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METHYLATED PHENANTHRENES ARE MORE POTENT THAN PHENANTHRENE IN A BIOASSAY OF HUMAN ARYL HYDROCARBON RECEPTOR (AhR) SIGNALING

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(Submitted 26 May 2014; Returned for Revision 5 July 2014; Accepted 13 July 2014)

Abstract: Alkylated polycyclic aromatic hydrocarbons (APAHs) are abundant in petroleum, but data regarding their toxicological properties are limited. A survey of all monomethylated phenanthrene structures revealed that they were 2 times to 5 times more potent than phenanthrene for activation of human aryl hydrocarbon receptor in a yeast bioassay. Phenanthrenes with equatorial methyl groups had the greatest potency. The greater potency of the methylated phenanthrenes highlights the need for more toxicological data on APAHs. *Environ Toxicol Chem* 2014;33:2363–2367. © 2014 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals, Inc. on behalf of SETAC. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

Keywords: Alkylated polycyclic aromatic hydrocarbons Aryl hydrocarbon receptor Phenanthrene Methylphenanthrene -
Petrogenic polycyclic aromatic hydrocarbons

INTRODUCTION

The presence of polycyclic aromatic hydrocarbons (PAHs) in the environment generates public health concerns because of their abundance, persistence, genotoxicity, carcinogenicity, and toxic effects on immune and reproductive systems and development [1,2]. Polycyclic aromatic hydrocarbons consist of 2 or more fused aromatic rings and are frequently generated by incomplete combustion of many carbonaceous materials [3–6]; they are also components of crude oil [7,8]. Most previous investigations have been directed toward unsubstituted PAHs, whereas methylated derivatives have not been specially distinguished and analyzed. However, methylated PAHs account for a relatively large proportion of total PAH contaminants in cases such as oil spills [9]. The ratio between a methylated PAH and an unmethylated PAH (phenanthrene, for instance) can be used to identify the sources of PAH contamination (i.e., whether it is of petrogenic or pyrogenic origin) [10]. Limited evidence has also shown that methylation on the aromatic rings could significantly change the toxicological properties of 3-ring PAHs [11,12]. Among the PAHs present in the environmental samples, phenanthrenes often are one of the most abundant families [8,13]. The parental form of phenanthrene is generally considered to be nontoxic, whereas a limited study of methylated phenanthrenes showed that they had weak mutagenicity or carcinogenicity in some model systems [14–16]. Despite the abundance and toxic significance of methylation substitutions among PAHs, information regarding the toxicity of methylated phenanthrenes is inadequate and far from systematic. Most data depend on a variety of experimental models or analysis of different steps that lead to toxic responses. When comparing the effects of phenanthrene and its methyl-substituted

derivatives, it is important to study these compounds systematically in a system that assesses specific steps in the processes that lead to toxic effects.

The toxicity of PAHs in mammals is mediated almost exclusively through the aryl hydrocarbon receptor (AhR) signaling pathway (for reviews, see Abel et al. [17] and Feng et al. [18]). The AhR is a ligand-activated transcription factor that directly binds and is activated by a wide variety of xenobiotics. It occurs in cells with its heterodimeric partner, the aryl hydrocarbon receptor nuclear translocator (ARNT), and together they form the aryl hydrocarbon receptor complex (AhRC). This complex binds a DNA sequence called xenobiotic responsive element (XRE) and mediates downstream gene regulation. In the presence of PAHs, AhR can be activated by ligand binding, which subsequently induces drug-metabolizing enzymes for metabolism or clearance of the foreign compounds. Polycyclic aromatic hydrocarbons often induce their own metabolism and clearance via changes in gene expression that are initiated by the ligand–AhRC complex. The metabolic induction initiated by the activated AhRC, however, sometimes creates PAH metabolites that form mutagenic adducts with DNA. The activation of the AhR is, therefore, an essential step for both PAH metabolism and toxicity. An example of this receptor-mediated relationship with toxicity is provided by the absence of benzo[a]pyrene-induced carcinogenesis in mice that are genetically engineered to lack AhR [19].

