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Hormonal Activity of Polycyclic Musks Evaluated by Reporter Gene Assay

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Synthetic musk fragrance compounds, such as polycyclic musks (PCMs), are a group of chemicals used extensively as personal care products, and can be found in the environment and the human body. PCMs, such as 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexa-methylcyclopenta-γ-2-benzopyran (HHCB) and 7-acetyl-1,1,3,4,4,6-hexamethyl-tetralin (AHTN), are known to have agonistic activities toward human estrogen receptor α (hERα) and hERβ, and have antagonistic activity toward the human androgen receptor (hAR), as shown in several reporter gene assays. However, little is known about the interaction of PCMs with the human thyroid hormone receptor (hTR), and the hormonal effects of other PCMs except for HHCB and AHTN. In this study, we focus on the interactions of six PCMs, namely, HHCB, AHTN, 4-acetyl-1,1-dimethyl-6-*tert*-butyl-indan (ADBI), 6-acetyl-1,1,2,3,3,5-hexamethylindan (AHMI), 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone (DPMI), and 5-acetyl-1,1,2,6-tetramethyl-3-isopropy-lindan (ATII) with hERα, hAR, and hTRβ by *in vitro* reporter gene assay using Chinese hamster ovary cells. All the samples were found to be agonists toward hERα, whereas no agonistic activities of these PCMs for hAR and hTRβ were observed. No antagonistic activities for hERα and hTRβ were observed at the concentrations tested. However, several PCMs, namely, HHCB, AHTN, ATII, ADBI, and AHMI, showed dose-dependent antagonistic activities for hAR, and the IC_{50} values of these compounds were estimated to be 1.0×10^{-7}, 1.5×10^{-7}, 1.4×10^{-7}, 9.8×10^{-6}, and 1.4×10^{-7} M, respectively. The results suggest that these PCMs interact with hERα and hAR but have no hormonal effect on hTRβ. This is the first report on the agonistic and antagonistic activities of ATII, ADBI, AHMI, and DPMI for hERα and hAR as determined by *in vitro* reporter gene assay using stably transfected Chinese hamster ovary cells.

1. Introduction

Synthetic musk fragrance compounds (nitro musk and polycyclic musk) belong to a family of chemicals used extensively as personal care products.[(1)] In particular, polycyclic musks (PCMs), namely, 1,3,4,6,7,8-hexahydro-4,6,6,7,8-hexa-methylcyclopenta-γ-2-benzopyran (HHCB), 7-acetyl-1,1,3,4,4,6-hexamethyltetralin (AHTN), 5-acetyl-1,1,2,6-tetramethyl-3-isopropy-lindan (ATII), 4-acetyl-1,1-dimethyl-6-*tert*-butyl-indan (ADBI), 6-acetyl-1,1,2,3,3,5-hexamethylindan (AHMI), and 6,7-dihydro-1,1,2,3,3-pen-

tamethyl-4(5H)-indanone (DPMI) are used as fragrance compounds in wash powder and cosmetics, with a worldwide production volume of approximately 6000 tons per year.[1,2] A recent study showed the occurrence of HHCB and AHTN in rivers, ocean, surface water, sewage sludge, sediment, fish, and mussels.[2–13] Typical high concentrations of these compounds in wastewater have been detected in the low μg/L range; in anthropogenically influenced surface waters, the levels of these compounds were reported to be in the ng/L range.[5,6,12] Our previous study has also demonstrated that relatively higher concentrations of PCMs, such as HHCB and AHTN, were detected in airborne particulate matter.[13]

Limited information has been reported on the hormonal effects of PCMs associated with the transcription or repression of novel steroid hormone receptors. A previous study showed that two PCMs, namely, HHCB and AHTN, have weak agonistic activities as demonstrated in human estrogen receptor α (hERα)- and β (hERβ)-dependent gene transcription assays.[14–16] Moreover, Schreurs *et al.* demonstrated that four PCMs, namely, AHTN, HHCB, AHMI, and ADBI, exhibit agonistic or antagonistic activities toward hER, hAR, and a progesterone receptor using the cell-based reporter gene assay.[15,16] However, there is no information on the interaction of PCMs toward the thyroid hormone receptor (TR). In addition, to date, agonistic and antagonistic activities toward hERs and hAR have already been well characterized in a few PCMs, such as HHCB and AHTN; however, limited information is currently available on the hormonal effects of other PCMs.[14–17] In this study, therefore, we investigated the agonistic and antagonistic activities of PCMs, namely, HHCB, AHTN, ATII, ADBI, AHMI, and DPMI, toward various nuclear receptors, namely, hERα, hAR, and hTRβ, by *in vitro* reporter gene assay using transfected Chinese hamster ovary cells.

