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Evaluation of tetrabromobisphenol A effects on human glucocorticoid and androgen receptors: a comparison of results from human- with yeast-based *in vitro* assays

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Abstract

The incidence of immune-related diseases increased over the last years in industrialized countries, suggesting a contribution of environmental factors. Impaired glucocorticoid action has been associated with immune disorders. Thus, there is an increasing interest to identify chemicals disrupting glucocorticoid action. The widely used flame retardant tetrabromobisphenol A (TBBPA) was reported earlier to potently inhibit glucocorticoid receptor (GR) and moderately androgen receptor (AR) activity in yeast-based reporter gene assays. To further characterize possible GR disrupting effects of TBBPA, transactivation experiments using a human HEK-293 cell-based reporter gene assay and cell-free receptor binding experiments were performed in the present study. Both, transactivation and GR binding experiments failed to detect any activity of TBBPA on GR function. Molecular docking calculations supported this observation. Additionally, the current study could confirm the antiandrogenic activity of TBBPA seen in the yeast assay, although the effect was an order of magnitude less pronounced in the HEK-293 cell-based system. In conclusion, TBBPA does not directly affect GR function and, considering its rapid metabolism and low concentrations found in humans, it is unlikely to cause adverse effects by acting through AR. This study emphasizes the use of cell-free assays in combination with cell-based assays for the *in vitro* evaluation of endocrine disrupting chemicals.

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Conflicts of Interest

The authors declare no conflict of interest.

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Keywords

Glucocorticoid receptor; androgen receptor; endocrine disrupting chemical; tetrabromobisphenol A; in vitro assay; toxicity

1 Introduction

The annual production of chemicals for industry, agriculture, personal care products, or food processing is continuously rising. Programs initiated by the authorities such as the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation of the European Union or the Endocrine Disruptor Screening Program (EDSP) of the U.S. Environmental Protection Agency aim to improve the safety assessment of man-made chemicals and thereby protect human health and the environment. In the past, extensive research focused on endocrine disrupting chemicals (EDCs) acting on sex steroid hormone action, with a major focus on estrogen receptor (ER) and androgen receptor (AR) function. In contrast, rather few studies addressed chemicals disrupting glucocorticoid action (Macikova et al. 2014; Nashev et al. 2012; Neel et al. 2013; Odermatt and Gumy 2008; Odermatt et al. 2006; Stavreva et al. 2012).

Glucocorticoids are regulating the immune system, cell proliferation and differentiation, brain function, energy metabolism, blood pressure and electrolyte balance. Due to their immunomodulatory effects, glucocorticoids are still among the most widely prescribed drugs to prevent graft rejection and to treat allergic or chronic inflammatory disease such as asthma, skin infections or rheumatoid arthritis (Baschant and Tuckermann 2010). Experiments in adult mice with keratinocyte-restricted GR inactivation showed an exacerbated inflammatory response to epidermal challenge, while postnatal mice displayed skin barrier defects and hallmarks of atopic dermatitis (Sevilla et al. 2013). Importantly, the incidence of atopic dermatitis and asthma has increased over the last decades, with significant variations between and within countries (Eder et al. 2006; Nutten 2015). Thus, exposure to EDCs that antagonize glucocorticoid receptor (GR) function may lead to impaired stress responses and immunosuppression and result in an enhanced susceptibility to allergies and aggravated inflammatory reactions. Environmental chemicals associated with disturbed GR function at human relevant concentrations are arsenic and dibutyltin (Gosse et al. 2014; Gumy et al. 2008; Kaltreider et al. 2001). Arsenic has been linked to type 2 diabetes, cardiovascular disease, several forms of cancer, and reproductive and developmental disorders (Abernathy et al. 1999), whereas dibutyltin is considered to be highly neuro- and immunotoxic (Jenkins et al. 2004; Whalen et al. 1999). Part of these effects might be due to interference with glucocorticoid action. Furthermore, exposure to polychlorinated biphenyls (PCBs) was observed to induce severe clinical symptoms including skin lesions, respiratory distress, neurologic disorders, disturbed liver function and suppression of cellular immunity (Nakanishi et al. 1985; Reggiani and Bruppacher 1985). Interestingly, several methyl sulfone metabolites have been shown to act as GR antagonists with IC₅₀ values in the low micromolar range (Johansson et al. 2005; Johansson et al. 1998). Thus, it has been hypothesized that they account at least partially for the respiratory symptoms (Grimm et al. 2015).

