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Methylated PACs Are More Potent Than Their Parent Compounds: A Study of Aryl Hydrocarbon Receptor–Mediated Activity, Degradability, and Mixture Interactions in the H4IIE-*luc* Assay

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Abstract: Twenty-six polycyclic aromatic compounds (PACs; including native polycyclic aromatic hydrocarbons [PAHs], hydroxylated PAHs, alkylated and oxygenated PAHs, and [alkylated] heterocyclic compounds) were investigated for their aryl hydrocarbon receptor (AhR)-mediated potencies in the H4IIE-*luc* bioassay. Potential degradabilities of PACs were investigated by use of various durations of exposure (24, 48, or 72 h), and various mixtures of PACs including PAHs, alkylated and oxygenated PAHs, and heterocyclic compounds were tested for their joint AhR-mediated potency. Additive behaviors of PACs in mixtures were studied by comparing observed mixture potencies with mixture potencies predicted by use of the concentration addition model. Methylated derivatives were more potent than their parent compounds in the H4IIE-*luc* assay. A time-dependent decrease in relative potency was observed for all AhR-active compounds, which may be indicative of in vitro biotransformation. Monomethylated compounds seemed to be more rapidly transformed than analogous unsubstituted compounds. In addition, the results showed that the predictive power of the concentration addition model increased with the number of compounds, suggesting additivity in multicomponent mixtures. Due to the greater potency of methylated derivatives and their ubiquitous occurrence, there is a need for further research on the toxicity and mixture behavior of these environmentally and toxicologically relevant compounds. *Environ Toxicol Chem* 2018;37:1409–1419. © 2018 SETAC

Keywords: Polycyclic aromatic compounds,; Relative potency factors,; In vitro bioassays,; Mixture toxicity

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are continuously introduced into the environment from both natural and human activities, including spills and pyrolysis of organic matter. Due to their close connection to historical industrial activities, PAHs are frequently present at relatively large concentrations in soils and sediments from industrial areas, such as former wood treatment facilities where creosote was used, or abandoned gas works plants (Murphy and Brown 2005; Saber et al. 2006; Lundstedt et al. 2007). The US Environmental Protection Agency has classified 16 PAHs as priority pollutants, and these are routinely analyzed in environmental monitoring programs and risk assessments of PAH-polluted sites. These sites can contain mixtures of PAHs and other important polycyclic aromatic compounds (PACs), such as alkyl-substituted PAHs and heterocyclic compounds, which are not included in analyses of the priority PAHs (Andersson and Achten 2015). Previous research has shown that risk assessments based only on priority PAHs underestimate the load of PAH contamination and overlook potential contaminants present in the environment (Andersson et al. 2009; Larsson et al. 2013). Alkylated PAHs and heterocyclic compounds have been detected in soils, rivers, and sediments (Lundstedt et al. 2007; Vondrácek et al. 2007; Wayland et al. 2008; Sun et al. 2014). Compositions of PACs in the environment depend on the source and age of contamination. Petrogenic sources such as crude oil or coals are usually dominated by alkylated PAHs, whereas pyrogenic sources can contain larger proportions of parent PAHs (Stout et al. 2015). For example, it has been reported that the alkylated derivatives of naphthalene, phenanthrene, fluorenes, fluoranthenes, dibenzothiophene, and chrysenes dominate the PAH profile of heavy fuel oil (a petrogenic source), whereas parent PAHs dominate the profile of creosote (a pyrogenic source; Stout et al. 2015).

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Binding to and activation of the aryl hydrocarbon receptor (AhR) by xenobiotic compounds are important initiating events that result in up-regulation of expression of messenger ribonucleic acid for mixed-function monooxygenase enzymes such as cytochrome P4501A1 (CYP1A1). In some compounds, such as PAHs, increased biotransformation can lead to tumorigenesis (Baird et al. 2005a). Like parent PAHs, some methylated PAHs have been shown to activate the AhR (Villeneuve et al. 2002; Machala et al. 2008; Marvanová et al. 2008) and have also been reported to promote tumorigenesis and to be mutagenic, carcinogenic, and (anti-) estrogenic (Santodonato 1997; Straif et al. 2005).

The use of compound-specific relative potency factors (REPs) is a well-established approach to evaluate biological activities and associated potential adverse outcomes of mixtures of chemicals acting via a common mode of action (Hilscherova et al. 2000). This approach assumes that the individual chemicals interact, often with a protein receptor in a concentration-additive manner. Relative potencies of chemicals are quantified compared with a reference compound (Villeneuve et al. 2002). This principle is well established for dioxin-like compounds: the potencies of compounds are related to the potency of the reference, which is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). To obtain the chemically derived TCDD equivalent of a mixture of AhR-activating compounds, the concentration of each compound is multiplied by its specific REP, and the resulting products are summed to a total chemically derived TCDD equivalent, reflecting the total AhR-mediated potential of the mixture.

A method for predicting the potency of a known mixture is the concentration addition model (Loewe and Muischnek 1926). The concentration addition model, like the toxic equivalency concept, is also based on additive behavior of constituents of a mixture. It has been reported to be an accurate reference model to predict toxicities of mixtures of chemicals that have the same or a similar mode of action (Faust et al. 2001; Backhaus et al. 2004). Additive behavior among PAHs has been reported previously (Larsson et al. 2012, 2014b).

The H4IIE-*luc* bioassay has been shown to be a suitable and valuable analytical tool for screening of AhR-activating compounds in environmental samples and mixture toxicity estimations of PACs in complex mixtures (Larsson et al. 2012, 2014a). The assay integrates the total potencies and mixture interactions of all AhR-activating compounds present in a sample. The measured AhR potency is expressed as bioassay-derived TCDD equivalents (Behnisch et al. 2001). With mass- or potency-balance analysis, which compares bioassay-derived TCDD equivalents with chemically derived TCDD equivalents based on REPs, it is possible to examine how much of the observed effects can be explained by the detected compounds. By selecting several durations of exposure, bioassays with liver cells or enzymes capable of biotransforming xenobiotics can also provide information about persistence and or lability of constituents of mixtures (Masunaga et al. 2004; Larsson et al. 2012, 2014b).

In the environment, PACs usually occur in mixtures, and their interactions might not be additive, such that the biological response might not be predicted based on a summation of partial contributions of individual chemicals. Instead of additive behavior, interactions between and among active and inactive compounds in mixtures can cause increased (supra-additive) or decreased (infra-additive) effects (Giesy and Kannan 1998). Therefore, potencies of mixtures depend not only on absolute and relative concentrations of individual constituent compounds in mixtures but also on interactions between non-AhR and AhR-active constituents in the mixture that affect their chemical activities at the receptor in an organism (Altenburger et al. 2003). Some of the interactions of mixtures of a number of PACs activating AhR-mediated responses have been reported previously (Barata et al. 2005; Larsson et al. 2014a), but little information has been available for mixtures of methylated PAHs and mixtures containing methylated PAHs, parent PAHs, and other PACs such as heterocyclic compounds.

In the present study, 26 native PAHs, hydroxylated PAHs, alkylated and oxygenated PAHs, and (alkylated) heterocyclic compounds were investigated for their AhR-mediated activity in the H4IIE-*luc* bioassay. The structures of the tested compounds can be found in the Supplemental Data (Figures S1 .1 and S1.2). Compounds were chosen based on expected occurrence in the environment and the availability of standards. Different exposure durations (24, 48, and 72 h) were selected to investigate the persistence (i.e., the potential metabolic degradability) of the tested compounds compared with their parent compounds, which is important from a toxicological point of view and for the risk assessment of environmental mixtures. An important aim of the present study was to examine PAC mixture interactions in the H4IIE-*luc* bioassay. Synthetic mixtures with various compositions of PAHs, alkylated PAHs, oxygenated PAHs, and heterocyclic compounds were chosen. To study additive behavior of PACs in the mixtures, observed mixture activities were compared with mixture activities predicted by the use of the concentration addition model. We tested several compounds, (e.g., 1,2,6-, 1,2,8-trimethylphenanthrene and 2,8-dimethyldibenzothiophene) that have not been investigated previously for their AhR-activating potency in the H4IIE-*luc* assay.

MATERIALS AND METHODS

Chemicals

Dimethylsulfoxide (DMSO; 99.5%, CAS no. 67-68-5), benzo[*b*]naphtho[2,1-*d*]furan (99.6%, CAS no. 239-30-5), 7-methylbenzo[*a*]anthracene (N/A, CAS no. 2541-69-7), triphenylene (99%, CAS no. 217-59-4), 7,12-dimethylbenzo[*a*]anthracene (99.9%, CAS no. 57-97-6), 7-methylbenzo[*a*]pyrene (96%, CAS no. 63041-77-0), chrysene (98.4%, CAS no. 218-01-9), and anthracene (97%, CAS no. 120-12-7) were obtained from Sigma-Aldrich. 2-Methylphenanthrene (>99%, CAS no. 2531-84-2), 2,4-dimethylphenanthrene (>99.5%, CAS no. 15254-64-5), 1,2,6-trimethylphenanthrene (>99.5%, CAS no. 30436-55-6), 1,2,8-trimethylphenanthrene (98.5%, CAS no. 20291-75-2), 2-hydroxychrysene (>99%, CAS no. 65945-06-4), 2-methoxychrysene (96.2%, CAS no. 63020-58-6), 6-ethylchrysene (99.3%, CAS no. 2732-58-3), 2,3-dimethyl-9,10-anthraquinone (>99%, CAS no.