2. Materials and Methods

2.1 *Chemicals*

HHCB, AHTN, ADBI, AHMI, DPMI, and ATII were obtained from Promochem (Teddington, UK). These reagents were dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd., Osaka, Japan) for the preparation of test solutions. The chemical structures of PCMs used in this study are shown in Fig. 1. Other solvents used were of reagent grade (Wako Pure Chemical Industries, Ltd.).

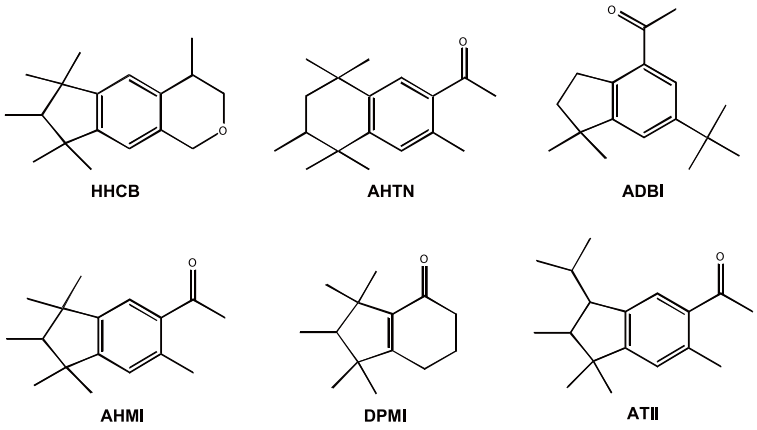

Fig. 1. Structures of PCMs used in this study. (HHCB) 1,2,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran; (AHTN) 6-acetyl-1,1,2,4,4,7-hexamethyltetraline; (ATII) 5-acetyl-1,1,2,6-tetramethyl-3-isopropylindan; (ADBI) 4-acetyl-1,1-dimethyl-6-*tert*-butyl-indan; (AHMI) 6-acetyl-1,1,2,3,3,5-hexamethylindan; (DPMI) 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone.

2.2 *Reporter gene assays for hERα, hAR, and hTRβ*

To evaluate the agonistic activity of various PCMs toward hERα, hAR, or hTRβ, reporter gene assay was carried out using Chinese hamster ovary cells (CHO-K1 cells, American Type Culture Collection), as described previously.[18–21]

Stock solutions of test chemicals were subjected to a 10-fold serial dilution with DMSO to prepare seven concentrations in the range of 10^{-1} to 10^{-7} M. These DMSO solutions were further diluted with DMEM/F-12 to give a final DMSO concentration of 0.1% (v/v). For the measurement of agonistic activity, the cultured cells were trypsinized and prepared at a density of 1.0×10^5 cells/ml in the assay medium, and seeded at 85 µl (for the hERα and hTRβ reporter gene assays) or 90 µl (for the hAR reporter gene assay) in 96-well plates. In the hERα and hTRβ reporter gene assays, the cells were transfected with a hERα or hTRβ expression plasmid, a reporter plasmid containing an estrogen-responsive element (ERE) or a thyroid hormone-responsive element (TRE), and phRL-TK (Promega, Madison, WI, U.S.A.), using the transfection reagent FuGene6 (Roche Diagnostics Co., Indianapolis, IN, U.S.A) according to the manufacturer's instruction. In the hAR reporter gene assay, namely, AR-Ecoscreen™, CHO-K1 cells express hAR and are stably transfected with a hAR-responsive luciferase reporter gene, which is based on the receptor-mediated mechanism of action.[19,21] After a 3-h transfection period (except for the hAR reporter gene assay), the prepared sample solution was added to the plates and incubated for 20 h at 37°C in each plate. The nominal test concentration of the samples applied to the cells ranged from 10^{-4} to 10^{-10} M. The controls, including the solvent control and 10^{-9} M 17β-estradiol: E_2 (for hERα), 10^{-8} M 5α-dihydroteststerone: DHT (for hAR), or 10^{-8} M 3,3',5-triiodothyronine: T3 (for hTRβ) as the positive controls were also incubated in each plate. Following a 20-h culture, the luciferase substrate, Steady-Glo™ (Promega), was added to all assay wells. After shaking at room temperature for 5 min, chemiluminescence intensity was measured using a Wallac 1420 ARVO SX multilabel counter (Perkin-Elmer, MA, U.S.A.).

