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Structural bisphenol analogues differentially target steroidogenesis in murine MA-10 Leydig cells as well as the glucocorticoid receptor

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ABSTRACT

Although much information on the endocrine activity of bisphenol A (BPA) is available, a proper human hazard assessment of analogues that are believed to have a less harmful toxicity profile is lacking. Here the possible effects of BPA, bisphenol F (BPF), bisphenol S (BPS), as well as the brominated structural analogue and widely used flame retardant tetrabromobisphenol A (TBBPA) on human glucocorticoid and androgen receptor (GR and AR) activation were assessed. BPA, BPF, and TBBPA showed clear GR and AR antagonism with IC_{50} values of 67 μM, 60 μM, and 22 nM for GR, and 39 μM, 20 μM, and 982 nM for AR, respectively, whereas BPS did not affect receptor activity. In addition, murine MA-10 Leydig cells exposed to the bisphenol analogues were assessed for changes in secreted steroid hormone levels. Testicular steroidogenesis was altered by all bisphenol analogues tested. TBBPA effects were more directed towards the male end products and induced testosterone synthesis, while BPF and BPS predominantly increased the levels of progestagens that are formed in the beginning of the steroidogenic pathway. The MA-10 Leydig cell assay shows added value over the widely used H295R steroidogenesis assay because of its fetal-like characteristics and specificity for the physiologically more relevant testicular Δ4 steroidogenic pathway. Therefore, adding an *in vitro* assay covering fetal testicular steroidogenesis, such as the MA-10 cell line, to the panel of tests used to screen potential endocrine disruptors, is highly recommendable.

1. Introduction

During the last decade much attention has been given to the hazard characterization and safety evaluation of endocrine disrupting compounds (EDCs). Considering the increasing list of potential EDCs, there is a strong need for alternative, rapid, and truly predictive *in vitro* screening assays. Consequently, many *in vitro* assays to assess for endocrine disrupting properties have been developed throughout the past years, such as the H295R steroidogenesis and several receptor-reporter gene transcriptional activation assays (Hecker et al., 2011; Bovee et al., 2007, 2011; Shen et al., 2009). Most of these assays, however, are directed towards effects in adults or puberty and less of these are focusing on effects early in life. For example, no appropriate *in vitro* model to test for effects on fetal steroidogenesis has been developed so far, while this is highly relevant with respect to masculinization of the male fetus. Furthermore, questions have been raised concerning the added value of the recently developed assays (Mantovani and Maranghi, 2005; van der Burg et al., 2011). Therefore, it seems

Abbreviations: 5αRed1, 5α-reductase type 1; 3β-HSD(1), 3β-hydroxysteroid dehydrogenase (type 1); 17β-HSD(3), 17β- hydroxysteroid dehydrogenase (type 3); cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; AR, androgen receptor; BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; c-Kit, receptor tyrosine kinase c-Kit (oncogene); CYP(s), cytochrome P450 enzyme(s); Cyp11A1, cytochrome P450 enzyme 11A1; Cyp17(A1), cytochrome P450 enzyme 17(A1); Cyp51, cytochrome P450 enzyme lanosterol 14α-demethylase; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; DOC, deoxycorticosterone; EC_{50}, half maximal effective concentration; EDCs, endocrine disruptors/disrupting chemicals/compounds; etio, etiocholanolone; FSH(R), follicle-stimulating hormone (receptor); GR, glucocorticoid receptor; H295R, human adrenocortical carcinoma cells; HMG-CoA red, HMG-CoA reductase; IC_{50}, half maximal inhibitory concentration; LH(R), luteinizing hormone (receptor); MM/L, minimal medium supplemented with L-leucine; MPW, masculinization programming window; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD, optical density; Por, cytochrome P450 oxidoreductase; (17-OH-)P4, (17-hydroxy-) progesterone; (17-OH-)P5, (17-hydroxy-) pregnenolone; RIA, radioimmunoassay; RT-qPCR, real time quantitative polymerase chain reaction; SD, standard deviation; SEM, standard error of the mean; StAR, steroidogenic acute regulatory protein; (α/β-)T, (alpha/beta-) testosterone; TBBPA, tetrabromobisphenol A.

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more appropriate to focus on the development of assays that cover crucial aspects within the reproductive system that are not covered yet by existing assays (Chapin et al., 2013; Piersma et al., 2013; van der Burg et al., 2011; Mantovani and Maranghi, 2005; Mantovani et al., 1999).

One of the most debated examples in the area of endocrine disruptors is bisphenol A (BPA) that is used to make plastics and epoxy resins. Associations between exposure to bisphenol A and the occurrence of several adverse health outcomes have been indicated, including breast and prostate cancer, metabolic syndrome, obesity, and subfertility (De Coster and van Larebeke, 2012). However, the scientific debate about causal relationships is still ongoing and it seems that there are sensitive and critical time windows for exposure to BPA early in life (Kundakovic et al., 2013; Vandenberg et al., 2013). BPA possesses estrogenic as well as anti-androgenic properties as a result of its binding affinity for these respective steroid hormone receptors (Kitamura et al., 2005; Lee et al., 2003; Paris et al., 2002). In addition, (genes encoding for) steroidogenic enzymes can be modulated by BPA (Zhang et al., 2011b). Even at low dose levels BPA can cause effects. For example, *in utero* exposure to BPA can instigate sex-specific epigenetic changes in the brain, which possibly underlie enduring effects on function and behavior concerning sexually dimorphic phenotypes (Kundakovic et al., 2013; Vandenberg et al., 2013). In order to protect the highly susceptible group of infants, the European Union decided to ban the usage of BPA in baby bottles in 2011. Since then, the European Food Safety Authority (EFSA) has been reevaluating the safety limits of BPA. Recently, also the Dutch Ministry of Health, Welfare and Sport released a report of the National Institute for Public Health and the Environment (RIVM) regarding the human and environmental health issues and regulatory perspectives of BPA (Bakker et al., 2014). Meanwhile, ever more compounds have been developed and used to replace BPA. Amongst these are the BPA analogues bisphenol F (BPF) and bisphenol S (BPS). As a result, the use of BPF and BPS in consumer products has gradually increased, supposedly as safer alternatives for BPA. Consequently, both compounds can nowadays be found in canned soft drinks and foods as well as thermal receipt paper (Becerra and Odermatt, 2012; Gallart-Ayala et al., 2011; Liao and Kannan, 2013; Vinas et al., 2010). Moreover, BPS has already been detected in human urine samples (Liao et al., 2012). Although much toxicological information on BPA is available, a proper human risk assessment of BPA analogues like BPF and BPS that are believed to have a less harmful toxicity profile is lacking. In our previous study we showed that BPA as well as the brominated structural analogue and widely used flame retardant tetrabromobisphenol A (TBBPA) affected efflux transporter activities and testosterone (T) secretion by murine MA-10 Leydig cells (Dankers et al., 2013). Noticeably, TBBPA has also been associated with *in vivo* endocrine and reproductive toxic effects (Van der Ven et al., 2008). Our previous findings were especially interesting with respect to the reported increase in male sub- and infertility, which has been observed in human populations in a number of industrialized countries. Exposure to EDCs has often been suggested as an important contributing factor to this observed increase in male sub- and infertility (Wong and Cheng, 2011; Jurewicz et al., 2009). In recent studies this decline in human male fertility is reflected by poor semen quality, a suggested decline in sperm count, and lowered testosterone levels in men (Andersson et al., 2008; Jorgensen et al., 2012; Travison et al., 2007).

