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Measured and predicted affinities of binding and relative potencies to activate the AhR of PAHs and their alkylated analogues

Sangwoo Lee [a], Woong-Hee Shin [b], Seongjin Hong [c], Habyeong Kang [a], Dawoon Jung [a], Un Hyuk Yim [d], Won Joon Shim [d], Jong Seong Khim [c], Chaok Seok [b], John P. Giesy [e,f,g], Kyungho Choi [a,*]

[a] School of Public Health, Seoul National University, Seoul, South Korea
[b] Department of Chemistry, Seoul National University, Seoul, South Korea
[c] School of Earth and Environmental Sciences & Research Institute of Oceanography, Seoul National University, Seoul, South Korea
[d] Oil and POPs Research Group, Korea Institute of Ocean Science and Technology (KIOST), Geoje, South Korea
[e] Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, SK, Canada
[f] Department of Zoology, and Center for Integrative Toxicology, Michigan State University, East Lansing, MI, USA
[g] Department of Biology & Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, Kowloon, Hong Kong Special Administrative Region

HIGHLIGHTS

- AhR activation potencies were evaluated by H4IIE-*luc* assay for 17 (alkylated) PAHs.
- AhR activation potencies were also estimated by an *in silico* dock model.
- ReP for AhR varied significantly by type of (alkylated) PAHs.
- Estimated binding distance in ligand binding domain was correlated with ReP.
- This *in silico* model could be used to predict toxicities mediated by AhR.

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) and their alkylated forms are important components of crude oil. Both groups of PAHs have been reported to cause dioxin-like responses, mediated by aryl hydrocarbon receptor (AhR). Thus, characterization of binding affinity to the AhR of unsubstituted or alkylated PAHs is important to understand the toxicological consequences of oil contamination on ecosystems. We investigated the potencies of major PAHs of crude oil, e.g., chrysene, phenanthrene and dibenzothiophene, and their alkylated forms ($n = 17$) to upregulate expression of AhR-mediated processes by use of the H4IIE-*luc* transactivation bioassay. In addition, molecular descriptors of different AhR activation potencies among PAHs were investigated by use of computational molecular docking models. Based on responses of the H4IIE-*luc in vitro* assay, it was shown that potencies of PAHs were determined by alkylation in addition to the number and conformation of rings. Potencies of AhR-mediated processes were generally greater when a chrysene group was substituted, especially in 1-methyl-chrysene. Significant negative correlations were observed between the *in vitro* dioxin-like potency measured in H4IIE-*luc* cells and the binding distance estimated from the *in silico* modeling. The difference in relative potency for AhR activation observed among PAHs and their alkylated forms could be explained by differences among binding distances in the ligand binding domain of the AhR caused by alkylation. The docking model developed in the present study may have utility in predicting risks of environmental contaminants of which toxicities are mediated by AhR binding.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) and their alkylated forms are common constituents of crude oil (Hong et al., 2012; Pampanin and Sydnes, 2013). For example, a range between 4.64 and 8.50 mg/g of total PAHs including alkylated PAHs was detected

* Corresponding author at: School of Public Health, Seoul National University, Gwanak, Seoul 151-742, South Korea.
E-mail address: kyungho@snu.ac.kr (K. Choi).

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in three types of cargo oils (Yim et al., 2011). Therefore PAHs and alkylated PAHs are frequently detected in the environment contaminated with spilled oil. In the environment, spilled oil is subject to several weathering processes, which encompass a variety of physical and biochemical alterations such as evaporation, photo-oxidation, solubilization, alkylation and microbial degradation (Neff et al., 2009). This process also causes significant changes in the chemical profile of the spilled oil, including the composition of alkylated PAHs (Baird et al., 2007; Yim et al., 2011).

PAHs are one of the major determinants of toxicity induced by oil exposure in oil spilled areas. Some of these compounds are either classified or suspected as potent mutagen, carcinogen, or teratogen (Collins et al., 1998; Hong et al., 2012; Machala et al., 2001b). In addition, numerous studies have shown that this group of compounds could induce dioxin-like responses via activation of aryl hydrocarbon receptor (AhR) (Eichbaum et al., 2014; Horii et al., 2009; Machala et al., 2001a,b; Villeneuve et al., 2002). Several PAHs such as benzo[*k*]fluoranthene, indeno[*1,2,3-cd*]pyrene, benzo[*a*]pyrene, and chrysene, as well as substituted PAHs such as 6-chlorochrysene and 4,7-dibromo-benz[*a*]anthracene have been reported as strong AhR agonists (Machala et al., 2001b; Villeneuve et al., 2000, 2002).

It has previously been reported that the composition of alkylated PAHs altered during weathering, and at the same time potency of AhR-mediated effects was influenced, in sediments contaminated with crude oil (Hong et al., 2012). Compared to PCDDs, PCDFs, PCBs, and other PAHs, relatively little is known about the toxicities of alkylated PAHs (Baird et al., 2007). Therefore, more studies on toxicity characteristics of PAHs and their alkylated forms are warranted in order to better understand hazards and risks of crude oil contamination in the environment.

The AhR protein, which belongs to the basic helix-loop-helix protein family, plays an important role in the toxicity pathway of dioxin-like compounds including PAHs (Eichbaum et al., 2014; Larsson et al., 2012). This ligand-dependent transcription factor responds to diverse ligands and plays a critical role not only in toxicity, but also in immune function, cardiovascular physiology, and xenobiotic metabolism (Ma, 2001; Motto et al., 2011; Xing et al., 2012). Interaction of chemicals with the AhR can influence expression of a large battery of genes and induce diverse biological or toxic effects in a wide range of species and tissues (Ma, 2001; Motto et al., 2011). Cytochrome P450 (CYP) 1A, a xenobiotic-metabolizing enzyme, is also regulated by AhR activation (Bak et al., 2013). Differences in sensitivities among species can be explained and thus predicted from the binding affinity of dioxin-like ligands to the AhR (Doering et al., 2014; Harvé et al., 2010; Prokipcak et al., 1990). In birds, amino acids at just two positions, 324 and 380 determine relative sensitivities among species (Farmahin et al., 2014). Relative potencies of ligands to activate the AhR are directly proportional to the affinity with which they bind to the AhR (Kramer and Giesy, 1999). The greater the proportion of occupancy on the receptor, the greater probability of the transformed receptor-ligand complex interacting with the dioxin response enhancer (DRE) on DNA (Farmahin et al., 2013; Lee et al., 2013; Larsson et al., 2014).

Computational prediction has become an important tool for exploring affinities of binding ligands and their receptor (Shin and Seok, 2012; Yuan et al., 2013). Studies based on various structure–activity relationships, such as quantitative structure–activity relationship (QSAR) have been conducted for dioxin-like compounds (Ashek et al., 2006; Beger and Wilkes, 2001; Li et al., 2011; Yang et al., 2009). Approaches based on molecular docking have proven helpful in investigating detailed intermolecular interactions (Shin and Seok, 2012; Wang et al., 2013; Yuan et al., 2013). However, approaches based on molecular docking have rarely been applied, because of the limitations of knowledge on the crystal structure of the AhR (Yuan et al., 2013).

The aims of this study were to: (1) determine relative potencies for activation of the AhR signal transduction pathway of major unsubstituted and alkylated PAHs by using the H4IIE-*luc* cell bioassay system, and (2) determine possible reasons for different potencies for activation of the AhR by the type of (alkylated) PAHs, employing computational molecular docking. GalaxyDock, which was used in the present study, is one of the recently developed docking methods that simultaneously consider receptor side-chain flexibility as well as ligand flexibility (Shin and Seok, 2012). This docking approach allows prediction of binding pose and binding affinity with fewer false positive results (Shin et al., 2013; Shin and Seok, 2012). Therefore, it has utility in predicting the AhR activation potency of a chemical. The results of this study will help develop a docking model that can accurately predict AhR activation potency and therefore AhR-mediated toxicities of chemicals.