6531-35-7), 2-hydroxy-9,10-anthraquinone (99.3%, CAS no. 605-32-3), 9(10H)-acridone (>99.5, CAS no. 578-95-0), 9-methylacridine (99%, CAS no. 611-64-3), dibenzothiophene (99.9%, CAS no. 132-65-0), 2-methyldibenzothiophene (>96.5%, CAS no. 20928-02-3), 2,8-dimethyldibenzothiophene (>96.5%, CAS no. 1207-15-4), 1-methylfluoranthene (98%, CAS no. 25889-60-5), 2,3-dimethylanthracene (99.8%, CAS no. 613-06-9), dinaphtho[1,2-*b*;1′,2′-*d*]furan (>96.0%, CAS no. 207-93-2), and 11H-benzo[*a*]carbazole (99.8%, CAS no. 239-01-0) were purchased from Chiron. 1-Methylchrysene (99.1%, CAS no. 3351-28-8), 2-methylchrysene (99.3%, CAS no. 3351-32-4), and 3-methylchrysene (99.3%, CAS no. 3351-31-3) were obtained from the Institute for Reference Materials and Measurements (Geel, Belgium). The reference compound TCDD (99.1%, CAS no. 1746-01-6) was obtained from AccuStandard. Stock solutions of the individual PACs were prepared in DMSO. Maximum concentrations of tested compounds varied due to solubility and visible cytotoxicity. Most compounds were tested at a concentration of 40 μM. However, 3-methylchrysene and dinaphtho[1,2-*b*;1′,2′-*d*]furan were tested at lesser concentrations to achieve a curve that would be appropriate for determining the REP values. Because of limited solubility, 9(10H-)acridone and 9-methylacridine were also tested at lesser concentrations. The compounds 2,3-dimethylanthracene, 2-hydroxy-9,-19-anthraquinone, 2,3-dimethyl-9,10-anthraquinone, 11H-benzo[*a*]carbazole, and benzonaphthol[2,1-*d*]furan had to be tested at lesser concentrations due to a limited sample volume. Maximum tested concentration in the assay can be found in the Supplemental Data, Table S2.1. All compounds used in the mixtures were prepared in DMSO at a concentration of 10 mM except for dibenzo[*a,h*]acridine, which was prepared at a concentration of 1mM because of limited solubility in DMSO. Six mixtures of PACs were prepared in different combinations of PACs (Table 1). Mixtures 3, 5, and 6 were mixed in equimolar concentration at a 1:1 ratio, resulting in total mixture concentrations of 10 mM. Mixture 1 (a binary mixture of dibenzothiophene and 2-methyldibenzothiophene) and mixture 4 (consisting of methylated PAHs) were mixed in equimolar concentrations at a 1:1 ratio and then diluted with DMSO to obtain the same effective concentrations of those compounds in the mixtures as in mixture 5. Mixture 2 contained 17 compounds including dibenz[*a,h*]acridine at a 1:1 ratio, resulting in a total mixture concentration of 9.5 mM. Mixtures were tested in a 1:4 dilution in the H4IIE-*luc* assay.

H4IIE-luc reporter-gene assay

The H4IIE-*luc* cell line is derived from a rat hepatoma cell line, which has been stably transfected with a firefly luciferase (luc) reporter construct (Murk et al. 1996). Assays with H4IIE-*luc* cells were conducted as described previously (Larsson et al. 2012). In brief, H4IIE-*luc* cells were maintained in α-minimum essential medium, supplemented with 10% fetal bovine serum at 37 °C, 5% CO_2, and 100% humidity. The H4IIE-*luc* cells were plated in triplicate wells at a density of 3 to 6×10^4 cells/well in 96-well plates and exposed after 24 h to a serial dilution of the reference chemical TCDD (0.4–300 pM) or solvent control (DMSO). Test compounds or mixtures were prepared as a serial dilution of 6 or 10 concentrations and were also tested in triplicate wells. The concentration of DMSO in test media exposure was 0.4%. Compounds were tested for 24, 48, or 72 h under the same conditions as those used for culturing. The cells were visually inspected for cytotoxicity before and after exposure to the PACs. Based on microscopic inspection, cytotoxicity was not observed at the concentrations tested.

After 24, 48, or 72 h of exposure, the exposure medium was removed, and the cells were washed twice with 100 μL phosphate-buffered saline. For assessment of luciferase activity,

TABLE 1: Composition of mixtures prepared in nonequivalent effect concentrations (ECs) and the EC level of each polycyclic aromatic compound (PAC) in the mixtures at the greatest concentration tested in the H4IIE-*luc* assay[a]

	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6
Chrysene		EC86				EC78
Benzo[*a*]anthracene		EC81				EC70
Benzo[*a*]pyrene		EC96				EC85
Benzo[*a*]anthracene-7,12-dione		EC18				
Naphtho[2,3-*a*]pyrene		EC94				EC89
1,2,6-Trimethylphenanthrene		EC20	EC52	EC39	EC39	EC39
1,2,8-Trimethylphenanthrene		EC25	EC51	EC41	EC41	EC41
1-Methylchrysene		EC105	EC120	EC115	EC115	EC115
2-Methylchrysene		EC110	EC127	EC121	EC121	EC121
3-Methylchrysene		EC77	EC89	EC85	EC85	EC85
7-Methylbenzo[*a*]anthracene		EC105	EC123	EC117	EC117	EC117
7-Methylbenz[*a*]pyrene		EC100	EC106	EC104	EC104	EC104
6-Ethylchrysene		EC88	EC108	EC102	EC102	EC102
1-Methylfluoranthene				EC9	EC9	
2,8-Dimethyldibenzothiphene		EC16	EC34	EC26	EC26	
Dibenz[*a,h*]acridine		EC95				
Benzo[*a*]fluorenone		EC28				
Dibenzothiophene	EC6				EC6	
2-Methyldibenzothiophene	EC6				EC6	
Greatest tested concentration [μM]	6.8	38	40	33.2	40	40

[a]The ECx values were calculated from the individual PAC concentration–response curves.

which is mediated via ligand activation of the AhR, steadylite substrate mix (PerkinElmer) was used. Cells were lysed with 25 μL of steadylite substrate mix and 25 μL phosphate-buffered saline, and plates were kept in the dark for 20 min to allow complete lysis and enzymatic reaction. For luciferase activity measurement, 30 μL of the cell lysates were transferred to a white 96-well microtiter plate. Luciferase activity was quantified by measuring light produced by use of a microplate luminometer (Fluostar Omega), and the data were evaluated with GraphPad Prism® Ver 5.01.

Calculations

REPs. Results of 3 to 6 independent experiments for each compound were included for the REP calculations. Results of assays were included in calculations when the following criteria were met: the concentration–response curve showed a sigmoidal curve with a positive slope; induction of luciferase was >20% of TCDD maximum induction; the maximum induction factor of the standard (TCDD) compared with the DMSO control was at least 6; and the standard deviation of the triplicate wells on the plate was ≤17%.

The REPs were calculated from the concentration–response curves by relating effect levels of the concentration–response curves of the tested PACs to the same effect level of the concentration–response curve of the reference compound TCDD (Equation 1).

$$\mathrm{REP}_i = \frac{\mathrm{TCDD\ EC}_x}{\mathrm{PAC\ EC}_x} \quad (1)$$

ECx describes the effect concentration of the standard or the compound, and *x* describes the response level of the effect concentration. For example, the REP50 was calculated by dividing the concentration of TCDD that produced 50% of maximum induction by the effect concentration of the PAC that induces 50% of the TCDD maximum induction. In the present study, the relative response factors REP20, REP25, REP50, and REP80 were calculated for PACs at effect concentrations of EC20, EC25, EC50, or EC80 at durations of exposure of 24, 48, or 72 h. To examine parallelism of the concentration–response curves of the TCDD standard and test compounds, variations in response over the range of effect concentrations of EC20 to EC80 (uncertainty range) were determined. Multiple point estimates over the range of effect concentrations of EC20 to EC80 can be used to identify uncertainties in the REP estimates (Villeneuve et al. 2000). The REPs of derivatives were compared with the REPs of parent compounds and derivatives from previous studies (Larsson et al. 2012, 2014b).

Prediction of AhR-mediated potencies of mixtures. The AhR-mediated potencies of mixtures were predicted by use of the concentration addition model, based on the concentration–response curves of methylated PAHs developed during the present study and the same curves of other PACs including native PAHs, oxy-PAHs and azaarenes that have been reported elsewhere (Larsson et al. 2012, 2014b). Data from 3 experiments were pooled and reanalyzed prior to prediction of potencies of mixtures. Pooling of data allows predictions to be based on 3 independent experiments instead of 1 and thus increases the precision of predictions. To ensure comparability of concentration–response curves and to enable pooling of data, the DMSO control was subtracted from the luciferase response to TCDD or tested PACs, and the responses were scaled from 0 to 100% of the TCDD maximum induction. Because compositions of mixtures were known, the concentration of each individual compound in a mixture can be described as a fraction p_i of the *i*th mixture component of the total concentration. The concentration addition model is mathematically expressed as Equation 2

$$\mathrm{ECx}_{\mathrm{mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{\mathrm{ECx}_i}\right)^{-1} \quad (2)$$

where *n* is the number of mixture components, $\mathrm{ECx}_{\mathrm{mix}}$ is the effect concentration of the mixture provoking *x*% effect, and ECx_i denotes the equivalent effect concentration of the *i*th mixture component that is needed to produce the effect *x* on its own.

Concentrations giving 10 to 100% mixture effect were calculated in increments of 5%, and concentration–effect pairs were plotted and analyzed by Graph Pad Prism Ver 5.01 with a sigmoidal concentration–response (variable slope) curve fitting.

Observed AhR-mediated mixture potency. The nonlinear curve regression (sigmoidal concentration–response equation) in GraphPad Prism Ver 5.01 was used to plot concentration–response functions for each PAC mixture. For comparability, expressions of luciferase for mixtures were normalized to the mean of the maximum induction of luciferase for TCDD. Prior to normalization, background light produced by the DMSO control was subtracted from both TCDD and the compound.