The testicular microenvironment plays a crucial role in mammalian spermatogenesis. The binding of the gonadotropins luteinizing hormone (LH) to the LH receptor (LHR) on Leydig cells and follicle-stimulating hormone (FSH) to the FSH receptor (FSHR) combined with activation of the androgen receptor (AR) in Sertoli cells are the essential factors in this process. Additionally, multiple cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes are involved in local steroidogenesis in Leydig cells, which is pivotal for regulating spermatogenesis in males (Payne et al., 1992; Payne and Hales, 2004). The male sex steroid testosterone (T) is the final product of testicular steroidogenesis. Besides its function in the fertility of the adult male, T also has a crucial task in fetal development and maturation. During the masculinization programming window (MPW) the fetal testes start to produce T, which assures correct phenotypic development of the male reproductive system (Scott et al., 2009). Recently, Silva et al. described the role of glucocorticoids in the maintenance of spermatogenesis and maturation of sperm in adulthood (Silva et al., 2014). Moreover, the human fetal adrenal gland produces cortisol at the start of the MPW, which evokes suppression of adrenal androgen production via a negative feedback loop, minimizing the potential for masculinization in the female fetus (Goto et al., 2006). A number of studies have shown that BPA as well as TBBPA can affect steroidogenesis *in vitro* and *in vivo* (Canton et al., 2005, 2006; Dankers et al., 2013; Kitamura et al., 2005; Roelofs et al., 2013; Van der Ven et al., 2008), and as a result could possibly act as human testicular toxicants. Unfortunately, the possible role of the glucocorticoid receptor (GR) in steroidogenesis as well as the potential effects of BPF and BPS on steroidogenesis remain rather unclear.

In the present *in vitro* study, we examined the effects of structural bisphenol analogues on several major endocrine factors involved in testicular functioning. Recombinant yeast cells expressing the AR or GR were used to study possible interactions of TBBPA, BPA, BPF, and BPS with these steroid hormone receptors. Furthermore, effects of BPF, BPS, and TBBPA on production of (sex) steroids in mouse MA-10 Leydig cells were evaluated. Also, effects on the expression of genes within the cholesterol biosynthesis and steroidogenesis pathway were assessed.

2. Materials and methods

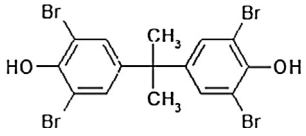
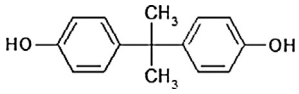
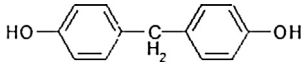
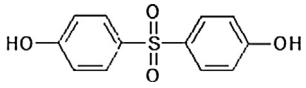
2.1. Chemicals

Table 1 displays the test compounds used: tetrabromobisphenol A (TBBPA; 97%, CAS# 79-94-7) was obtained from Sigma–Aldrich Co. (Zwijndrecht, The Netherlands), bisphenol A (BPA; 99.5%, CAS# 80-05-7) was purchased at Dr. Ehrenstorfer GmbH (Augsburg, Germany), and bisphenol F (BPF; >99.0%, CAS# 620-92-8) and bisphenol S (BPS; >98.0%, CAS# 80-09-1) were acquired from TCI Europe N.V. (Zwijndrecht, Belgium). Stock solutions were prepared in DMSO. The maximum solvent concentration for exposures was 0.1% v/v.

2.2. Yeast AR and GR bioassays

The recombinant yeasts used in the present study were constructed by Dr. T. Bovee (RIKILT, Wageningen UR). These yeasts stably express human androgen (AR) or glucocorticoid (GR) receptors and express yeast enhanced green fluorescent protein (yEGFP) as reporter protein when exposed to androgens or (gluco) corticosteroids, respectively (Bovee et al., 2007, 2011). Three days before exposure, cytosensor cultures were prepared by inoculating yeasts on agar of a minimal medium supplemented with L-leucine (#L8912; Sigma–Aldrich Co.) Agar plates were incubated at 30 °C for two days for colonies to form and then stored at 4 °C. One day before exposure, overnight cultures were prepared by inoculating one colony of yeast in 15 mL minimal medium supplemented with 120 mg L-leucine (MM/L) and containing 20 g dextrose (D-glucose, #215530; Becton Dickinson B.V., Breda, The Netherlands), 5 g ammoniumsulfate (#A4418; Sigma–Aldrich

Table 1
Overview of the test compounds used in this study, including their structural formulas (prepared with MDL ISIS™/Draw 2.5, MDL Information Systems, Inc. San Leandro, CA, USA) and log *P* values (derived from http://www.chemicalize.org/).

Bisphenol analogue	Abbreviation	Structure	Log *P*
Tetrabromobisphenol A	TBBPA		7.12
Bisphenol A	BPA		4.04
Bisphenol F	BPF		3.46
Bisphenol S	BPS		2.32

Co.), and 1.7 g yeast nitrogen base (#233520; Becton Dickinson B. V.) in 1 L Milli-Q water. Overnight cultures were kept at 30 °C with orbital shaking at 225 rpm. At the late log phase, cultures were diluted in MM/L to an optical density (OD) of 0.05 measured at 630 nm on a spectrophotometer (Shimadzu UV-160A; Shimadzu Benelux, 's-Hertogenbosch, The Netherlands). For exposure, aliquots of 200 μL/well of this yeast suspension were plated into the inner 60 wells of 96-well plates with V-shaped bottom (#651201; Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands). The remaining outer wells were filled with sterile water. Yeast suspension aliquots were exposed to the controls and test compounds by addition of 2 μL of stocks dissolved in DMSO. For both AR and GR agonistic studies several concentrations of BPA (10 nM–300 μM), BPF, BPS (10 pM–1 mM), and TBBPA (10 pM–100 μM) were tested. As positive controls for AR and GR activation, testosterone (T; 100 pM–1 mM) and dexamethasone (DEXA; 100 nM–1 mM) were used, respectively. To study the AR or GR antagonistic properties of the selected test compounds, a range of concentrations of BPA (AR: 10 nM–300 μM; GR: 1 nM–300 μM), BPF (AR: 10 nM–300 μM; GR: 1 nM–1 mM), BPS (AR and GR: 1 nM–1 mM), and TBBPA (AR: 1 nM–1 mM; GR: 1 nM–100 μM) were co-exposed with either T (100 nM) for AR or DEXA (60 μM) for GR measurements, respectively. Yeast cultures were exposed for 24 h at 30 °C with orbital shaking at 125 rpm. Fluorescence (excitation at 485 nm and emission at 530 nm) was measured at 0 and 24 h with the POLARstar Galaxy (BMG Labtech GmbH). Also, OD (at 595 nm) was measured at these time points to check if cytotoxicity occurred.

