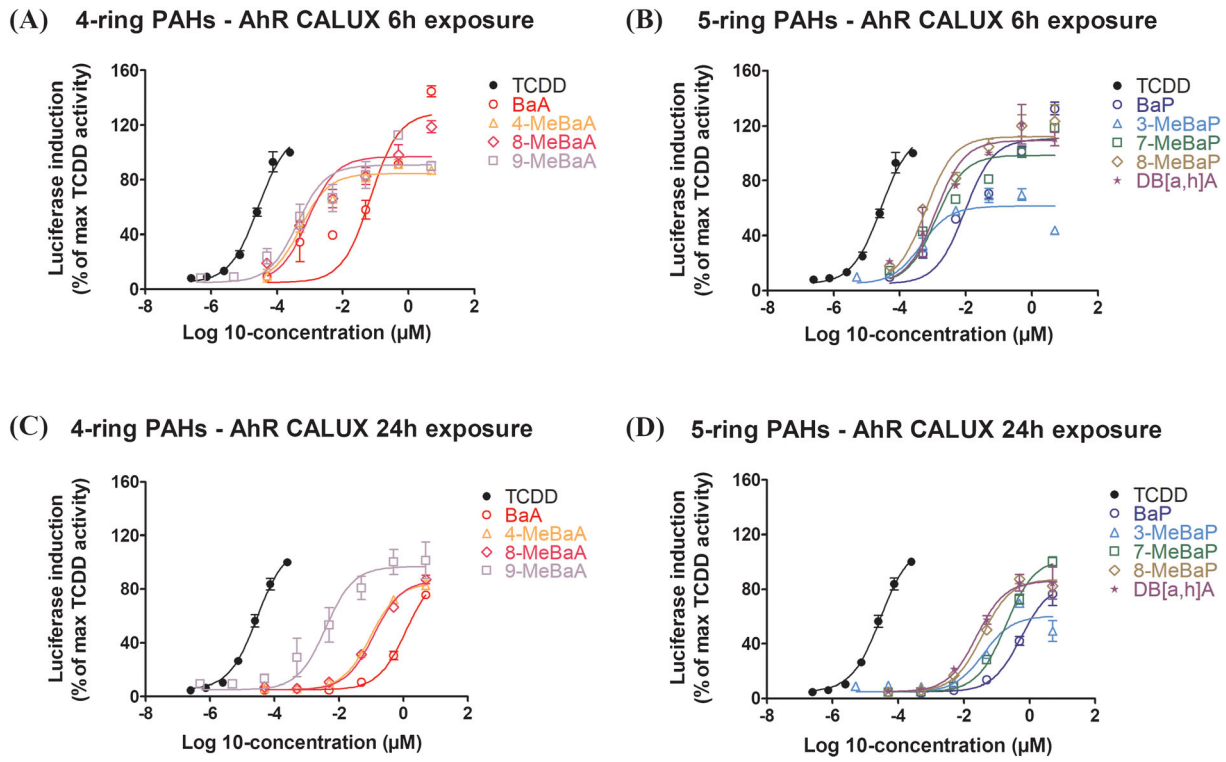

FIGURE 2 Concentration-dependent effects of TCDD and the nine unsubstituted and methylated PAHs tested in the AhR CALUX agonist assay following 6 h (A,B) or 24 h (C,D) exposure. The highest luciferase induction of the positive control TCDD was set at 100%. Results represent data from three independent experiments and are presented as mean ± SEM.

their respective parent compounds BaA and BaP following both 6 and 24 h exposure. The cytotoxicity results (Supporting Information 3) showed that no cytotoxicity was observed in the H4IIE.luc cells when exposed to the same concentrations of test compounds as tested in the AhR CALUX assay.

3.2 | Effects of test compounds on the ER-α

In the U2OS CALUX assay, the direct interaction between test compounds and the firefly luciferase enzyme might result in stabilization of the luciferase enzyme, which lead to increased luciferase activity without an underlying increased gene expression (Sotoca et al., 2010). Test compounds might also unspecifically inhibit the luciferase induction in the U2OS cells without exerting cytotoxicity. Thus, the U2OS-cytotox CALUX assay (see results in Supporting Information 4) was performed to check and correct for the nonspecific induction or inhibition of luciferase activity by each test compound. Results obtained in the ER-α/RAR CALUX assay were then corrected for this nonspecific activity by dividing the luciferase induction activity obtained in the U2OS ER-α/RAR assay by the corresponding results obtained in the U2OS-cytotox CALUX assay, for each test compound after which the corrected luciferase induction was calculated. The corrected U2OS ER-α data are presented in Figure 3, and the uncorrected data are presented in Supporting Information 5. In addition, the cell viability results obtained in the WST-1 assay (Supporting Information 3) showed that no cytotoxicity was observed in the U2OS cells when exposing to the same concentrations of test compounds as the U2OS CALUX assays. As shown in Figure 3A and B, six PAHs (i.e., BaA, 4-MeBaA, 8-MeBaA, 9-MeBaA, BaP, and 3-MeBaP) were able to induce estrogenic activity in the ER-α CALUX agonist assay, ranging from 10% (induced by BaP) to 75% (induced by 8-MeBaA) of the maximum E2-induced response. Among these six PAHs, only BaA and 8-MeBaA induced more than 50% luciferase induction, whereas the other four PAHs induced less than 50% luciferase induction, which indicates a rather weak ER-α agonist activity at the concentrations tested. The EC50 values of BaA and 8-MeBaA in the ER-αCALUX agonist assay were 27.00 and 11.99 μM, respectively (Table 1). Three 5-ring PAHs (i.e., 7-MeBaP, 8-MeBaP, and DB[a,h]A) tested negative in the ER-α CALUX agonist assay. In the ER-α CALUX antagonist assay, none of the PAHs was able to induce substantial anti-estrogenic activity (Figure 3C,D).