In the present study, we systematically explored all monomethylated phenanthrene structures and 1 dimethylated phenanthrene (3,6-dimethylphenanthrene) for their ability to activate the AhR, using a yeast reporter assay system [20]. Each methylated derivative was compared with the unsubstituted phenanthrene control, and all of the methylated derivatives showed enhanced AhR-activation capability relative to phenanthrene. In addition, the variation in the shapes of the dose–response curves implied that even the position of methyl groups affected the AhR activation process. To our knowledge, the present study is the first to compare the AhR signaling

All Supplemental Data may be found in the online version of this article.

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Published online 16 July 2014 in Wiley Online Library
(wileyonlinelibrary.com).

DOI: 10.1002/etc.2687

potentials of substituted phenanthrenes in a unified experimental system.

MATERIALS AND METHODS

Chemicals

All available monomethylation positions around the ring of phenanthrene backbone (purities at 98% or higher) were purchased from the following sources: phenanthrene (Sigma Aldrich), 1-methylphenanthrene (1-MP; Crescent Chemical), 2-methylphenanthrene (2-MP; Sigma Aldrich), 3-methylphenanthrene (3-MP; BOC Sciences), 4-methylphenanthrene (4-MP; Chem Service), 9-methylphenanthrene (9-MP; Crescent Chemical), and 3,6-dimethylphenanthrene (3,6-DMP; AccuStandard). Their structures are shown in Figure 1. Each compound was dissolved as 10 mM stock in dimethyl sulfoxide (DMSO) and diluted to make working solutions ranging from 1×10^{-2} to 1×10^{-6} M.

Cell culture

An engineered *Saccharomyces cerevisiae* yeast strain, YCM3, was used; it was derived from the W303a yeast strain and is made available to investigators through the American Type Culture Collection (strain MYA-3637). Full-length human AhR and ARNT genes were inserted onto chromosome III under the control of the galactose promoter. The YCM3 strain was transformed with a LacZ reporter plasmid that is expressed after interaction with ligand-activated AhRC (Figure 2). This modification process was described in a previous study [20]. The yeast cells were maintained in a glucose-containing medium (0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented with 0.01% uracil, adenine, leucine, and histidine), and the bioassay was performed in a galactose-containing medium (2% galactose substituted for glucose in the medium described above). All medium components were purchased from Sigma Aldrich.

Yeast bioassay

The YCM3 yeast cells were diluted into the galactose medium to a final absorbance at 600 nm ($A_{600\text{nm}}$) of 0.04, plated into 96-well plates at 200 μL /well, and treated with various concentrations of PAHs for 18 h at 30 °C. During this time, the human AhR and ARNT were expressed in the presence of galactose and activated by PAH ligands in the cell to form the AhRC. The AhRC subsequently bound to xenobiotic response elements (XREs) on the plasmid and activated reporter gene (LacZ) expression. The expression level of the reporter gene was subsequently detected by a colorimetric LacZ assay. Briefly,

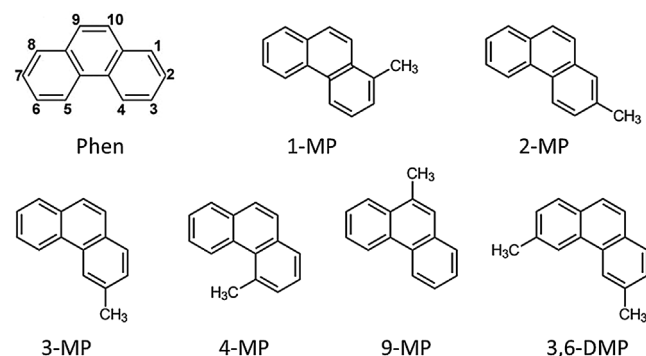


Figure 1. Structure of phenanthrene (Phen) and methylated derivatives investigated in the present study. MP = methylphenanthrene; 3,6-DMP = 3,6-dimethylphenanthrene.