2.3. MA-10 Leydig cell culture

Mouse Leydig tumorigenic cells (MA-10) were kindly provided by Dr. Mario Ascoli (University of Iowa, Iowa City, Iowa, USA) (Ascoli, 1981). Propagation of cells was performed as described previously by Dankers et al. (2013). In short, cells were grown in 1:1 Dulbecco's Modified Eagle Medium/F-12 nutrient mixture (Ham) with phenol red (DMEM/F-12 1:1, #11320; Gibco, Life Technologies Europe BV, Bleiswijk, The Netherlands) supplemented with 15% HyClone (#SH30068.03; Thermo Fisher Scientific, Waltham, USA), 2% HEPES (1 M) (#15630; Gibco), and 1% penicillin/streptomycin (#15140; Gibco). Cells were maintained at 37 °C in a humidified atmosphere (95%) with 5% CO_2. Medium was refreshed 24 h prior to subculturing cells twice weekly. At least 45 min prior to use, flasks and plates were coated at room temperature with 0.1% gelatin (Attachment Factor Protein; Gibco).

2.4. Cytotoxicity assay

Cell viability of MA-10 cells after exposure to test compounds was determined by performing cytotoxicity assays. Therefore, the capacity of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by the mitochondrial enzyme succinate dehydrogenase was measured. Following exposure, media were removed and cells were incubated with MTT (1 mg/mL) for 30 min at 37 °C in a humidified atmosphere (95%) with 5% CO_2. After aspiration 1 mL isopropanol was added at room temperature in order to extract the formed blue colored formazan (Denizot and Lang, 1986). At a wavelength of 595 nm absorbance was measured spectrophotometrically (POLARstar Galaxy, BMG Labtech GmbH).

2.5. Testosterone secretion assay

T secretion assays were performed as described previously by Roelofs et al. (2014). Briefly, MA-10 cells were plated 24 h prior to exposure at a density of 2.0×10^5 cells/well/1 mL in 24-well plates. Cells were exposed to a range of non-cytotoxic concentrations of BPS (0.01–30 μM), BPF, and TBBPA (0.01–100 μM) for 48 h. Afterwards, the media were removed and stored at −20 °C until further analysis. Measurements of T secretion in the media were performed with a commercially available T radioimmunoassay (T RIA) kit according to producer's instructions (#IM1119; analytical sensitivity = 0.025 ng/mL; Beckman Coulter GmbH, Krefeld, Germany). 8-Bromoadenosine 3′,5′-cyclic monophosphate (cAMP; (100 μM)), a second messenger, which induces the expression of genes of steroidogenic enzymes, was used as a positive control for induction of T secretion.

2.6. Assessment of the steroid profile

For assessment of the steroid profile via a metabolomics approach, MA-10 cells were plated in 6-well plates at a density of 6.0×10^5 cells/well/3 mL 24 h prior to exposure. Cells were exposed for 48 h to BPF, BPS, and TBBPA (10 μM), as well as cAMP (100 μM), which was used as a positive control for steroid secretion. After exposure, medium of each well was transferred separately to a 10 mL Greiner tube and stored at −80 °C until further analysis.

Analysis of samples was performed at RIKILT Wageningen UR (Wageningen, The Netherlands). Determination of steroidogenic profiles was performed using GC-MS/MS analysis as described by Rijk et al. (2012). A 300 μL aliquot per sample was filled up with Milli-Q water till 3 mL and spiked with an internal standard mixture. Next, samples were subjected to solid-phase extraction (SPE) using a C18 column (500 mg, 3 mL, Varian Bond Elute, Harbor City, CA, USA), which was previously conditioned with methanol. The column was washed with Milli-Q water and a mix of acetonitrile and water (35:65, v/v), respectively. Free steroids were then eluted with acetone, the eluent was evaporated under nitrogen gas and reconstituted in 100 μL methanol and 2 mL TRIS-buffer (0.1 M, pH 9.5), after which a liquid–liquid extraction was performed with *n*-pentane. After centrifugation for 5 min at $3000 \times g$, the organic layer was collected in a glass tube. This extraction procedure was repeated and the combined organic fraction was evaporated until dryness at 40 °C under a gentle stream of nitrogen gas. Each dried sample was redissolved in 0.5 mL ethanol, transferred into a derivatization-vial and evaporated until dryness. The dried samples were derivatized by adding 25 μL of the derivatization reagent MSTFA++ followed by an 1 h incubation at 60 °C. Finally, the derivatized mixture was evaporated and reconstituted in 25 μL iso-octane. Steroid hormones were analyzed using a Varian 1200 L triple quadruple mass spectrometer equipped with a CP8400 autosampler and a CP-3800 GC. The VF-17MS GC column (L = 30 m, id = 0.25 mm, df = 0.25 μm) was obtained from Varian. Two microliter of the purified samples or standard solutions were injected onto the GC column at a pulsed pressure of 30 psi. The temperature program was started at 110 °C (constant for 1 min), whereafter it was increased 20 °C per minute till 240 °C (held for 1.5 min), and subsequently increased 1 °C per minute to 244 °C, followed by an increase of 25 °C per minute to 340 °C (held for 2 min). The helium flow was kept constant at 1.0 mL per minute. The GC–MS/MS was operated in electron ionization (EI) mode using multiple reaction monitoring (MRM). The programs Cluster and Treeview were used to visualize the changes in steroid profiles plotted in a heat map as fold changes compared to the DMSO control (Eisen et al., 1998).

2.7. Gene expression

Gene expression studies were performed according to the method previously described by Roelofs et al. (2014). In brief, MA-10 cells were plated 24 h prior to exposure in 12-well plates at a density of 6.0×10^5 cells/well/2 mL. Exposure to BPF, BPS, and TBBPA (10 μM), as well as the positive control cAMP (100 μM) was continued for 6 h. Total RNA was isolated from exposed MA-10 cells by chloroform-phenol extraction using RNA InstaPure according to producer's instruction (Eurogentec, Liège, Belgium). The purity and concentration of the isolated RNA was determined spectrophotometrically at absorbance wavelengths of 230, 260, and 280 mm using a NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA. RNA samples were diluted to a concentration of 66.7 μg/mL by the addition of RNAse free water and kept at −80 °C until further use. cDNA was prepared from 15 μL RNA (66.7 μg/mL) with 4 μL iScript reaction mix and 1 μL iScript reverse transcriptase from the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc. Veenendaal, The Netherlands) and real time quantitative polymerase chain reaction (RT-qPCR) was performed with a mixture of 7.5 μL iQ SYBR green supermix (Bio-Rad Laboratories, Veenendaal, The Netherlands), 0.6 μL forward primer (FP) (10 μM), 0.6 μL reverse primer (RP) (10 μM), 0.3 μL RNAse free water, and 6 μL cDNA diluted till the appropriate concentration for each primer pair (Table A.1).