RESULTS

Relative efficacies and potencies of PACs in the H4IIE-luc assay

To examine time-dependent effects in the H4IIE-*luc* assay, REPs of 26 PACs, including alkylated, hydroxylated, and oxygenated PAHs and heterocyclic compounds were determined for 3 durations of exposure (Supplemental Data S2, Tables S2.1–2.3). Uncertainty ranges for REPs based on EC20 to EC80 were determined for all 14 compounds that achieved complete concentration–response curves (>80% of the TCDD maximum induction). Uncertainty ranges were less than 10 except for 6 compounds. The REPs derived for 3-methylchrysene, dinaphtho[1,2-*b*;1′,2′-*d*]furan, 7-methylbenzo[*a*]anthracene, and 2-methylchrysene during 24 h of exposure had uncertainty ranges of 40, 11, 21, and 11, respectively, whereas the uncertainty ranges of the REPs for 1,2,6-trimethylphenanthrene and 1,2,8-trimethylphenanthrene during 48 h of exposure were 13 and 51, respectively.

All alkylated PAHs elicited AhR-mediated effects (>30% of the TCDD maximum induction) after 24 h of exposure (Figure 1). A full concentration–response curve (>80% of the TCDD maximum induction) was obtained by 3-methylchrysene after 24 h of exposure, whereas responses of luciferase to 1-methylfluoranthene, 2,3-dimethylanthrancene, 2-methylphenanthrene, and 2,4-dimethylphenanthrene were less than 60% of the TCDD maximum induction. The compounds 1,2,6-trimethylphenanthrene, 1,2,8-trimethylphenanthrene, 1-methylchrysene, 2-methylchrysene, 6-ethylchrysene, 7-methylbenzo[*a*]anthracene, 7,12-dimethylbenzo[*a*]anthracene, and 7-methylbenzo[*a*]pyrene elicited up-regulation of luciferase expression (117–180% of the TCDD maximum induction) after 24 h of exposure, with REP values ranging from 10^{-6} to 10^{-3}. A maximum observed response of a compound that is greater than the maximum observed efficacy of the reference compound is termed superefficacy. Efficacy is the maximum response achievable by a compound. Based on 24 h of exposure, the following rank order of REP values (based on comparisons of EC25 values) for alkylated PAHs was observed: 3-methylchrysene > 2-methylchrysene > 7-methylbenzo[*a*]pyrene > 1-methylchrysene > 7-methylbenzo[*a*]anthracene > 7,12-dimethylbenzo[*a*]anthracene > 6-ethylchrysene > 1,2,8-trimethylphenanthrene > 1,2,6-trimethylphenanthrene > 2,4-dimethylphenanthrene

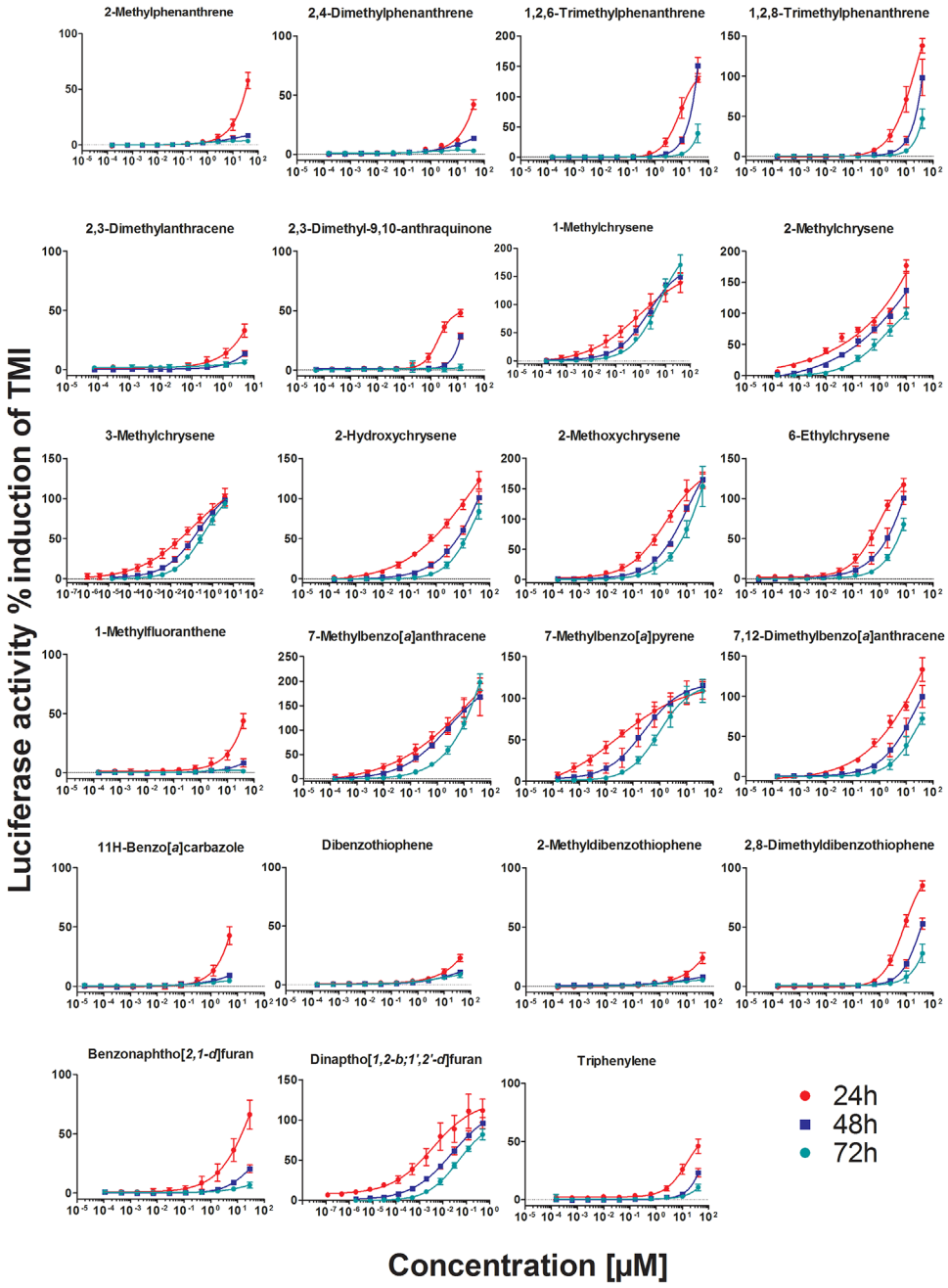

FIGURE 1: Concentration–response curves of aryl hydrocarbon receptor–mediated response of tested polycyclic aromatic compounds at 24, 48, and 72 h of exposure in the H4IIE-*luc* assay. Data represent the results of 3 to 6 independent experiments with 3 replicates of each tested concentration. Error bars represent the mean value and the standard deviation. TMI = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin maximum induction.

> 2-methylphenanthrene, with values ranging from 10^{-7} to 10^{-3}.

Of the tested heterocyclic compounds, only 2,8-dimethyldibenzothiophene and dinaphtho[1,2-*b*;1′,2′-*d*]furan exhibited full concentration–response curves (>80% of the TCDD maximum induction) at 24 h of exposure (Figure 1). Benzo[*b*]naphtho[2,1-*d*]furan, 11H-benzo[*a*]carbazole, and 2-methyldibenzothiophene elicited 66, 46, and 24% of the TCDD maximum induction, respectively. 9-Methylacridine and 9-(10H)acridone exhibited minimal AhR-mediated activity after 24 h of exposure (<10% of the TCDD maximum induction), and the REPs were not calculable. The REP value of the derivative 2,8-dimethyldibenzothiophene was 78-fold greater than the REP value of the parent compound dibenzothiophene. Based on 24 h of exposure, the following rank order of REPs (based on calculation at EC25) for heterocyclic compounds was found: dinaphtho[1,2-*b*;1′,2′-*d*]furan > benzo[*b*]naphtho[2,1-*d*]furan > 2,8-dimethyldibenzothiophene > 11H-benzo[*a*]carbazole > 2-methyldibenzothiophene > dibenzothiophene > 9-methylacridine = 9-(10H)acridone, with values ranging from 10^{-8} to $10^{-2.}$ The other compounds studied, 2-hydroxychrysene and 2-methoxychrysene, elicited superefficacies that were 123 and 162% of the TCDD maximum induction, whereas 2-hydroxy-9,10-anthraquinone was a weak inducer (8% of the TCDD maximum induction). The PAH triphenylene and the dimethylated derivative dimethyl-9,10-anthraquinone exhibited efficacies of 46 and 48% of the TCDD maximum induction, respectively.

Longer durations of exposure (48 or 72 h) resulted in lesser potencies of tested chemicals (Figure 2 and Supplemental Data, S1 and Tables S2.1–2.3). The REPs of monomethylated PAHs decreased faster than the REPs of parent compounds and other derivatives with increasing exposure duration (Figure 2). The REPs of tested PACs based on the EC25 decreased significantly after 48 h of exposure ($p < 0.05$, Student's *t* test) except for benzo[*b*]naphtho[2,1-*d*]furan. After an additional 24 h of exposure (from 48 to 72 h), significant decreases in REPs based on the EC25 ($p < 0.05$, Student's *t* test) were observed for all compounds except for 6-ethylchrysene and 7,12-dimethylbenzo[*a*]anthracene. Superefficacy was observed for 1-methylchrysene, 2-methoxychrysene, 2-methylchrysene, 7-methylbenz[*a*]anthracene, and 7-methylbenz[*a*]pyrene also after 48 or 72 h of exposure. In contrast, lesser efficacy was observed during extended durations of exposure (24–72 h) for 1,2,6-trimethylphenanthrene, 1,2,8-trimethylphenanthrene, 2,8-dimethyldibenzothiophene,6-ethylchrysene, and 7,12-dimethylbenzo[*a*]anthracene. Dibenzothiophene, 2-methyldibenzothiophene, 11H-benzo[*a*]carbazole, 2,3-dimethyl-9,10-anthraquinone, 2,4-dimethylanthracene, 2-methylphenanthrene, 2,4-dimethylphenanthrene, and triphenylene had no quantifiable AhR-mediated effects after 48 or 72 h of exposure (TCDD maximum induction < 25%).

