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Effects of polycyclic musks HHCB and AHTN on steroidogenesis in H295R cells

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HIGHLIGHTS

- HHCB and AHTN can inhibit progesterone and cortisol production.
- HHCB and AHTN can down-regulate *3βHSD2* and *CYP21*.
- HHCB and AHTN can up-regulate *CYP17*, *CYP11B1* and *CYP11B2*.
- AHTN can influence steroidogenesis by affecting *MC2R* expression.

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ABSTRACT

1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(γ)-2-benzopyran (HHCB) and 7-acetyl-1,1,3,-4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN) are widely used in personal care products. Previous studies showed that HHCB and AHTN can be found in various environmental matrices and have potential endocrine disrupting effects. However, the effects on adrenocortical function of HHCB and AHTN are not fully understood. This study evaluated the influences of HHCB and AHTN on seven steroid hormones (progesterone, aldosterone, cortisol, 17α-OH-progesterone, androstenedione, 17β-estradiol, and testosterone) and 10 genes involved in steroidogenic pathways (*HMGR*, *StAR*, *CYP11A1*, *3βHSD2*, *CYP17*, *CYP21*, *CYP11B1*, *CYP11B2*, *17βHSD*, and *CYP19*) using the H295R cell line in the absence and presence of 8-Br-cAMP. *MC2R* transcription on the cell membrane was also examined to further investigate the effects of HHCB and AHTN on adrenal steroidogenesis. The results demonstrated that HHCB and AHTN could inhibit progesterone and cortisol production mainly by the suppression of *3βHSD2* and *CYP21*. Meanwhile, high concentrations of AHTN can affect the sensitivity of H295R cells to ACTH by disrupting *MC2R* transcription. Overall, the results indicate that high concentrations of HHCB and AHTN can affect steroidogenesis *in vitro* using the H295R cell line.

1. Introduction

Polycyclic musks (PCMs), predominantly 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(γ)-2-benzopyran (HHCB) and 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN), are widely used as essential fragrance ingredients in cosmetics, perfumes, laundry cleaning detergents and other consumer products. Due to their widespread use, lipophilicity, and persistence, PCMs have been detected in various environmental matrices including freshwater, seawater, sediments, biota, and even humans (Zeng et al., 2008; Hu et al., 2010, 2011; Hutter et al., 2010). The wide distribution of PCMs and their polycyclic nature raise the question whether PCMs have the potential to disturb endocrine functions. There are increasing evidences that PCMs could exert estrogenic, anti-estrogenic, anti-androgenic, and anti-progestagenic activities, and they could also cause reproductive disorder and population decline of the polychaete (*Capitella species* I) (Seinen et al., 1999; Bitsch et al., 2002; Schreurs et al., 2004, 2005a, 2005b; Yamauchi et al., 2008; Ramskov et al., 2009). Most previous studies are focused on the reproductive effects, but less attention is paid to other endocrine organs, such as the adrenal gland. Because of high lipid content, copious blood supply and steroidogenic capacity, the adrenal gland is likely to accumulate lipophilic chemicals (Ribelin, 1984). Thus, it is important to assess the adrenocortical toxicity of HHCB and AHTN.

The adrenal gland is one of the most important endocrine organs in human and, unlike the gonads, essential for survival (Sanderson, 2006). All steroidogenic processes take place in the adrenal cortex, which is responsible for production of three major types of steroid hormones: mineralocorticoids, glucocorticoids, and the sex hormones (Li and Wang, 2005). The H295R cell line,

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derived from an adrenocortical carcinoma, preserves the intact steroidogenic pathways in the normal adrenal cortex and allows the measurement of alterations in gene transcription, enzyme activity and hormone production at the same time, which makes it a unique bioassay model for evaluating the effects of chemicals on steroidogenic pathways and adrenocortical toxicity (Fig. 1) (Gazdar et al., 1990; Johansson et al., 2002; Oskarsson et al., 2006; Hecker and Giesy, 2008). Additionally, H295R cells treated with forskolin (a cAMP inducer) could make the steroidogenic transcript profiles more similar to those in normal human adrenal cells, which further suggests that the H295R cell line is particularly suitable for the risk assessment of adrenocoritical toxicity (Oskarsson et al., 2006). Activation of cAMP is also critical for the investigation of adrenocorticotropic hormone (ACTH)'s effect on the steroidogenic process under biological conditions. ACTH is released from the pituitary gland and stimulates the production of steroid hormones by activating adenylyl cyclase to elevate cAMP after binding to melanocortin 2 receptor (*MC2R*) on the cell membrane (Sewer and Waterman, 2003; Li and Wang, 2005).