3.3 | Effects of test compounds on the RAR

The luciferase induction obtained in the RAR CALUX assay (for both agonist and antagonist activity) corrected using the respective results from the U2OS-cytotox CALUX assay is presented in Figure 4, and the uncorrected data are presented in the Supporting Information 5. It can be seen from Figure 4 that none of the PAHs tested was able to induce RAR agonistic or antagonistic activity.

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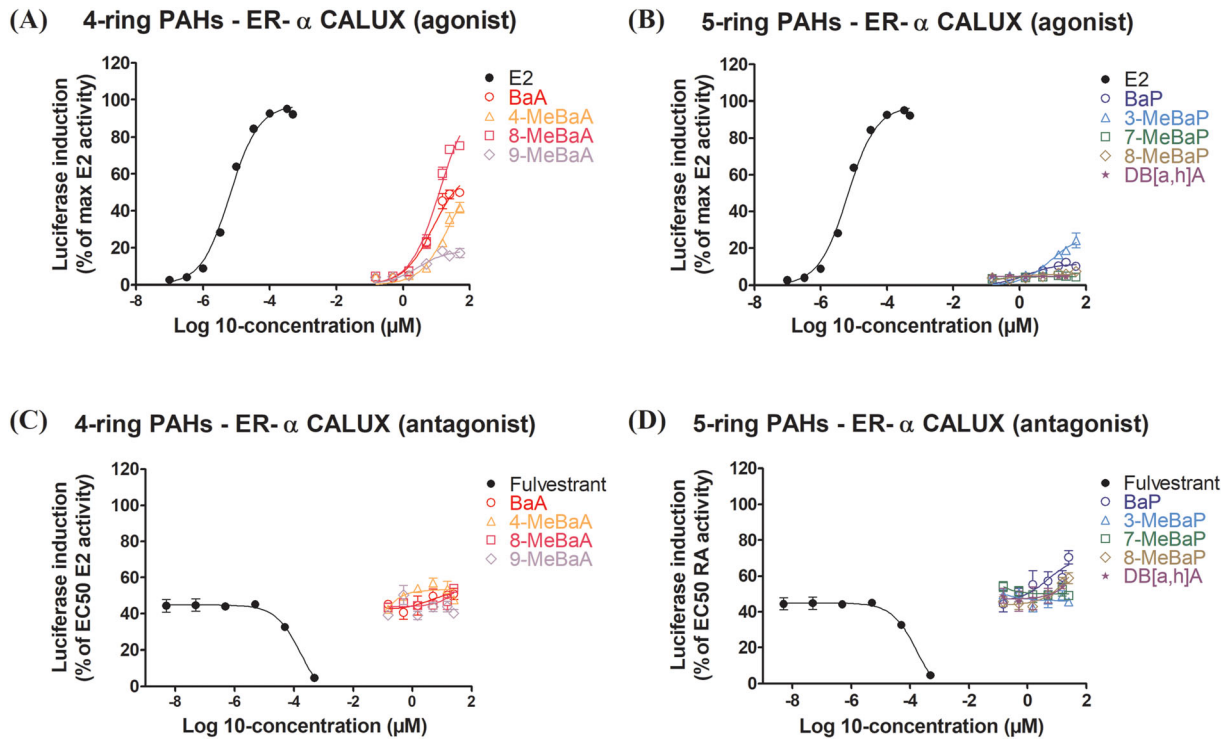

FIGURE 3 (A–D) Concentration-dependent effects of E2, fulvestrant, and the nine unsubstituted and methylated PAHs tested in the ER-α CALUX (agonist/antagonist) assay. The highest luciferase induction of the positive control E2 was set at 100% for the agonist assay, and the luciferase induction at the EC50 of E2 was set at 50% for the antagonist assay. Results represent data from three independent experiments and are presented as mean ± SEM.