Observed and predicted AhR-mediated potency in mixtures of PACs

In the present study, AhR-mediated potencies of 6 mixtures consisting of various combinations of native PAHs, methylated PAHs, oxygenated PAHs, dibenzothiophenes, and azaareenes were determined by use of the H4IIE-*luc* assay. All mixtures elicited AhR-mediated effect except for the noneffective binary mixture 1 (Figures 3 and 4), which was composed of the weak AhR inducers dibenzothiophene and 2-methyldibenzothiophene. Superefficacy was observed for all AhR-active mixtures. The concentration addition model was used to predict

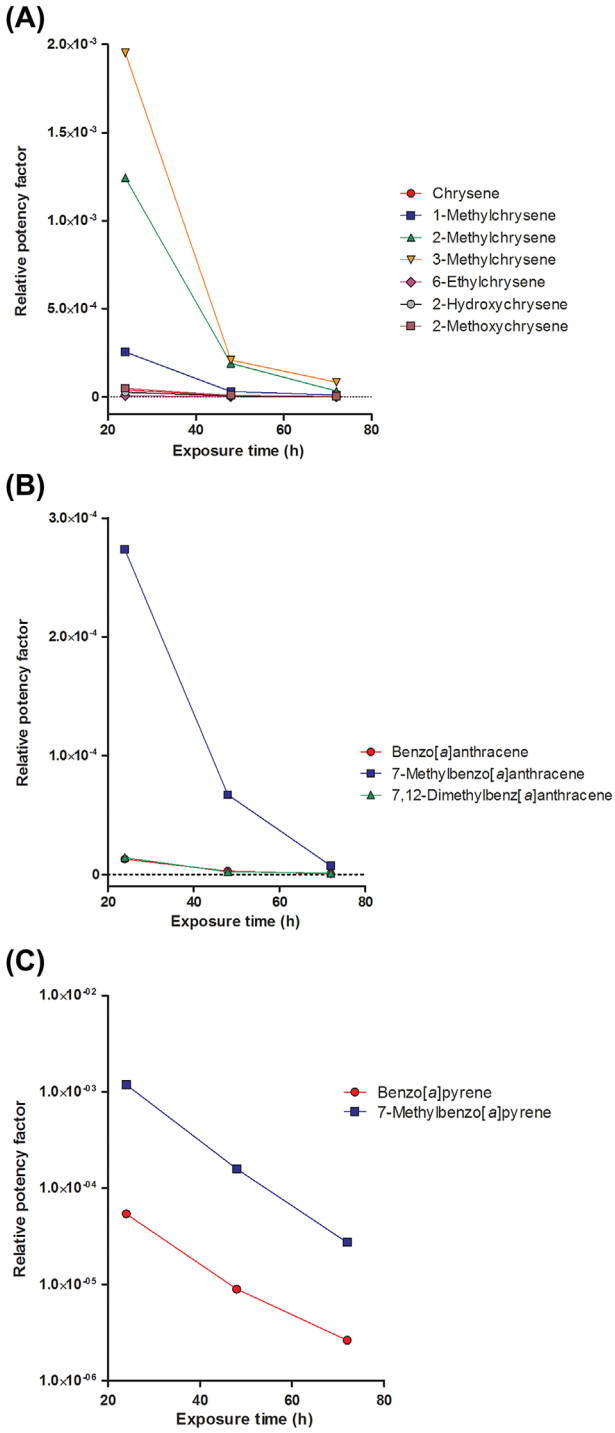

FIGURE 2: Comparison of the exposure–time-dependent relative potency factors of the parent compound based on the 25% effect concentration of (**A**) chrysene, (**B**) benzo[*a*]anthracene, and (**C**) benzo[*a*]pyrene and their derivative(s).

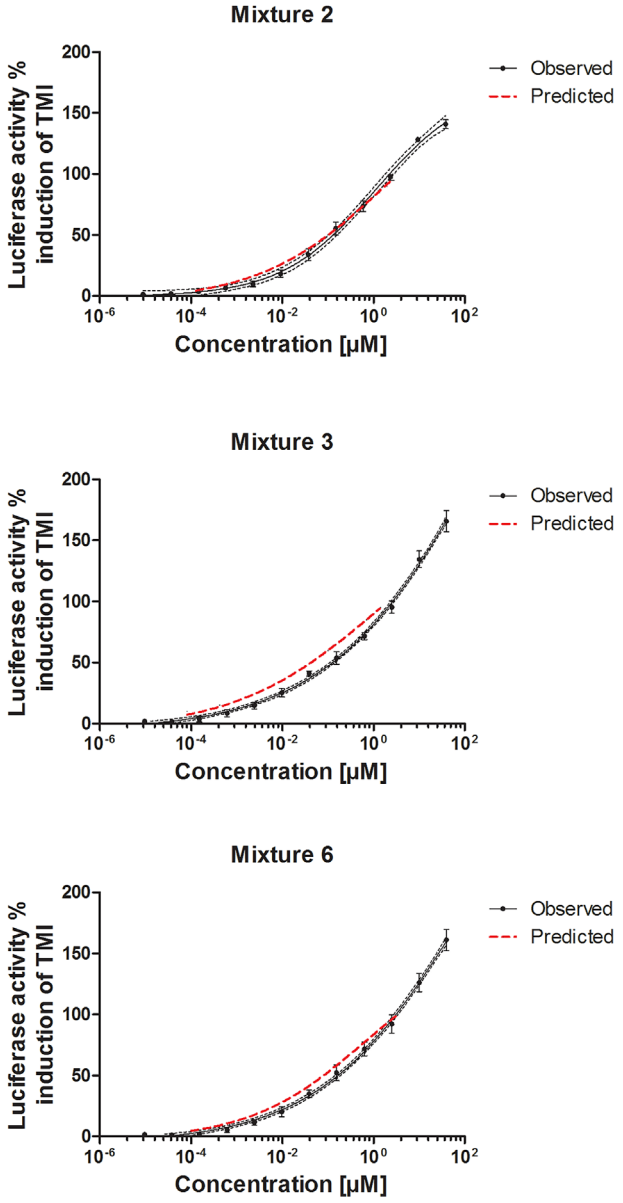

FIGURE 3: Observed and predicted aryl hydrocarbon receptor–mediated activity of polycyclic aromatic compound mixtures after 24 h of exposure in the H4IIE-*luc* assay. The observed data are based on 3 independent experiments consisting of 3 replicates of each exposure concentration. The predicted data obtained by the concentration addition model are based on 3 to 6 independent experiments for each of the individual compounds with triplicate exposures for each concentration. Error bars represent standard deviations, and dashed lines give the 95% confidence intervals of the observed curve. TMI = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin maximum induction.

AhR-mediated potencies of 3 mixtures (mixtures 2, 3, and 6). The prediction was based on the relative predominance of the compounds in the mixtures and their individual concentration–response curves determined in the present or previous studies (Larsson et al. 2012, 2014b). Predictions based on the concentration addition model were limited to the maximum effect observed for the compound with the least luciferase induction in each mixture. Interactions among constituents in mixtures were investigated by comparing potencies of mixtures with potencies predicted by the concentration addition model

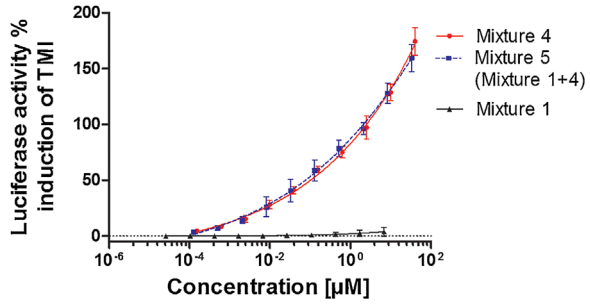

FIGURE 4: Impact of the noneffective mixture 1 (dibenzothiophene and 2-methyldibenzothiophene) on aryl hydrocarbon receptor–mediated response of a mixture of methylated polycyclic aromatic hydrocarbons (mixture 4). Mixture 5 consists of mixtures 1 and 4. Concentration–response curves are based on 3 independent experiments consisting of 3 replicates of each concentration. TMI = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin maximum induction.

(Figure 3). In general, absolute differences between observed and predicted AhR-mediated potencies of mixtures were small. The potency of mixture 2, composed of 16 PACs including PAHs, oxy-PAHs, alkylated PAHs, and N- and S-heterocyclic compounds was slightly overestimated by the concentration addition model in the lesser response portion of the curve, whereas additivity was observed at greater concentrations of the mixture (indicated by an overlapping 95% confidence interval). The observed EC50 value was similar to the predicted EC50 value for the mixture. The AhR-mediated potency of mixture 3, composed of 9 methylated PAHs, was overestimated by the concentration addition model, and the observed EC50 was 2.4-fold greater than the predicted EC50. The observed EC50 of mixture 6, composed of 12 native PAHs and methylated PAHs, was 2.1-fold greater than the predicted EC50 of the mixture. However, the curve of the predicted effect of the mixture overlapped the confidence interval of the observed effect in the lesser and greater effect ranges of the concentration potency curve for the mixture.

The AhR-mediated effect of a binary mixture (mixture 1) composed of S-heterocyclic compounds (dibenzothiophene and 2-methyldibenzothiophene), which was ineffective in activating the AhR, was tested in the H4IIE-*luc* assay. The mixture exhibited no significant AhR-mediated effect (Figure 4). To investigate whether the presence of ineffective dibenzothiophenes altered potencies of mixtures of AhR-active PACs, a mixture composed of 10 methylated PAHs (mixture 4) was tested alone or in combination with mixture 1 (mixture 5). The concentration–response curves of mixture 4 and mixture 5 (the latter composed of mixtures 1 and 4) were similar in shape, and the EC50 of mixture 4 was similar to that of the EC50 of mixture 5.