2. Materials and methods

2.1. Test chemicals

Three PAHs that are frequently detected in crude oil and in oil contaminated sediments, e.g., chrysene, phenanthrene, and dibenzothiophene (Hong et al., 2012; Jung et al., 2013; Yim et al., 2011), and their major alkylated forms, including four alkylated chrysenes, seven alkylated phenanthrenes, and three dibenzothiophenes, were chosen as target chemicals ($n = 17$), and were purchased from Supelco (Bellefonte, PA, USA), Aldrich (St. Louis, MO, USA) and Chiron (Trondheim, Norway) (Table S1). Commercial availability of standard chemicals was also considered in choosing target PAHs. All the unalkylated PAHs and their analogous alkylated PAHs were dissolved in dimethyl sulfoxide (DMSO) and the concentration of the solvent was set at 0.1% in the bioassay. Maximum concentrations of PAHs tested in the *in vitro* bioassay were as great as 1 μg/mL medium, but varied slightly because of the limitations in the amount of standard available (Table S1). Physicochemical characteristics of the tested PAHs and their alkylated forms are summarized in Table S1.

2.2. in vitro assay

Potencies of target PAHs to activate the AhR signal transduction pathway were measured by use of the H4IIE-*luc in vitro* assay. H4IIE-*luc* cells were cultured in low glucose Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; Sigma), 100 U/mL penicillin, 10 mg/mL streptomycin (Sigma) at 37 °C and in a 5% CO_2 atmosphere. The H4IIE-*luc* cell bioassay was conducted according to Khim et al. (1999) and Lee et al. (2013) with minor modifications. Trypsinized cells from a culture plate were diluted to a density of approximately 8.0×10^4 cells/mL, and 250 μL was plated into each of the 60 interior wells of 96 well microplates. After incubation overnight, culture medium was removed and test chemicals or standards were dosed. For dosing, test chemicals or standards were added to culture medium at 0.1% v/v, and then 250 μL of the dosed media were allocated to each well. For each chemical or control, six concentrations were prepared with 3-fold serial dilutions. All the exposures were performed in triplicates in the same assay plate. A separate exposure with triplicates was performed and comparable responses were confirmed, e.g., consistently greater or negligible dioxin-like responses for specific tested chemicals. After 72 h exposure, luciferase activities were measured with a commercial

kit, Britelite™ Plus (PerkinElmer, Waltham, MA, USA) using a Tecan infinite® 200 microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Viability of cells and overall cytotoxicity of samples were determined by use of the WST assay (Roche Applied Science, Indianapolis, IN, USA) following the protocol of the manufacturer. Concentration–response curves for all tested compounds are shown in Fig. S5 of Supplementary Information.

2.3. Calculation of dioxin-like responses

Dioxin-like responses were calculated following the method of Hong et al. (2012) and Horii et al. (2009). Mean relative luminescence units were converted to a percentage of the maximum response (%-$TCDD_{max}$) observed for a standard containing 100 nM (=100%-$TCDD_{max}$) 2,3,7,8-TCDD. To determine relative dioxin-like potency of a given chemical, TCDD-EQ, or TCDD standard equivalent, was derived. The TCDD-EQ is expressed in ng/g, which is the amount of 2,3,7,8-TCDD (in ng) equivalent to a potency by one gram of a given chemical. The maximum TCDD-EQ (TCDDmaxEQ) was derived from the dose that exhibited the maximum response (% TCDD-max) among multiple doses tested for a given PAH, and was used to conservatively indicate the dioxin-like potency of the tested chemical (Hong et al., 2012). Relative potencies (RePs, unitless) were determined directly from the sample dose–response relationships for each compound and standard curve generated from the range of 2,3,7,8-TCDD dilutions (1.2, 3.7, 11, 33, and 100 pM). ReP_{20}, ReP_{50} and ReP_{80} were determined at the doses of a given chemical of which responses are equivalent to 20%, 50%, and 80% response levels of the maximum 2,3,7,8-TCDD concentration (100 nM TCDD) in standard curves, respectively (Hong et al., 2012; Lee et al., 2013). If the deviation between ReP_{20}, ReP_{50}, and ReP_{80} is within an order of magnitude, generally the use of ReP_{50} is considered as reliable (Horii et al., 2009).

2.4. in silico molecular modeling

The GALAXY protein modeling package (Shin et al., 2014) was used for prediction of the conformation of the AhR and for determining affinities of binding from receptor-ligand docking of each ligand. First, the three-dimensional structure of the ligand binding domain (LBD) of the AhR was predicted from the sequence of amino acids in the rat AhR (UNIPROT accession code P41738) by using the GalaxyTBM template-based modeling program (Ko et al., 2012). Two crystal structures 4F3L (sequence identity = 28%, residues 245–361) and 3RTY (sequence identity = 18%, residues 147–339) were selected as templates. Docking was then performed by using GalaxyDock (Shin et al., 2013). A docking box on the receptor was constructed large enough to cover the important residues (R282, H285, F318, I319, H320, C327, I332, M334, A375, F398, F400) that were revealed by previous mutagenesis experiments (Goryo et al., 2007; Pandini et al., 2007). Among those residues, two aromatic residues of H285 and F318 were recognized as the most important for ligand binding (Goryo et al., 2007; Pandini et al., 2007). Distances between the nearest aromatic ring of ligand, i.e., PAHs, and each of the ligand binding domains, i.e., F318 and H285, were calculated from the predicted binding pose generated by GalaxyDock, and the sum of the two binding distances was used as a representative parameter to compare with the results of the *in vitro* cell line assay.

2.5. Statistical analysis

To compare the measurements from *in vitro* assays with predictions from *in silico* assay, linear regression modeling was performed with SPSS 20.0 for Windows® (SPSS, Chicago, IL, USA). Assumption of normality was tested using Shapiro–Wilk's normality test, and for non-parametric data, Spearman's rank correlation analysis was conducted. In addition, the Kruskal–Wallis test was carried out to determine the differences among groups divided by binding distance. The results were considered statistically significant if $p < 0.05$.

3. Results

3.1. Binding affinity of PAHs in H4IIE-luc in vitro assay

Binding potencies of a given PAH were significantly altered by alkylation, but the difference in binding affinity could not be simply explained by number of alkylations (Table 1). % TCDDmax and TCDDmaxEQ of PAHs were calculated as relative binding potency parameters based on the H4IIE-*luc in vitro* assay (Table 1). Among phenanthrenes, 1,2,6,9-tetramethyl-phenanthrene and parent phenanthrene were relatively potent inducers of AhR-mediated responses with 83.0% and 32.0% of TCDDmax, respectively. While unsubstituted dibenzothiophene elicited AhR-mediate responses, all the alkylated dibenzothiophenes did not exhibit any AhR-mediated potencies. Chrysene and its alkylated forms elicited generally greater AhR-mediated responses compared to those measured for the rest of the tested PAHs. The responses obtained from chrysenes ranged from 77.9% to 145.3% of TCDDmax. The greatest relative potency for AhR activation was observed in 1-methyl-chrysene, of which TCDDmaxEQ was calculated to be 6.58 log ng/g.

Unsubstituted and alkylated chrysenes were a million times less potent than 2,3,7,8-TCDD, with ReP_{50} values ranging between 9.5×10^{-6} and 8.0×10^{-7}, which are relatively large potency among the tested PAHs. Among the chrysenes, 3-methyl chrysene

Table 1
TCDDmaxEQs and relative potency (RePs) of unsubstituted and alkylated PAHs.