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The expression of three cholesterol biosynthesis genes was studied: cytochrome P450 enzyme lanosterol 14α-demethylase (Cyp51), HMG-CoA reductase (HMG-CoA red), and cytochrome P450 oxidoreductase (Por). Also, the expression of eight steroidogenic genes was studied: steroidogenic acute regulatory protein (StAR), cytochrome P450 enzyme 11A1 (Cyp11A1), cytochrome P450 enzyme 17A1 (Cyp17A1), 3β-hydroxysteroid dehydrogenase type 1 (3β-Hsd1), and 17β-hydroxysteroid dehydrogenase type 3 (17β-Hsd3), 5α-reductase type 1 (5αRed1), receptor tyrosine kinase c-Kit (c-Kit; stem cell factor with proposed role in spermatogenesis) (Zhang et al., 2011a), and luteinizing hormone receptor (LHr). As a reference gene β-actin was used, similarly to previous studies with this cell line (Dankers et al., 2013; Roelofs et al., 2014). β-Actin gene expression in MA-10 cells was not statistically significantly affected by the exposures in this study and the mean Cq value between assays was 18.26 ± 0.35. All primers span an exon-exon junction to ensure mRNA amplification only and were run through National Center for Biotechnology Information (NCBI) Blast (nucleotide non-redundant database) to confirm specificity. Efficiency was also determined and was between 90–115% for all primer pairs. Table A.1 shows the sequences of the primer pairs used. The mixtures were placed in the CFX Connect™ (Bio-Rad Laboratories, Inc.) and firstly heated till 95 °C for 3 min, following 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 45 s. Subsequently, a melt curve was run to ensure the exclusion of primer dimers and other non-specific products formed during the RT-qPCR. The gene expression of each sample was expressed as threshold cycle (Ct), normalized to the reference gene β-actin (ΔCt). For each sample the fold induction relative to the DMSO control was calculated. A maximum technical replicate variance of 0.5 cycles was established, which corresponds to a change of 1.4-fold in expression. Therefore, any change in gene expression that was between 0.7 and 1.4-fold was considered to be within experimental variation and thereby uncertain.

2.8. Data analysis

Experiments were performed in duplicate (T secretion assays) or triplicate (yeast AR and GR bioassays, gene expression experiments, and steroid profile) and within each independent experiment each concentration was tested in duplicate (steroid profile) or triplicate (yeast AR and GR bioassays, gene expression experiments, and T secretion assays). The results are depicted as the mean with standard deviation (SD; steroid profile) or standard error (SEM; yeast AR and GR bioassays, T secretion assays, and gene expression experiments) of replicates within each experiment. Data calculations were made using GraphPad Prism 6.0 (GraphPad Software Inc. San Diego, USA). Statistical significance of differences of the mean as compared to the control was calculated using a two-tailed unpaired Students' *t*-test (for single concentrations) or a one-way ANOVA and post-hoc Dunnett's test (for concentration curves). Statistically significant differences were considered when $P < 0.05$.

3. Results and discussion

3.1. Yeast receptor (AR and GR) bioassays

Effects of structural bisphenol analogues, TBBPA, BPA, BPF, and BPS, on androgen (AR) and glucocorticoid (GR) receptor activation were assessed in specific yeast receptor bioassays. Only non-cytotoxic concentrations of the compounds were used. Both receptors responded well upon stimulation of the receptor with their corresponding known agonist, *i.e.* testosterone (T) for the AR and dexamethasone (DEXA) for the GR, with EC_{50} values of 58 nM and 74 μM, respectively (Fig. A.1). None of the bisphenol analogues tested showed agonistic effects on either AR or GR activity (data not shown). AR activity in yeast cells evoked by the addition of T (100 nM) was significantly and concentration-dependently inhibited by BPA, BPF, and TBBPA with IC_{50} values of 39 μM, 20 μM, and 982 nM, respectively (Fig. 1A). Also, GR activity in yeast cells stimulated with DEXA (60 μM) was significantly and concentration-dependently inhibited by BPA, BPF, and TBBPA with corresponding IC_{50} values of 67 μM, 60 μM, and 22 nM, respectively (Fig. 1B). Thus, BPA, BPF, as well as TBBPA showed clear antagonistic properties towards both the AR and GR. In contrast, BPS did not have an antagonistic effect on either AR or GR activity. For both receptors, TBBPA was the most potent antagonist with IC_{50} values in the nanomolar range, whereas BPA and BPF had apparent IC_{50} values in the micromolar range.

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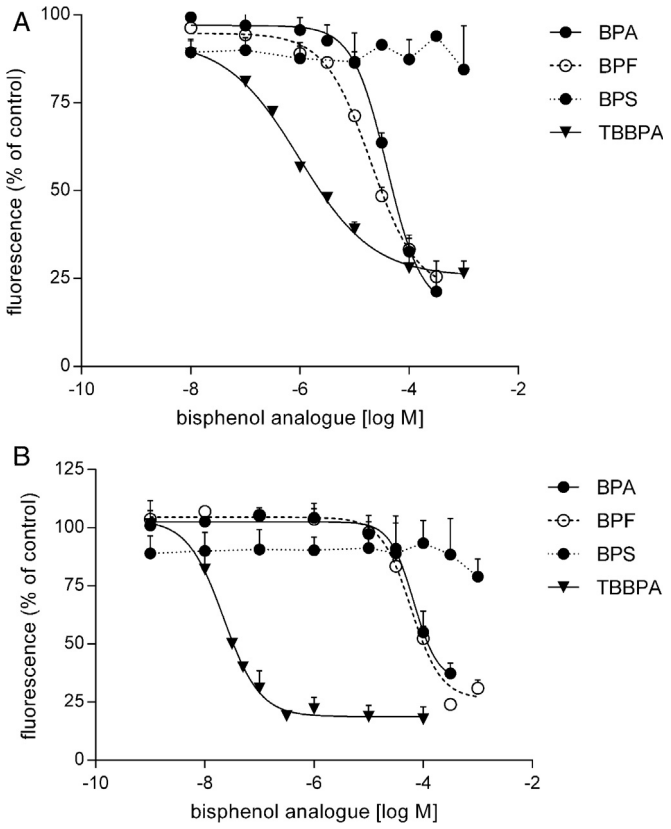

Fig. 1. (A) AR and (B) GR response measured as relative fluorescence as compared to vehicle-treated yeast cells after a 24-hour exposure to a concentration curve of BPA and the three selected analogues in combination with testosterone (100 nM) for AR and dexamethasone (60 μM) for GR measurements, respectively. Data are represented as means ± SEM with $N = 3$ and $n = 9$.