DISCUSSION

Estimation of REPs is based on assumptions that concentration–response curves of the tested chemical and the reference compound achieve equal efficacy and that slopes of the concentration–response curves are similar, ensuring parallelism of the curves (Lee et al. 2013). Violations of these assumptions

can lead to inaccurate REPs. The uncertainty range, that is, the magnitude of difference in REPs estimated across a range usually between EC20 and EC80, can serve as a confidence interval for REPs (Hilscherova et al. 2000). Uncertainty ranges for most REPs derived for 14 compounds, which elicited full efficacy (>80% of the TCDD maximum induction), were generally small (by a factor $\leq$10) for most estimates of REPs between EC20 and EC80. This finding indicates parallel slopes of the concentration–response curves of the PAC and the corresponding reference compound TCDD. Thus, the REPs derived in the present study should be suitable for use in assay-specific potency-balance analysis. Uncertainty ranges of 3-methylchrysene, 7-methylbenzo[*a*]anthracene, and 2-methylchrysene after 24 h of exposure and 1,2,6-trimethylphenanthrene and 1,2,8-trimethylphenanthrene after 48 h of exposure were greater than 10. In this case of nonparallel concentration–response relationships, single-point REP estimates, for example, REPs based on EC25, can be used in assay-specific potency-balance analysis. However, potency-balance analysis should be applied with careful consideration of the limitations associated with the uncertainties of REPs.

In the present study, REPs of derivatives of PAHs were compared with the REPs of the analogous parent compounds chrysene, benzo[*a*]anthracene, and benzo[*a*]pyrene determined previously by Larsson et al. (2012). The methylated derivatives of chrysene, benzo[*a*]anthracene, and benzo[*a*]pyrene that we tested had significantly greater potencies compared with the parent compounds ($p < 0.5$, Student's *t* test). The presence of a methyl group of PAHs appears to increase the potency after 24 h of exposure compared with an ethyl group, a methoxy group, or a hydroxyl group (Figure 2). Methylated PAHs and some other derivatives are potent agonists of the AhR. Some of them are even more potent than analogous parent compounds. However, hazards and risks posed by PAHs are usually assessed by quantification of only the 16 priority PAHs. Due to their presence and potency, methylated PAHs and heterocyclic compounds can contribute importantly to the unexplained AhR-mediated potency in environmental samples (Keiter et al. 2008; Andersson et al. 2009; Larsson et al. 2013). Methylated PAHs along with heterocyclic aromatic compounds, such as dinaphthofuranes, were identified as major CYP1A-inducing compounds in a contaminated sediment (Brack and Schirmer 2003). Dinaphtho-[1,2-*b*;1′,2′-*d*]furan was the most potent AhR-activating compound in the present study, which is consistent with results of a previous study indicating that dinaphthofuranes are potent inducers of ethoxyresorufin-*O*-deethylase activity in rainbow trout liver cells (RTLW1-cells; Brack and Schirmer 2003). However, the other *O*-heterocyclic compound tested in the present study (benzo[*b*]naphtho[2,1-*d*]furan) was more than 10^4 times less potent than was dinaphtho[1,2-*b*;1′,2′-*d*]furan at all exposure durations. Comparison of benzo[*b*]naphtho[2,1-*d*]furan with the parent compound benzo[*a*]fluorene (Larsson et al. 2014b) showed that benzo[*b*]naphtho[2,1-*d*]furan was significantly less potent than its parent PAH and the oxygenated PAH benzo[*a*]fluorenone (Larsson et al. 2014b) after 24 h of exposure ($p < 0.5$, Student's *t* test). Dibenzothiophene and its methylated derivatives were also weak AhR inducers. However, the dimethylation of dibenzothiophene increased the potency of the parent compound. The derivatives of acridine, 9-methylacridine and 9-(10H)acridone, tested in the present study failed to induce AhR-mediated response in the H4IIE-*luc* assay, which is consistent with results of a previous study, in which those compounds failed to induce ethoxyresorufin-*O*-deethylase activity in breast cancer cells (Lam et al. 2018). However, the small concentrations tested in the present study might be a reason why those compounds were not able to activate the AhR.

Up-regulation of AhR-responsive gene expression including CYP1A1 results in a greater ability to biotransform the inducing compounds, which then engenders their excretion and detoxification (Baird et al. 2005b; Nebert et al. 2004). Longer exposure resulted in a decrease in AhR-mediated potency for all AhR-active compounds, which indicates that the compounds were likely biotransformed by H4IIE-*luc* hepatocytes. Time-dependent changes in REPs in the H4IIE-*luc* assay of the PACs studied differed among compounds depending on their structure and functional group. The results of the present study suggest a potentially greater degradability of monoalkylated PAHs than of unsubstituted PAHs in H4IIE-*luc* hepatocytes. However, in the environment, alkylated PAHs have been reported to be less degradable than unsubstituted PAHs (Lundstedt et al. 2003). This might be because alkylated derivatives are more lipophilic and are therefore stronger sorbed to organic matter. Despite the time-dependent decreases in the REPs of the compounds, some of the derivatives were relatively potent after 72 h of exposure. The decline in REPs or luciferase activity with increased exposure duration is assumed to be a consequence of metabolic degradation of the PAHs in the cells, resulting from the induction of CYP1A1. This assumption is supported by the findings of previous studies (Jones et al. 2000; Larsson et al. 2014b). However, declining REPs can also result from differences in cell density in the wells, potential changes in cell activity because of crowding, or a loss of PACs due to evaporation or adsorption to the plastic wells. Furthermore, chemicals could interfere with the luciferase gene directly via a mechanism that is independent of the AhR.

The REPs of the methylated compounds along with dibenzothiophene after 24 h of exposure were comparable to those determined in earlier studies (Table 2; Vondrácek et al. 2007; Machala et al. 2008; Marvanová et al. 2008; Hinger et al. 2011). The REPs of 2-methylphenanthrene, 7-methylbenzo[*a*]anthracene, and 7,12-dimethylbenzo[*a*]anthracene obtained in the present study were similar compared with those from previous studies (Table 2). The REPs of the 3 monomethylated chrysenes (1-methylchrysene, 2-methylchrysene, and 3-methylchrysene) obtained in the present study were 11- to 19-fold greater than those reported by Machala et al. (2008). To the best of the authors' knowledge, except for 1- and 3-methylchrysene, 6-ethylchrysene, and dibenzothiophene, which have been tested at 72 h of exposure (Lee et al. 2015), the methylated PAHs and heterocyclic compounds included in the present study had not been previously tested for longer term exposures (48 or 72 h) in the H4IIE-*luc* assay. The REPs in both studies are in a similar range, but test conditions such as the DMSO concentration differed between the studies.

TABLE 2: Comparison of relative potency factors (REPs) after 24-h exposure based on 25% effect concentration (EC25) for polycyclic aromatic compounds from the present study in the H4IIE-*luc* assay and from previous studies

	REP25	REP25
2-Methylphenanthrene	1.0×10^{-07}	3.8×10^{-07}[a]
1-Methylchrysene	2.4×10^{-04}	1.2×10^{-05}[b]
2-Methylchrysene	1.2×10^{-03}	7.7×10^{-05}[b]
3-Methylchrysene	2.0×10^{-03}	4.9×10^{-04}[b]
7-Methylbenzo[*a*]anthracene	1.0×10^{-04}	4.4×10^{-04}[c]
7,12-Dimethylbenzo[*a*]anthracene	4.1×10^{-06}	5.4×10^{-06}[c]
Dibenzothiophene	4.5×10^{-08}	9.2×10^{-08}[d]

[a]Vondrácek et al. (2007).
[b]Machala et al. (2008).
[c]Marvanová et al. (2008).
[d]Hinger et al. (2011).

Organisms are rarely exposed to a single chemical in the environment. Therefore, it is important to describe and be able to predict toxic potencies of mixtures of environmentally relevant compounds and obtain important background information that can be used in assessment of complex environmental samples. In the present study, AhR-mediated mixture potencies were investigated by use of the concentration addition model, which is based on the assumption of additive behavior of the mixture components. Interactions in mixtures were investigated by comparison of potencies measured in the H4IIE-*luc* assay with potencies predicted by use of the concentration addition model. The concentration addition model was able to predict the AhR-mediated potency of the mixture composed of 16 PACs (mixture 2), but tended to slightly overestimate the AhR-mediated potencies of 2 other mixtures (3 and 6), which were composed of 9 and 12 compounds, respectively. The results suggest infra-additive effects in mixtures 3 and 6, which indicates a weak inhibitory effect on the AhR-mediated potency in the mixtures. Both infra- and supra-additive AhR-mediated effects of mixtures of PACs have been observed previously (Larsson et al. 2012, 2014a). Additive interactions of AhR agonists were suggested for mixture 2, composed of 16 PACs. This result is in agreement with the results of other studies, which have shown that the predictive power of the concentration addition model increased with the increasing number of compounds in the mixture (Warne and Hawker 1995; Kortenkamp et al. 2009). Multicomponent mixtures can elicit both infra- and supra-additive effects simultaneously, but the overall effect might be near additivity because those effects cancel each other out (Kortenkamp et al. 2009). The concentration–response curve of mixture 5 (composed of mixtures 1 and 4) differed only marginally compared with mixture 4, which suggests that the noneffect mixture containing the weak AhR inducers dibenzothiophene and 2-methyldibenzothiophene will not affect the AhR-mediating potency of methylated PAHs.

Superefficacy is reported in the present study and in previous studies (Seidel et al. 2001; Larsson et al. 2014b). However, the responsible mechanism is not yet well understood; different mechanisms have been proposed such as inhibition of AhR degradation by proteolysis, leading to an increase in the intracellular levels of ligand-activated AhR (Ma and Baldwin 2002). This has been proposed to enhance AhR-dependent gene expression. It has also been proposed that inhibition of expression or enhanced degradation of a labile repressor enhances AhR-dependent gene transcription (Ma and Baldwin 2002). Whether the studied compounds are able to inhibit the synthesis of the repressor or AhR degradation or if other mechanisms are involved is unknown. In addition, the impact of such compounds in mixtures needs to be studied further.