In the reporter gene assay for assessing antagonistic activity, the cultured cells were prepared according to the same procedure as described in the measurement of agonistic activity, and DMEM/F-12 containing 10^{-9} M E_2 (for hERα), 5×10^{-10} M DHT (for hAR) or 10^{-8} M T3 (for hTRβ) was used for the final sample dilutions to detect the inhibition of luciferase activity.

Regarding the toxicity of PCMs to CHO-K1 cells, the toxicological property of PCMs was evaluated following the procedure of Satoh *et al.*[19] CHO-K1 cells that are stably transfected with the luciferase gene pcDNA-luc (cLuc-EcoScreen™ cells) and stably express luciferase without induction were used in this study.

2.3 *Data analysis*

Data were presented as the mean and standard deviation (S.D.) of triplicate assays. For the agonistic activity, the 50% effective concentration (EC_{50}) and the 10% effective concentration (EC_{10}) values were calculated by determining the concentration of the test chemical corresponding to 50% and 10% of its maximum luciferase activity of the positive control. For determining the antagonistic activity, the 50% inhibition concentration (IC_{50}) for the antagonistic assay was calculated by determining the concentration of the test chemical corresponding to 50% inhibition of the 5×10^{-11} M E_2-induced estrogen activity, 5×10^{-10} M DHT-induced androgenic activity, and 1×10^{-8} M T3-induced luciferase activity for thyroid hormonal activity. The toxicity value represents a decrease of 50% in absorbance at 595 nm between DMSO and the test compound.

3. Results

All the PCMs showed agonistic activity for hERα in a dose-dependent manner (Fig. 2) when the concentration was less than 10^{-5} M, and the agonistic activity of these compounds was approximately 10^4 to 10^5 smaller than that of E_2 as a positive control. The EC_{10} values of HHCB, AHTN, ATII, ADBI, AHMI, and DPMI were estimated as 6.8×10^{-7}, 3.6×10^{-7}, 5.8×10^{-8}, 1.3×10^{-6}, 3.9×10^{-7}, and 1.1×10^{-6} M, respectively (Table 1). Among these PCMs, the highest agonistic activity for hERα was observed in ATII as estimated from the EC_{10} and EC_{50} values. On the other hand, no antagonistic activity of the PCMs for hERα was observed at the concentrations tested in this study (Table 2).

No agonistic activity for hAR in all the PCMs used in this study was detected (Table 1). In contrast, the PCMs except for DPMI exhibited antagonistic activity for hAR at the concentration ranges tested in this study (Fig. 3), and the IC_{50} values for

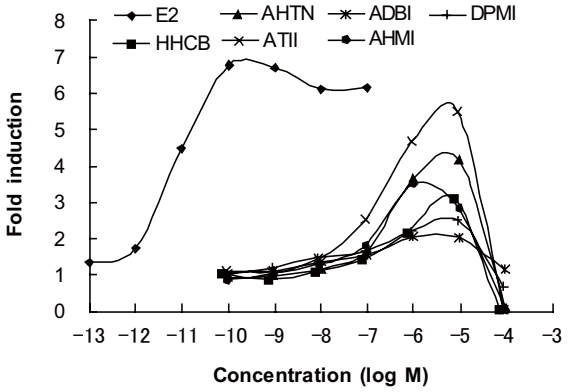

Fig. 2. Agonistic activity of PCMs for hERα as determined by reporter gene assay using stably transfected CHO-K1 cells. Data are presented as the mean of triplicate assays.