With respect to the receptor antagonism described in the present study, it is clear that BPA and BPF show effects within the same order of magnitude, which is in line with previous work from Rosenmai et al. (2014). It was noted that TBBPA was a very potent *in vitro* antagonist for especially the GR, but also for the AR, compared to the other structurally-related bisphenols (Table 1). The AR antagonizing effects seen in yeast cells exposed to BPA and BPF are consistent with earlier reports (Kitamura et al., 2005; Wetherill et al., 2007; Li et al., 2010; Rosenmai et al., 2014; Wang et al., 2014). However, we did not observe the AR antagonistic trend previously described for BPS (Kitamura et al., 2005; Rosenmai et al., 2014). We are the first to observe antagonistic properties of TBBPA on AR activity, while in previous studies TBBPA did not show an effect on AR activity, neither agonistic nor antagonistic (Kitamura et al., 2005; Li et al., 2010). These earlier studies were conducted with either a mouse fibroblast cell line (NIH3T3) or a yeast two-hybrid assay system both with a reporter gene, luciferase or β-galactosidase, respectively, using dihydrotestosterone (DHT) as an AR substrate (EC_{50} = 10 pM or 13 nM, respectively) (Kitamura et al., 2005; Li et al., 2010). Our studies were performed with recombinant yeasts containing a reporter protein using T as a ligand for the AR (EC_{50} = 58 nM). The different cell systems and distinct composition of the assays used by each research group, may explain the differences in findings that have been reported.

To our knowledge, we are the first to describe GR antagonistic effects of BPA, BPF and TBBPA as well as the absence of an effect of BPS. Sargis et al. found BPA to be a GR agonist at a concentration of 1 μM in 3T3-L1 preadipocytes transiently transfected with a luciferase reporter construct (Sargis et al., 2010). The transient character of the transfection method within their assay as well as the possible differences in the role of the GR in the process of adipogenesis, might explain the difference with our results. Moreover, luciferase assays are known to suffer from aspecific effects, for example when compounds degrade or stabilize luciferase (Sotoca et al., 2010). Elevated levels of glucocorticoids have been found to suppress T levels in adult males (Hardy et al., 2005). Also, inhibition of androsterone production occurred in rat progenitor Leydig cells *in vitro* via suppression of steroidogenic acute regulatory protein (StAR) and 3B-HSD by glucocorticoids through a GR-mediated mechanism (Xiao et al., 2010). Furthermore, *in vivo* rat Leydig cell apoptosis increases when corticosterone levels are raised under the influence of the GR, thereby lowering serum T levels (Chen et al., 2012). It remains to be investigated how the GR antagonism of bisphenols we found could affect testicular steroidogenesis *in vivo*. Because the GR is involved in the regulation of spermatogenesis, compounds affecting GR activity might hamper adequate sperm production (Silva et al., 2014). This is clearly a mode of action that has so far remained unrecognized in the research on EDCs and male fertility in particular. Future experimental studies should confirm if this antagonism is also applicable for the *in vivo* situation at relevant (human) dose levels.

3.2. Differential modulation of testosterone secretion

At first, we measured the sex hormone endpoint of male steroidogenesis in MA-10 Leydig cells, namely testosterone (T) secretion, after exposure to bisphenol analogues (Fig. 2). T secretion by MA-10 cells after exposure to the DMSO control (0.1% v/v) was 4.5 ± 1.1 pg/mL, which was comparable to the level observed in our previous study with the same cell line (Dankers et al., 2013). Exposing cells to the positive control cAMP (100 μM) induced T secretion 1241-fold compared to vehicle-treated cells (data not shown) and was also in the same range as reported previously (Dankers et al., 2013; Roelofs et al., 2014).

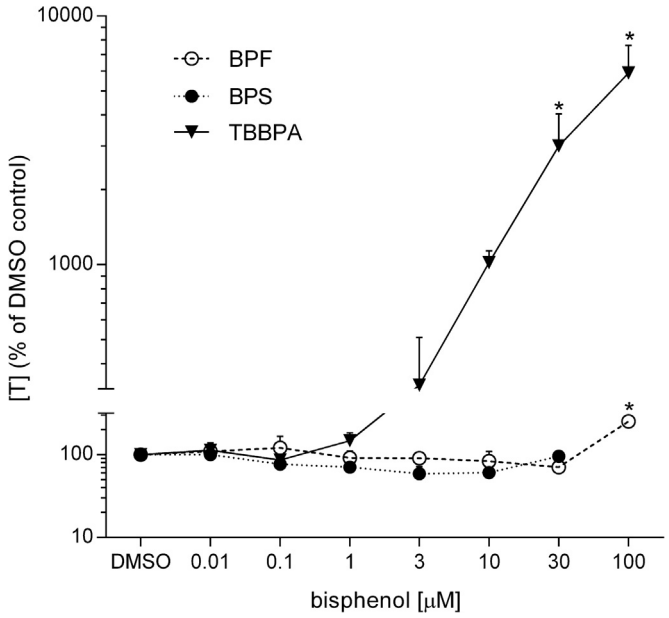

Fig. 2. Testosterone secretion by MA-10 cells after a 48-hour exposure to various concentrations of BPF, BPS, and TBBPA. Testosterone was measured using a commercially available RIA. Data are represented as means ± SEM of two independent experiments that were performed in triplicate. Significance was assessed using a one-way ANOVA test followed by Dunnett's post hoc test. *Significantly different from DMSO control-treated cells ($P < 0.05$).

In this study, exposure to each of the three selected bisphenol analogues in concentration ranges of 0.01–30 μM for BPS, and up to 100 μM for BPF and TBBPA showed that BPF and TBBPA increased T secretion, whereas BPS did not affect T secretion by MA-10 cells at the highest concentration tested (Fig. 2). The increase in T secretion by BPF and TBBPA was 2.5-fold and 59-fold, respectively, at the highest concentration tested (100 μM) (Fig. 2). BPA was previously shown to concentration-dependently increase T levels in the media of MA-10 cells up to 6-fold of control levels at 30 μM (Dankers et al., 2013). In line with our *in vitro* findings, a single, high dose of BPA (125 mg/kg) was found to transiently increase testicular T production in 18-day old mice (Song et al., 2002). While another study showed a decrease in rat testicular T production, Leydig cell numbers and steroidogenic enzymes at BPA exposure of >100 mg/kg in Wistar/ST rats after 6 weeks of exposure starting at prepubertal age (Nakamura et al., 2010). Yet studies with low doses of BPA (<40 μg/kg) suggest a decrease in circulating T levels in mice and rats or no effect upon prenatal and early pubertal exposure (Richter et al., 2007; Delclos et al., 2014). The decline in testicular T production in abovementioned studies was consistent with a reduced number of Leydig cells upon *in utero* exposure and subsequent reduced steroidogenic capacity. For TBBPA and especially BPS and BPF, less information on testicular effects is available. TBBPA studies typically show testicular effects, *i.e.* increased testicular weight and testicular adenomas, but no effects on plasma T levels or sperm (Van der Ven et al., 2008; Serbus et al., 2014). On the other hand, interactions of TBBPA with hormone-mediated pathways are found *in vitro* (Colnot et al., 2014). No *in vivo* studies were found that described *in vivo* testicular effects of BPS and BPF. Yet some studies describe interactions with steroidogenesis *in vitro*. Recently, BPA, BPF, as well as BPS showed a decreased secretion of basal T in human and mouse but not rat fetal testis explant cultures (Eladak et al., 2014). Clearly, a better mechanistic insight in the actions of bisphenol analogues is needed at relevant (human) dose levels.

