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Oestrogenic and androgenic activity of oxybenzone and methylparaben *in vitro*

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Motivated by emerging concerns about health hazards associated with various industrial chemicals, this study investigated the disruption of endocrine system using well established *in vitro* assays. Due to the lack of scientific data on adverse effects of chemicals used in personal care products (PCPs), the focus was placed on oestrogenic and androgenic action of photostabiliser oxybenzone and preservative methylparaben. To this end we relied on *in vitro* assays for oestrogen and androgen receptor activation based on HeLa-9903 and AR-EcoScreen GR KO M1 cell lines to determine dose response according to respective OECD Test Guidelines 455 and 458. Our findings clearly demonstrate that both chemicals act as oestrogen receptor agonists and androgen receptor antagonists, raising additional concerns about health risks for humans posed by excessive and widespread use of such chemicals in PCPs.

KEY WORDS: agonist; antagonist; AR-EcoScreen GR KO M1; endocrine disruption; HeLa-9903; hormone receptors; personal care products

Endocrine disrupting chemicals (EDCs) pose significant health risks to the global population due their potential to interfere with the endocrine system. Many such endocrine disruptive effects are associated with the activation of nuclear hormone receptors, oestrogen and androgen in particular, as they alter hormone responsive genes (1).

The growing body of evidence linking EDCs to adverse health effects has resulted in two strategic EU documents, namely the Resolution on a comprehensive EU framework on endocrine disruptors (2) and the European Commission Communication on endocrine disruptors (3). The mechanisms of EDC action are highly complex and involve interactions with oestrogen, androgen, thyroid, and other hormone receptors. In agonistic interactions, ECDs mimic natural hormones and activate hormone receptors. In antagonistic interactions EDCs block hormone receptor activation and thus disrupt the function of natural hormones (4). EDC action can disrupt the synthesis, release, transport, metabolism, or elimination of natural hormones crucial for maintaining homeostasis and affect neurological, reproductive, and metabolic development and function, which may, in turn, lead to disorders such as diabetes, obesity, infertility, and hormone-dependent cancers (5). In addition to their complex modes of action, these chemicals do not adhere to traditional dose-response dynamics and their adverse effects can become apparent only years after initial exposure (6, 7).

To ensure successful implementation of strategic risk management for EDCs, the Organisation for Economic Co-operation and Development (OECD) developed test guidelines (TG) for quick screening and detection of androgen and oestrogen receptor agonists and antagonists (8, 9) contained in numerous pesticides, pharmaceuticals, preservatives, plasticisers, photostabilisers, and other products people eat, drink, or use for personal care (10, 11).

Among these chemicals, oxybenzone and methylparaben have gained much attention due to their widespread use in personal care products (PCPs). Oxybenzone (benzophenone-3) is commonly used as UV filter in sunscreens, cosmetics, plastics, and paints (12, 13). It can penetrate the skin and placental barrier and has been detected in different human samples such as urine, plasma, breast milk, and amniotic fluid (14–16). Recently, the Commission Regulation (EU) 2022/1176 was amended with conclusions made by the Scientific Committee for Consumer Safety (SCCS) (17) that oxybenzone is consumer-safe if its concentration as a UV filter in face creams, hand creams, and lipsticks does not exceed 6 % or it does not exceed 0.5 % in cosmetic products in which it protects the cosmetic formulation. Furthermore, the EU Joint Research Centre has proposed the inclusion of oxybenzone in the 5th Watch List of the Water Framework Directive in the group of sunscreen agents, which are already listed in the current WL (EU 2022/1307) to ensure that enough high-quality monitoring data are collected for risk assessment (18).

Methylparaben is often used as a preservative in PCPs, food, and pharmaceutical products (19), and its presence has been confirmed in human plasma, urine, breast, and placental tissue (20–23). In addition, *in vitro* and *in vivo* studies have shown its

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oestrogenic and anti-androgenic action (24, 25). The SCCS recently issued an opinion, limiting its safe-use concentration to 0.4 % in cosmetic products (26). The European Chemicals Agency (ECHA) has included both oxybenzone (13) and methylparaben (27) in its Endocrine disruptor assessment list (28).