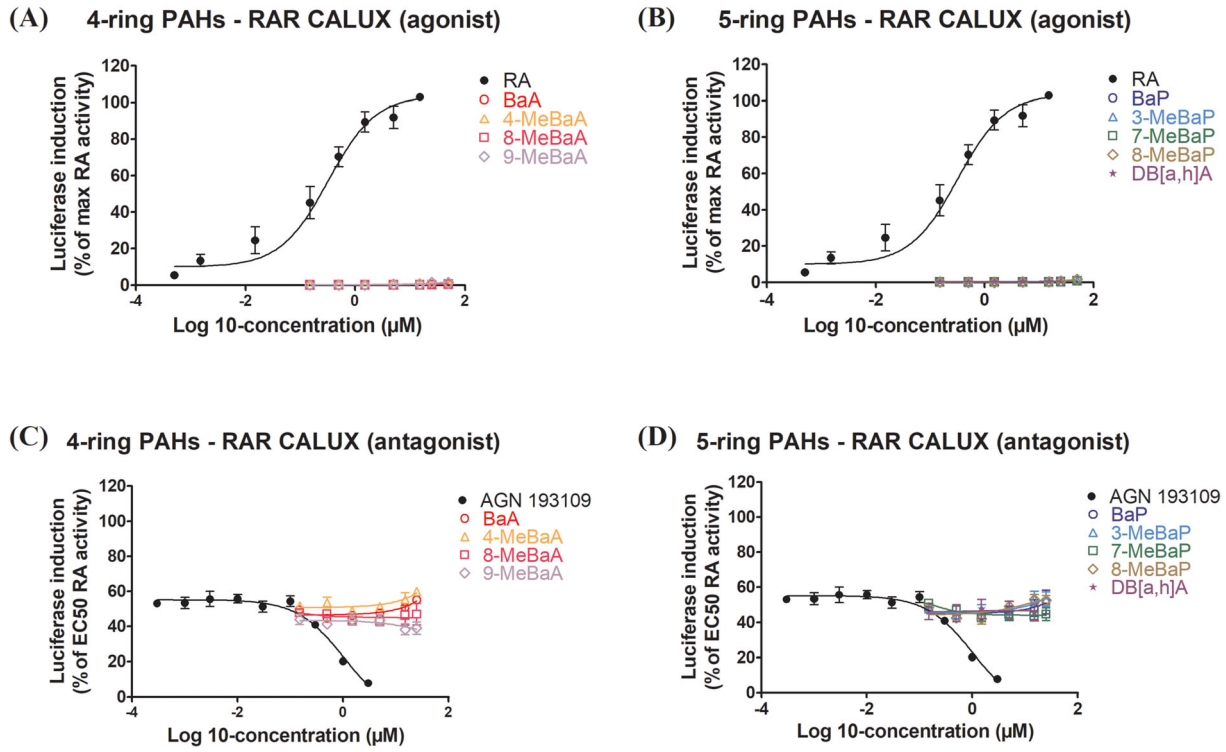

FIGURE 4 (A–D) Concentration-dependent effects of RA, AGN 193109, and the nine PAHs tested in the RAR CALUX (agonist/antagonist) assay. The highest luciferase induction of the agonist positive control RA was set at 100% for the agonist assay, and the luciferase induction at the EC50 of RA was set as 50% for the antagonist assay. Results represent data from three independent experiments and are presented as mean ± SEM.

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3.4 | Counteracting effects of PAH-induced developmental toxicity in the ZET by the AhR antagonist (CH223191)

As depicted in Figures 5 and 6, all PAHs tested, except 7-Me-BaP, induced developmental retardations in zebrafish embryos, shown by reduction of extended-GMS score and embryo survival in the ZET at 96 hpf. The current results are consistent with the ZET results we reported previously (Fang et al., 2022). As shown in Figure 5A, the extended-GMS score upon exposure to 15 μM BaA was not significantly affected with by the addition of CH223191; however, the severity of several developmental retardations, for example, pericardial edema, yolk sac edema, and deformed body shape, induced by 15 μM BaA was slightly reduced in the presence of CH223191 (Figure 6A). Furthermore, CH223191 fully counteracted the embryo lethality induced by 50 μM BaA (Figure 5A) but had no effect on the observed developmental retardations (Figure 6A). For 4-MeBaA (Figure 5B), the addition of CH223191 significantly counteracted only the embryo lethality induced at the concentration of 15 μM but not of 50 μM 4-MeBaA. Similar to BaA, even though the addition of CH223191 did not result in a higher extended-GMS score following exposure to 15 μM 8-MeBaA in the ZET (Figure 5C), the severity of pericardial edema, yolk sac edema, deformed body shape, and deformed head and jaw were reduced (Figure 6C). The addition of CH223191 successfully counteracted the effect of unhatched embryos and reduced the severity of yolk sac edema induced by 15 and 50 μM 9-MeBaA (Figure 5D, Figure 6D).