3.3. Steroid hormone profiling

To provide more insight in the effects of BPS, BPF and TBBPA on Leydig cell steroidogenesis, steroid levels of MA-10 cells were further evaluated at a single non-toxic concentration (10 μM) using a metabolomics approach. Changes in hormone levels are represented in Fig. 3 as (A) a table with the absolute levels and (B) a heat map of three independent experiments, showing the effects of these compounds on overall steroid production in these cells. Additionally, Fig. A.2 shows bar graphs with the relative levels of each separate steroid hormone assayed. Analysis of all the steroid hormones secreted by vehicle-treated MA-10 Leydig cells revealed that these cells display a fetal-like testicular steroidogenic profile, instead of an adult hormone synthesis pathway (Fluck et al., 2011; Scott et al., 2009). Based on these steroid profiles, an overview of the active steroidogenic pathways in the MA-10 cells was compiled (Fig. A.3).

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.tox.2015.01.003.

Our finding of the presence of an alternative pathway leading to the formation of DHT (dotted arrows), next to the conventional pathway for the generation of androgens in the testes, is noticeable. In literature, this alternative pathway is described as the so-called "backdoor pathway" (Kamrath et al., 2012; Fluck et al., 2011). This "backdoor pathway" involves the enzyme 5α-reductase, which converts 17-hydroxy-progesterone (17-OH-P4) to androsterone. From this step onwards, androstanediol and eventually DHT are formed. Interestingly, exposure of MA-10 cells to cAMP did not result in any androsterone secretion, suggesting that this "backdoor pathway" is not stimulated by cAMP. However, this "backdoor pathway" is mentioned as one of the more pronounced pathways for fetal testicular hormone production (Fluck et al., 2011). Possible steroidogenic effects of EDCs on fetal testes are highly important for development later in life. Thus, an *in vitro* system like the MA-10 cells is of particular importance to study direct effects on fetal testicular steroidogenesis. Based on the results of the present study, the Leydig MA-10 cell line would be a valuable addition to existing screening methods for steroidogenesis like the human adrenocorticocarcinoma cell line H295R (Hecker et al., 2011). We clearly show here for the first time, that the steroidogenic pathway in murine testicular MA-10 cells resembles the *vivo* testicular situation better and is very different from the adrenal H295R steroidogenic pathway (Rijk et al., 2012; van Duursen et al., 2013). In testicular steroidogenesis, the Δ4 pathway is the main steroidogenic pathway via formation of androstenedione, instead of the Δ5 pathway, which runs via DHEA and is the main route seen in the adrenals (Sanderson, 2006; Vihko and Ruokonen, 1974). This is also reflected by the present study, where DHEA was only measured in media of MA-10 cells exposed to cAMP, a second messenger and inducer of several pathways enhancing steroidogenesis in an unspecific manner. Furthermore, DOC, corticosterone, and 11-deoxycortisol were found, which are all corticoids possible of activating the GR. MA-10 cells, like other Leydig cells, are known to possess the GR. Also, in Sertoli cells the GR is expressed and Sertoli cell-mediated GR actions support normal testicular function (Hazra et al., 2014). Its presence in multiple testicular cell types renders the GR a potentially important target for testicular toxicity, which is already stressed by the GR antagonism we observed in the yeast bioassays after exposure to the bisphenols as stated above (Section 3.1). Additionally, differential steroidogenic profiles in the human H295R cell line and the murine MA-10 cell line could indicate species-differences in steroidogenic pathways. The extent of species differences in the fetal testicular steroidogenic pathway of human, mouse, and other species are under debate (Scott et al., 2009; Sanderson, 2006; Vihko and Ruokonen, 1974). Interestingly,

A

DMSO	cAMP	BPF	BPS	TBBPA	Steroid hormone
0,37 ± 0,18	20,06 ± 2,67 *	1,12 ± 0,20 *	0,77 ± 0,17 *	0,39 ± 0,08 #^	P5
1,55 ± 0,25	0,27 ± 0,17 *	1,64 ± 0,28	1,48 ± 0,28	1,53 ± 0,21	17-OH-P5
1,77 ± 0,70	29,27 ± 0,23 *	8,55 ± 1,07 *	7,14 ± 1,36 *	6,43 ± 2,74 *	P4
0,11 ± 0,02	3,71 ± 0,58 *	0,17 ± 0,06	0,09 ± 0,04	0,69 ± 0,12 *#^	17-OH-P4
0,06 ± 0,01	10,07 ± 1,05 *	0,09 ± 0,02	0,08 ± 0,02	0,14 ± 0,04 *	DOC
0,00 ± 0,01	0,47 ± 0,43	0,00 ± 0,00	0,02 ± 0,02	0,00 ± 0,00	corticosterone
0,00 ± 0,01	0,09 ± 0,02 *	0,00 ± 0,00	0,00 ± 0,00	0,01 ± 0,01	11-deoxycortisol
0,51 ± 0,14	0,00 ± 0,00 *	0,66 ± 0,26	0,56 ± 0,29	0,37 ± 0,28	etio/androsterone
0,04 ± 0,02	0,13 ± 0,09	0,08 ± 0,02	0,06 ± 0,01	0,03 ± 0,01 #^	androstanediol
0,00 ± 0,01	0,17 ± 0,13	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	DHEA
0,21 ± 0,13	17,61 ± 1,06 *	0,44 ± 0,28	0,22 ± 0,14	3,00 ± 1,29 *#^	androstenedione
0,15 ± 0,13	0,56 ± 0,06 *	0,08 ± 0,07	0,03 ± 0,03	0,17 ± 0,29	E1
0,09 ± 0,02	1,73 ± 0,47 *	0,14 ± 0,02	0,11 ± 0,04	0,30 ± 0,10 *#^	α-T
0,19 ± 0,05	15,43 ± 3,75 *	0,27 ± 0,10	0,20 ± 0,09	1,82 ± 1,01 *^	β-T