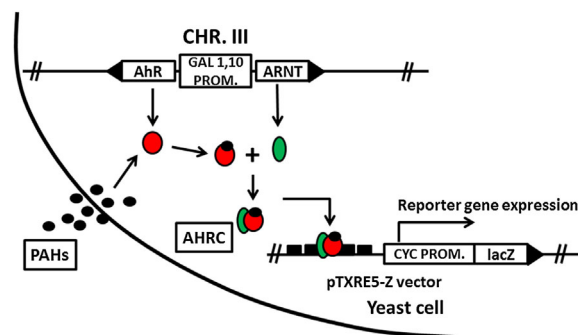


Figure 2. Schematic diagram of yeast bioassay. Circles represent aryl hydrocarbon receptor (AhR). Ovals represent aryl hydrocarbon receptor nuclear translocator (ARNT). Black dots represent the polycyclic aromatic hydrocarbons (PAHs) used as agonists in the bioassay. AhR and ARNT genes are inserted in yeast chromosome III under the control of the galactose promoter, and the reporter gene (LacZ) is regulated by 5 copies of the xenobiotic response elements (XRE) constructed in a plasmid, pTXRE5-Z. Adapted from Alnafisi et al. [22]. CHR. III = chromosome III; GAL 1,10 PROM = galactose promoter; AHRC = aryl hydrocarbon receptor complex; CYC PROM = cytochrome C promoter. [Color figure can be viewed in the online issue which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com)]

after 18 h of incubation, yeast cells were suspended by pipetting, and a 25- μL aliquot from each well was transferred into another 96-well assay plate and mixed with 225 μL of substrate solution (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 1 mM MgCl_2 , 10 mM KCl, 0.2% sarkosyl, 1 mM dithiothreitol, and 0.4 mg/mL ortho-nitrophenyl- β -galactoside; pH 7.0). The assay plate was incubated for approximately 5 min at 37 °C, and then 100 μL of a 1-M Na_2CO_3 solution was added to stop the reaction and enhance the yellow color. Cell densities were determined from the first 96-well plate by measuring $A_{600\text{nm}}$, and the color intensities of LacZ reactions were determined by $A_{405\text{nm}}$ from the corresponding wells of the second plate. Each analysis included a positive control (1 μM β -naphthoflavone), a negative control (1% DMSO), and a relatively control (100 μM phenanthrene).

Calculations

The LacZ units were calculated based on the following formula

$$\text{LacZ unit} = \frac{A_{450 \text{ Sample}} - A_{450 \text{ empty}}}{A_{600 \text{ Sample}} - A_{600 \text{ empty}}} \times \frac{1000 \text{ units}}{\text{cell volume (mL)} \times \text{reaction time (min)}}$$

where $A_{450 \text{ Sample}}$ is the measurement of a ligand-treated well at 405-nm absorbance; $A_{450 \text{ empty}}$ is the measurement of a well that contains only medium and no cells; $A_{600 \text{ Sample}}$ is the measurement of yeast cell density of a ligand-treated well at 600-nm absorbance; $A_{600 \text{ empty}}$ is the measurement of a well that contains only medium and no cells; cell volume is the amount of cell suspension added per well in mL (typically 0.025 mL); and reaction time is the time period of the colorimetric reaction (typically 5 min). The LacZ units were calculated as a percentage relative to the positive control (β -naphthoflavone signal set as 100%), and the dose-response curves were calculated using fitting routines provided in SlideWrite Plus for Windows (Ver 7.01; Advanced Graphics Software) and a 2-parameter curve

$$y = m + a0 \times x / (a1 + x)$$

where m is the $\text{LacZ}_{\text{DMSO}}$ value from the assay plate, $a0$ is the maximum effect, and $a1$ is the half-maximum effective