Table 1
Agonistic activities of PCMs for human estrogen receptor α (hERα), androgen receptor (hAR), and thyroid hormone receptor β (hTRβ) by reporter gene assay using stably transfected CHO-K1 cells.

Chemical	hERα		hAR		hTRβ	
	EC_{10} (M)	EC_{50} (M)	EC_{10} (M)	EC_{50} (M)	EC_{10} (M)	EC_{50} (M)
E_2*	1.8×10^{-12}	1.2×10^{-11}	—	—	—	—
DHT**	—	—	1.3×10^{-11}	8.4×10^{-10}	—	—
T3***	—	—	—	—	6.3×10^{-11}	3.4×10^{-10}
HHCB	6.8×10^{-7}	Weak****	ND	ND	ND	ND
AHTN	3.6×10^{-7}	2.0×10^{-6}	ND	ND	ND	ND
ATII	5.8×10^{-8}	3.2×10^{-7}	ND	ND	ND	ND
ADBI	1.3×10^{-6}	Weak****	ND	ND	ND	ND
AHMI	3.9×10^{-7}	2.2×10^{-6}	ND	ND	ND	ND
DPMI	1.1×10^{-6}	Weak****	ND	ND	ND	ND

The 50% effective concentration (EC_{50}) and the 10% effective concentration (EC_{10}) values were calculated by determining the concentration of the test chemical corresponding to 50% and 10% of its maximum luciferase activity of the positive control. ND: agonistic activity not detected; —: agonistic activity not determined; *E_2: 17β-estradiol (positive control for hERα); **DHT: 5α-dihydroteststerone (positive control for hAR); ***T3: 3,3',5-triiodothyronine (positive control for hTRβ); ****Weak: EC_{50} values not calculated but weak agonistic activity detected.

Table 2
Antagonistic activities and toxicity effects of PCMs for human estrogen receptor α (hERα), androgen receptor (hAR), and thyroid hormone receptor β (hTRβ) as determined by transfected reporter gene assay with CHO-K1 cells.

Chemical	hERα		hAR		hTRβ	
	IC_{50} (M)	Toxicity (M)	IC_{50} (M)	Toxicity (M)	IC_{50} (M)	Toxicity (M)
HHCB	ND	$> 7.5\times10^{-6}$	1.0×10^{-7}	$> 7.5\times10^{-6}$	ND	$> 7.5\times10^{-6}$
AHTN	ND	$> 9.8\times10^{-6}$	1.5×10^{-7}	$> 9.8\times10^{-6}$	ND	$> 9.8\times10^{-6}$
ATII	ND	$> 9.0\times10^{-6}$	1.4×10^{-7}	$> 9.0\times10^{-6}$	ND	$> 9.0\times10^{-6}$
ADBI	ND	$> 9.8\times10^{-5}$	9.8×10^{-6}	$> 9.8\times10^{-5}$	ND	$> 9.8\times10^{-5}$
AHMI	ND	$> 9.5\times10^{-5}$	1.4×10^{-7}	$> 9.5\times10^{-6}$	ND	$> 9.5\times10^{-5}$
DPMI	ND	$> 9.0\times10^{-5}$	$> 1.6\times10^{-6}$	$> 9.0\times10^{-6}$	ND	$> 9.0\times10^{-5}$

The 50% inhibition concentration (IC_{50}) was calculated by determining the concentration of the test chemical corresponding to 50% inhibition of the 5×10^{-11} M E_2-induced estrogen activity, 5×10^{-10} M DHT-induced androgenic activity and 1×10^{-8} M T3-induced luciferase activity for thyroid hormonal activity. ND: antagonistic activity not detected. The toxicity level represents a decrease of 50% in absorbance at 595 nm between DMSO and the test compound.