Chemical[a]	% $TCDD_{max}$ (%)	$TCDD_{max}EQ$ (log ng/g)	ReP_{20}	ReP_{50}	ReP_{80}
Chrys	111.7	4.90	4.4×10^{-7}	9.7×10^{-7}	2.1×10^{-6}
1-M-Chrys	145.3	6.58	2.5×10^{-6}	7.7×10^{-6}	2.4×10^{-5}
3-M-Chrys	77.9	5.05	5.9×10^{-6}	9.5×10^{-6}	1.5×10^{-5}
6-E-Chrys	97.3	4.43	3.5×10^{-7}	8.0×10^{-7}	1.8×10^{-6}
1,3,6-TM-Chrys	85.7	4.60	1.4×10^{-6}	2.5×10^{-6}	4.6×10^{-6}
Phen	32.0	2.50	1.8×10^{-8}	2.9×10^{-8}	4.6×10^{-8}
1,2,6,9-TeM-Phen	83.0	4.31	1.1×10^{-6}	2.7×10^{-6}	6.5×10^{-6}
DiBZTP	15.2	2.01	7.8×10^{-9}	1.1×10^{-8}	1.6×10^{-8}

Relative potency (ReP) values of individual PAHs and alkylated PAHs for AhR-mediated activity were calculated based on EC_{20} (ReP_{20}), EC_{50} (ReP_{50}), and EC_{80} (ReP_{80}), compared to the potency of 2,3,7,8-TCDD.
For chemicals with % TCDDmax < 0 were not shown here. For the estimates of dioxin-like responses of these chemicals, refer to Table S2 of Supplementary Information.
[a] Full name and abbreviation of tested chemicals: chrysene (chrys); 1-methylchrysene (1-M-Chrys); 3-methylchrysene (3-M-Chrys); 6-ethylchrysene (6-E-Chrys); 1,3,6-trimethylchrysene (1,3,6-TM-Chrys); phenanthrene (Phen); 1,2,6,9-tetramethylphenanthrene (1,2,6,9-TeM-Phen); dibenzothiophene (DiBZTP).

exhibited the greatest relative potency ($ReP_{50} = 9.5 \times 10^{-6}$). ReP_{50} estimates of phenanthrene and dibenzothiophene were 2.9×10^{-8}, and 1.1×10^{-8}, respectively.

3.2. Computational modeling of three-dimensional structure of AhR

Since a reliable crystal structure of AhR is not currently available, in order to estimate the affinity of binding between AhR and PAHs by molecular docking, a three-dimensional structure of rat AhR was constructed (Fig. S1). Two crystal structures (4F3L and 3RTY) were selected as templates by use of the HHSearch re-scoring scheme of GalaxyTBM (Ko et al., 2012; Soding, 2005). When the model structure was submitted to the MolProbity server (Chen et al., 2010) for quality validation, 95.9% of backbone φ/ψ angles were located in the favored region of the Ramachandran plot, and 3.4% and 0.7% were in the allowed region and the outlier region, respectively. The Ramachandran plot of this model is presented in Fig. S2. The quality of the predicted structure was determined to be superior to that of the homology model of AhR used in the previous study (Yuan et al., 2013). For docking of ligands on proteins, accuracy of protein side chain conformations as well as backbone structure are important. Based on MolProbity, the percentage of side chain rotamer outliers for the model of the AhR was relatively small (3.1%).

3.3. Molecular docking results and comparison with in vitro assay

Results of docking simulations, including estimated binding free energy, and the binding distance between ligands and LBD of the AhR were summarized for 17 PAHs in Table 2. The predicted free energy of binding between the AhR and 6-ethyl-chrysene was the least (−9.13 kcal/mol), which indicated a greater affinity of binding. The estimated free energy of 1-methyl-chrysene, which showed the greatest potency for inducing signal transduction based on the results of the *in vitro* H4IIE-*luc* assay, was −8.59 kcal/mol. Among the PAHs, unsubstituted chrysene was determined to have the shortest total binding distance to binding domains (10.08 Å).

Free energy was associated neither with log TCDDmaxEQ nor with log ReP (Fig. 1). A weak negative relationship was observed with log TCDDmaxEQ ($\beta = -0.180$), however statistical significance was not observed ($p > 0.05$). With log ReP, no meaningful association was observed (Fig. 1).

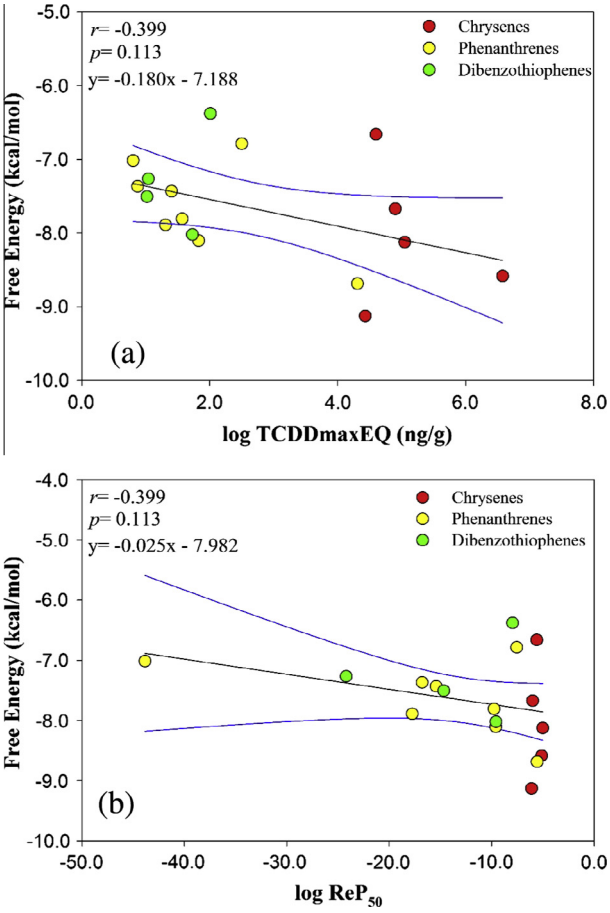

Fig. 1. Relationship between the results of *in vitro* assay (TCDDmaxEQ and ReP_{50}) and *in silico* assay (free energy). (a) TCDDmaxEQ against free energy (kcal/mol), (b) ReP_{50} against free energy (kcal/mol).

Significant negative associations were observed between sum of the binding distances and log TCDDmaxEQ ($\beta = -0.683$, $p = 0.011$, $r = -0.602$), and between log ReP and the sum of the binding distance ($\beta = -0.121$, $p = 0.024$, $r = -0.545$) (Fig. 2). Moreover, when the sums of two distances of each PAH were divided into three groups depending on the distances, e.g., 'Near' as 10.0–11.5 Å

Table 2
Free energy (ΔG) and binding distance between the ligand (unsubstituted and alkylated PAHs) and the respective ligand binding domain (LBD).

Chemical	Free energy (kcal/mol)	Binding distance to LBD (Å)		
		Total distance	Distance to H285	Distance to F318
Chrys	−7.67	10.08	3.90	6.18
1-M-Chrys	−8.59	11.37	5.14	6.22
3-M-Chrys	−8.13	10.78	5.04	5.75
6-E-Chrys	−9.13	11.39	4.78	6.61
1,3,6-TM-Chrys	−6.66	12.28	4.76	7.52
Phen	−6.79	10.41	4.39	6.02
2-M-Phen	−7.01	13.27	5.16	8.11
3-M-Phen	−7.43	10.35	4.38	5.97
1,2-DM-Phen	−7.37	12.11	5.16	6.95
1,6-DM-Phen	−7.89	15.71	6.19	9.52
1,2,6-TM-Phen	−8.11	12.36	5.51	6.85
1,2,9-TM-Phen	−7.81	12.36	5.19	7.18
1,2,6,9-TeM-Phen	−8.69	12.39	5.47	6.92
DiBZTP	−6.38	10.35	4.24	6.10
2-M-DiBZTP	−7.27	18.72	8.26	10.46
2,4-DM-DiBZTP	−7.51	18.26	8.36	9.90
2,4,7-TM-DiBZTP	−8.02	12.74	6.05	6.69