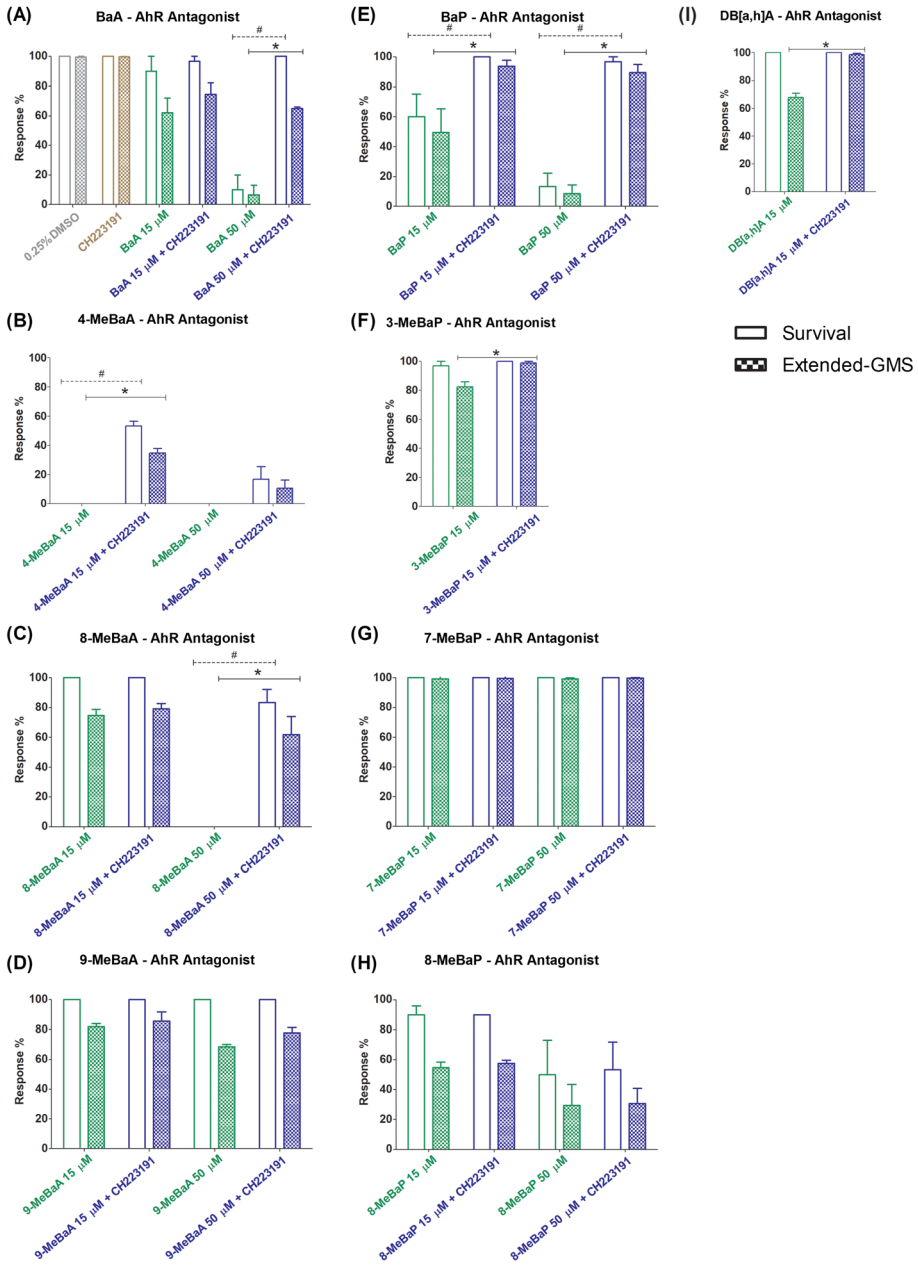

FIGURE 5 (A–I) Effects of the nine PAHs on embryo survival (open bars) and extended-GMS (shaded bars) with the absence (green bars) or presence (blue bars) of CH223191 (an AhR antagonist). 0.25% DMSO (grey bars) and only CH223191 (brown bars) were used as controls for the experiment. Results represent data from three independent experiments and are presented as mean ± SEM. The significant differences ($p < 0.05$) in embryo survival and extended-GMS in the absence or presence of CH223191 are represented by hashtag (#) and asterisk (*) symbols, respectively.

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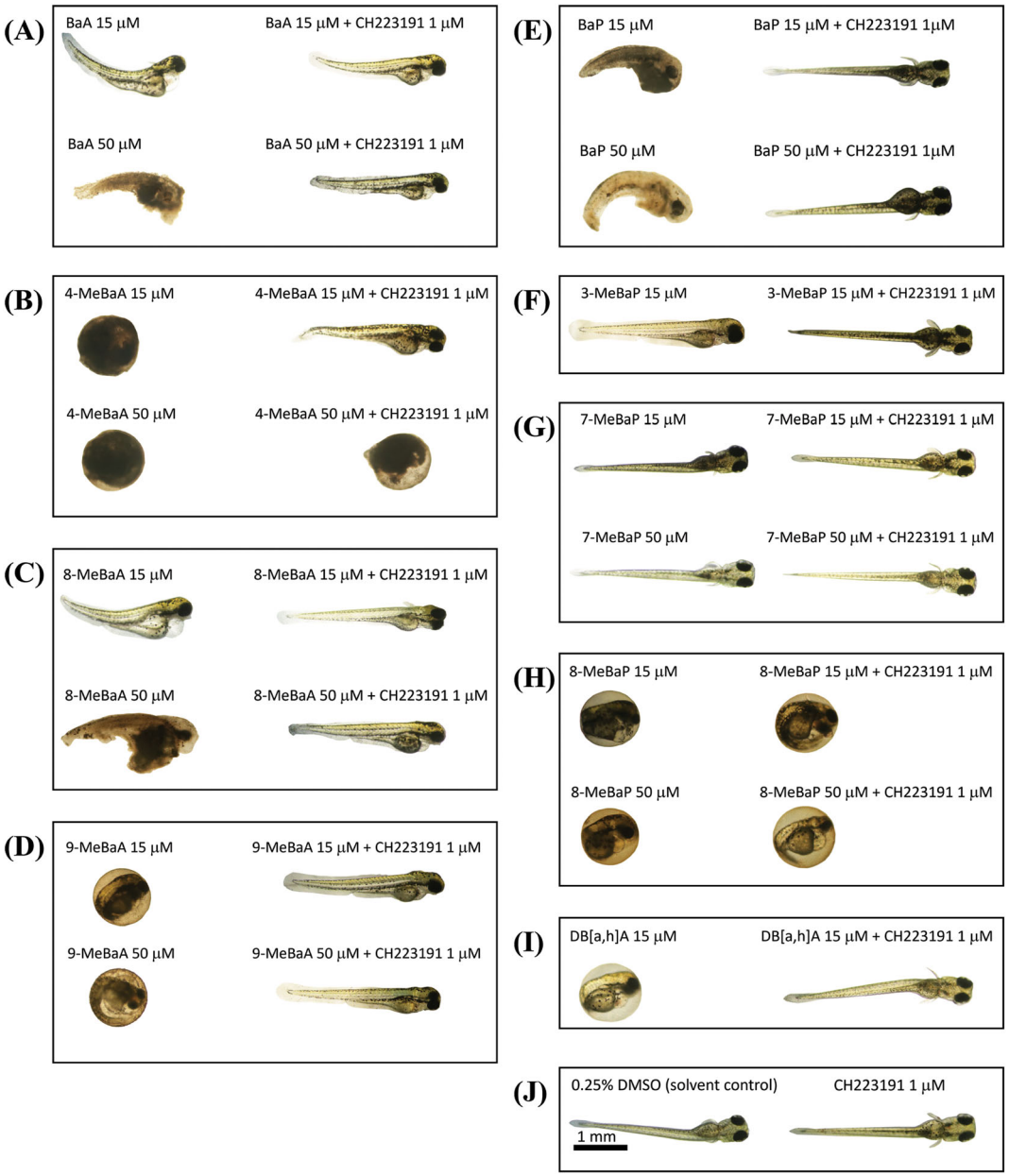

FIGURE 6 (A–I) Zebrafish embryos morphology at 96 hpf upon exposure to nine PAHs under study with and without CH223191 at 96 hpf. (J) Representative images of zebrafish embryos exposed to 0.25% DMSO (solvent control) and 1 μM CH223191 at 96 hpf. Scale bar = 1 mm.