Motivated by these recent regulatory activities, our aim was to evaluate the androgenic and oestrogenic activities of oxybenzone and methylparaben. This is the first study of the kind to strictly follow the respective OECD TG No. 458 (8) and OECD TG 455 (9) to provide science-based information for risk assessment and management of consumer products containing oxybenzone and methylparaben that can help in regulatory decision-making.

MATERIALS AND METHODS

Cell cultures

The AR-EcoScreen GR KO M1 cell line was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Osaka, Japan). This cell line is characterised by stable expression of both human androgen receptor reporter gene and firefly luciferase gene, making it appropriate for detecting (anti-) androgens. Additionally, this mutant cell line has improved specificity for androgen receptor because of glucocorticoid receptor (GR) knockout (29). Cells were cultured in tissue culture-treated T75 flasks (Sarstedt, Nümbrecht, Germany) and maintained according to the JCRB protocol. DMEM/F-12 (Gibco) supplemented with 10 % (v/v) foetal bovine serum (FBS, Sigma Aldrich, Steinheim, Germany), hygromycin (25 μg/mL, InvivoGen, San Diego, CA, USA), and phleomycin D1 (Zeocin®, 50 μg/mL, InvivoGen, San Diego, CA, USA) were used as cell culture media. Cells were grown at 37 °C and 5 % CO_2 until reaching ca. 90 % confluence, at which point they were used for experiments.

The HeLa-9903 cell line, designed to screen (anti-)oestrogens, as it is transfected with the oestrogen receptor α reporter gene construct, was also purchased from the JCRB Cell Bank and cultured in the same flasks as AR-EcoScreen GR KO M1 cells at 37 °C and 5 % CO_2. For cell propagation, we used the MEM cell culture medium (Gibco, Grand Island, NY, USA) supplemented with 10 % (v/v) charcoal-stripped FBS (Sigma Aldrich, Steinheim, Germany), 60 μg/mL kanamycin sulphate (Sigma Aldrich, Steinheim, Germany), and 1 % L-glutamine (Sigma Aldrich, Steinheim, Germany). At ca. 90 % confluence, cells were used for experiments.

Cytotoxicity evaluation

Dose-response cytotoxicity of methylparaben (Sigma Aldrich) and oxybenzone (Sigma Aldrich) was evaluated with the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Kit (MTS) assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells were seeded in clear flat bottom 96-well plates (Eppendorf, Hamburg, Germany) at a density of $4x10^4$ cells per well in 100 μL of cell culture medium (CCM). After incubation at 37 °C and 5 % CO_2 for 24 h, the cells were treated with oxybenzone or methylparaben in the doses of 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 μmol/L. Cells treated with 10 % (v/v) DMSO were used as positive control, while untreated cells were considered negative control. After the 24-hour treatment, the medium was removed, cells washed with phosphate buffered saline (PBS) three times, and the MTS reagent was added to each well. Cell plates were then incubated at 37 °C and 5 % CO_2 for another 2 h, and the quantity of formazan product (directly proportional to the number of living cells) measured by absorbance at 490 nm using a SpectraMax iD3 microplate reader (Molecular Devices, San Jose, CA, USA). The results are expressed as the mean percentage of live cells compared to negative control with standard deviation of six replicates from two individual experiments.

Methylparaben and oxybenzone dose selection for receptor activity determination

Our dose selection started with *in vitro* studies from the literature, including our previously published study (30–32). As there are no published data on biological effects of methylparaben and oxybenzone on HeLa-9903 and AR-EcoScreen GR KO M1 cells, we used the range between 10^{-5} and 10^2 μmol/L for both chemicals, as the doses in this range did not reduce cell viability by more than 20 % in either cell model. Namely, to evaluate the agonistic or antagonistic activity against oestrogen and androgen receptors, hormone receptors need to be functional, and they are only functional in viable cells.

It was not possible to test higher doses and to determine IC_{50} doses due to the poor solubility of test substances in aqueous media.