B

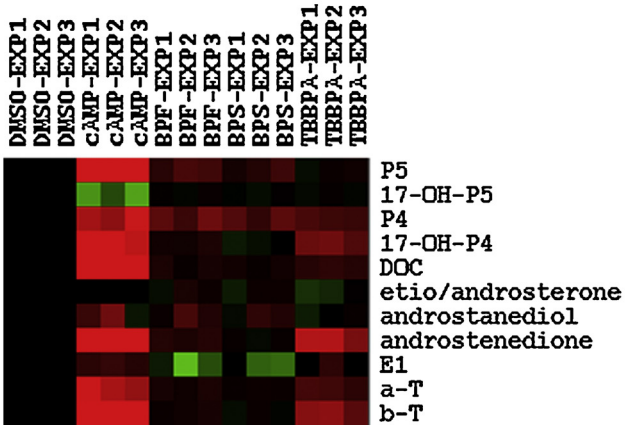

Fig. 3. Steroid hormone secretion by MA-10 cells exposed for 48 h to cAMP (100 μM), BPF, BPS, or TBBPA (10 μM). (A) Table with absolute steroid hormone levels (ng/mL). Data are represented as means ± SD of three independent experiments that were performed in duplicate. Significance was assessed by means of a Student's *t*-test. *, #, or ^ Significantly different from DMSO control-, BPF-, or BPS-treated cells, respectively ($P < 0.05$). (B) Heat map showing fold changes of individual experiments compared with DMSO control-treated cells. Color scales range from bright red to bright green corresponding to up- or downregulation of the steroid hormone, respectively. Maximum brightness represents a fold-change of ≥4 (2Log ratios of ≤−2 or ≥2). Mass amplitudes showing a fold-change <1.5 (2Log ratios <0.58) are presented in black. EXP, experiment; (17-OH-)P5, (17-hydroxy-) pregnenolone; (17-OH-)P4, (17-hydroxy-) progesterone; DOC, deoxycorticosterone; etio/androsterone, etiocholanolone/androsterone; DHEA, dehydroepiandrosterone; E1, estrone; α/a- or β/b-T, alpha- or beta-testosterone, respectively.

a recent study by Johnson et al. (2012) shows that there is high resemblance between mouse and human fetal xenografts regarding testicular steroidogenesis. As a result, studies using murine testes can offer great advantages for mechanistic studies of testicular development and for exploring the mechanisms by which compounds exert testicular toxic effects in humans (O'Shaughnessy and Fowler, 2011).

Exposure of MA-10 cells to the vehicle (DMSO) control demonstrated that progesterone (P4), the main precursor of T in the testes, is the predominant hormone secreted. This finding is supported by earlier studies with this cell line (Schwarzenbach et al., 2003; Ascoli, 1981; Ascoli and Puett, 1978). Hormone levels found in the MA-10 cell medium after exposure to the DMSO control ranged between 0.04 and 1.77 ng/mL for androstanediol and P4, respectively. A difference in total T levels after exposure to the DMSO control was found between measurements with the RIA and metabolomics approach, showing a higher level (approximately 60-fold) measured in the samples analyzed by GC–MS/MS compared to the T RIA samples. This difference is most likely due to the difference in analytical method used, where the GC–MS based method is much more sensitive, specific, and accurate. Furthermore, these measurements are a snapshot of the situation in the medium of the cell at that exact moment. This will affect the hormone levels over time and therefore should be considered with some margin of uncertainty. Exposure of MA-10 cells to cAMP (100 μM) showed a significant increase of almost all steroids assessed, except for 17-hydroxy-pregnenolone (17-OH-P5), which showed a decreased level compared to the control, and etiocholanolone/androsterone (etio/androsterone), which was even absent in the media samples analyzed. However, corticosterone and androstanediol levels did not significantly change after cAMP exposure. Also, only after cAMP exposure, dehydroepiandrosterone (DHEA), that reflects the preferred Δ5 pathway, was measured, next to the Δ4 pathway-derived androstenedione, which indicates that both steroidogenic routes are present in this cell line. This is also supported by high levels of P4 and 17-OH-P5 in vehicle-control exposed samples, indicating high 3β-HSD and CYP17 activity, of which 3β-HSD especially is needed for conversion of Δ5 to Δ4 hormones. The presence of estrone (E1) indicated some level of estrogen formation.

Compared to DMSO control-treated cells, several hormone levels increased after exposure to the bisphenol analogues. In the case of BPF and BPS an increase of pregnenolone (P5) and P4 levels was observed, whereas TBBPA exposure caused an induction of P4, 17-OH-P4, deoxycorticosterone (DOC), androstenedione, α-T as well as β-T levels (Fig. A.2). When comparing the effects of the different bisphenols exposures mutually, only TBBPA showed statistically significant changes in hormone levels when compared to either BPF or BPS exposure. TBBPA showed an increase in androstenedione and α-T as well as a decrease of P5, 17-OH-P4, and

androstanediol as compared to BPF-exposed cells. Compared to BPS, TBBPA exposure resulted in an increase in 17-OH-P4, androstenedione, α-T, and β-T levels, but also a decrease in the levels of P5 and androstanediol. Comparing the effects of TBBPA, BPF and BPS, those of TBBPA were more directed towards the male end products of T synthesis, while BPF and BPS predominantly changed the levels of progestagens that are formed in the beginning of the steroidogenic pathway (*cf.* Fig. A.3). The difference in effects on steroidogenesis caused by TBBPA versus bisphenol A analogues exposure, possibly originating from differences in their mechanism of action, already points out that validation of the MA-10 cell assay by different classes of compounds in necessary. Furthermore, combining multiple assays covering specific modes of action of the endocrine system could advance our knowledge on the way EDCs exert their differential effects (van der Burg et al., 2011). A recent study by Rosenmai et al. (2014) described metabolomics in the human adrenocortical H295R cell line upon exposure to several structural bisphenol A analogues. They also showed a marked increase in progestagens for BPF (mainly P4) and BPS (mainly 17-OH-P4), but no effect of BPA. Interestingly, we observed no difference in potency between BPF and BPS for induction of P4 levels and in our study 17-OH-P4 was not statistically significantly affected. Also, a decrease in DHEA was described upon BPA, BPS and especially BPF exposure of H295R cells, while secretion of this metabolite was not detectable in our study with MA-10 cells. Moreover, the "backdoor pathway" steroids etio/androsterone or androstanediol were not described in the study of Rosenmai et al. (2014). The differences in bisphenol analogue effects found between H295R cells and MA-10 cells again underline the importance of combining both assays as tissue- and possibly also species-differences in steroidogenic pathways can exist as described above that might influence the outcome of a study.

An increase in testicular T production could theoretically lead to a more masculine phenotype because of its promoting role in the development and differentiation of the male organs (Scott et al., 2009). However, no *in vivo* studies were found that clearly found such an effect upon TBBPA exposure (Van der Ven et al., 2008; Lilienthal et al., 2008). Moreover, as described above, the timing of exposure plays an important role on the ultimate effect. Increased prenatal progestagen levels have been associated with a delay in parturition (Hashimoto et al., 2010; Condon et al., 2003; da Fonseca et al., 2003; Guillette et al., 1991; Wilson et al., 1981). Vinggaard et al. found that higher levels of testicular P4 in male rat fetuses upon exposure of the pregnant dams to the fungicide prochloraz were linked with delayed parturition (Vinggaard et al., 2005). In addition, both androgens and progestagens function as anti-gonadotropins through a negative feedback loop within the (adult) hypothalamic-pituitary-gonadal (HPG) axis, thereby reducing FSH and LH levels. As a result, this may lead to an adverse reproductive outcome later in life (Bandivdekar et al., 2000; Brady et al., 2003). Whether this could also be a pathway for (developmental) toxicity by BPF and BPS needs to be confirmed by an *in vivo* study.