concentration (EC₅₀). The EC₅₀s were calculated from the curve as an indicator of AhR activation capacity. Relative EC₅₀ and relative 25% maximum effective concentration (EC₂₅) values were further introduced to compare potencies of various methylated phenanthrenes. These values (relative EC₅₀ and relative EC₂₅) were defined as the concentrations of the total PAH at which the AhR was activated to 50% or 25% of the activation observed in the 100- μ M phenanthrene control, respectively. The 100- μ M concentration was chosen because it was the maximum that could be achieved in the assay. The relative EC₅₀ and relative EC₂₅ and the errors associated with these values were calculated from the fitted curves. A detailed description of these calculations is provided in the Supplemental Data. The significances of the differences between relative EC₅₀s and relative EC₂₅s of the different methylated phenanthrenes were determined using analysis of variance (ANOVA) followed by a posttest based on Student–Newman–Keuls analysis. Analyses were performed using GraphPad Instat 3.

RESULTS

We employed a yeast bioassay (Figure 2) to measure the ability of various methylated phenanthrenes to induce human AhR activation [20]. In this assay, full-length AhR and ARNT were engineered into the yeast chromosome under the control of galactose promoter so that their expression could be switched on or off by medium constituents (galactose or glucose). *Saccharomyces cerevisiae* expresses endogenous Hsp70 and Hsp90 proteins that are structurally and functionally similar to their counterparts in vertebrates [21], and these chaperones help regulate proper folding and expression of the human AhR and ARNT. The hydrophobic chemicals tested in the present study are assumed to cross the cell membrane by passive diffusion, and previous studies have shown that the yeast cell wall does not impede the transport process when the test compounds are smaller than 760 Da [22]. All compounds used in these assays fall below this molecular weight cut-off. After entering the cytoplasm, the test compounds bind to AhR and ARNT, whose synthesis is enhanced by the presence of galactose in the culture medium, and form the active complex AhRC. During the 18-h induction process, YCM3 yeast cells were incubated with varying concentrations of phenanthrene and its methylated derivatives, and their signaling activities were measured via a colorimetric substrate. We used 1% DMSO as the solvent control, because all the PAHs were dissolved in this vehicle. The positive controls were β -naphthoflavone (β -NF), which previously has been reported as a strong agonist of AhR [22,23]. Because we also wanted to compare unsubstituted phenanthrene with the methylated derivatives, a control of 100 μ M phenanthrene was also included in each assay. In addition to activating the AhR, some of the tested compounds also inhibited cell accumulation (described in more detail below). Differences in cell growth at the end of the 18-h incubation period were normalized by measuring each culture's absorbance at 600 nm. Data points were excluded if the tested compound inhibited cell accumulation by more than 50% relative to the DMSO control.

Figure 3 shows dose–response curves obtained when the various substituted phenanthrenes were tested in the yeast bioassay. The curve fits had an r^2 of greater than 0.99 for the 2-, 3-, 4-, and 9-methylated derivatives; the r^2 values for the 1-MP and 3,6-DMP curves were 0.97 and 0.96, respectively. These data demonstrate that the methyl group and its position on the phenanthrene backbone clearly affected the ability of each

