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Assessment of androgen receptor agonistic/antagonistic effects on 25 chemicals in household applicants by OECD *in vitro* stably transfected transcriptional activation assays

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HIGHLIGHTS

- AR agonistic/antagonistic effects on 25 chemicals in household applicants are assessed.
- Two *in vitro* stably transfected transcriptional activation assays are applied.
- α-Dodecyl-ω-hydroxypoly (oxyethylene) has been determined as AR antagonist.
- 3-Iodo-2-propynyl butylcarbamate also exhibited a weak AR antagonistic effect.
- This report firstly provides information about their AR agonist/antagonistic effects.

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ABSTRACT

The aim of this study is to assess the androgen receptor (AR) agonistic/antagonistic effects on various chemicals, which are used in household products including cleaning agents and wetted tissues by *in vitro* OECD test guideline No. 458 (using AR-EcoScreen™ cell line) and the me-too test method (using 22Rv1cell line), which was adopted as OECD project No. 4.99. All chemicals were not determined as AR agonists. However α-dodecyl-ω-hydroxypoly (oxyethylene) and 3-iodo-2-propynyl butylcarbamate have shown a weak AR antagonistic effects with IC_{50} values of 2.18 ± 0.12 and 4.26 ± 0.17 μg/ml via binding affinity to AR in only 22Rv1/mouse mammary tumor virus using AR transcriptional activation assay, because of their different cytotoxicity on each applied cell line. This report firstly provides information about agonistic/antagonistic effects against human AR of various chemicals including surfactants and biocides by OECD *in vitro* stably transfected transcriptional activation assays. However, further *in vivo* and human model studies are needed to confirm their adverse effects.

1. Introduction

During the past several decades, a number of synthetic chemicals were developed to meet a wide variety of industrial needs, and they were easily released into the environment (UNEP, 2004). Unfortunately, various chemicals such as bisphenols, phthalates and parabens are considered endocrine disrupting chemicals (EDCs), since they can exhibit the adverse effect on the mammalian endocrine system (Schug et al., 2011). Especially, nonylphenolis the most abundant derivatives of alkylphenol polyethoxylate compounds that mimics the biological activity of estrogens by binding to estrogen receptor affecting reproduction (Brown and Reinhard, 2003). Alkylphenol polyethoxylate compounds are widely used as non-ionic surfactants in detergents, emulsifiers and cosmetics (Cevdet et al., 2009). EDCs can be exposed to human by oral consumption of food and water or by using cosmetics and various household applicants including cleaning agents (Giulivo et al.,

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2016). EDCs act together to exert adverse activity at low exposure level (Schug et al., 2011; Vandenberg et al., 2013), and they have life-long effects and consequences for the next generation (Annamalai and Namasivayam, 2015). Therefore, risk assessment of EDCs is complicated by the observed low dose effects and non-monotomic dose responses (Vandenberg et al., 2012). EDCs can also show the adverse effect on endocrine function by endocrine-related receptors agonist and antagonist or by disrupting the synthesis of hormones (Annamalai and Namasivayam, 2015; Schug et al., 2011; Shanle and Xu, 2011; Vandenberg et al., 2013). For such reasons, various experts have typically estimated that exposure to EDCs are likely to contribute substantially to disease and dysfunction throughout life, with costs in the hundreds of billions of Euros or US dollars per year (Trasande et al., 2015).

To address the rising global concerns about EDCs, there is a critical need to screen chemicals that exert hormone-like activities. Therefore, worldwide organizations including Europe, the United States, Japan, and Republic of Korea have developed international standardization methods for detection of potential endocrine disrupting activities. The development of test guidelines for testing and assessment of EDCs was initiated in 1997 by the Organization for Economic Co-operation and Development (OECD), and this cooperation has been managed by the Endocrine Disruptors Testing and Assessment (EDTA) task force (OECD, 2010, 2005). The OECD approved the updated OECD conceptual framework for testing and assessment of EDCs in 2012 (OECD, 2012). This document describes five levels of test guidelines to determine the endocrine disruptors, and the *in vitro* test methods including cell based transcriptional activation (TA) assay are outlined in level 2 to provide data about the selected endocrine mechanisms (OECD, 2012). In addition, the United States Environmental Protection Agency (EPA) applied TA assay to confirm the interactions between EDCs and hormonal systems (USEPA, 2011).