In vitro testing systems are widely applied in the safety assessment process, and the importance of such testing systems increased by the decision that chemicals used in body care products can no longer be tested in animals. Cell-based assays are used to investigate potential EDCs. Many assays, including the ER α β -lactamase reporter gene assay in HEK-293 cells, the ER α luciferase reporter gene assay in BG-1 ovarian cells and the multiplexed ER reporter gene assay in HepG2 cells that are also used in the ToxCast21 testing battery (Rotroff et al. 2014), are based on the receptor-mediated activation of a reporter gene in human cell lines. Alternatively, green-fluorescence protein reporter gene assays in yeast cells are used for the detection of potential EDCs due to the low costs and high throughput capacity of these assays (Gaido et al. 1997; Routledge and Sumpter 1996). Yeast strains expressing AR and progesterone receptor (PR) are also available (Baker 2001). However, the limitations of these assays need to be taken into account for data interpretation. The OECD issued a guidance document for the evaluation of EDCs in which they excluded the YES and YAS due to limited cell membrane permeability, transport issues and insufficient ability to discriminate between agonists and antagonists (OECD 2012).

In a recent study Roelofs *et al.* investigated the widely used flame retardant tetrabromobisphenol A (TBBPA) for its potential to interfere with GR function using recombinant yeast cells expressing the human GR and a GR-dependent green fluorescence protein (Roelofs et al. 2015). They reported a highly potent GR antagonistic effect of TBBPA with an IC₅₀ value of 22 nM. However, these authors did not provide additional experiments to support their data from the yeast GR reporter gene assay. Nevertheless, TBBPA was recently shown to decrease the secretion of interferon- γ from immune cells (Almughamsi and Whalen 2016). As possible mechanism, activation of the ERK1/2 MAPK pathway and of P38 kinase by TBBPA has been suggested. Since there is a functional interaction between GR activity and the MAPK pathway, and glucocorticoids are well-known modulators of inflammatory cytokines and chemokines, a block of GR function by TBBPA may disturb the regulation of the immune system.

TBBPA covers more than half of the global annual production of brominated flame retardants (Law et al. 2006). It is mainly applied in the laminate coating of printed circuit boards, where it is covalently bound to a polymer matrix and cannot be released into the environment (ECB 2006). Nevertheless, up to 20% of the manufactured TBBPA is used as an additive flame retardant in electronic equipment, furniture, building and construction materials, packaging, and consumer products (ECB 2006). Since additive flame retardants do not chemically react with their coated basis, they are prone to leach out of this matrix.

The aim of the current study was to re-evaluate the impact of TBBPA on GR function by performing cell-free receptor binding experiments and transactivation experiments in a human HEK-293 cell-based assay. Additionally, the effects of TBBPA on AR-mediated transactivation and on binding to AR were investigated.

2 Materials and methods

2.1 Chemicals and reagents

[1,2,4,6,7-³H]-dexamethasone and [1,2,6,7-³H]-testosterone were purchased from American Radiolabeled Chemicals (St. Louis, MO). Cell culture medium and other chemicals were obtained from Sigma Aldrich (Buchs, Switzerland).

2.2 Cell culture

Human Embryonic Kidney-293 cells (HEK-293) cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/l glucose, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, pH 7.4, and 10% MEM non-essential amino acid solution. Sf9 cells were cultured at 27°C in EX-Cell 420 medium supplemented with 10% FBS and 100 U/ml penicillin/streptomycin.