In order to evaluate the effects of the tested PCMs on steroidogenic pathways and adrenocortical toxicity, we exposed H295R cells to HHCB and AHTN at different concentrations in the absence and presence of 1 mM 8-Br-cAMP (a cAMP analog) for 48 h. Production of seven steroid hormones (progesterone, aldosterone, cotisol, 17α-OH-progesterone, androstenedione, testosterone, and 17β-estradiol) and transcription of ten genes encoding the steroidogenic enzymes (*HMGR*, *StAR*, *CYP11A1*, *3βHSD2*, *CYP17*, *CYP21*, *CYP11B1*, *CYP11B2*, *17βHSD*, and *CYP19*) were investigated. *MC2R* transcription on the cell membrane was also examined to further elucidate the effects of HHCB and AHTN on adrenal steroidogenesis.

2. Materials and methods

2.1. Chemicals

Tonalide (AHTN, 98%) was obtained from LGC-Promochem and Galaxolide (HHCB, 95%) was from Beijing Wohai Global Technology Co., Ltd. 8-Bromo-cyclic adenosine monphosphate (8-Br-cAMP) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO, 99.9%) was purchased from Amresco (Shanghai, China).

2.2. Cell culture

The H295R human adrenocortical carcinoma cell line was purchased from the American Type Culture Collection (ATCC#CRL-2128, Manassas, VA, USA) and cultured in 75 cm^2 petri dish with 12 mL of DMEM/F-12 (HyClone, SH30023.01B) at 37 °C containing 95% air and 5% CO_2 atmosphere. The medium was supplemented with 1% insulin–transferrin–selenium-G (ITES-G, Gibco BRLm, 41400-045), 1% penicillin–streptomycin (GibcoBRL, 15140-122), and 2.5% Nu-Serum (BD Bioscience, 35500). The medium was refreshed two or three times a week and cells were detached from the culture dish for subculture. Cells were used between passages 5 and 10 after thawing from liquid nitrogen.

2.3. Cell exposure

Test chemicals were dissolved in DMSO and the final concentration of vehicle, DMSO, in the exposure medium was 0.1% (v/v). For cell viability measurements, H295R cells were seeded in a 96-well plate at a concentration of 6×10^5 cells mL^{-1} in 200 μL of medium per well. After 24 h, cells were exposed to 0.25, 2.5, 25 μM of tested chemicals dissolved in DMSO and the medium was refreshed with phenol red-free DMEM/F-12 medium (HyClone, SH30272.01) supplemented with 1% ITES-G, 1% penicillin–streptomycin, and 2.5% charcoal stripped fetal bovine serum (Biological industries, 04-201-1A). After 48 h of exposure, cell viability was evaluated by Alamar Blue assay. Briefly, resazurin was added directly to the 96-well plates to a final concentration of 10 μM without removing the medium. After incubating for 2 h at 37 °C, 100 μL of medium was transferred to a 96-well black polystyrene microplate, and the fluorescence signal (530 nm/590 nm, excitation/emission) was subsequently measured using spectral scanning multimode reader (Thermo Scientific Varioskan Flash).

For the measurement of hormone and gene transcription, the initial plating cell density was 1.2×10^6 cells $well^{-1}$ in 2.5 mL of cell medium per well of a six-well plate. 1 mM 8-Br-cAMP was added to mimic the ACTH upsurge according to previous studies

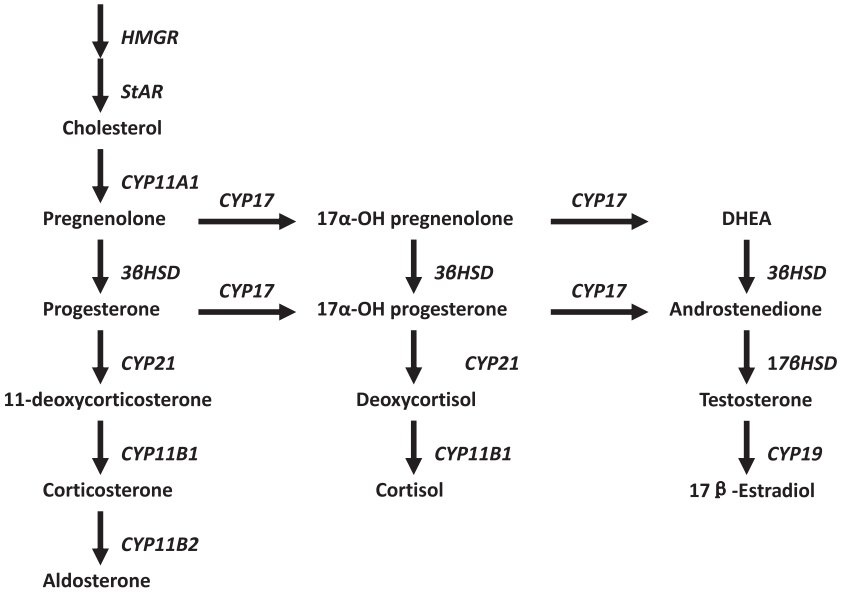

Fig. 1. Steroidogenic pathways in H295R cells. Enzymes are in italics and hormones are in bold. CYP indicates cytochrome P450; HSD indicates hydroxysteroid hydrogenase; DHEA indicates dehydroepiandrosterone. Modified based on the diagram of Furuta et al. (2008), Ma et al. (2011) and Li et al. (2004).