Both embryo lethality and developmental retardations of the parent compound BaP were significantly counteracted by CH223191 as shown by an increase in the extended-GMS score and the survival rates; however, a slightly enlarged yolk sac could still be observed in the zebrafish embryos when co-exposed with 50 μM BaP and CH223191 (Figures 5E, 6E). For 3-MeBaP, the addition of CH223191 counteracted all developmental retardations induced by this test compound. 7-MeBaP tested negative in the ZET, with or without the presence of an AhR antagonist. Of the nine tested PAHs, 8-MeBaP was the most potent one to induce developmental retardations in zebrafish embryos (Fang et al., 2022), and as shown in Figures 5H & 6H, its developmental toxicity in the ZET could not be counteracted by the addition of 1 μM CH223191. Finally, the observed developmental toxicity induced by 15 μM DB[a,h]A in the ZET was successfully counteracted by the addition of CH223191 (Figures 5I, 6I).

The AhR antagonist CH223191 used in the present study was previously reported to be a selective AhR antagonist ligand for TCDD (Zhao et al., 2010). To ensure that the antagonist can be used to counteract PAH-mediated effects on the AhR, the antagonizing effect of CH223191 on the observed PAH-induced AhR activity in the AhR CALUX assay was investigated by co-exposing the H4IIE.luc cells with each PAH at their EC50s, in the absence or presence of 100 μM CH223191. Results obtained (Figure 7) show that CH223191 successfully counteracted the AhR-mediated gene expression induced by

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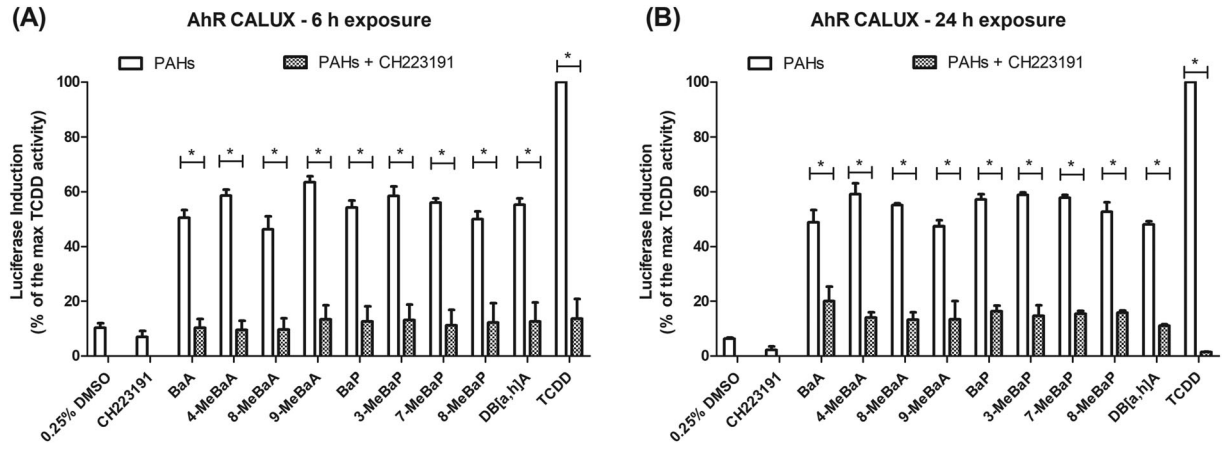

FIGURE 7 Counteracting effect of CH223191 on the AhR-mediated gene expression by the nine PAHs following (A) 6 or (B) 24 h exposure in the AhR CALUX assay. 0.25% v/v DMSO, CH223191 only (100 μM) and TCDD (0.25 nM) were used as controls for the experiment. Results represent data from three independent experiments and are presented as mean ± SEM. The significant difference ($p < 0.05$) in response (i.e., luciferase induction), in the absence or presence of CH223191, is represented by asterisk (*) symbols.

each PAH tested in the AhR CALUX assay after both 6 and 24 h exposure, without inducing cytotoxicity (Supporting Information 3). This indicates that CH223191 is not only able to inhibit TCDD-induced AhR-mediated activities but also PAH-induced AhR-mediated gene expression.

3.5 | Counteracting effects of PAH-induced developmental toxicity in the ZET by the ER-α antagonist (fulvestrant)

PAHs under study that showed substantial estrogenic activities (≥50% response of max E2 activity; Figure 3A) in the ER-α CALUX assay (i.e., BaA and 8-MeBaA) were tested in the ZET, in the absence or presence of 3 μM of the ER-α antagonist fulvestrant. As shown in Figure 8, co-exposure of zebrafish embryos with fulvestrant did not counteract the developmental retardations induced by 15 μM of BaA but fully counteracted the embryo lethality induced by 50 μM BaA at 96 hpf although most developmental retardations observed, for example, pericardial and yolk sac edemas were still present or not affected by the addition of fulvestrant (Figure 9A). For 8-MeBaA, at 96 hpf fulvestrant significantly counteracted the developmental retardations induced by 15 μM 8-MeBaA (e.g., reduced severity of pericardial and yolk sac edemas) but did not counteract the embryo lethality induced by 50 μM BaA (Figures 8 and 9B).