2.3 Competitive cell-free receptor binding assay

Recombinant human glucocorticoid receptor α (GR) baculovirus stock was produced using the Bac-to-Bac expression system and recombinant GR was expressed in Sf9 cells according to the instructions by the manufacturer (Invitrogen, Carlsbad, CA). Human androgen receptor (AR) was transiently expressed in HEK-293 cells upon transfection by the calcium phosphate precipitation method and cultivation for 72 h. Cells transfected with pcDNA3 vector served as control.

For preparation of lysates, cells were collected in ice-cold PBS and centrifuged for 10 min at $3000 \times g$ and 4°C, resuspended in binding buffer (50% glycerol, 10 mM EDTA, 50 mM sodium tungstate, 50 mM HEPES, pH 7.4) and homogenized with 50 strokes using a glass puller. The lysates were centrifuged ($16'000 \times g$, 10 min, 4°C) and supernatants stored at -80°C.

Recombinant human GR lysates were incubated in the presence of 10 nM [1,2,4,6,7-³H]-dexamethasone and unlabeled competitor (either 10 μ M or 100 nM cortisol or 10 μ M TBBPA) for 4 h at 16°C. Human AR lysates were incubated with 10 nM [1,2,6,7-³H]-testosterone and unlabeled competitor (either testosterone or TBBPA at different concentrations). Unbound ligand was separated by adding 5% dextran coated charcoal into the binding buffer, followed by incubation at 4°C for 10 min and centrifugation for 10 min at $3200 \times g$ and 4°C. The receptor bound fraction of radioligand in supernatants was measured by scintillation counting.

2.4 GR- and AR-dependent transactivation assay

HEK-293 cells (100,000 cells/well for GR and 200,000 cell/well for AR transfection) were seeded in poly-L-lysine coated 24-well plates, incubated for 24 h and transiently transfected using calcium phosphate precipitation. For the GR transactivation assay, cells were transfected with plasmid for human GR (0.3 μ g/well), pCMV-lacZ β -galactosidase transfection control (0.05 μ g/well) and TAT3-TATA luciferase reporter (0.45 μ g/well). For the AR transactivation assay cells were transfected with human AR (0.4 μ g/well), pCMV-lacZ β -galactosidase control (0.025 μ g/well) and TAT3-TATA luciferase reporter (0.375 μ g/

well). At 6 h post-transfection cells were washed with DMEM and incubated for another 18 h. The cells were then washed with steroid-free DMEM (cDMEM) and cultivated for 3 h at 37°C. This culture medium was then replaced with fresh cDMEM containing steroid hormones (10, 25 and 100 nM cortisol for GR, 10 nM testosterone for AR), receptor antagonist (1 μ M RU486 for GR and 0.5 μ M flutamide for AR) or TBBPA (10 μ M or 1 μ M), followed by incubation for 24 h. Cells were lysed in 60 μ L of tropix lysis solution (Applied Biosystems, Foster City, CA) supplemented with 0.5 μ M dithiothreitol and frozen at -80°C for at least 20 min. Luciferase activity was determined in 20 μ L lysate adding 100 μ L of D-luciferin-firefly substrate solution at a final concentration of 0.47 mM D-luciferin, 53 mM ATP, 0.27 mM coenzyme A, 0.13 mM EDTA, 33.3 mM dithiothreitol, 8 mM MgSO₄, 20 mM tricine, pH 7.8. β -galactosidase activity was measured in 20 μ L lysate using the Tropix kit. Samples were measured using a SpectraMax-L luminometer (Molecular Devices, Devon, UK).