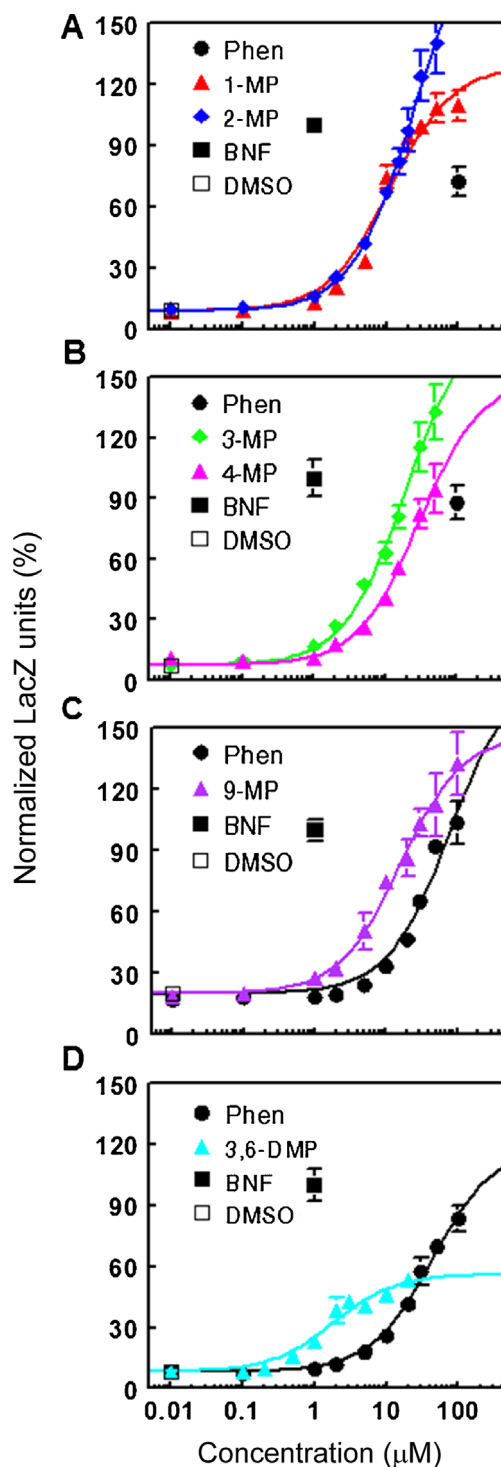


Figure 3. The ability of methylated phenanthrenes to activate the aryl hydrocarbon receptor. Data are expressed as the percentage of the maximum activity (1 μ M β -naphthoflavone [BNF] set as 100%). Data points represent the mean \pm standard deviation ($n=4$); at least 3 independent experiments were performed for each tested compound. Dimethyl sulfoxide (DMSO; 1%) was included as a negative control; 100 μ M phenanthrene (Phen) treatment was included in each assay plate for calculation of relative effective concentration, 50%. MP = methylphenanthrene; DMP = dimethylphenanthrene. [Color figure can be viewed in the online issue which is available at [wileyonlinelibrary.com](http://www.wileyonlinelibrary.com)].

individual compound to activate the AhR and thus regulate the downstream reactions. The methylated derivatives also showed variations in their ability to saturate the bioassay, as shown in Figure 3A. For 1-MP, the response began to show signs of

saturation at 30 μM , and there was no statistically significant difference in the bioassay response at 50 μM and 100 μM . In contrast, the bioassay continued to respond to 2-MP with an almost linear dose-response curve at concentrations up to 50 μM . The toxicity of 2-MP to the yeast cells at higher concentrations precluded data collection above this concentration. At concentrations above 50 μM (Figure 3B), 3-MP and 4-MP were also toxic to the yeast cells, and these concentrations also failed to saturate the assay response. Unsubstituted phenanthrene and the 9-MP derivative were less toxic to the yeast cells, and at 100 μM , both of these compounds led to some degree of saturation in the bioassay (Figure 3C). The dose-response curve for the only commercially available dimethylphenanthrene, 3,6-DMP (Figure 3D), had a completely different shape than the rest of the curves and failed to achieve more than approximately 50% response in the assay.

To make more precise comparisons between the potencies of these compounds, we calculated relative EC50 and relative EC25 values, which were based on the concentrations that activated 50% and 25% of 100 μM phenanthrene effects, respectively. These values provided better comparisons among the test compounds for 3 reasons. First, determination of relative EC50 compensated for any variations among multiple assay plates. Second, the relative EC values provided a way to compare each derivative directly with the planar phenanthrene. Third, the relative EC50 and relative EC25 avoided a determination of the maximum effect of each chemical because cytotoxicity of some compounds precluded data collection at higher concentrations. The relative EC50 values of various phenanthrene derivatives are shown in Figure 4A. The most potent activators were 1-MP and 2-MP, with relative EC50s of 4.0 μM and 4.6 μM , respectively. The relative EC50 of 3-MP showed an intermediate value (5.8 μM), whereas the 9-MP and 4-MP were the least potent in the assay, with relative EC50s of 7.8 μM and 11.7 μM , respectively. An ANOVA of the entire data set yielded a $p < 0.0001$, indicating that the differences observed among these compounds were highly significant. Posttest analysis showed that the relative EC50s of all the methylated phenanthrenes were significantly different ($p < 0.001$) from the unmethylated compound, and there were also significant differences in the relative EC50s of the 3 most potent monomethylated phenanthrenes (1-MP, 2-MP, and 3-MP) and the least potent methylated phenanthrene (4-MP). The 3,6-DMP was also significantly more potent than 4-MP, but its relative EC50 value was not significantly different from those of other monomethylated phenanthrenes.