Determination of androgen receptor activity

Agonist and antagonist androgen receptor activity was identified with the AR-reporter gene assay conducted according to the OECD protocol TG 458 (8). Prior to experiments, AR-EcoScreen GR KO M1 cells were seeded in a medium supplemented with 5 % (v/v) charcoal-stripped FBS instead of normal FBS to reduce interferences from serum hormones. White opaque flat-bottom 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) were used, and the cells were seeded at the density of 10^4 cells per well in 100 μL of CCM. After 24 h of incubation, cells were treated with eight non-cytotoxic doses (100, 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 μmol/L) of oxybenzone or methylparaben for another 24 h.

Cells treated with dihydrotestosterone (DHT) were used as positive control, hydroxyflutamide (HF) was applied as AR antagonist control, while untreated cells served as negative control. After the 24-hour treatment, CCM was discarded, and the cells were washed thoroughly with PBS. The cell lysate was prepared in accordance with protocol for Promega luciferase assay system (E1500, Promega, Madison, WI, USA) by adding 20 μL of cell

culture lysis reagent diluted five times with distilled water. After 20-min centrifugation at 15 × *g* the luminescence intensity was measured using the same SpectraMax iD3 microplate reader. Luciferase assay reagent was freshly prepared every time and added with injector system in the volume of 100 μL per well.

Determination of oestrogen receptor activity

Agonists and antagonist oestrogen receptor activity was identified with the ER-reporter gene assay according to the OECD TG 455 (9) using HeLa-9903 cells. Briefly, the cells were seeded at a density of $1x10^4$ cells per well in white opaque flat-bottom 96-well plates and incubated for at 37 °C and 5 % CO_2 3 h, upon which time test and control substances were added in the volume of 50 μL/well and incubated for another 24 h. Oxybenzone or methylparaben were applied in the doses of 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 μmol/L. Cells treated with 17 β-oestradiol (E2) served as positive control and non-treated cells as negative control. After the 24-h treatment, the cells were prepared for luciferase activity measurement as described above for the AR-reporter gene assay.

Data analysis

All experiments were run in triplicate and repeated twice. The results are expressed as means with standard deviations. Data analysis was run on GraphPad Prism6 (GraphPad Software, San Diego, CA, USA). The benchmark dose (BMD) was calculated from the Excel spreadsheets provided as supplementary material for both OECD TG 455 and 458, which sets the benchmark response (BMR) for both AR and ER agonistic and antagonistic activity. The BMR for AR antagonism is set to 30 % inhibition of AR activity (IC_{30}) induced by 500 pmol/L DHT. For the substance to be considered ER agonist, it has to achieve 10 % or more of the 1 nmol/L E2 ER activity (PC_{10}). Thus, the BMD for AR antagonism corresponds to IC_{30}, while the BMD for ER agonism corresponds to PC_{10}.

RESULTS AND DISCUSSION

Cytotoxicity

Figures 1 and 2 clearly show that none of the selected oxybenzone and methylparaben doses were cytotoxic to HeLa-9903 and AR-EcoScreen GR KO M1 cells. Both cell types had more than 90 % viability compared to negative control, which is why we used the entire selected dose range for both test substances in subsequent determination of androgen and oestrogen receptor activity.