2.5 Docking of TBBPA into the ligand binding pocket of GR

Docking was performed using the GOLD version 5.2 software (Chambridge Crystallographic Data Centre, Cambridge, UK) (Jones et al. 1997). This program applies a genetic algorithm for the identification of accurate docking poses for small molecules into the binding pocket of a protein. The crystal structure with the Protein Data Bank (PDB) entry 3H52 [DOI: [10.1016/j.jmb.2009.11.011](https://doi.org/10.1016/j.jmb.2009.11.011)] was chosen for the GR protein due to the antagonistic conformation. First, the co-crystallized ligand (RU486, also known as mifepristone) was deleted from the binding site and re-docked into the GR binding pocket to investigate whether GOLD could restore the original binding orientation and therefore validate the docking settings (RMSD value of 0.057). The binding site was centered on the primary amine nitrogen of Gln570 (x -48.19; y 16.58; z -36.62) surrounded by a 10 Å sphere. GoldScore was selected as scoring function. Interactions found by the docking solutions between the ligand and the GR binding pocket were evaluated using LigandScout 3.12 (inte:ligand GmbH, Vienna, Austria). This program automatically analyzes the observed interaction pattern between the docked ligand and the protein, based on the chemical functionalities, the geometric distances and the angles between neighboring structures (Wolber and Langer 2005).

2.6 Statistical analysis

Data were analyzed using *t*-test in the GraphPad Prism 5 software. Values represent mean $\pm$ SD.

3 Results

3.1 Assessment of TBBPA effects on GR binding and transcriptional activity

In order to evaluate the potent antagonistic activity of TBBPA on GR (IC₅₀ 22 nM) that was observed in a recombinant yeast reporter gene assay by Roelofs *et al.* (Roelofs et al. 2015), a transactivation assay in HEK-293 cells was employed in the present study. For this purpose, HEK-293 cells were transiently transfected with human GR and a GR-dependent luciferase reporter gene (Fig. 1A). As expected, cortisol at different concentrations induced the GR-dependent expression of the luciferase activity; however, TBBPA did not act as an antagonist

and no decrease in the cortisol-dependent GR activation, using 10 nM, 25 nM and 100 nM cortisol, could be observed when cells were co-treated with 10 μ M TBBPA. A slight, statistically significant, increase in reporter gene activity was observed upon co-treatment with 10 nM cortisol and 10 μ M TBBPA. This may be explained by competition of TBBPA with cortisol for unspecific binding sites. Unlike TBBPA, the antagonist RU486 at 1 μ M completely blocked the cortisol-induced GR activation. Also, TBBPA was not able to induce GR-mediated activation of the luciferase reporter gene, even at the high concentration of 10 μ M. Thus, an interference of TBBPA with GR-mediated transactivation could not be demonstrated in this human cell-based assay.

To exclude limited cellular uptake or potential efflux of TBBPA as a reason for the lack of effect on GR, a competitive receptor binding assay was performed under cell-free conditions. Lysates of cells expressing recombinant human GR were co-incubated with 10 nM [3 H]-dexamethasone and unlabeled competitor (either different concentrations of cortisol (F) or 10 μ M TBBPA), followed by determination of the GR bound fraction of [3 H]-dexamethasone (Fig. 1B). TBBPA at a concentration of 10 μ M did not substantially displace the radiolabeled dexamethasone from the receptor, in contrast to the known ligand cortisol. These results indicate that TBBPA has no direct effect on GR ligand binding and does not affect GR transcriptional activity.