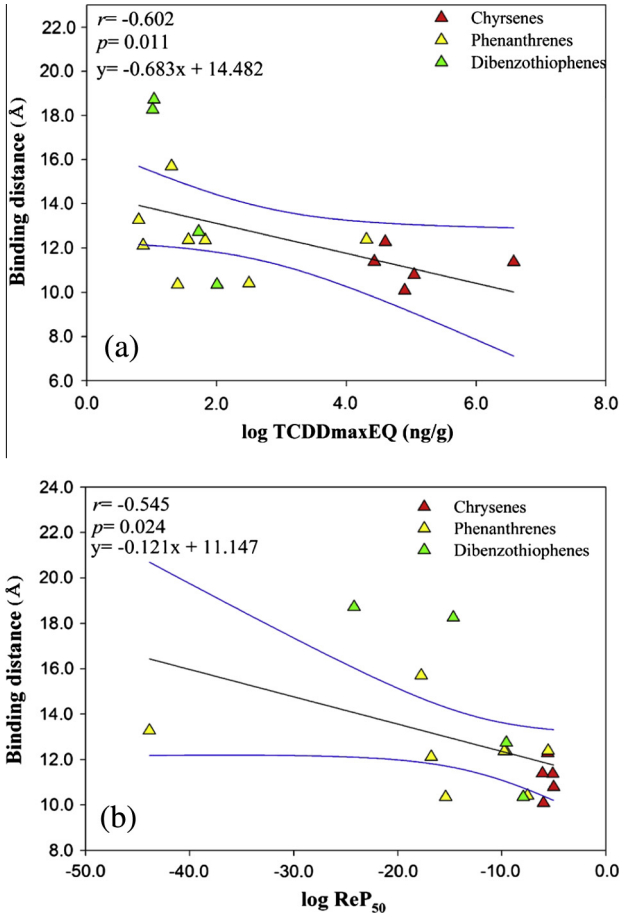

Fig. 2. Relationship between the results of *in vitro* assay (TCDDmaxEQ and ReP_{50}) and *in silico* assay (sum of binding distances to two binding sites (H285, and F318). (a) TCDDmaxEQ against binding distance (Å), (b) ReP against binding distance (Å).

between ligand and binding domain of receptor; 'Mid' as 11.6–13.0 Å; or 'Far' >13.1 Å, the 'Far' distance group, significantly lesser values for both TCDDmaxEQ and ReP were observed for 'Far' group compared to the other groups (Fig. 3).

4. Discussion

4.1. H4IIE-luc in vitro assay

Available literature data are scarce, therefore direct comparison with our data is not easy. However, dioxin-like potencies estimated for the target PAHs in the present study are expected to be comparable to those available elsewhere. One example is chrysene. The ReP_{50} estimate for chrysene determined in this study was 9.7×10^{-7} which is 1.3- and 2.4-fold different from the previous estimates using the H4IIE-*luc* system, reported by Villeneuve et al. (2002) and Larsson et al. (2012), respectively.

RePs derived for the tested PAHs varied not only by the type of PAH but by alkylation (Table 1). Chlorination, bromination or alkylation of PAHs and naphthalenes have been reported to affect the relative potency for AhR activation of PAHs (Horii et al., 2009; Villeneuve et al., 2000). The position of substitution was important in determining potency of a given alkylated PAH. Importance of the position of the chlorine or bromine atom on the PAH molecules, by estimating and comparing ReP values and toxic equivalencies of dioxin-like compounds (TEQs) of chlorinated and brominated PAHs including fluorine, phenanthrene and chrysene have been previously estimated for compounds in environmental matrices (Horii et al., 2009). Moreover, naphthalenes, which consist of two fused benzene rings, showed greater ReP when chlorinated ($ReP_{50} = 2.6 \times 10^{-3}$ for 1,2,3,4,6,7-hexa chlorinated-naphthalene) (Villeneuve et al., 2000). In phenanthrene, methylation resulted in different AhR induction equivalency factors (Vondracek et al., 2007). In several fishes, different toxicity potentials have been reported due to alkylation of PAHs. For example, in zebrafish embryo, 7-isopropyl-1-methylphenanthrene or retene, showed greater cardiotoxicity compared to unsubstituted phenanthrene (Scott et al., 2011). In addition, in Japanese medaka (*Oryzias latipes*), dimethylated PAHs exhibited different toxic potential on embryonic development compared to those of unsubstituted PAHs (Rhodes et al., 2005).

4.2. Comparison between in vitro measurement and in silico modeling

The observation of effects of alkylation of PAH on potencies to upregulate AhR-mediated signal transduction, could not be easily predicted by simple alkylation properties such as number of alkylation on PAHs. In the present study, since lesser free energy of the

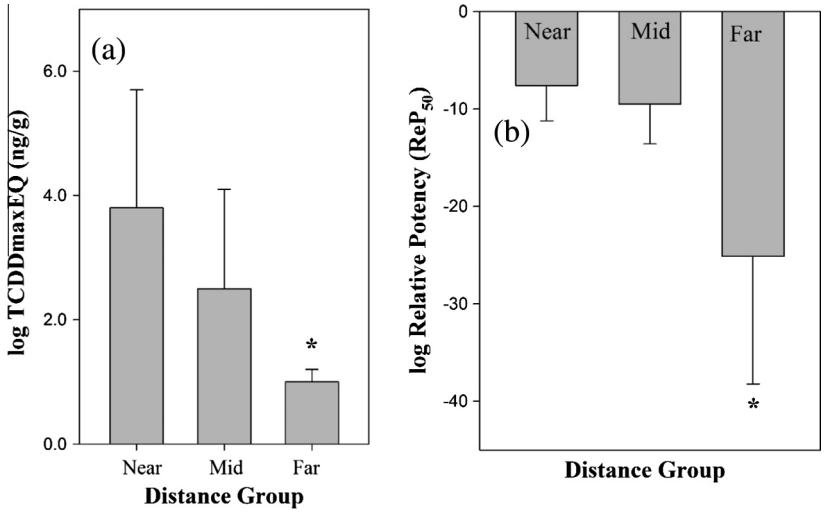

Fig. 3. Different AhR binding activity according to the binding distance (Near: >10.0, ⩽11.5; Mid: >11.5, ⩽13.0; Far: >13.0 (unit: Å)). Asterisk indicates significant difference between the distance groups ($p < 0.05$).

binding between ligand and receptor means greater docking potential, and thus greater occupancy of the receptor, the free energy of binding between ligands and the AhR, which was predicted using the GalaxyDock flexible protein–ligand docking program, was employed to explain the experimental TCDDmaxEQ values or ReP_{50}s (Fig. 1). Free energy values have been widely used to estimate docking potential (Bak et al., 2013; Baker and Chandsawangbhuwana, 2012; Dolenc et al., 2011; Motto et al., 2011) and to develop Quantitative Structure Activity Relationship (QSAR) models (Li et al., 2011, 2010; Yang et al., 2011). To validate the new AhR homology model, Motto et al. (2011) compared free energies of binding predicted by the model with empirical values of pEC_{50} and demonstrated good correlation ($R^2 = 0.81$). A significant correlation ($R = 0.586$, $p < 0.01$) between estrogenic activity of 20 anthroquinones, determined in a yeast transactivation assay of estrogen potency and free energy of binding ($E_{binding}$) predicted from molecular docking simulations was reported (Li et al., 2010). Based on this observation, Li et al. (2010) applied $E_{binding}$ as a key parameter to develop a QSAR model for anthraquinones. Similarly, potentials of energy of binding, expressed as U-dock, have been used to predict the ligand binding potency (Bak et al., 2013). Unlike our expectation, however, the associations between the free energy of binding between ligands and the AhR, and experimental TCDDmaxEQ values ($r = -0.399$, $p = 0.113$) and ReP_{50}s were weak and statistically insignificant (Fig. 1), implying limited utility of this parameter in explaining differential dioxin-like potencies among PAHs.