Because toxicity prevented us from testing higher concentrations that would have maximized signaling for some of the compounds under study, we also examined relative EC25 values as a method for comparing compounds of varying solubility and toxicity [24]. When relative EC25 values were examined, as shown in Figure 4B, all of the methylated phenanthrenes were again significantly more potent than unmethylated phenanthrene. The 3,6-DMP was significantly more potent than the 3-MP, 4-MP, and 9-MP in the yeast bioassay. The order of potencies of the monomethylated derivatives remained as noted for their relative EC50 values. In addition, a significant difference between 1-MP and 9-MP was observed.

DISCUSSION

The present study was initiated because of our laboratories' interest in comparing the toxicity of PAHs from petrogenic versus pyrogenic sources. We chose to examine an early step of

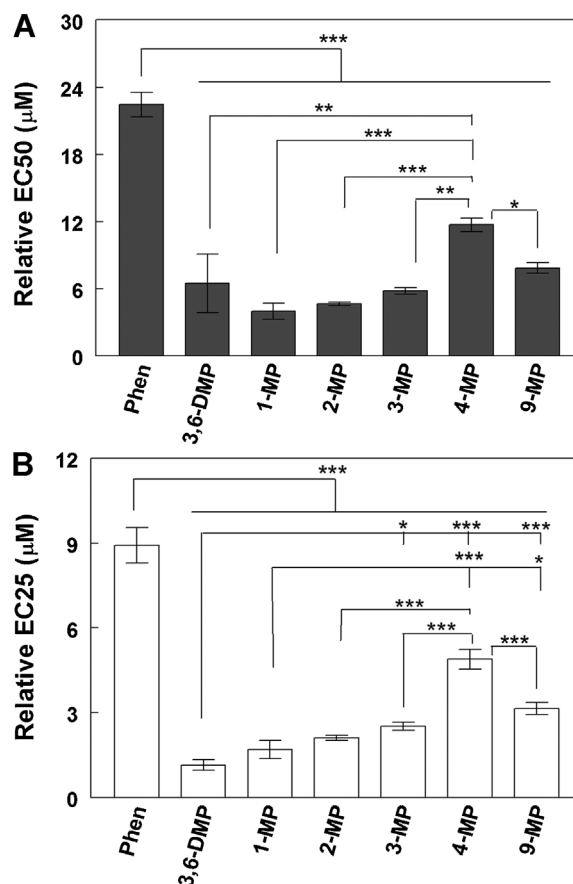


Figure 4. Relative half-maximum effective concentration (EC50; A) and relative 25% effective concentration (EC25; B) values for unsubstituted and substituted phenanthrenes. The relative EC50 and relative EC25 values are defined as the concentrations of the methylated phenanthrene that induced 50% and 25%, respectively, of the activity observed after treatment with 100 μM phenanthrene (Phen) in the yeast bioassay. Data are expressed as mean \pm standard error of the mean. Significance, as determined by a posttest based on Student-Newman-Keuls analysis, is indicated as follows: *** $p < 0.001$; ** $0.001 > p < 0.01$; * $0.01 > p < 0.05$. 3,6-DMP = 3,6-dimethylphenanthrene; MP = methylphenanthrene.