3.2 Structural modeling of ligand binding to GR

To computationally elucidate the results for TBBPA obtained in the transactivation and binding assays, docking calculations were performed to investigate the interactions formed with the GR ligand binding site and therefore the potential to trigger a molecular switch of the helix 12. In the X-ray crystal structure, the GR antagonist RU486 (mifepristone) induces a relocation of the helix 12 position to reshape the coactivator towards a corepressor site with the possibility to bind nuclear receptor corepressor (NCoR) (Schoch et al. 2010) (Fig. 2A and B). TBBPA adopts a position similar to GR agonists without covering the side pocket compared to the dimethyl amino phenyl moiety of RU486 in order to interact with the helix 12 and induce a position change (Fig. 2C and D). RU486 was found to form hydrophobic contacts with Ile756 and Met752 of helix 12 as well as with Leu2263 and Ile2266 of the NCoR motif. The only interaction with the helix 12 and TBBPA found is a hydrophobic interaction with Ile756, but with considerable larger distances compared to RU486. Furthermore, the interactions with the GR binding site observed for TBBPA are mainly of hydrophobic nature with one additional hydrogen bond (H-bond) involving the sulfur atom of Met560. H-bonds including a sulfur center are generally considered as weak H-bonds compared to interactions with oxygen or nitrogen groups. The same interaction pattern was also found when docking TBBPA into a GR crystal structure with an agonistic conformation (data not shown).

3.3 Effect of TBBPA on human androgen receptor

The earlier study by Roelofs *et al.* reported an antiandrogenic activity of TBBPA with an IC₅₀ value of 982 nM in the yeast AR-dependent reporter gene assay (Roelofs et al. 2015). In order to compare these earlier observations from the yeast GR and AR reporter gene assays with our human cell-based systems, an AR transactivation assay in HEK-293 cells

was conducted (Fig. 3A). Treatment of the cells with 10 nM testosterone resulted in an approximately eight fold increase in luciferase activity. Co-treatment with the antagonist flutamide partially reduced the testosterone-mediated reporter gene activation. TBBPA did not exert an antiandrogenic effect at a concentration of 1 μ M; however, at 10 μ M it reduced the testosterone-dependent AR activation by 37%. Moreover, at 10 μ M TBBPA showed weak agonist properties and resulted in the activation of the AR-dependent luciferase reporter gene. Furthermore, a cell-free competitive binding assay was conducted with cell lysates expressing recombinant AR (Fig. 3B). TBBPA exhibited a concentration-dependent displacement of the radiolabeled testosterone, whereby 10 μ M TBBPA displaced 62% of the AR-bound [3 H]-testosterone. At 1 μ M TBBPA, which is close to the IC₅₀ value reported by Roelofs et al. (Roelofs et al. 2015), 31% of [3 H]-testosterone were displaced from the AR. Thus, these results could confirm the antiandrogenic activity of TBBPA, although the effect was less pronounced in the human cell-based system compared with the yeast assay.

Discussion

The results of the present study suggest that TBBPA does not affect GR function. TBBPA showed neither agonistic nor antagonistic effects in the GR transactivation assay in HEK-293 cells, and TBBPA was not able to displace the radiolabeled ligand dexamethasone from the GR binding site in a cell-free assay. In addition, molecular modeling was applied to study possible interactions of TBBPA with residues of the GR ligand binding pocket. Although TBBPA was not excluded from the GR ligand binding pocket due to steric hindrance, key stabilizing interactions that are formed with the agonist dexamethasone (not shown) or the antagonist RU486 (Fig. 2) are absent in the case of TBBPA. The antagonistic effect of TBBPA observed by Roelofs *et al.* on AR in the yeast assay with an IC₅₀ of 982 nM (Roelofs et al. 2015) could also be detected in the HEK-293 cell-based assay in the present study, although the effect was much weaker with an estimated IC₅₀ >10 μ M. TBBPA was found to displace the bound radiolabeled ligand testosterone from AR with an estimated IC₅₀ between 5-10 μ M. Thus, at least ten fold weaker activity of TBBPA towards AR was found in the human cell-based assay compared with the yeast assay.