CONCLUSIONS

Our results suggest that alkylated PAHs and naphthofuranes can be a potential risk to the environment and human health due to their potential toxicity. Time-dependent decreases in REPs were observed for all active compounds; the decreases differed depending on molecular structure and functional group. The predictive power of the concentration addition model increased with numbers of mixture components, and the best accuracy was found for the multicomponent mixture composed of native, methylated, and oxygenated PAHs and N- and S-heterocyclic compounds. This finding shows additive interactions of the compounds and that the concentration addition model is a powerful and valuable tool for prediction of AhR-mediated activity of mixtures in the H4IIE-*luc* bioassay.

The risk of PAHs is usually assessed by analysis of the 16 priority PAHs; such an analysis can overlook other important contaminants. Due to similar sources of origin or formations from parent PAHs, alkylated PAHs, oxygenated PAHs, and heterocyclic compounds are present in the environment and are thus ubiquitous. The results of the present study indicate that several PACs (e.g., alkylated PACs, hydroxylated PAHs, and naphthofurans) can contribute significantly to the AhR-mediated potencies observed in samples from contaminated areas. The use of reporter gene assays such as the H4IIE-*luc* assay as a complementary tool to chemical analysis avoids an underestimation of the risk, because such assays integrate total potencies and mixture interactions of all compounds present in a sample that act via a common mode of action. Toxicological information on alkylated PAHs and heterocyclic compounds is currently limited, and further studies are required to investigate their mode of actions and joint potencies in mixtures. The individual REPs determined in the present study are suitable for use in potency-balance analysis and risk assessment of complex mixtures of PACs.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4087.

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Data availability—Data are available on request to the corresponding author (Monika.Lam@oru.se) or the last author (Maria.Larsson@oru.se).

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Running head: Methylated PAHs are more potent than their parent compounds

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Supporting information: Methylated PAHs are more potent than their parent compounds –a study on AhR-mediated activity, degradability and mixture interactions in the H4IIE-luc assay

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S1. Structures of studied compounds

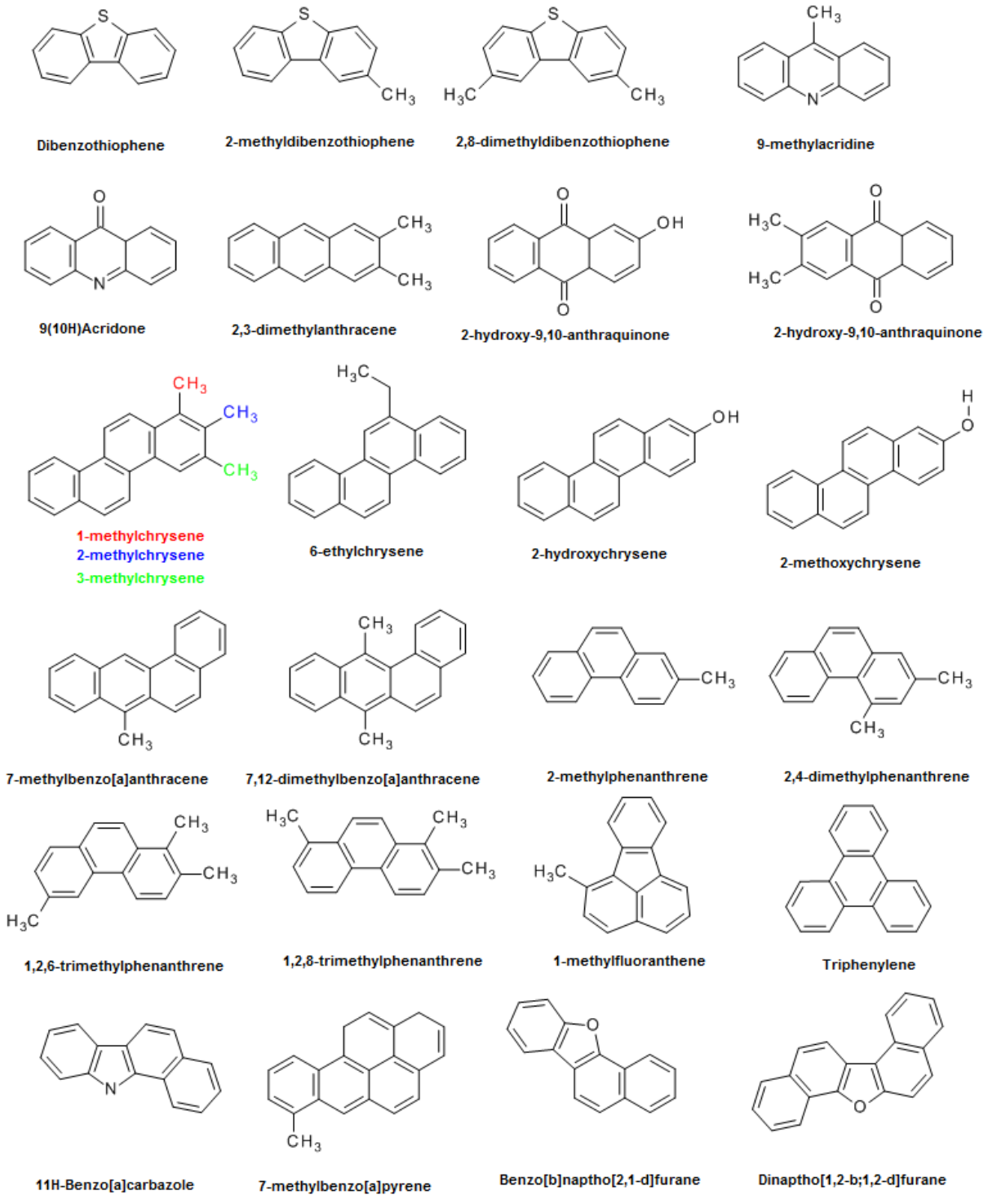

Figure S1.1 Structure of compounds in this study

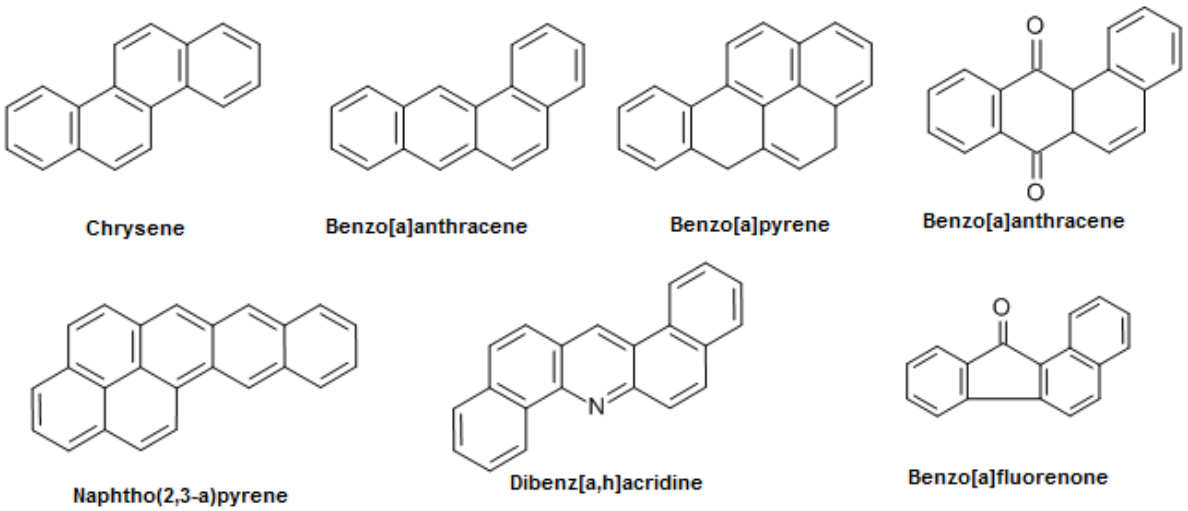

Figure S1.2 Structure of additional compounds studied in the mixture studies.

S2. Relative potency factor derived from 24, 48 and 72 h

Table S2.1. Relative potency factors (REP) (mean of three to six independent experiment) based on the effective concentration (EC_{25}, EC_{50} and EC_{20}-EC_{80}) for polycyclic aromatic compounds (PACs) derived from 24 h exposure of H4IIE-*luc* assay.

			24 h		
	$Conc_{max}$[µM]	TMI_{max} (%)	EC_{25}	EC_{50}	EC_{20}-EC_{80}
Dibenzothiophene	40	23	$4.6x10^{-08}$ ($\pm1.4x10^{-08}$)	nq	$5.2x10^{-08}$ - nq ($\pm1.4x10^{-08}$ - nq)
2-Methyldibenzothiophene	40	24	$5.2x10^{-08}$ ($\pm1.3x10^{-08}$)	nq	$6.0x10^{-08}$ -nq ($\pm1.3x10^{-08}$-nq)
2,8-Dimethyldibenzothiophene	40	85	7.6x10-^{07} ($\pm1.2x10^{-07}$)	$8.9x10^{-07}$ ($\pm1.0x10^{-07}$)	$7.4x10^{-07}$-$1.1x10^{-06}$($\pm1.3x10^{-07}$-2.5x10-^{07})
9-Methylacridine	3.4	8	nq	nq	nq
9(10H)-Acridone	2.6	8	nq	nq	nq
2,3-Dimethylanthracene	5	33	$1.1x10^{-06}$ ($\pm7.8x10^{-07}$)	nq	$1.2x10^{-06}$ - nq ($\pm3.2x10^{-07}$ - nq)
2-Hydroxy-9,10-anthraquinone	20	4	nq	nq	nq
2,3-Dimethyl-9,10-anthraquinone	13	48	$4.1x10^{-07}$ ($\pm1.5x10^{-07}$)	nq	$4.0x10^{-07}$ -nq ($\pm1.6x10^{-07}$-nq)
1-Methylchrysene	40	140	$2.5x10^{-04}$ ($\pm1.7x10^{-05}$)	$9.9x10^{-05}$ ($\pm2.2x10^{-05}$)	$3.5x10^{-04}$ -$6.9x10^{-05}$ ($\pm5.6x10^{-05}$-$2.8x10^{-05}$)
2-Methylchrysene	40	155	$1.2x10^{-03}$ ($\pm4.2x10^{-04}$)	$3.9x10^{-04}$ ($\pm2.3x10^{-04}$)	$1.7x10^{-03}$ -$1.6x10^{-04}$ ($\pm5.7x10^{-04}$-$1.5x10^{-04}$)

Continuation of table S2.1. Relative potency factors (mean of three to six independent experiment) based on the effective concentration (EC_{25}, EC_{50} and EC_{20}-EC_{80}) for polycyclic aromatic compounds (PACs) derived from 24 h exposure of H4IIE-*luc* assay.