A significant negative association between total binding distance between the ligand and the two binding domains and relative potency for AhR activation calculated from the *in vitro* assay ($r = -0.602$, $p = 0.011$ with TCDDmaxEQ, Fig. 2a) suggests that the binding distance could be successfully employed to explain differential dioxin-like potencies of the tested PAHs. In addition, the group with a binding distance larger than 13.0 Å ('Far') exhibited significantly lesser TCDDmaxEQ than other groups (Fig. 3). Two LBDs in the AhR, i.e., H285 and F318 that were used for calculation of the distances (Figs. S3 and S4) were considered as possible key binding domain sites in the mouse AhR according to the results of previous studies (Goryo et al., 2007; Pandini et al., 2007). However, key binding domains of AhR could be different by species. For avian species, amino acids sites at 324 and 380 in AhR are sensitively responsive to dioxin-like compounds (Farmahin et al., 2013).

Binding distance has been used as a tool for docking scoring and for evaluation of contact between ligand and LBD (Baker and Chandsawangbhuwana, 2012; Gosavi et al., 2013; Park et al., 2011). Alteration of binding distance between ligand and each binding domain accounts for changes in estrogenic potency of 4-methyl-2,4-bis(4-hydrozyphenyl)pent-1-ene (MBP), a bisphenol A (BPA) metabolite, compared to that of its unsubstituted compound (Baker and Chandsawangbhuwana, 2012). Based on the result of Spearman correlation analysis which showed a slightly greater correlation coefficient with the binding distance to F318, this site appears to be more important LBD of the AhR.

There is an uncertainty in the ReP values for the PAHs with lesser dioxin-like potencies. When the dioxin-like response of a given chemical is sufficiently great, e.g., >10% TCDDmax, then the variation among ReP_{20}, ReP_{50}, and ReP_{80} is generally narrow, and the use of ReP_{50} as a parameter for indicating dioxin-like potency is considered as reliable (Horii et al., 2009). For Figs. 1–3, however, we used the ReP estimates from all the chemicals even with negative % TCDDmax, and showed the relationships between biological activities and *in silico* based chemical descriptors (e.g., free energy or binding distance), in order to consider the impact of less potent chemicals in the regression model. While the use of RePs from less potent PAHs in the regression model might lack quantitative accuracy (Fig. 2b), this observation clearly supports the negative association of binding distance with TCDDmaxEQ (Fig. 2a).

In the *in silico* assay, binding distance showed better correlation with the results of the *in vitro* assay, compared to the free energy of binding. Correlations of the binding distance calculated with log TCDDmaxEQ or log ReP derived from the *in vitro* assay were generally significant, suggesting that the binding distance can be employed to explain the experimental relative potency for AhR activation of PAHs. Validation studies with more chemicals with AhR activation are necessary. The results of this study suggest that this computational docking model has a utility as a potential measure to predict the AhR mediated risks of chemicals and environmental samples.

4.3. Conclusions

Depending on alkylation of PAHs, relative potency of PAHs for AhR activation was different in the H4IIE-*luc in vitro* assay. Among three major PAHs and their alkylated forms, greater relative potency for AhR activation was observed with chrysenes, especially 1-methyl-chrysene. Alteration of the relative potency for AhR activation could not be easily explained by number of alkylations. The binding distance between the ligand and the LBD of AhR calculated by *in silico* modeling showed significant negative correlations with the observed relative potency for AhR activation determined in the H4IIE-*luc* cells. The computational docking model presented here is a promising tool to predict binding affinities and therefore has a potential to be applied to estimate AhR mediated risks of environmental contaminants.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.05.033.

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Supplementary Information

Measured and predicted affinities of binding and relative potencies to activate the AhR of PAHs and their alkylated analogues

[1]Sangwoo Lee, [2]Woong-Hee Shin, [3]Seongjin Hong, [1]Habyeong Kang, [1]Dawoon Jung, [4]Un Hyuk Yim, [4]Won Joon Shim, [3]Jong Seong Khim, [2]Chaok Seok, [5,6,7]John P. Giesy, [1*]Kyungho Choi

1 School of Public Health, Seoul National University, Seoul, South Korea

2 Department of Chemistry, Seoul National University, Seoul, South Korea

3 School of Earth and Environmental Sciences & Research Institute of Oceanography, Seoul National University, Seoul, South Korea

4 Oil and POPs Research Group, Korea Institute of Ocean Science and Technology (KIOST), Geoje, South Korea

5 Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, SK, Canada

6 Department of Zoology, and Center for Integrative Toxicology, Michigan State University, East Lansing, MI, USA

7 Department of Biology & Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, Kowloon, Hong Kong, SAR, China

Supplementary Tables

Table S1.

Table S2.

Supplementary Figures

Figure S1. Modeled crystal structure of rat aryl hydrocarbon receptor.

Figure S2. Ramachandran analysis. (a) general case, (b) isoleucine and valine, (c) pre-proline, (d) glycine, (e) *trans*-proline, (f) *cis*-proline. 95.5% (141/147) of all residues were in favored (98%) regions and 99.3% (146/147) of all residues were in allowed (>99.8%) regions. There was one outlier: 392 ARG (144.6, 130.1).

Figure S3. Relationship between *in vitro* assay (TCDDmaxEQ) and *in silico* assay (binding distance (Å) to (a) H285 and (b) F318).

Figure S4. Relationship between *in vitro* assay (ReP_{50}) and *in silico* assay (binding distance (Å) to (a) H285 and (b) F318).

Figure S5. Concentration-Response curves for all the tested PAHs. (a) Chrysene group, (b) Phenanthrene group, (c) Dibenzothiophene group)

Table S1. List of unsubstituted and alkylated PAHs tested in the present study

Chemical[a]	CAS RN.	$LogK_{OW}$	Molecular Weight (g/mol)	Maximum Tested Concentration	Vendor
Chrys	218-01-9	6.06	228.28	1 µg/ml	Supelco
1-M-Chrys	3351-28-8	6.42	242.31	0.2 µg/ml	Chiron
3-M-Chrys	3351-31-3	6.42	242.31	0.2 µg/ml	Chiron
6-E-Chrys	2732-58-3	6.88	256.34	1 µg/ml	Chiron
1,3,6-TM-Chrys	NA	7.44	270.37	1 µg/ml	Chiron
Phen	85-01-8	4.90	178.23	1 µg/ml	Aldrich
2-M-Phen	2531-84-2	5.34	192.26	1 µg/ml	Chiron
3-M-Phen	832-71-3	5.34	192.26	1 µg/ml	Chiron
1,2-DM-Phen	20291-72-9	5.64	206.28	0.5 µg/ml	Chiron
1,6-DM-Phen	20291-74-1	5.64	206.28	0.5 µg/ml	Chiron
1,2,6-TM-Phen	30436-55-6	6.03	220.31	0.5 µg/ml	Chiron
1,2,9-TM-Phen	146448-88-6	6.03	220.31	0.5 µg/ml	Chiron
1,2,6,9-TeM-Phen	204256-39-3	6.48	234.34	0.5 µg/ml	Chiron
DiBZTP	132-65-0	4.52	184.26	1 µg/ml	Aldrich
2-M-DiBZTP	20928-02-3	5.04	198.28	0.5 µg/ml	Chiron
2,4-DM-DiBZTP	31317-18-7	5.47	212.31	0.5 µg/ml	Chiron
2,4,7-TM-DiBZTP	216983-03-8	6.03	226.34	0.5 µg/ml	Chiron

[a] Full name and abbreviation of tested chemicals: chrysene (chrys); 1-methylchrysene (1-M-Chrys); 3-methylchrysene (3-M-Chrys); 6-ethylchrysene (6-E-Chrys); 1,3,6-trimethylchrysene(1,3,6-TM-Chrys); phenanthrene (Phen); 2-methylphenanthrene (2-M-Phen); 3-methylphenanthrene (3-M-Phen); 1,2-dimethylphenanthrene (1,2-DM-Phen); 1,6-dimehtylphenanthrene (1,6-DM-Phen); 1,2,6-trimethylphenanthrene (1,2,6-TM-Phen), 1,2,9-trimethylphenanthrene (1,2,9-TM-Phen); 1,2,6,9-tetramethylphenanthrene (1,2,6,9-TeM-Phen); dibenzothiophen (DiBZTP); 2-methyldibenzothiophen (2-M-DiBZTP); 2,4-dimethyldibenzothiophen (2,4-DM-DiBZTP); 2,4,7-trimethyldibenzothiophen (2,4,7-TM-DiBZTP). NA: Not available.