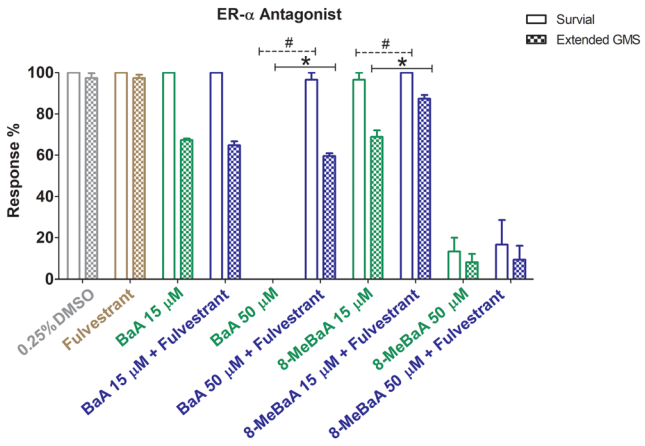

FIGURE 8 Effects of BaA and 8-MeBaA on embryo survival (open bars) and extended-GMS (shaded bars) with the absence (green bars) or presence (blue bars) of fulvestrant (a potent ER-α antagonist). 0.25% DMSO (grey bars) and fulvestrant (brown bars) were used as controls for the experiment. Results represent data from three independent experiments and are presented as mean ± SEM. The significant differences ($p < 0.05$) in embryo survival and extended-GMS in the absence or presence of fulvestrant are represented by hashtag (#) and asterisk (*) symbols, respectively.

4 | DISCUSSION

The present study evaluated the AhR, ER-α and RAR-mediated activities of a series of 4- and 5-ring unsubstituted and monomethylated PAHs using CALUX reporter gene assays and the potential role of these receptors in mediating the observed PAH-induced developmental toxicity in the ZET. The results demonstrate that all tested PAHs induced a strong AhR agonist activity in the AhR CALUX assay; no (ant)agonist activity in the RAR CALUX assay; no anti-estrogenic activity in the ER-α antagonist CALUX assays; and only two out of nine PAHs tested (i.e., BaA and 8-MeBaA) were able to induce estrogenic activity (≥50% of E2 max activity) in the ER-α agonist CALUX assay. It is worth mentioning that in the AhR CALUX assay, all monomethylated 4- and 5-ring PAHs tested induced stronger AhR-mediated activities compared to their respective unsubstituted parent PAHs. The role of AhR and ER-α in PAH-induced developmental toxicity was further investigated by co-exposing zebrafish embryos to the selected PAHs and an AhR antagonist (CH223191) or an ER-α

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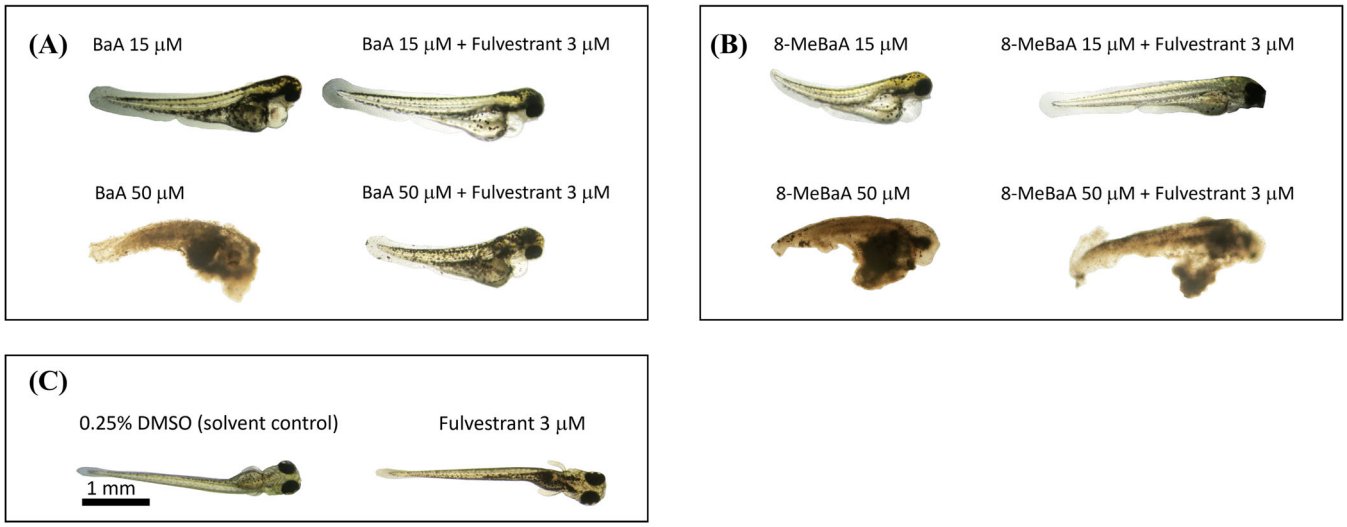

FIGURE 9 (A–B) Zebrafish embryo morphology at 96 hpf upon exposure to BaA and 8-MeBaA with the absence or presence of fulvestrant. (C) Representative images of zebrafish embryos exposed to 0.25% (solvent control) and 3 μM fulvestrant at 96 hpf. Scale bar = 1 mm.

antagonist (fulvestrant). The co-exposure to PAHs with CH223191 reduced the PAH-induced developmental toxicity in the ZET to various degrees, except for 8-MeBaP for which the developmental toxicity was not significantly counteracted by CH223191. Moreover, the observed embryo lethality and developmental retardations induced by the estrogenic PAHs (i.e., BaA and 8-MeBaA), could be counteracted to a certain extent by the addition of fulvestrant in the ZET. These findings suggested that both ER-α and AhR may partially be involved in mediating the PAH-induced developmental toxicity observed in the ZET.