The responses of nuclear receptors exert important roles in TA assays (Ozgyin et al., 2015; Shang et al., 2002). When ligands with hormonal functions bind to the nuclear receptors within cells, the ligand-receptor complex undergoes a conformational change that leads to a transcriptional response (Maire et al., 2010). Considering this mechanism, TA assays can be used to detect the potential hormonal activity of exogenous chemicals (Lee et al., 2012, 2014; Maire et al., 2010; Wang et al., 2014). OECD also has developed and validated the TA assays for determination of the androgen receptor (AR) agonist and antagonist. The TA assay using the AR-EcoScreen™ cell line was developed by Japan (Araki et al., 2005), and there was firstly adopted as OECD Test Guideline No. 458 in 2016. Furthermore, TA assays using the 22Rv1/mouse mammary tumor virus (MMTV) cell line (Sun et al., 2016) and U2-OS cell line (Burga et al., 2010) were suggested to OECD for the establishment of a performance-based test guideline (PBTG) with AR-EcoScreen™ AR TA. Consequently, the main purpose of our study is to assess the AR agonistic/antagonistic effects on chemicals in household applicants, whose AR agonistic and antagonistic effects are not yet known, by *in vitro* OECD test guideline No. 458 and 22Rv1/MMTV AR TA (OECD project 4.99).

2. Materials and methods

2.1. Chemicals and reagents

The 25 test substances used in this study are shown in Table 1. *N,N*-dimethyl-1-dodecanamine-N-oxide and D-limonene were evaluated as ethanolic solutions (Merck, Darmstadt, Germany). Ethylenediaminetetraacetic acid was dissolved in distilled water prior to use. All other test substances were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) for analysis. All stock solutions were prepared in consideration of each chemical's solubility. Briefly, each test chemical was diluted in DMSO or appropriate solvent at log-serial scale until any cloudiness or precipitation was noticeable. For conducting AR TA assays, 5α-dihydrotestosterone (DHT, Wako, Osaka, Japan), mestanolone (Sigma-Aldrich, St. Louis, MO), di-(2-ethylhexyl)phthalate (DEHP, Sigma-Aldrich, St. Louis, MO), hydroxyflutamide (Sigma-Aldrich, St. Louis, MO), and bisphenol A (BPA, Sigma-Aldrich, St. Louis, MO) were obtained commercially. The solvents, used in dissolving the chemicals, were used as a vehicle control.

2.2. Cell culture

2.2.1. AR-Ecoscreen™ cell line

The AR-Ecoscreen™ cell line was purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cells were propagated in Eagle's minimum essential medium: phenol red-free DMEM/F-12 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 5% v/v fetal bovine serum (FBS), zeocin (200 μg/mL), hygromycin (100 μg/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL). For assay, the cells were cultured in phenol red free DMEM/F-12 supplemented with 5% v/v dextran-coated, charcoal-treated (DCC)-FBS (PAA, Australia), penicillin (100 units/mL), and streptomycin (100 μg/mL). The cells were maintained in a 5% CO_2 incubator at 37 °C.

2.2.2. 22Rv1/MMTV cell line

The 22Rv1/MMTV cell line was established in our previous study (Sun et al., 2016). Cells were propagated in RPMI1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco, Grand Island, NY), 1 X antibiotic-antimycotic (Gibco, Grand Island, NY), and 2 mM GlutaMax™ (Gibco, Grand Island, NY) in a humidified 5% CO_2 atmosphere at 37 °C. For analysis, the cells were cultured in phenol red-free RPMI 1640 (Gibco, Grand Island, NY) supplemented with 5% DCC-FBS (PAA, Australia), 1X antibiotic-antimycotic (Gibco, Grand Island, NY) and GlutaMAX™ (Gibco, Grand Island, NY). The cells were maintained in a 5% CO_2 incubator at 37 °C.

2.3. Luciferase assays

2.3.1. AR-Ecoscreen™ AR TA assay

The cells (1×10^4 cells/well) were seeded in a series of standard 96-well microplates (Nunc, MA, USA) and then pre-incubated for 24 h at 37 °C in an atmosphere of 5% CO_2. The cells were exposed to serially diluted test solutions as well as a reference control. After incubation for 24 h, the media were removed from each well and replaced with luciferase assay solution (100 μL/well) (Steady-Glo® Luciferase Assay System, Promega, Madison, WI), followed by incubation for 5 min at room temperature. Luciferase activity was measured using a MicroBeta2 2450 plate counter (Perkin-Elmer, Waltham, MA). For antagonist assays, cell viability was assessed after the evaluation of luciferase activity by measuring the Renilla luciferase activity according to the manufacturer's instructions (Promega, Madison, WI). For the quality control, the responses of the cell line were continuously monitored using the control wells in each assay plate. The response for the agonist control (DHT) with 10 nM (for agonist test) and 500 pM (for antagonist test) should be at least 6.4 and 5.0, respectively. The fold-induction corresponding to the PC_{10} value of the concurrent PC (10 nM DHT) should be greater than 1 + 2 standard deviation (SD) of the fold-induction value (= 1) of the VC.

2.3.2. 22Rv1/MMTV AR TA assay

The cells (3×10^4 cells/well) were seeded in 96-well culture plates (Corning, Corning, NY). After incubation for 48 h, the control agents and serially diluted test compounds for the AR agonist/