			24h		
	$Conc_{max}$[µM]	TMI_{max} (%)	EC_{25}	EC_{50}	EC_{20}-EC_{80}
3-Methylchrysene	4	101	$2.0x10^{-03}$ ($\pm1.2x10^{-03}$)	$3.5x10^{-04}$ ($\pm1.5x10^{-04}$)	$3.3x10^{-03}$ -$8.2x10^{-05}$ ($\pm2.3x10^{-03}$-$4.1x10^{-05}$)
6-Ethylchrysene	40	117	$5.0x10^{-06}$ ($\pm1.4x10^{-06}$)	$4.2x10^{-06}$ ($\pm7.5x10^{-07}$)	$5.5x10^{-06}$ -$4.9x10^{-06}$ ($\pm1.8x10^{-06}$-$1.4x10^{-06}$)
2-Hydroxychrysene	40	123	$2.6x10^{-05}$ ($\pm4.2x10^{-06}$)	$8.8x10^{-06}$ ($\pm9.5x10^{-07}$)	$3.7x10^{-05}$ -$5.3x10^{-06}$ ($\pm7.1x10^{-06}$-$6.4x10^{-07}$)
2-Methoxychrysene	40	162	$4.9x10^{-05}$ ($\pm1.3x10^{-05}$)	$3.2x10^{-05}$ ($\pm4.1x10^{-06}$)	$5.9x10^{-05}$ -$3.9x10^{-05}$ ($\pm1.5x10^{-05}$-$4.8x10^{-06}$)
7-Methylbenz[a]anthracene	40	180	$2.7x10^{-04}$ ($\pm1.6x10^{-04}$)	$1.0x10^{-04}$ ($\pm4.8x10^{-05}$)	$3.9x10^{-04}$ -$8.1x10^{-05}$ ($\pm2.6x10^{-04}$-$3.5x10^{-05}$)
7,12-Dimethylbenz[a]anthracene	40	133	$1.4x10^{-05}$ ($\pm3.3x10^{-06}$)	$5.7x10^{-06}$ ($\pm8.0x10^{-07}$)	$1.9x10^{-05}$ -$4.2x10^{-06}$ ($\pm5.5x10^{-06}$-$5.5x10^{-07}$)
2-Methylphenanthrene	40	57	$1.5x10^{-07}$ ($\pm5.7x10^{-08}$)	nq	$1.6x10^{-07}$ -nq ($\pm6.2x10^{-08}$-nq)
2,4-Dimethylphenanthrene	40	42	$1.3x10^{-07}$ ($\pm1.7x10^{-08}$)	nq	$1.2x10^{-07}$ -nq ($\pm2.0x10^{-08}$-nq)
1,2,6-Trimethylphenanthrene	40	128	$9.4x10^{-07}$ ($\pm1.8x10^{-07}$)	$1.5x10^{-06}$ ($\pm3.5x10^{-07}$)	$8.6x10^{-07}$ -$3.2x10^{-06}$ ($\pm1.5x10^{-07}$-$8.0x10^{-07}$)
1,2,8-Trimethylphenanthrene	40	138	$1.0x10^{-06}$ ($\pm2.4x10^{-07}$)	$1.3x10^{-06}$ ($\pm4.1x10^{-07}$)	$1.0x10^{-06}$ -$2.8x10^{-06}$ ($\pm2.3x10^{-07}$-$1.0x10^{-06}$)
1-Methylfluoranthene	40	44	$1.7x10^{-07}$ ($\pm3.8x10^{-08}$)	$1.8x10^{-07}$ ($\pm4.6x10^{-08}$)	$1.8x10^{-07}$ -nq ($\pm2.3x10^{-07}$-nq)

Continuation of table S2.1. Relative potency factors (mean of three to six independent experiment) based on the effective concentration (EC_{25}, EC_{50} and EC_{20}-EC_{80}) for polycyclic aromatic compounds (PACs) derived from 24 h exposure of H4IIE-*luc* assay.

			24h		
	$Conc_{max}$[µM]	TMI_{max} (%)	EC_{25}	EC_{50}	EC_{20}-EC_{80}
Triphenylene	40	46	$2.9x10^{-07}$ ($\pm5.7x10^{-08}$)	$1.9x10^{-07}$ ($\pm6.5x10^{-08}$)	$3.1x10^{-07}$ -nq ($\pm3.6x10^{-08}$-nq)
11H-Benzo[a]carbazole	5	43	$1.0x10^{-06}$ ($\pm1.6x10^{-07}$)	nq	$9.9x10^{-07}$ -nq ($\pm1.6x10^{-07}$-nq)
7-Methylbenz[a]pyrene	40	109	$1.2x10^{-03}$ ($\pm4.9x10^{-04}$)	$3.2x10^{-04}$ ($\pm1.2x10^{-04}$)	$1.6x10^{-03}$ -$7.7x10^{-05}$ ($\pm7.4x10^{-04}$-$3.1x10^{-05}$)
Benzonaphtho[2,1-d]furan	29	66	$6.9x10^{-07}$ ($\pm3.9x10^{-07}$)	$4.3x10^{-07}$ ($\pm1.1x10^{-07}$)	$8.3x10^{-07}$ -$3.8x10^{-07}$ ($\pm5.5x10^{-07}$-$5.8x10^{-08}$)
Dinaphtho[1,2-b;1',2'-d]furan	0.5	107	$3.0x10^{-02}$ ($\pm1.8x10^{-02}$)	$8.2x10^{-03}$ ($\pm5.3x10^{-03}$)	$5.1x10^{-02}$ -$4.5x10^{-03}$ ($\pm3.0x10^{-02}$-$4.4x10^{-03}$)

nq = not quantifiable; $\%TMI_{max}$ = %TCDD maximum induction of highest tested concentration of the tested compound

Table S2.2. Relative potency factors (REP) (mean of three to six independent experiment) based on the effective concentration (EC_{25}, EC_{50} and EC_{20}-EC_{80}) for polycyclic aromatic compounds (PACs) derived from 48 h exposure of H4IIE-*luc* assay.

			48h		
	$Conc_{max}$[µM]	TMI_{max} (%)	EC_{25}	EC_{50}	EC_{20}-EC_{80}
Dibenzothiophene	40	11	nq	nq	
2-Methyldibenzothiophene	40	8	nq	nq	
2,8-Dimethyldibenzothiophene	40	53	$3.3x10^{-07}$ ($\pm 7.5x10^{-08}$)	$4.3x10^{-07}$ ($\pm 1.6x10^{-07}$)	$3.2x10^{-07}$ -nq ($\pm 5.6x10^{-08}$-nq)
9-Methylacridine	3.4	3	nq	nq	nq
9(10H)-Acridone	-	-	-	-	-
2,3-Dimethylanthracene	5	13	nq	nq	nq
2-Hydroxy-9,10-anthraquinone	-	-	-	-	-
2,3-Dimethyl-9,10-anthraquinone	13	28	$3.4x10^{-07}$ ($\pm 5.9x10^{-08}$)	nq	2.9x10-07(±3.8x10-08)
1-Methylchrysene	40	149	$3.0x10^{-05}$ ($\pm 8.3x10^{-06}$)	$3.3x10^{-05}$ ($\pm 8.3x10^{-06}$)	$3.3x10^{-05}$ -$4.0x10^{-05}$ ($\pm 1.0x10^{-05}$-$8.1x10^{-06}$)
2-Methylchrysene	40	203	$1.9x10^{-04}$ ($\pm 5.0x10^{-05}$)	$6.1x10^{-05}$ ($\pm 1.7x10^{-06}$)	$2.7x10^{-04}$ -$4.7x10^{-05}$ (±8.9x10-05-$1.2x10^{-05}$)
3-Methylchrysene	4	98	$2.1x10^{-04}$ ($\pm 5.9x10^{-05}$)	$9.9x10^{-05}$ ($\pm 2.5x10^{-05}$)	$2.7x10^{-04}$ -$6.9x10^{-05}$ ($\pm 8.5x10^{-05}$-$9.2x10^{-07}$)
6-Ethylchrysene	40	100	$1.6x10^{-07}$ ($\pm 8.2x10^{-07}$)	$1.7x10^{-06}$ ($\pm 5.5x10^{-07}$)	$1.7x10^{-06}$ -$3.9x10^{-06}$ ($\pm 9.7x10^{-07}$-$2.2x10^{-06}$)

Continuation of table S2.2 Relative potency factors (REP) (mean of three to six independent experiment) based on the effective concentration (EC_{25}, EC_{50} and EC_{20}-EC_{80}) for polycyclic aromatic compounds (PAC) derived from 48 h exposure of H4IIE-*luc* assay.