Table S2. TCDDmaxEQs and relative potency (RePs) of unsubstituted and alkylated PAHs which were used in comparison with *in silico* analysis

Chemical[a]	% $TCDD_{max}$ (%)	$TCDD_{max}EQ$ (log ng/g)	ReP_{20}	ReP_{50}	ReP_{80}
Chrys	111.7	4.90	4.4×10^{-7}	9.7×10^{-7}	2.1×10^{-6}
1-M-Chrys	145.3	6.58	2.5×10^{-6}	7.7×10^{-6}	2.4×10^{-5}
3-M-Chrys	77.9	5.05	5.9×10^{-6}	9.5×10^{-6}	1.5×10^{-5}
6-E-Chrys	97.3	4.43	3.5×10^{-7}	8.0×10^{-7}	1.8×10^{-6}
1,3,6-TM-Chrys	85.7	4.60	1.4×10^{-6}	2.5×10^{-6}	4.6×10^{-6}
Phen	32.0	2.50	1.8×10^{-8}	2.9×10^{-8}	4.6×10^{-8}
2-M-Phen	< 0	0.80 [b]	2.6×10^{-33} [b]	1.4×10^{-44} [b]	7.3×10^{-56} [b]
3-M-Phen	< 0	1.40 [b]	7.5×10^{-14} [b]	3.9×10^{-16} [b]	2.1×10^{-18} [b]
1,2-DM-Phen	< 0	0.87 [b]	1.2×10^{-14} [b]	1.6×10^{-17} [b]	2.2×10^{-20} [b]
1,6-DM-Phen	< 0	1.31 [b]	2.5×10^{-15} [b]	1.8×10^{-18} [b]	1.3×10^{-21} [b]
1,2,6-TM-Phen	< 0	1.82 [b]	1.1×10^{-9} [b]	2.5×10^{-10} [b]	6.0×10^{-11} [b]
1,2,9-TM-Phen	< 0	1.57 [b]	6.8×10^{-10} [b]	1.7×10^{-10} [b]	4.3×10^{-11} [b]
1,2,6,9-TeM-Phen	83.0	4.31	1.1×10^{-6}	2.7×10^{-6}	6.5×10^{-6}
DiBZTP	15.2	2.01	7.8×10^{-9}	1.1×10^{-8}	1.6×10^{-8}
2-M-DiBZTP	< 0	1.04 [b]	5.9×10^{-20} [b]	6.2×10^{-25} [b]	6.5×10^{-30} [b]
2,4-DM-DiBZTP	< 0	1.02 [b]	3.2×10^{-13} [b]	2.1×10^{-15} [b]	1.4×10^{-17} [b]
2,4,7-TM-DiBZTP	< 0	1.73 [b]	1.1×10^{-9} [b]	2.6×10^{-10} [b]	6.7×10^{-11} [b]

[a] Full name and abbreviation of tested chemicals: chrysene (chrys); 1-methylchrysene (1-M-Chrys); 3-methylchrysene (3-M-Chrys); 6-ethylchrysene (6-E-Chrys); 1,3,6-trimethylchrysene(1,3,6-TM-Chrys); phenanthrene (Phen); 2-methylphenanthrene (2-M-Phen); 3-methylphenanthrene (3-M-Phen); 1,2-dimethylphenanthrene (1,2-DM-Phen); 1,6-dimehtylphenanthrene (1,6-DM-Phen); 1,2,6-trimethylphenanthrene (1,2,6-TM-Phen), 1,2,9-trimethylphenanthrene (1,2,9-TM-Phen); 1,2,6,9-tetramethylphenanthrene (1,2,6,9-TeM-Phen); dibenzothiophen (DiBZTP); 2-methyldibenzothiophen (2-M-DiBZTP); 2,4-dimethyldibenzothiophen (2,4-DM-DiBZTP); 2,4,7-trimethyldibenzothiophen (2,4,7-TM-DiBZTP). [b]Values were calculated from insufficient bioassay responses (i.e., <0% TCDDmax), and were used only for assessing the relationship between the dioxin-like responses and the molecular descriptors of the tested chemicals. ReP values with insufficient bioassay responses were estimated by extrapolation.

Relative potency (ReP) values of individual PAHs and alkylated PAHs for AhR-mediated activity were calculated based on EC_{20} (ReP_{20}), EC_{50} (ReP_{50}), and EC_{80} (ReP_{80}), compared to the potency of 2,3,7,8-TCDD.

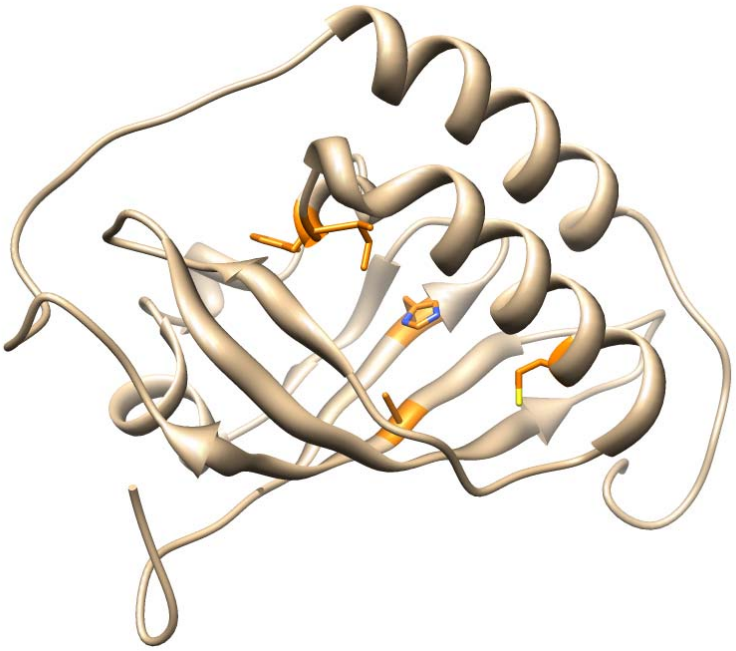
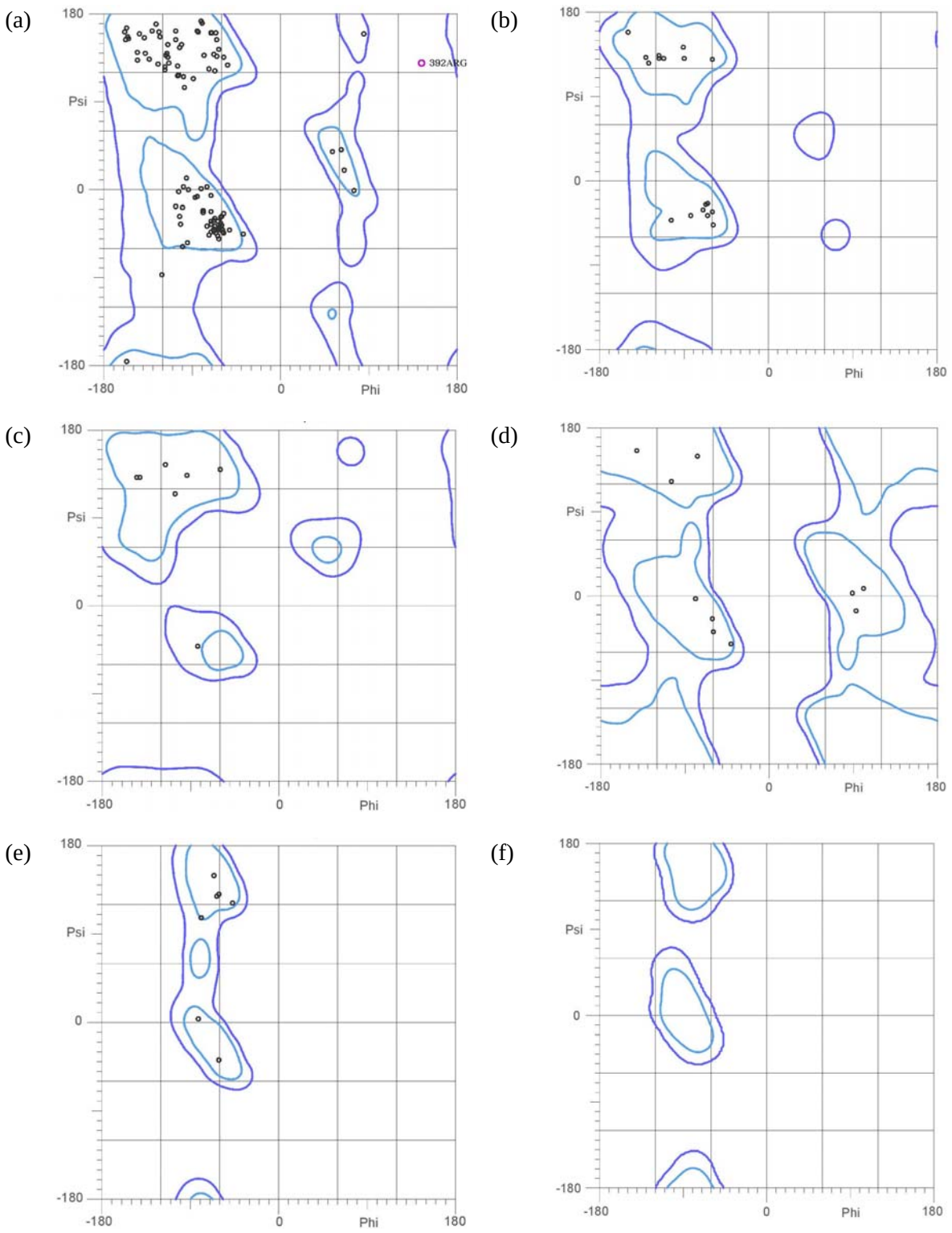