The discrepancy of the current study with the potent antagonistic activity of TBBPA on GR (IC₅₀ 22 nM) measured in the yeast GR assay by Roelofs *et al.* (Roelofs et al. 2015) may be due to limitations of the yeast assay. The applied yeast GR assay showed a very low sensitivity to the potent ligand dexamethasone and a very high concentration of 60 μ M was used to activate the reporter gene, an issue that has been observed before in yeast (Garabedian and Yamamoto 1992; Picard et al. 1990). The low sensitivity of the yeast GR assay is probably a result of limited access of dexamethasone to the cytosolic compartment. The yeast ATP-binding cassette transporter PDR5 was found to modulate the intracellular amount of dexamethasone (Kralli et al. 1995). Lack of PDR5 activity in a mutant yeast strain led to increased intracellular dexamethasone concentration and an increased response to glucocorticoids. Interestingly, PDR5 represents a functional homologue of the human P-glycoprotein (P-gp), and TBBPA was shown to inhibit P-gp, although at very high concentrations with an IC₅₀ value of 25 μ M (Dankers et al. 2013). Considering the supposed anti-glucocorticoid effect of TBBPA, it is unlikely that TBBPA at concentrations below 1 μ M inhibits PDR5, and a hypothesis to be tested in future experiments includes the induction

of the expression of PDR5 or another transport protein that catalyzes the efflux of glucocorticoids from the yeast cytosolic compartment. Transport was found to be at least partly responsible for the distinct GR-ligand response in yeast (Kralli et al. 1995). Another important factor impacting on steroid hormone receptor activity is the availability of chaperones and co-activator/co-repressor proteins. Although yeast does not have steroid receptors, it expresses two heat shock proteins (HSP82 and HSC82) that might fulfill at least in part the function of HSP90 in controlling the folding of human steroid hormone receptors (Chang and Lindquist 1994). Nevertheless, the limitations of the low sensitivity of the yeast receptor assays have been addressed by introducing the co-chaperones FKBP52 and HIP, resulting in enhanced GR reporter gene activation (Nelson et al. 2004; Riggs et al. 2003).

Rather than directly modulating GR function it is more likely that TBBPA might act on pathways downstream of GR. Several studies suggested that TBBPA interacts with mitogen-activated protein kinase (MAPK) pathways. Exposure of natural killer (NK) cells to TBBPA led to the activation of the MAPKs ERK1/2 and P38 as well as their upstream activator MAPK kinases (Cato et al. 2014). This appeared to be involved in the TBBPA-mediated reduction of the lytic function of NK cells (Kibakaya et al. 2009). Additionally, a study in murine RAW 264.7 macrophage showed that TBBPA induced COX2, TNF α , IL-1 β and IL-6 expression through enhanced phosphorylation of Akt, the MAPKs p38, ERK1/2 and JNK, as well as transcriptional activation of NF- κ B and AP-1 (Han et al. 2009). Furthermore, TBBPA was reported to induce reactive oxygen species formation by an ERK1/2-dependent pathway (Reistad et al. 2005; Reistad et al. 2007).

A majority of *in vitro* studies on TBBPA-mediated effects used concentrations in the lower micromolar range. Thus, evaluation of relevant concentrations observed in the human body is crucial for its risk assessment. TBBPA blood serum concentrations in the general population were assessed in different studies worldwide including two large Canadian biomonitoring surveys (Canada 2012). One study evaluated 50,599 serum samples of which all analyzed samples displayed TBBPA concentrations below the limit of detection (LOD) of 0.03 ng/L serum. The second study determined TBBPA concentrations in plasma samples of 771 Inuit participants. In 5% of the measured subjects, TBBPA was found at concentrations ranging from 10–480 ng/L (0.08 - 3.5 nM). A Belgian study involving 515 participants detected maximal TBBPA serum levels of 0.186 ng/L (Kicinski et al. 2012). Measurements in human milk displayed average TBBPA levels of 0.06 - 4.11 ng/g fat (EFSA 2011). Limited data are available for personnel with occupational exposure to TBBPA. Two studies examining the TBBPA serum levels of computer technicians and workers at electronic dismantling plants revealed serum concentrations up to 7.4 pmol/g fat (EFSA 2011; Jakobsson et al. 2002). The low systemic bioavailability of TBBPA after oral exposure can be explained by the extensive first-pass metabolism by conjugation with glucuronic acid or sulfate (Kuester et al. 2007; Schauer et al. 2006). The TBBPA conjugates are preferentially translocated into the bile and excreted by the feces (Colnot et al. 2014). Therefore, TBBPA does not bioaccumulate and the concentrations measured in the general population are several orders of magnitude lower than the concentrations causing the reported biochemical effects in the *in vitro* experiments.