3.4. Expression of genes involved in cholesterol biosynthesis and steroidogenic pathways

Subsequently, we investigated whether effects seen on steroid secretion by MA-10 cells are reflected by altered cholesterol biosynthesis or steroidogenic gene expression after cAMP, BPF, BPS,

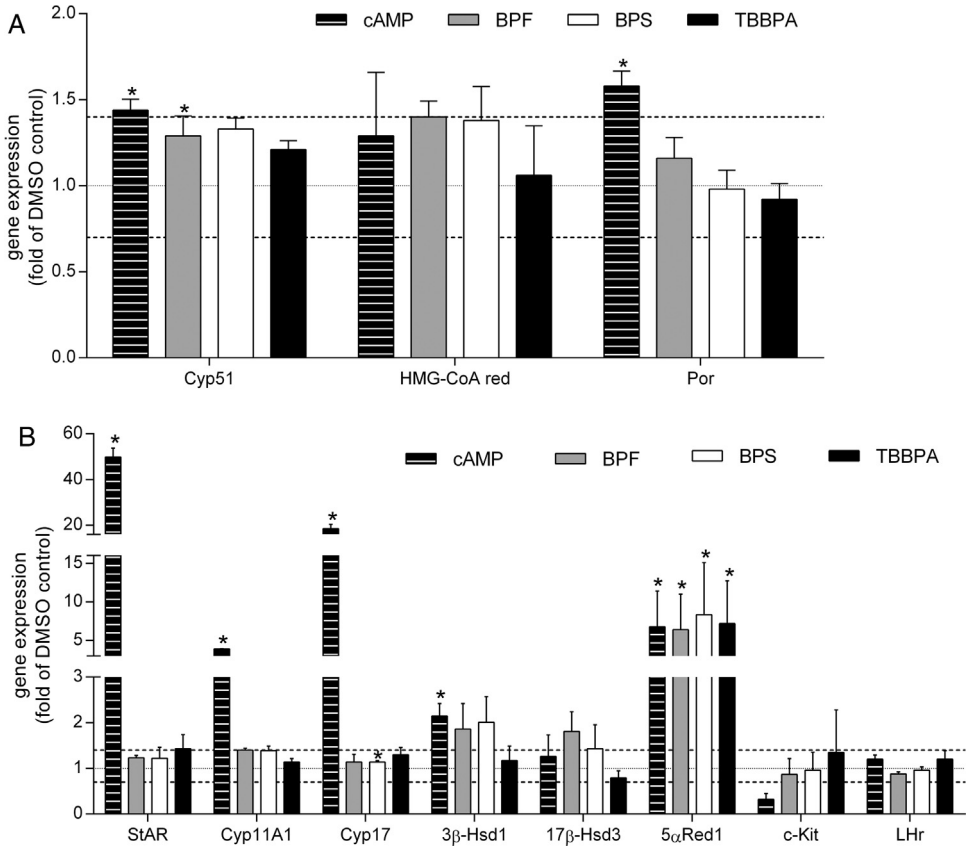

Fig. 4. Expression of genes involved in (A) cholesterol biosynthesis or (B) steroidogenesis in MA-10 cells after a 6-hour exposure to cAMP (100 μM) or one of the selected bisphenol analogues (10 μM). Data are represented as means ± SEM of three independent experiments that were performed in triplicate. Significance was assessed by means of a Student's *t*-test. *Significantly different from DMSO control-treated cells ($P < 0.05$). The gray dotted line indicates the reference level of the DMSO control. Dashed lines indicate the upper and lower limits of uncertainty of 1.4 and 0.7-fold difference compared to the DMSO control, respectively.

or TBBPA exposure. cAMP (100 μM) caused a statistically significant increase in gene expression of Cyp51 (1.4-fold), Por (1.6-fold), StAR (50-fold), Cyp11A1 (3.9-fold), Cyp17A1 (19-fold), 3β-Hsd1 (2.2-fold), and 5αRed1 (6.8-fold) whereas HMG-CoA red, 17β-Hsd3, c-Kit and LHr gene expression levels were not affected (Fig. 4). The rather unspecific manner in which cAMP upregulated most of the genes involved in cholesterol biosynthesis and steroidogenesis is also reflected in its steroid profile (Fig. 3). The increase in P4 together with StAR gene expression after exposure of MA-10 cells to cAMP has been reported previously (Schwarzenbach et al., 2003). None of the bisphenol analogues upregulated the StAR gene expression, which suggests that their effects on testicular steroidogenesis are not mediated by a cAMP-dependent pathway.

Most notable was the upregulation of 5αRed1 gene expression by 6.4-, 8.4-, and 7.2-fold by BPF, BPS, and TBBPA, respectively (Fig. 4B). This is in line with our findings pointing towards the existence of the physiologically relevant fetal "backdoor pathway" in MA-10 cells. Additionally, Flück et al. found that the 5αRed1 gene is expressed more in fetal than in adult testes (Fluck et al., 2011), which supports our finding that the MA-10 Leydig cell line represents a more fetal-like testicular steroidogenesis pattern. Statistically significant upregulation of Cyp51 gene expression by BPF (Fig. 4A), as well as Cyp17A1 by BPS (Fig. 4B) were also observed in our study, yet these lie within the uncertainty range of the study design. Together, the results from the gene expression and steroid production suggest that effects of bisphenol analogues on steroidogenesis are caused by catalytic modulation of *e.g.* CYP17 activity. Yet the upregulation of 5αRed1 gene expression suggests a redirection of steroidogenesis, which might lead to important effects in fetal testis development and function that warrant further investigation.

4. Conclusions

The present study shows for the first time that bisphenols have differential effects on the steroidogenesis in MA-10 Leydig cells. Our results indicate that BPF and BPS are not necessarily 'safer' alternatives compared to BPA regarding their endocrine modulating capacity. This conclusion is in line with results of a recent study by Rosenmai et al. (2014). Moreover, TBBPA seems to elicit the highest endocrine modulating potential with strong induction of Leydig cell testosterone secretion and GR antagonism in the nanomolar range. The limited information on GR modulating effects with respect to male reproductive toxicity warrant further *in vivo* studies. The MA-10 Leydig cell assay appears to be particular useful to study effects of suggested EDCs on fetal testicular steroidogenesis, which differs substantially from adrenal steroidogenesis. Therefore, it is highly recommendable to consider the use of an *in vitro* assay covering the fetal testis, such as the MA-10 cell line, next to the existing H295R steroidogenic assay for screening for potential EDCs.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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