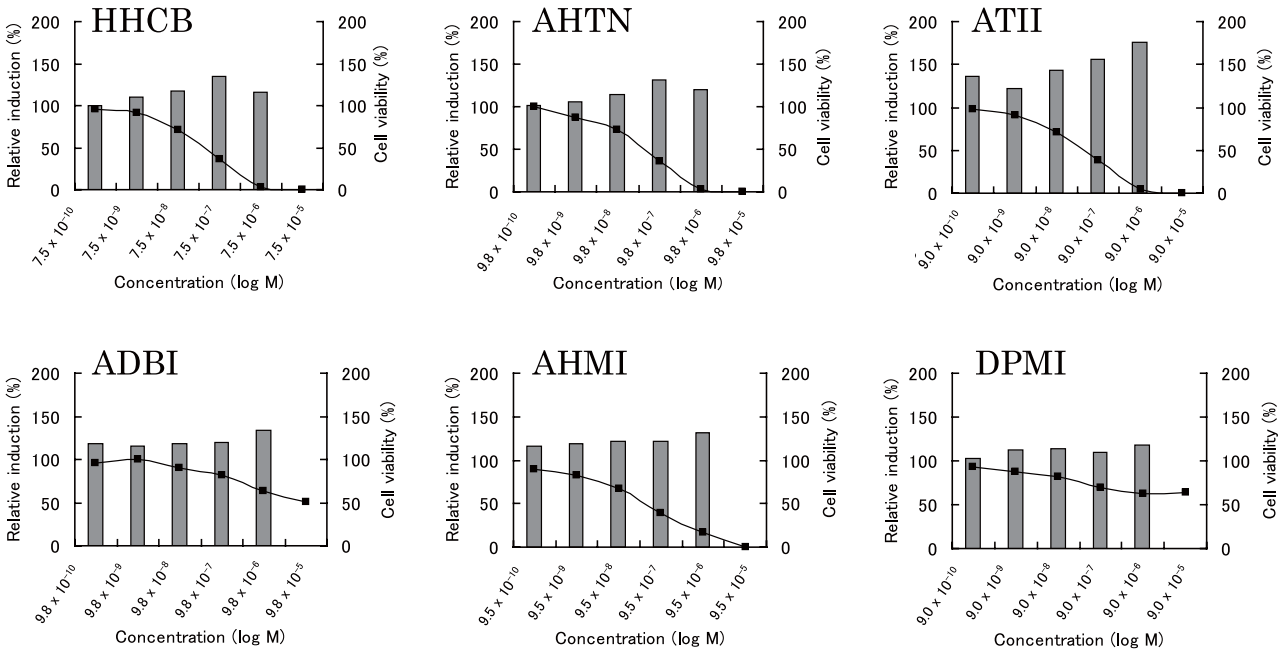

Fig. 3. Antagonistic activity (black bar) and toxicity effects (black symbol) of PCMs for hAR as determined by reporter gene assay using stably transfected CHO-K1 cells. Data are presented as the mean of triplicate assays.

HHCB, AHTN, ATII, ADBI, and AHMI were estimated to be 1.0×10^{-7}, 1.5×10^{-7}, 1.4×10^{-7}, 9.8×10^{-6}, and 1.4×10^{-7} M, respectively (Table 2).

By using an *in vitro* reporter gene assay, treatments with PCMs did not induce hTRβ-mediated transcriptional activity (Table 1). In addition, no antagonistic activities for hTRβ were observed for all the PCMs at the concentration ranges tested in this study (Table 2).

4. Discussion

In this study, the agonistic and antagonistic activities of six PCMs (HHCB, AHTN, ATII, ADBI, AHMI, and DPMI), which are ubiquitously present in the environment, for hERα, hAR, or hTRβ were assessed by *in vitro* reporter gene assay using stably transfected CHO-K1 cells.

Seinen *et al.* evaluated the agonistic activities of AHTN and HHCB for hERα and hERβ.[14] Weak agonistic activities of AHTN and HHCB were observed by reporter gene assay using hERα-transfected HEK293 cells but both compounds did not activate hERβ, and their potencies were about six orders of magnitude lower than that of the endogenous ligand E_2. In response to 0.1 nM E_2, the hERα-transfected HEK293 cells exhibited a maximum induction response of 130-fold, whereas AHTN and HHCB caused a maximal induction of 20-fold at 50 μM. In addition, Schreurs *et al.* also showed the weak agonistic activities of AHTN and HHCB by reporter gene assay using hERα-transfected HEK293 cells.[17] In this study, we used hERα-transfected CHO-K1 cells to assess the agonistic activities of AHTN and HHCB. As a result, AHTN and HHCB were found to be agonists toward hERα, which is in accordance with previous studies.[14,17]