			48h		
	$Conc_{max}$[µM]	TMI_{max} (%)	EC_{25}	EC_{50}	EC_{20}-EC_{80}
2-Hydroxychrysene	40	101	$4.4x10^{-06}$ ($\pm 2.2x10^{-06}$)	$3.9x10^{-06}$ ($\pm 2.6x10^{-06}$)	$5.0x10^{-06}$ -$6.4x10^{-06}$ ($\pm 2.4x10^{-06}$-$6.0x10^{-06}$)
2-Methoxychrysene	40	165	$9.2x10^{-06}$ ($\pm 3.6x10^{-07}$)	$1.4x10^{-05}$ ($\pm 6.1x10^{-06}$)	$9.2x10^{-06}$ -$4.8x10^{-05}$ ($\pm 4.9x10^{-07}$-$3.3x10^{-05}$)
7-Methylbenz[a]anthracene	40	168	$8.1x10^{-05}$ ($\pm 4.5x10^{-05}$)	$3.9x10^{-05}$ ($\pm 1.5x10^{-05}$)	$8.1x10^{-05}$ -$4.0x10^{-05}$ ($\pm 4.5x10^{-05}$-$2.2x10^{-05}$)
7,12-Dimethylbenz[a]anthracene	40	100	$2.0x10^{-06}$ ($\pm 6.7x10^{-07}$)	$1.9x10^{-06}$ ($\pm 3.5x10^{-07}$)	$2.2x10^{-06}$ -$3.0x10^{-06}$ ($\pm 7.7x10^{-07}$-$4.0x10^{-07}$)
2-Methylphenanthrene	40	9	nq	nq	nq
2,4-Dimethylphenanthrene	40	14	nq	nq	nq
1,2,6-Trimethylphenanthrene	40	151	$5.6x10^{-07}$ ($\pm 4.5x10^{-08}$)	$1.4x10^{-06}$ ($\pm 5.4x10^{-07}$)	$4.7x10^{-07}$ -$6.4x10^{-06}$ ($\pm 1.9x10^{-08}$-$4.9x10^{-06}$)
1,2,8-Trimethylphenanthrene	40	98	$3.8x10^{-07}$ ($\pm 6.0x10^{-08}$)	$8.3x10^{-07}$ ($\pm 1.8x10^{-07}$)	$3.2x10^{-07}$ -$1.7x10^{-05}$ ($\pm 5.1x10^{-08}$-$3.3x10^{-05}$)
1-Methylfluoranthene	40	8	nq	nq	nq
Triphenylene	40	23	nq	nq	nq
11H-Benzo[a]carbazole	5	9	nq	nq	nq
7-Methylbenz[a]pyrene	40	115	$1.6x10^{-04}$ ($\pm 7.6x10^{-05}$)	$8.6x10^{-05}$ ($\pm 4.9x10^{-05}$)	$2.0x10^{-04}$ -$6.3x10^{-05}$ ($\pm 9.6x10^{-05}$-$4.9x10^{-05}$)

Continuation of table S2.2 Relative potency factors (REP) (mean of three to six independent experiment) based on the effective concentration (EC_{25}, EC_{50} and EC_{20}-EC_{80}) for polycyclic aromatic compounds (PAC) derived from 48 h exposure of H4IIE-*luc* assay.

			48h		
	$Conc_{max}$[µM]	TMI_{max} (%)	EC_{25}	EC_{50}	EC_{20}-EC_{80}
Benzonaphtho[2,1-d]furan	29	25	$1.5x10^{-07}$ ($\pm 1.0x10^{-07}$)	nq	$1.1x10^{-07}$-nq ($\pm 8.2x10^{-08}$-nq)
Dinaphtho[1,2-b;1',2'-d]furan	0.5	96	$2.5x10^{-03}$ ($\pm 5.8x10^{-04}$)	1.2x10-03 ($\pm 5.6x10^{-05}$)	$3.2x10^{-03}$-$5.2x10^{-04}$ ($\pm 9.5x10^{-04}$-$9.6x10^{-06)}$

nq = not quantifiable; $\%TMI_{max}$ = % TCDD maximum induction of highest tested concentration of the tested compound

Table S2.3. Relative potency factors (REP (mean of three to six independent experiment) based on the effective concentration (EC_{25}, EC_{50} and EC_{20}-EC_{80}) for polycyclic aromatic compounds derived from 72 h exposure of H4IIE-*luc* assay.

			72h		
	$Conc_{max}$[µM]	TMI_{max} (%)	EC_{25}	EC_{50}	EC_{20}-EC_{80}
Dibenzothiophene	40	9	nq	nq	nq
2-Methyldibenzothiophene	40	5	nq	nq	nq
2,8-Dimethyldibenzothiophene	40	28	nq	nq	$1.72x10^{-07}$ - nq ($\pm 5.4x10^{-08}$-nq)
9-Methylacridine	3.4	2	nq	nq	nq
9(10H)-Acridone	-	-	-	-	-
2,3-Dimethylanthracene	5	6	nq	nq	nq
2-Hydroxy-9,10-anthraquinone	-	-	-	-	-
2,3-Dimethyl-9,10-anthraquinone	13	2	nq	nq	nq
1-Methylchrysene	40	170	$9.8x10^{-06}$ ($\pm 3.4x10^{-06}$)	$1.5x10^{-05}$ ($\pm 6.7x10^{-06}$)	$9.6x10^{-06}$ -$4.2x10^{-05}$ ($\pm 3.1x10^{-06}$-$2.5x10^{-05}$)
2-Methylchrysene	40	170	$3.2x10^{-05}$ ($\pm 1.4x10^{-05}$)	$1.7x10^{-05}$ ($\pm 3.1x10^{-06}$)	$4.3x10^{-05}$ -$2.5x10^{-05}$ ($\pm 2.0x10^{-05}$-$1.1x10^{-05}$)
3-Methylchrysene	4	93	$8.4x10^{-05}$ ($\pm 4.8x10^{-06}$)	$6.2x10^{-05}$ ($\pm 6.6x10^{-06}$)	$9.1x10^{-05}$ -$3.8x10^{-05}$ ($\pm 6.6x10^{-06}$-$6.8x10^{-06}$)
6-Ethylchrysene	40	67	$4.8x10^{-07}$ ($\pm 1.2x10^{-07}$)	$7.7x10^{-07}$ ($\pm 2.7x10^{-07}$)	$4.5x10^{-07}$ -$1.9x10^{-06}$ ($\pm 1.0x10^{-07}$-$1.5x10^{-06}$)

Continuation of Table S2.3. Relative potency factors (mean of three to six independent experiment) based on the effective concentrations (EC_{25}, EC_{50} and EC_{20}-EC_{80}) for polycyclic aromatic compounds derived from 72 h exposure of H4IIE-*luc* assay.

			72h		
	$Conc_{max}$[µM]	TMI_{max} (%)	EC_{25}	EC_{50}	EC_{20}-EC_{80}
2-Hydroxychrysene	40	84	$2.0x10^{-06}$ ($\pm1.7x10^{-06}$)	$2.6x10^{-06}$ ($\pm2.0x10^{-06}$)	$1.9x10^{-06}$ -$4.9x10^{-06}$ ($\pm1.6x10^{-06}$-$4.1x10^{-06}$)
2-Methoxychrysene	40	154	$3.8x10^{-06}$ ($\pm1.7x10^{-06}$)	$5.4x10^{-06}$ ($\pm3.1x10^{-06}$)	$3.8x10^{-06}$ -$1.5x10^{-05}$ ($\pm1.7x10^{-06}$-$1.3x10^{-05}$)
7-Methylbenz[a]anthracene	40	191	$7.4x10^{-06}$ ($\pm7.6x10^{-07}$)	$6.5x10^{-06}$ ($\pm1.0x10^{-06}$)	$8.2x10^{-06}$ -$1.1x10^{-05}$ ($\pm9.5x10^{-07}$-$4.1x10^{-06}$)
7,12-Dimethylbenz[a]anthracene	40	72	$1.1x10^{-06}$ ($\pm6.2x10^{-07}$)	$1.2x10^{-06}$ ($\pm6.0x10^{-07}$)	$1.0x10^{-06}$ -$1.1x10^{-06}$ ($\pm6.1x10^{-07}$-$7.2x10^{-07}$)
2-Methylphenanthrene	40	3	nq	nq	nq
2,4-Dimethylphenanthrene	40	3	nq	nq	nq
1,2,6-Trimethylphenanthrene	40	39	$1.6x10^{-07}$ ($\pm7.0x10^{-08}$)	nq	$1.4x10^{-07}$ -nq ($\pm5.1x10^{-08}$-nq)
1,2,8-Trimethylphenanthrene	40	47	$1.8x10^{-07}$ ($\pm3.5x10^{-09}$)	nq	$1.6x10^{-07}$ -nq ($\pm1.1x10^{-08}$-nq)
1-Methylfluoranthene	40	1	nq	nq	nq
Triphenylene	40	11	nq	nq	nq
11H-Benzo[a]carbazole	5	4	nq	nq	nq
7-Methylbenz[a]pyrene	40	108	$2.7x10^{-05}$ ($\pm9.4x10^{-06}$)	$2.3x10^{-05}$ ($\pm5.8x10^{-06}$)	$2.9x10^{-05}$ -$2.0x10^{-05}$ ($\pm1.0x10^{-05}$-$6.4x10^{-06}$)

Continuation of Table S2.3. Relative potency factors (mean of three to six independent experiment) based on the effective concentrations (EC_{25}, EC_{50} and EC_{20}-EC_{80}) for polycyclic aromatic compounds derived from 72 h exposure of H4IIE-*luc* assay.

			72h		
	$Conc_{max}$[µM]	TMI_{max} (%)	EC_{25}	EC_{50}	EC_{20}-EC_{80}
Benzonaphtho[2,1-d]furan	29	7	nq	nq	nq
Dinaphtho[1,2-b;1',2'-d]furan	0.5	80	$5.6x10^{-04}$ ($\pm 4.5x10^{-05}$)	$3.7x10^{-04}$ ($\pm 7.9x10^{-05}$)	$6.1x10^{-04}$ –nq ($\pm 6.7x10^{-05}$-nq)

nq = not quantifiable; $\%TMI_{max}$ = % TCDD maximum induction of highest tested concentration of the tested compound