Figure S1.

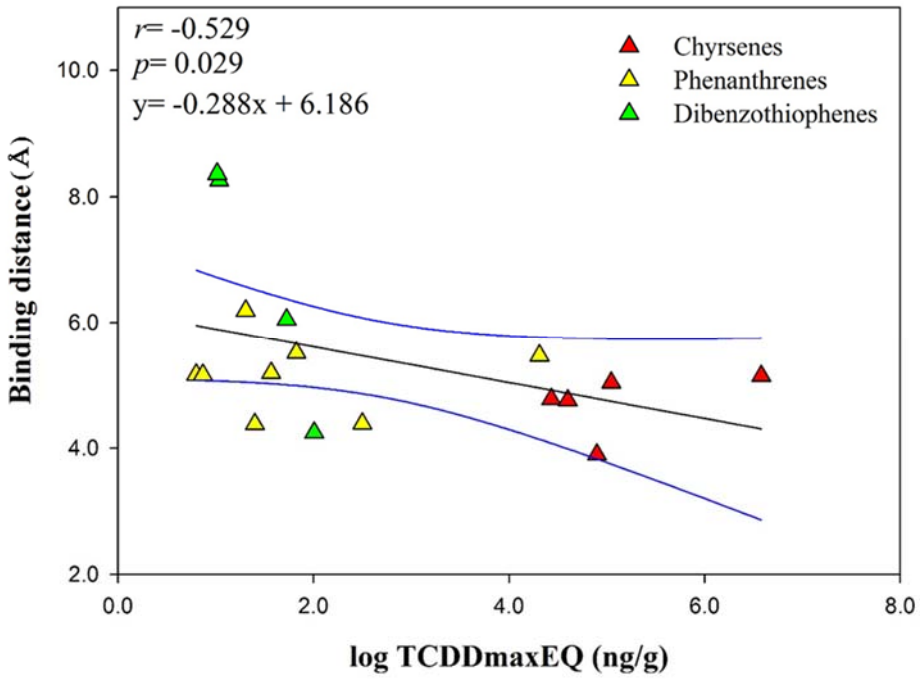
74



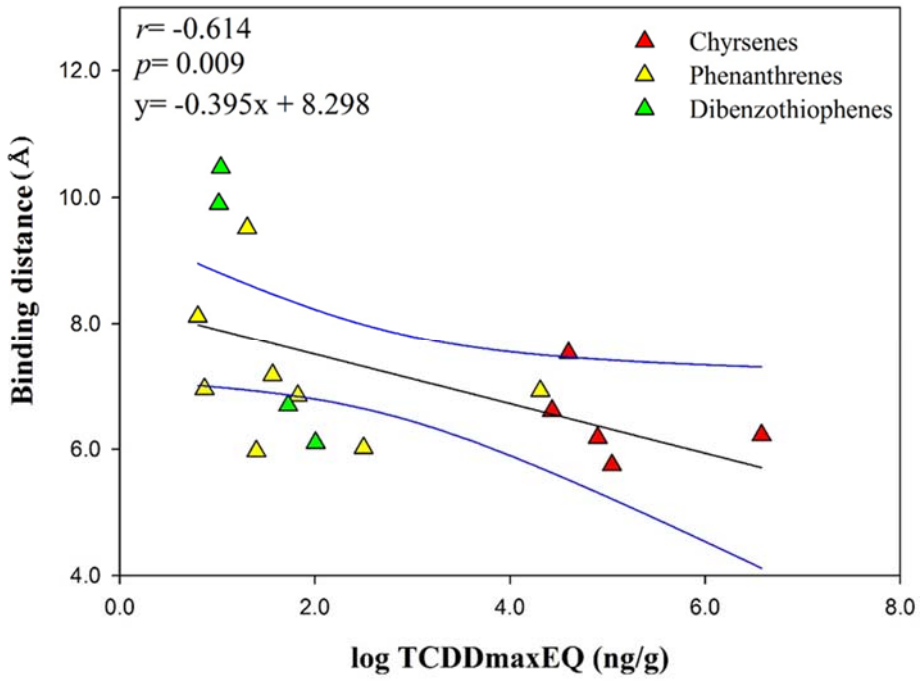
75 Figure S2.

76

(a)



(b)