However, the International Agency for Research on Cancer (IARC) recently classified TBBPA as probably carcinogenic to humans (group 2A) (Grosse et al. 2016), based on rodent carcinogenicity studies by the U.S. National Toxicology Program (NTP). A significantly increased incidence of uterine adenocarcinomas, adenomas and malignant mixed Müllerian tumors was found in female Wistar Han rats receiving 500 or 1000 mg TBBPA/kg body weight (bw)/day in a two-year gavage study (Dunnick et al. 2015; NTP 2013). At such high concentrations, the enzymes for sulfation and glucuronidation may become saturated and conjugation of TBBPA likely competes with that of endogenous mediators such as estrogens, resulting in a higher concentration of free estrogens which stimulate cell proliferation and promote tumor growth (Lai et al. 2015; Wikoff et al. 2016). In support of this, several *in vitro* and computational studies provided evidence that TBBPA inhibits estrogen sulfotransferases (Gosavi et al. 2013; Hamers et al. 2006; Kester et al. 2002). However, the TBBPA serum concentrations measured in humans differ by several orders of magnitude from the plasma concentrations that compromised sulfation of TBBPA in rats. Thus, at the exposure levels seen in humans TBBPA is expected to be conjugated and excreted before it can cause adverse effects. Moreover, TBBPA toxicity studies in rats addressing non-cancer endpoints indicated no considerable effects on histopathology, organ and body weights, reproductive and developmental parameters, behavior and neuropathology, clinical signs, serum chemistry or mortality rate (reviewed by (Colnot et al. 2014; Lai et al. 2015)). The only treatment-related alteration in rats was limited to decreased circulating thyroxine (T₄) serum levels, the inactive form of thyroid hormones. However, no concomitant changes of active triiodothyronine (T₃) and compensatory increase of thyroid stimulating hormone (TSH) were noted, as well as any evidence of histopathological changes of the thyroid tissue. As this observation was only found in rats, it is suggested to be species-specific and related to hepatic induction of T₄-uridine diphosphate glucuronyl transferase (UDP-GT) enzymes which increase T₄ metabolism in the liver. Due the non-biologically relevant doses of TBBPA applied in these studies and the absence of other thyroid-related effects, this effect is not considered to be of relevance for human health (Canada 2012; ECB 2006). Therefore, to our knowledge, so far no effects of concern for human health have been reported for TBBPA.

4 Conclusion

In vitro and *in vivo* risk assessment studies of potential EDCs should include a careful consideration of human exposure and toxicokinetic parameters for the determination of systemically relevant compound concentrations. To avoid biased results in mode-of-action studies, the limitations and applicability of the *in vitro* testing systems need to be considered. Yeast GR screens are highly limited because of their low sensitivity towards glucocorticoids, probably due to efflux transport proteins. This may lead to false negative or false positive results, as exemplified in the current study for TBBPA. Using cell-free receptor binding and human cell-based transactivation assays, the results of the current study show no direct effect of TBBPA on GR function and very weak antagonistic effects on AR. Considering its rapid metabolism and low concentrations found in humans, TBBPA is unlikely to cause adverse effects by acting through GR or AR. The current study emphasizes

the combined use of cell-based and cell-free assays for the assessment of chemical effects on steroid hormone receptors.