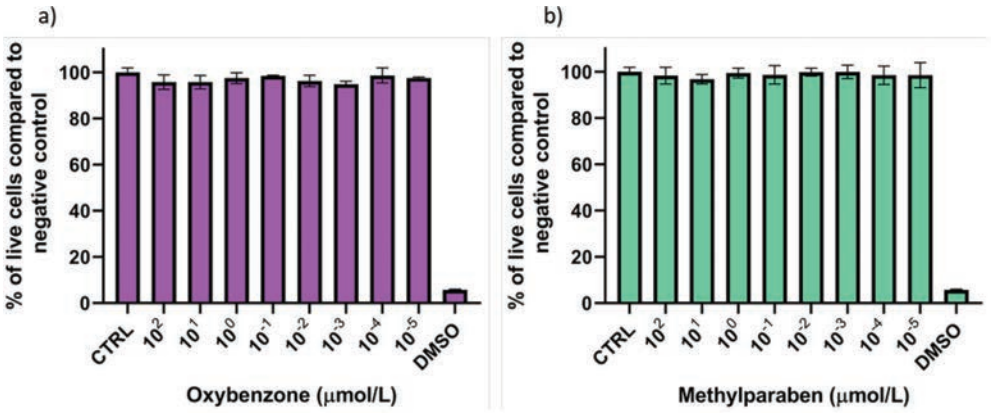

Figure 1 Viability of HeLa-9903 cells treated with: a) oxybenzone and b) methylparaben. The results are given as mean % of live cells vs negative control (untreated cells) and denote means with standard deviations of six replicates from two individual experiments. Cells treated with 10 % (v/v) DMSO served as positive control

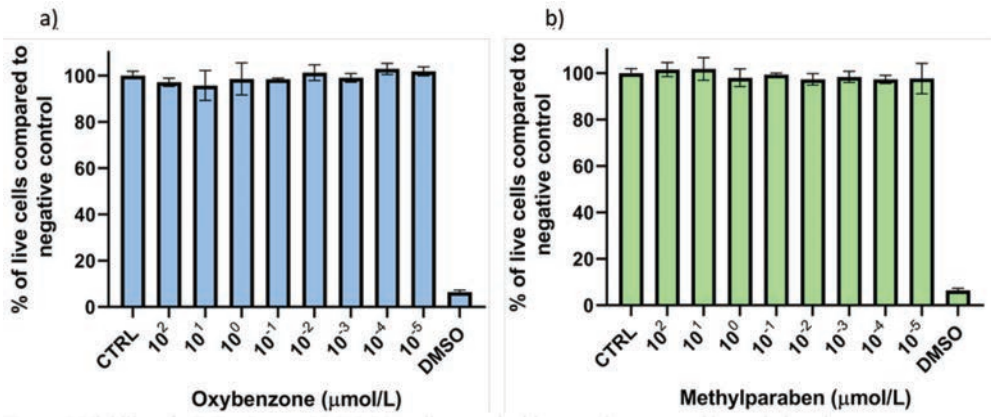

Figure 2 Viability of AR-EcoScreen GR KO M1 cells treated with: a) oxybenzone and b) methylparaben. The results are given as mean % of live cells vs negative control (untreated cells) and denote means with standard deviations of six replicates from two individual experiments. Cells treated with 10 % (v/v) DMSO served as positive control

Androgen receptor activity

None of the tested doses of either methylparaben and oxybenzone exhibited androgenic (AR agonistic) effects in AR-EcoScreen GR KO M1 cells (Figure 3), but most doses of either substance had the antagonistic effects against AR (Figure 4). The BMDs for AR antagonism (calculated as IC_{30}, i.e. concentration that inhibits 30 % of AR activity induced by 500 pmol/L DHT) were 5.01 μmol/L for oxybenzone and 13.8 μmol/L for methylparaben.

Our results for anti-androgenic effects of methylparaben are in line with the report issued by Chen et al. (30), who examined androgenicity of different parabens in 2933Y cells in a dose range similar to ours (10^{-4} μmol/L to 10^{1} μmol/L) and found that methylparaben inhibited the testosterone response by 40 %. As for anti-androgenicity findings of oxybenzone, our results are consistent with the Sung et al. (33) report for Sprague-Dawley rats *in vivo*, and their *in vitro* cytotoxicity findings in cells associated with the male reproductive system compared to normal non-reproductive cells. Ma et al. (31) also found the antagonistic action of oxybenzone in MDA-kb2 cells in the dose range they tested (10^{-2} μmol/L to 10^{2} μmol/L). The dose which inhibited DHT response by 50 % (IC_{50}) in their study was 28.5 μmol/L. The dose range we tested incorporates doses from these two studies and also includes doses found in human plasma and serum samples (14, 21).