PAH interaction after arrival in the cytoplasm, the binding and subsequent activation of the AhR signaling. This process is important to subsequent PAH toxicity in a wide variety of cell types and was therefore a reasonable indicator for a potency comparison of phenanthrenes with varying methyl substituents. We chose the modified yeast model system to study the human AhR activation process for 2 reasons. First, AhR is a widely studied receptor that binds to PAHs and induces subsequent changes in gene expression that in turn determine toxic effects. Second, the toxicological mechanism of PAHs can be complicated, and understanding the process one step at a time may be more useful than directly applying a more complicated system (mammalian cell culture or a whole animal) with many blinded steps.

The present data suggested that the presence of methyl groups on phenanthrene rings increased their potency in the AhR activation and signaling processes. This result may mean that the methylated derivatives thus have greater toxic potential than phenanthrene. In addition, the position of methyl modification may also play a role in toxicity. The methylphenanthrenes with the methyl group in equatorial positions (1-MP, 2-MP, and 3-MP) seem to have the highest potencies, whereas the derivatives with the methyl group in the bay region (4-MP) or on the back (9-MP) are not as active in this assay. A similar AhR-activation pattern was observed in a rat liver system, in

which 1-MP, 2-MP, and 3-MP were also stronger than 9-MP in the AhR activation process; of the methyl derivatives tested, all were stronger agonists than the parental phenanthrene [16]. These findings also parallel research with polychlorinated biphenyls and dioxins, some of which are strong agonists of AhR. In the case of these compounds, extensive chemical structural studies indicate that chlorines at equatorial positions enhance binding affinity for the receptor and thus increase potency. Thus, our data are consistent with chemical structural data for polychlorinated biphenyls and dioxins that explain interactions with AhR.

The only commercially available dimethylated phenanthrene tested in the present study, 3,6-DMP, which has 2 equatorial substituents, was significantly more potent than the 3-MP, 4-MP, and 9-MP when EC25s were compared. The 3,6-DMP also produced a signal in the bioassay that was only approximately 50% of that produced by phenanthrene and the monomethylated derivatives. The simplest explanation for this reduced signal was decreased solubility of 3,6-DMP compared with phenanthrene and monomethyl derivatives, because its estimated water solubility is approximately 10-fold lower than that of phenanthrene and approximately 3-fold lower than that of the monomethylated phenanthrenes [25]. However, benzo[a]pyrene, which is estimated to be 10-fold to 50-fold less soluble than 3,6-DMP [25], has been shown to be a very potent activator in this yeast bioassay and to produce a maximum response similar to that of phenanthrene [22]. An alternate explanation may be that 3,6-DMP is a partial agonist. Further studies are under way to elucidate the nature of the AhR-mediated response to dimethyl phenanthrenes in human cells.

At present, the available toxicological information about methylated phenanthrenes, or any other methylated PAH derivatives, is quite limited. Future studies will target the next step in the AhR pathway, which is induction of cytochrome P450 gene family (CYP) expression. Well-known target genes of AhRC include CYP1A1, CYP1A2, CYP1B1, and several phase II enzymes that are involved in xenobiotic metabolism. Once expressed, these P450s can oxidize the PAHs to transform them into metabolites that may be genotoxic. The PAH-DNA adducts may be useful as markers for PAH activity in the future studies as well.

In summary, the methylated phenanthrenes are generally recognized to be abundant among the PAHs of petrogenic origin, and they activate AhR to induce transcriptional signaling more potently than does phenanthrene. Future studies are still required to reveal their toxicology in detail. However, considering the lack of data relevant to methylated PAHs in general, the present study may serve as a model to study the methylated PAHs systematically and provide insight into their mechanistic effects.

SUPPLEMENTAL DATA

Tables S1–S3. (24 KB DOC).

Acknowledgment—The present study was supported by a grant from the US National Institute of Environmental Health Sciences (U01/U19 IU19 ES20677), the Tulane University Cancer Center, and the Louisiana Cancer Research Consortium.

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