All tested PAHs induced AhR-mediated activities after both 6 and 24 h exposure with EC50 values being substantially lower after 6 h of exposure than after 24 h of exposure, indicating a transient AhR activation. A similar transient AhR activation was observed previously for individual PAHs (Machala et al., 2001) and PAH-containing substances (Kamelia et al., 2021). The transient AhR activation is considered to be a protective adaptive response to many ligands, including PAHs, whereas persistent AhR induction may affect normal biological processes including embryonic development in vertebrates (Hankinson, 1995; Larigot et al., 2018; Puga et al., 2002). Additionally, our data is in line with the data reported in published studies using yeast, rat, mice, or human cell lines (Boonen et al., 2020; Lam et al., 2018; Machala et al., 2001; Marvanová et al., 2008; Sun et al., 2014; Trilecová et al., 2011; Vondráček et al., 2017), where monomethylated PAHs were shown to be more potent AhR inducers than their parent PAHs (see an overview of the reported receptor-mediated activities induced by unsubstituted and alkylated PAHs in Supporting Information 6). This indicates that the addition of a methyl substituent on the aromatic ring of PAHs could increase the AhR-mediated activity of these substances compared to their parent PAHs. However, such an observation does not always result in an increased in vitro developmental toxicity potency of these substances. Our previous study (Fang et al., 2022) showed that the addition of a methyl substituent on the aromatic ring of a PAH, depending on its position, could either increase or decrease the developmental toxicity of the corresponding PAHs. Taken together, the AhR activation by PAHs, as quantified in the AhR CALUX assay, should not directly be translated into their developmental toxicity potency because the AhR reporter gene assay is merely a cell-based screening assay that lacks the physiological feedback mechanisms and metabolism.

Developmental retardations, including yolk sac edema, pericardial edema, and delayed development such as unhatched embryos, were noted following exposure to the (un)substituted PAHs under study. These manifestations are consistent with the typical AhR-mediated developmental effects observed in zebrafish embryos upon exposure to some PAHs and/or PAH-containing substances (Billiard et al., 1999; Fang et al., 2022; Goodale et al., 2013; Kamelia et al., 2019; Knecht et al., 2017; Puga et al., 2005; Wincent et al., 2015). To further evaluate the role of the AhR in mediating the PAH-induced developmental toxicity in the ZET, co-exposure experiments were conducted where zebrafish embryos were exposed to each individual PAH with or without the AhR antagonist, CH223191. CH223191 has been reported to antagonize the TCDD-induced AhR-mediated activity without AhR agonist activity or estrogenic activity in HepG2 human hepatoma cells and MCF-7 human breast cancer cells (Kim et al., 2006). However, another study reported that CH223191 had high selectivity to the TCDD-related AhR ligands and might not inhibit the AhR-mediated activity induced by PAHs (Zhao et al., 2010). In this previous study, the AhR-dependent reporter gene expression by TCDD at 1 nM was almost fully inhibited by CH223191 at 10 μM, whereas the activity of PAHs at equally effective concentrations of 0.1 to 1 μM was not inhibited. This apparent selectivity for TCDD may be due to the experimental conditions chosen with an 10,000-fold excess of CH223191 to TCDD and an only 10- to 100-fold excess of CH223191 to the PAHs. Thus, this apparent

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selectivity for TCDD may have been due to the higher ratio between inhibitor and agonist in the case of TCDD, while the lower ratio between inhibitor and agonist in the case of PAHs hampers effective inhibition. Thus, in the present study, CH223191 was tested at 100 μM in the AhR CALUX assay. The co-exposure experiment in the AhR CALUX assay (Figure 7) shows that CH223191 could not only inhibit TCDD-induced AhR-mediated gene expression but also PAH-induced AhR-mediated gene expression. This indicates that CH223191 is a suitable AhR antagonist to evaluate the potential role of the AhR in PAH-induced developmental toxicity in both the AhR CALUX assay and the ZET. Results obtained in the ZET show that the presence of CH223191 substantially reduced the severity or even fully counteracted the developmental retardations (i.e., yolk sac edema, pericardial edema, and deformed body shape) induced by seven out of nine PAHs tested (i.e., BaA, 4-MeBaA, 8-MeBaA, 9-MeBaA, BaP, 3-MeBaP, and DB[a,h]A). This observation is in line with the results of several studies reported that knockout of the AhR2, the primary isoform of AhR responsible for PAH-induced developmental toxicity in zebrafish, abated TCDD- and PAH-induced pericardial and yolk sac edemas in zebrafish embryos (Billiard et al., 2006; Goodale et al., 2012; Incardona et al., 2006; Van Tiem & Di Giulio, 2011). 8-MeBaP was the only PAH for which the observed developmental retardations in zebrafish embryos were not substantially neutralized by the addition of CH223191. 8-MeBaP is the most potent PAH to induce AhR-mediated activity, as tested in the AhR CALUX, which suggests that a higher concentration of CH223191 or a more potent AhR antagonist may be needed to counteract the observed retardations induced by this substance in the ZET. However, changes to the experimental design appeared not practically possible because 1 μM was already the highest concentration of CH223191 that did not affect the normal development of zebrafish embryos until 96 hpf (see Supporting Information 1). Overall, our results support the notion that the developmental toxicity as observed with some PAHs is partially AhR-mediated.