Oestrogen receptor activity

Figure 5 shows that methylparaben and oxybenzone can be considered oestrogen receptor agonists, as both induced almost 60 % of E2 response at the highest doses selected. The BMD values for ER agonism for oxybenzone and methylparaben were 3.87 μmol/L and 3.94 μmol/L, respectively. Since both compounds exhibited agonistic activity on the oestrogen receptor, the antagonistic assay was not performed.

A similar response for oxybenzone was reported by an *in vitro* study using a recombinant yeast assay, while no oestrogenic effects were seen *in vivo* (34). Methylparaben, in turn, was oestrogenic in immature rats tested with a uterotrophic assay (35) and also stimulated proliferation in oestrogen-dependent MCF-7 cells (36). MCF-7 cell proliferation assay is another well-known method for identifying estrogenic compounds (37). In another study (38), methylparaben in the dose range of 2–500 μmol/L increased proliferation of MCF-7 cells, acted as the agonist of the oestrogen receptor in the MLVN cells, and promoted oestradiol secretion in H295R cells.

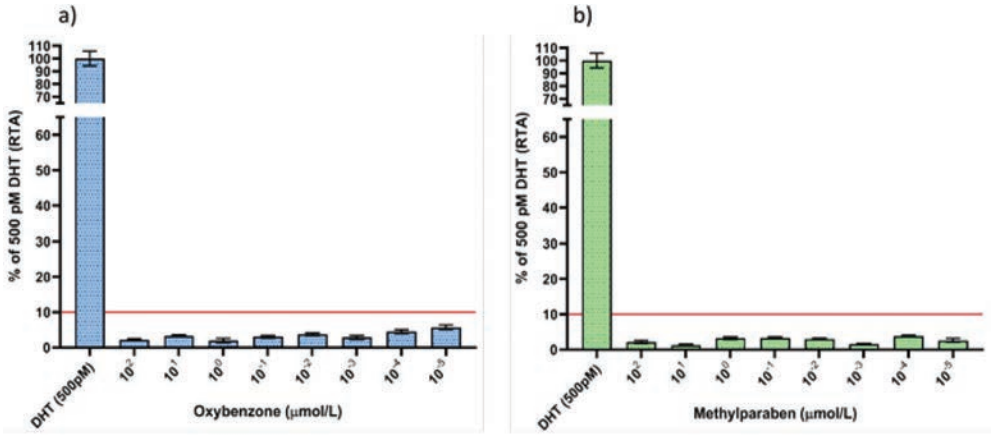

Figure 3 Androgenic activity of a) oxybenzone and b) methylparaben in the AR-EcoScreen GR KO M1 cell line. The results are given as mean % of inhibition vs positive control (500 pmol/L dihydrotestosterone) and denote means with standard deviations (error bars) of six replicates from two individual experiments. Standard deviations are given as error bars

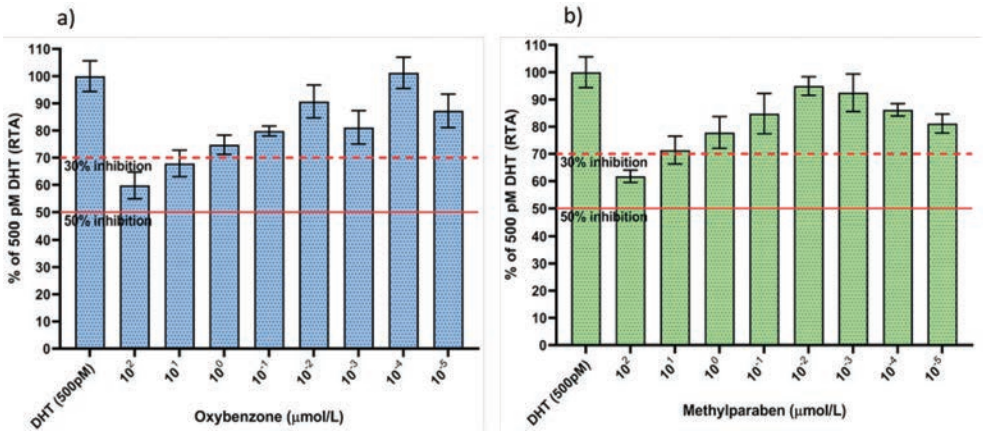

Figure 4 Anti-androgenic activity of a) oxybenzone and b) methylparaben in the AR-EcoScreen GR KO M1 cell line. The results are given as mean % of inhibition vs positive control (500 pmol/L dihydrotestosterone) and denote means with standard deviations (error bars) of six replicates from two individual experiments. Standard deviations are given as error bars