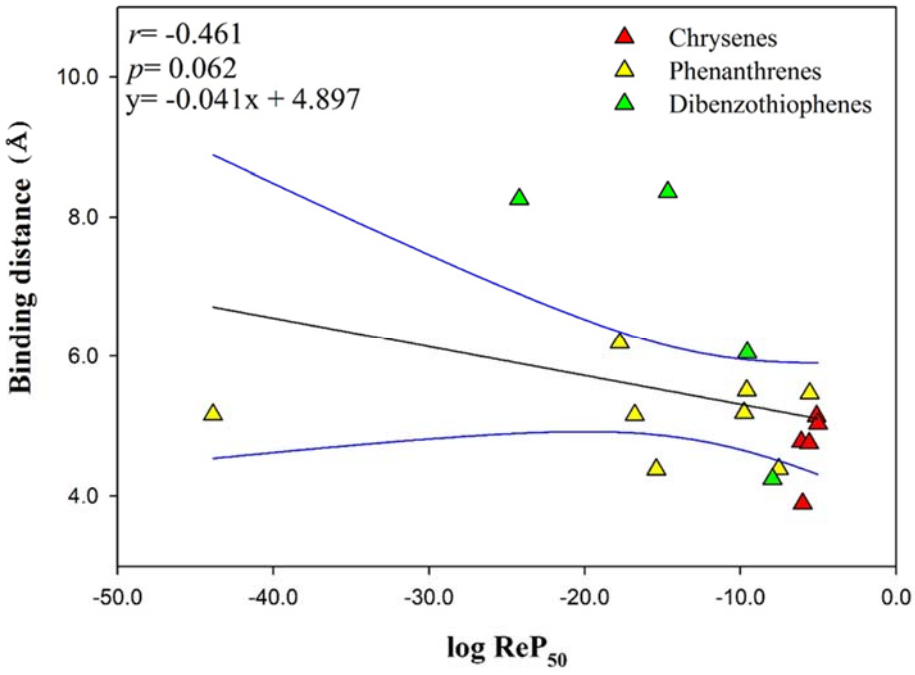


77 Figure S3.

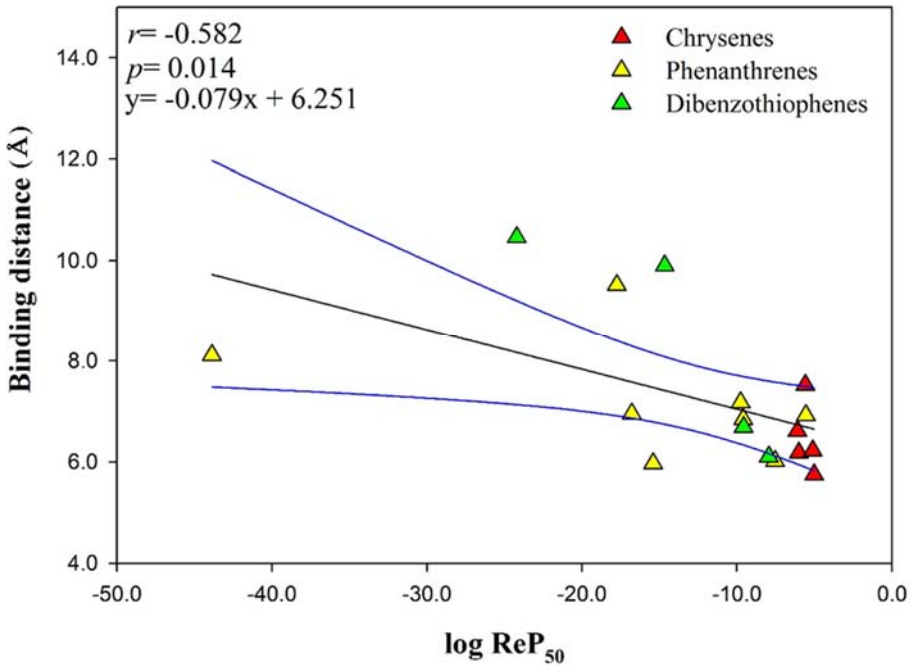
78

79

(a)



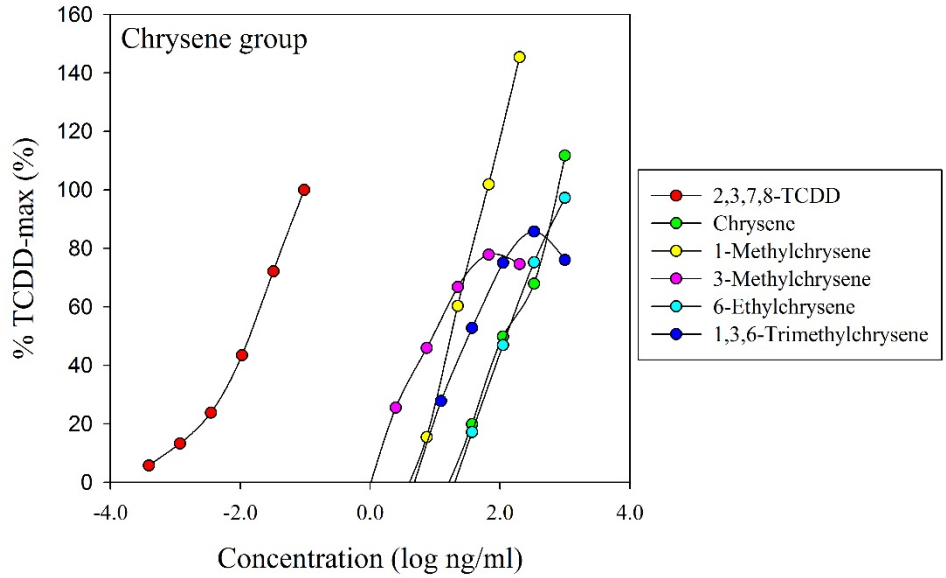
(b)



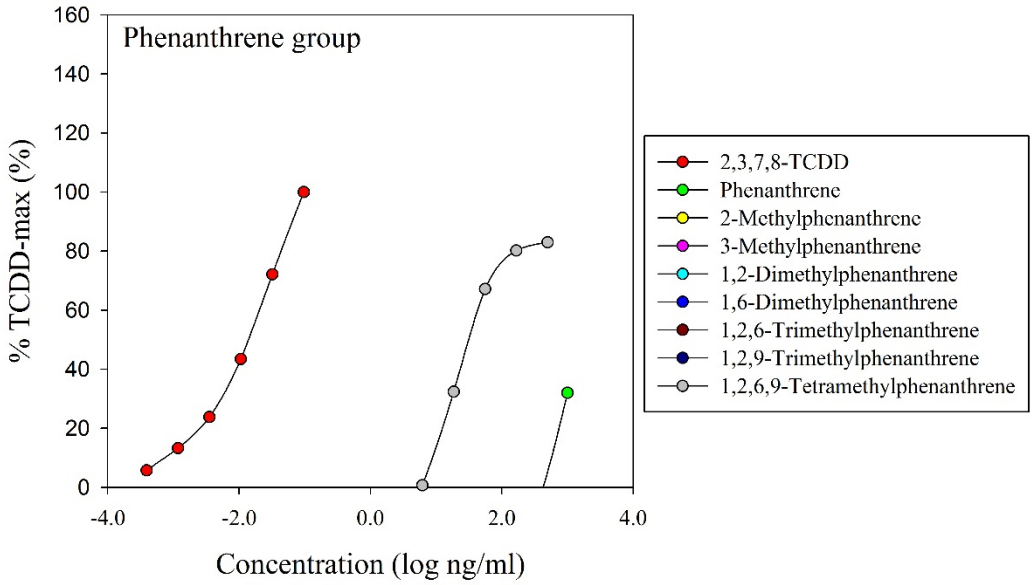
80 Figure S4.

81

(a)



(b)



(c)

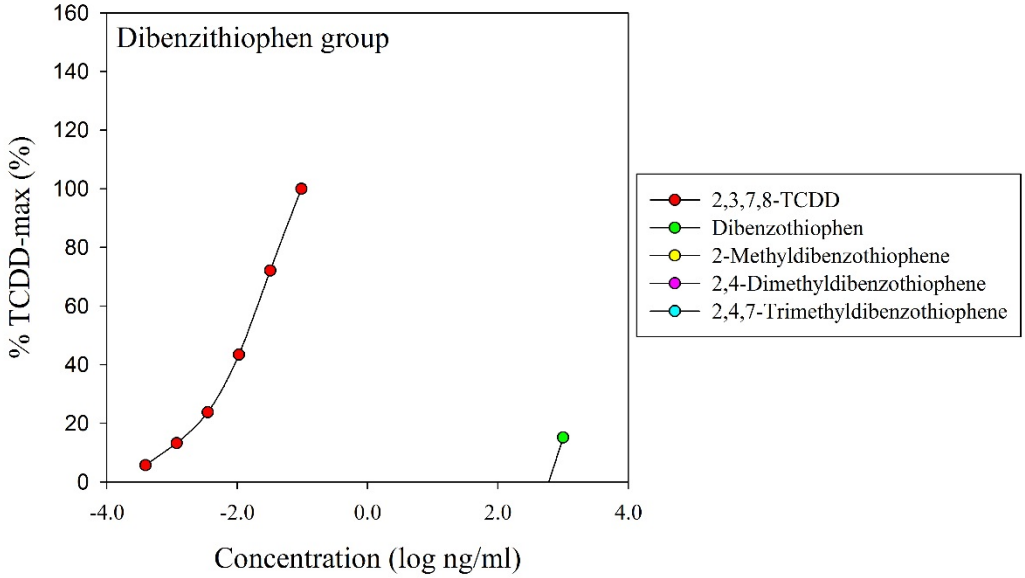


Figure S5.