Of the nine PAHs tested, only BaA and 8-MeBaA showed substantial estrogenic activity in the ER-α CALUX assay. Some discrepancy of (anti)estrogenic activities of PAHs tested between results obtained in the present study and available literature data was noted (see an overview of the ER-α-mediated activities induced by unsubstituted and alkylated PAHs in Supporting Information 6). For example, BaP was reported to be a strong estrogenic compound (Boonen et al., 2020; Charles et al., 2000; Fertuck, Kumar, et al., 2001; Lam et al., 2018), whereas in the present study, BaP showed only a weak estrogenic activity. A possible explanation for this discrepancy might be related to the different metabolic capabilities present in the different used cell lines. The human breast carcinoma (VM7lucE42) cell line used by Lam et al. (2018) contains CYP450, which might result in the bioactivation of BaP into its major hydroxylated metabolite 3-hydroxy-BaP (3-OHBaP), which was shown to induce more estrogenicity than its parent BaP (Charles et al., 2000; Kamelia et al., 2020). Thus, a higher estrogenic activity might be observed for BaP when using a test system that is capable of metabolic activation, which does not hold for the U2OS cell line used in the present study.

The ER-α antagonist fulvestrant only counteracted the embryo lethality induced by 50 μM BaA and the developmental retardations induced by 15 μM 8-MeBaA such as craniofacial deformities and kinked tail, which are the typical ER-α-mediated developmental effects in zebrafish (Adam et al., 2021; Ahi et al., 2016; Kishida et al., 2001; Wester & Vos, 2003). One of the possible explanations for this observation could be that 8-MeBaA has a lower EC50 than BaA in activating the ER-α in the ER-α CALUX assay (Table 1), pointing at a potential higher binding affinity of 8-MeBaA for ER-α than of BaA; hence, the ER-α antagonist fulvestrant counteracted the developmental retardations induced by 8-MeBaA only at a lower concentration (i.e., 15 μM) while being potent enough to counteract the BaA-induced effects at 50 μM.

Published studies reported that the well-characterized RAR endogenous ligand, retinoic acid, can induce developmental toxicity, for example, causing craniofacial malformation in rodent embryos (Morriss-Kay, 1993) and failure to hatch in zebrafish embryos (Herrmann, 1995). None of the nine PAHs under study showed RAR agonist or antagonist activity, indicating that the PAH-induced developmental toxicity might be RAR-independent. Besides AhR, ER-α, and RAR, other receptors such as the androgen receptor (AR) also play a role in the developmental processes (Kelce & Wilson, 1997). Previous studies showed that the DMSO extracts of some PAH-containing substances induced AR antagonist effect in the U2OS AR antagonist CALUX assay (Kamelia et al., 2018). However, the IC50s for this AR antagonist activity showed no meaningful correlation with the 4–5 ring PAHs content ($R^2 = 0$) present in those substances. Moreover, a poor correlation ($R^2 = 0.02$) was found between the IC50s obtained in the AR antagonist CALUX assay of the DMSO extracts of PAH-containing substances and their corresponding in vitro developmental toxicity potency, as evaluated in the mEST (Kamelia et al., 2018, 2017). Based on the above findings, it can be assumed that the AR does not play an important role in mediating the developmental toxicity induced by the 4- and 5-ring PAHs. Therefore, the AR-CALUX assay was not included in the present study.

Nominal concentrations of chemicals with hydrophobic property such as PAHs were reported to be significantly higher than their free concentrations in in vitro test systems, including the ZET (Groothuis et al., 2015; Knöbel et al., 2012). Lipophilic compounds may partition to the cell culture medium constituents, for example, serum protein and lipid, and to the plastic, which consequently affect their biologically effective concentration at the molecular target in vitro. In light of this, an in vitro chemical distribution model developed by Kramer et al. (Kramer et al., 2012, 2022 (manuscript in preparation); Groothuis et al., 2015) was used to estimate actual concentrations of the nine PAHs in the CALUX assays and ZET applied (Supporting Information 9–12). The outcome of these model predictions shows that in the CALUX assays, free concentrations of the tested PAHs are substantially lower than their nominal concentrations, and that a large portion of the PAHs is predicted to bind to the cells and the plastic well plates. For the ZET, which contains no serum in the exposure medium (i.e., egg water), PAHs were predicted to bind to the zebrafish embryos and the plastic well plates. However, it should be noted that

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the relative potencies of the nine PAHs to induce AhR-mediated or ER-α-mediated activities remain the same when using the modeled free fraction of PAHs in the CALUX assays instead of the nominal concentrations (Supporting Information 13–15). The considerable differences between the free available concentration and the nominal concentration should be taken into consideration when using in vitro assay results for example for reverse dosimetry to predict in vivo dose response curves.

To conclude, the present study evaluated the in vitro receptor-mediated activities of nine (un)substituted PAHs and the role of these receptors in mediating the observed developmental retardations by these substances in the ZET. Obtained results show that all nine tested PAHs were strong AhR ligands and only two out of the nine PAHs induced substantial estrogenic activity, whereas none of the tested PAHs showed anti-estrogenic activity or interacted with the RAR. Co-exposure experiments reveal that developmental retardations observed in zebrafish embryos following exposure to the selected PAHs were counteracted to a certain extent by the addition of an AhR or ER-α antagonist. This observation supports the hypothesis that the AhR and/or ER-α may play a role in mediating the developmental toxicity of PAHs. Moreover, the addition of a methyl substituent on the aromatic ring of PAHs appeared to result in increased in vitro AhR-mediated activity, which was not necessarily accompanied by increased but in some cases rather by decreased in vitro developmental toxicity, indicating that AhR activation does not present the only mode of action underlying in vitro developmental toxicity in the ZET.

CONFLICT OF INTEREST

Lenny Kamelia is employed by Shell Global Solutions International B. V., a member company of Concawe, and is totally free (by contract) to design and conduct research and express her own scientific opinion without any obligation towards either Shell or Concawe. The current findings are not intended to constitute any product endorsement. The other authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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