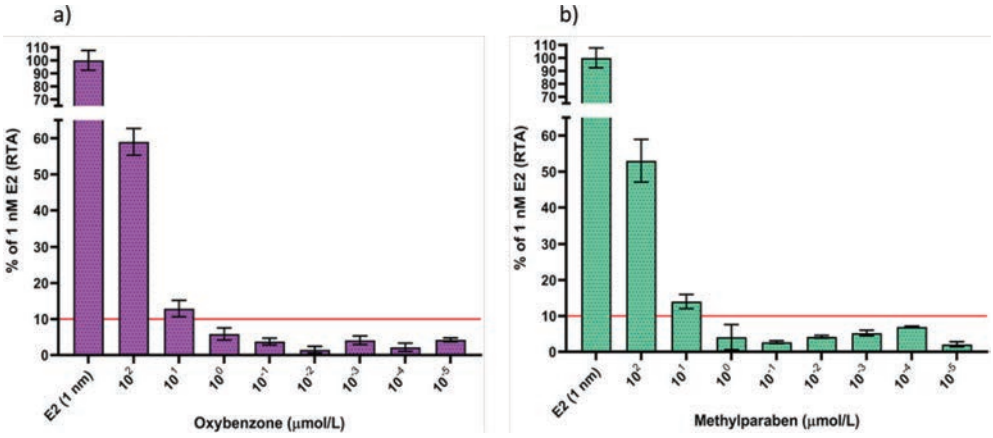

Figure 5 Estrogenic activity of a) oxybenzone and b) methylparaben in HeLa-9903 cells. The results are given as mean % of inhibition vs positive control (1 nmol/L 17 β-oestradiol) and denote means with standard deviations (error bars) of six replicates from two individual experiments. Standard deviations are given as error bars

CONCLUSION

This research confirms the endocrine disrupting properties of oxybenzone and methylparaben *in vitro*. Both substances acted as oestrogen receptor agonists and androgen receptor antagonists. Considering that these chemicals can disrupt the endocrine system and harm human health, their use must be strictly regulated.

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Estrogena i androgena aktivnost oksibenzona i metilparabena u *in vitro* uvjetima

Sve veća zabrinutost zbog nepovoljna zdravstvenog učinka raznih industrijskih kemikalija potaknula je provedbu ovog istraživanja, koje je usmjereno na moguće poremećaje endokrinog sustava. Zbog nedostatka znanstvenih podataka o štetnim učincima kemikalija koje se koriste u proizvodima za osobnu njegu (PCP, od engl. *personal care products*), istražena je estrogenska i androgena aktivnost fotostabilizatora oksibenzona i konzervansa metilparabena dobro uspostavljenim i regulatorno prihvaćenim *in vitro* testovima. Uz striktno pridržavanje smjernica Organizacije za ekonomsku suradnju i razvoj (OECD) za testiranje aktivacije estrogenskih i androgenih receptora, primijenjeni su OECD testovi broj 455 i 458, koji se temelje na staničnim linijama HeLa-9903 i AR-EcoScreen GR KO M1. Dobiveni rezultati jasno su pokazali da obje kemikalije djeluju kao agonisti estrogenskih receptora i antagonisti androgenih receptora u *in vitro* uvjetima, stoga je potrebna sveobuhvatna procjena rizika za ljudsko zdravlje zbog pretjerane i široko rasprostranjene uporabe takvih kemikalija u PCP-ovima.

KLJUČNE RIJEČI: agonist; antagonist; endokrina disrupcija; higijenski proizvodi; hormonski receptori