A few studies were performed to determine the agonistic activities of PCMs except for AHTN and HHCB for both hERs. Schreurs *et al.* demonstrated that none of the five tested musks, namely, AHTN, HHCB, ADBI, AETT, and AHMI, showed clear ER agonism on both hERα and hERβ in HEK293 cells.[16] In this study, however, ATII, ADBI, AHMI, and DPMI showed agonistic activity for hERα as demonstrated by *in vitro* reporter gene assay using stably trasfected CHO-K1 cells, suggesting that the relative estrogenic potencies of PCMs decreased in the order of ATII > AHTN = AHMI > HHCB > ADBI = DPMI. To our knowledge, this is the first report on the agonistic activities of ATII, ADBI, AHMI, and DPMI for hERα.

A previous study demonstrated that AHTN, HHCB, AHMI, and ADBI have agonistic and antagonistic activities for various nuclear receptors, such as hERα, hERβ, hAR, and progesterone (hPR).[15,16] In a reporter gene assay using AR CALUX® cells to assess antagonistic activity, the IC_{50} values of AHTN, HHCB, and AHMI were estimated to be 3.6×10^{-6}, 2.9×10^{-6}, and 2.7×10^{-6} M, respectively, whereas the IC_{50} of ADBI was not determined. In the reporter gene assay used in this study, however, the IC_{50} of ADBI was estimated to be 9.8×10^{-6} M, suggesting that this compound shows an antagonistic activity for hAR. For detecting the antagonistic activities of PCMs, namely, AHTN, HHCB, and AHMI (IC_{50} values in this study: 1.5×10^{-6}, 1.0×10^{-7}, and 1.4×10^{-7} M, respectively) for hAR, the *in vitro* reporter gene assay using stably transfected CHO-K1 cells showed a higher sensitivity than other *in vitro* assays, such as the CALUX cell assay. The CHO-K1 cells used in this study express hAR and are stably trasfected with a hAR-responsive luciferase reporter gene, which is based on the receptor-mediated mechanism of action.[19] A previous study has demonstrated that this assay system could detect a wide range of organic/inorganic compounds, indicating its high sensitivity and rapidness for screening various hormonally active agents.[19] Therefore, the reporter gene assay used in this study could be a useful tool for screening hormonally active agents including PCMs. Additionally, an antagonistic activity of ATII for hAR was found in this study. To our knowledge, this is the first report on the antagonistic activities of ATII and ADBI for hAR and hERα.

In other studies, several chemical compounds have been assessed for their interaction with the thyroid hormone receptor. A few hydroxyl-polychlorinated biphenyl xeno-thyroid hormones have been found, namely 3',4,6-trichlorobiphenyl-3-ol, 2',4,5',6-tetrachlorobiphenyl-2-ol, 2',3,3',5-tetrachlorobiphenyl-2-ol, 2',3,3',6-tetrachlorobiphenyl-4-ol, 3,3',5,5',6-pentachlorobiphenyl-2-ol, 2,3',4,5',6-pentachlorobiphenyl-3-ol,[22] bisphenol A related compounds in 3,3',5,5'-tetrabromobisphenol A, and 3,3',5,5'-tetrachlorobisphenol A.[23] These results indicate that lipophilic groups

must be present at the 3,5-position of the outer ring to optimize binding. In this study, none of the test compounds showed hTRβ transactivation and repression (Tables 1 and 2). As for the reason for the nonresponsiveness, the chemical structures of the PCMs did not affect the agonistic and antagonistic activities toward hTRβ.

In conclusion, the hormonal effects of PCMs, namely, HHCB, AHTN, ATII, ADBI, AHMI, and DPMI were demonstrated using various hormone receptor-dependent reporter gene assays. To our knowledge, this is the first report on the agonistic and antagonistic activities of ATII, ADBI, AHMI, and DPMI for hERα and hAR as determined by *in vitro* reporter gene assay using stably transfected CHO-K1 cells.

Acknowledgments

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