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Abbreviations

AR	androgen receptor
EDCs	endocrine disrupting chemicals
EDSP	Endocrine Disruptor Screening Program
ER	estrogen receptor
GR	glucocorticoid receptor
HSP	heat shock protein
IARC	International Agency for Research on Cancer
LOD	limit of detection
MAPK	mitogen-activated protein kinase
NCoR	nuclear receptor corepressor
NK cell	natural killer cell
NTP	U. S. National Toxicology Program
PCBs	polychlorinated biphenyls
PDB	Protein Data Bank
P-gp	P-glycoprotein
PR	progesterone receptor
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
T3	triiodothyronine
T4	thyroxine
TBBPA	tetrabromobisphenol A
TSH	thyroid stimulating hormone

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Fig. 1. Effect of TBBPA on GR binding and activity.

(A) Impact of TBBPA on GR transactivation in HEK-293 cells transiently transfected with plasmids for human GR, a corticosteroid receptor-sensitive luciferase reporter gene and a galactosidase transfection control. Cells were incubated for 24 h in the presence of 10 nM, 25 nM and 100 nM cortisol (F), 1 μ M mifepristone (RU486) or different concentrations of TBBPA. The luciferase reporter activity was normalized to the internal galactosidase control. Data were normalized to vehicle control (0.2% DMSO) and represent mean $\pm$ SD from three independent experiments each performed in triplicates. (B) Displacement of 10 nM radiolabeled [3 H]-dexamethasone from GR by unlabeled competitor (either cortisol (F) or TBBPA). Sf9 lysates expressing recombinant human GR were incubated with 10 nM [3 H]-dexamethasone and either 10 μ M or 100 nM unlabeled cortisol (F) or 10 μ M TBBPA until the binding equilibrium was reached. Values were normalized to the maximal binding of 10 nM [3 H]-dexamethasone and represent the remaining [3 H]-dexamethasone bound to GR. ns (not significant); * $p < 0.05$.

Fig. 2. Docking of TBBPA and mifepristone into the ligand binding site of GR.

Co-crystallized mifepristone (RU486) (A) and docked TBBPA (B) in complex with the GR ligand binding domain and a receptor-interacting motif of the nuclear receptor corepressor (NCoR) in an active antagonistic conformation (PDB code 3H52, domain A). The automatically generated pharmacophore maps the important structural details of the ligand for binding (yellow spheres: hydrophobic interactions and red and green arrows with spheres are H-bond interactions). Amino acid residues involved in ligand binding are depicted as sticks. Whereas the interactions formed by RU486 (marked yellow spheres) trigger a molecular switch of the helix 12 to reshape the corepressor site (red arrow), TBBPA is not able to cover this side pocket and form the required interactions to modulate helix 12. Two-dimensional representation of the binding interactions of RU486 (C) and TBBPA (D). The color code corresponds with part A and C.

Fig. 3. Effect of TBBPA on AR binding and activity.

(A) Impact of TBBPA on AR transactivation in HEK-293 cells transiently transfected with plasmids for human AR, an AR-sensitive luciferase reporter gene and a galactosidase transfection control. Cells were incubated for 24 h in the presence of 10 nM testosterone (Testo), 0.5 μ M flutamide (Fluta) or different concentrations of TBBPA. The luciferase reporter activity was normalized to the internal galactosidase control. Data were normalized to vehicle control (0.2% DMSO) and represent mean $\pm$ SD from four independent experiments each performed in triplicates. (B) Displacement of radiolabeled [3 H]-testosterone from the AR by unlabeled competitor (either testosterone or TBBPA). HEK-293 lysates expressing AR were incubated with 10 nM [3 H]-testosterone and various concentrations of unlabeled testosterone or TBBPA until the binding equilibrium was reached. Values were normalized to the maximal binding at 10 nM [3 H]-testosterone and represent the AR bound fraction of [3 H]-testosterone. ns (not significant), * p 0.05, ** p 0.01, **** p 0.0001.