Are Structural Analogues to Bisphenol A Safe Alternatives?

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Background: Bisphenol A (BPA) is a chemical with widespread human exposure suspected of causing low-dose effects. Thus, a need for developing alternatives to BPA exists. Structural analogues of BPA have already been detected in foods and humans. Due to the structural analogy of the alternatives, there is a risk of effects similar to BPA.

Objectives: The aim was to elucidate and compare the hazards of bisphenol B (BPB), bisphenol E (BPE), bisphenol F (BPF), bisphenol S (BPS) and 4-cumylphenol (HPP) to BPA.

Methods: In vitro studies on steroidogenesis, receptor activity, and biomarkers of effect, as well as Quantitative Structure-Activity Relationship (QSAR) modeling.

Results: All test compounds caused the same qualitative effects on estrogen receptor and androgen receptor activities, and most of the alternatives exhibited potencies within the same range as BPA. Hormone profiles for the compounds indicated a specific mechanism of action on steroidogenesis which generally lead to decreased androgen, and increased estrogen and progestagen levels. Differential effects on corticosteroid synthesis were observed suggesting a compound-specific mechanism. Overall, BPS was less estrogenic and antiandrogenic than BPA, but BPS showed the largest efficacy on 17α-hydroxyprogesterone (17α-OH progesterone). Finally, there were indications of DNA damage, carcinogenicity, oxidative stress, effects on metabolism, and skin sensitization of one or more of the test compounds.

Conclusions: Interference with the endocrine system was the predominant effect of the test compounds. A substitution of BPA with these structural analogues should be carried out with caution.

Key words: bisphenol A; BPA analogues; in vitro; QSAR; steroidogenesis.

Bisphenol A (BPA) is a chemical used in many applications including production of polymer products such as polycarbonate plastics and epoxy resins which are used in various food contact materials (EFSA, 2006). BPA can furthermore be used as an additive (Geens et al., 2011), e.g., in thermal papers for receipts (Liao and Kannan, 2011; Mendum et al., 2011) and have been found in recycled paper (Geens et al., 2011; Vinggaard et al., 2000).

Total BPA levels (conjugated + free) have been measured in more than 90% of urine samples from the U.S. population (n = 2517; Calafat et al., 2008) and the population of seven Asian countries (n = 296; Zhang et al., 2011b) between 2003–2004 and 2006–2010, respectively, suggesting that the vast majority of the population is exposed. Furthermore, conjugated and/or unconjugated BPA has been detected in human amniotic fluid (Edlow et al., 2012; Yamada et al., 2002), umbilical cord blood (Chou et al., 2011; Lee et al., 2008), and placenta (Cao et al., 2012; Jimenez-Diaz et al., 2010) suggesting that the fetus is exposed. Diet is estimated the main source of human exposure followed by thermal paper (EFSA, 2013).

BPA has been widely investigated with respect to its toxicological hazard and has shown low-dose effects including disturbed mammary gland development (Moral et al., 2008), changes in normal behavioral development (Xu et al., 2010), and changes in obesity associated parameters (Miyawaki et al., 2007) in rodents. BPA is well known for its estrogenic activity (Gould et al., 1998; Grignard et al., 2012; Kitamura et al., 2005; Krishnan et al., 1993; Paris et al., 2002), but other mechanisms of action have also been reported such as human pregnane X receptor (PXR) agonism (Sui et al., 2012), effects on steroid hormone synthesis (Zhang et al., 2011a), and androgen receptor (AR) antagonism (Kitamura et al., 2005; Lee et al., 2003a; Paris et al., 2002; Vinggaard et al., 2008), all of which contribute to the overall potential of BPA to interfere with hormone systems.

In 2010, the Canadian Government prohibited the import, sale, and advertisement of polycarbonate baby bottles containing BPA (Government of Canada, 2010), and in 2011, the European Union prohibited the use of BPA in the manufacture of polycarbonate feeding bottles for infants (The European Commission, 2011). Thus, an incentive for developing alternative substances exists. Structural analogues such as bisphenol S (BPS) have already been found in canned soft drinks, canned foods (Gallart-Ayala et al., 2011; Vinas et al., 2010), and thermal receipt papers (Becerra and Odermatt, 2012; Liao et al., 2012b). Bisphenol B (BPP) has been found in canned tomatoes (Grumetto et al., 2008), canned soft drinks, and canned beers.
In addition, BPS (free and conjugated) has been detected in 81% of urine samples from the general population collected from USA and seven Asian countries \( (n = 315; \) Liao et al., 2012a), and BPB has been found in 28% of blood sera from Italian endometriotic women \( (n = 58; \) Cobellis et al., 2009).

Generally limited information on the hazards of these BPA analogues is available. However, as a consequence of the structural similarity of these alternatives to BPA, there is a risk that they could lead to similar adverse effects. Thus, an urgent need for investigating the hazards of the alternatives to BPA exists. In the present study, the overall aim was to compare the hazard of BPB, bisphenol E (BPE), bisphenol F (BPF), BPS, and 4-cumylphenol (HPP) to BPA by characterizing their toxicological profiles in silico and in vitro. The toxicological profiling included acute and local effects, endocrine disruption, teratogenicity, genotoxicity, carcinogenicity, and effects on metabolism.

**METHODS**

**In Silico Profiling**

Two QSAR modeling tools, MultiCASE (version 2.4.1.4) and Leadscope (version 3.04-10) were applied. The individual QSAR models are described in Supplementary materials 1 and in Dybdahl et al. (2012), Jensen et al. (2008, 2011), Jonsdottir et al. (2012), and Vinggaard et al. (2008). Experimental data for compounds included in the training set of QSAR models for a given endpoint are indicated in Table 1.

MultiCASE is a statistical model system that aims to discover fragment combinations, called biophores/biophobes for active/inactive molecules, relevant for an observed effect. Further MultiCASE identifies modulators, such as physiochemical properties, which may affect the probability of a fragment being a biophore/biophobe. Warnings are given in predictions if a fragment is not represented in the training set, or if a contradictory modulator is present in a prediction. Warnings were considered an indication that the molecule was outside the model applicability domain. MultiCASE predictions are reported as positive or negative.

Leadscope uses a library of 27,000 structural features typically found in small drug molecules and eight calculated molecular descriptors for QSAR modeling. The best correlated features are selected when building a predictive model. In this study, a compound is in domain if there is ≥30% structural similarity with a training set compound and if the compound contains at least one structural feature from the model. Predictions are provided as probabilities \( (p) \) for the presence of a given effect. In this study, \( p \geq 0.7 \) is considered a positive prediction and \( p \leq 0.3 \) a negative prediction.

**In Vitro Profiling**

Test chemicals. BPA, BPB, BPE, BPF, BPS, and HPP stock solutions of 40mM were prepared in dimethyl sulfoxide (DMSO). BPA, BPF, BPS, and HPP were purchased from Sigma-Aldrich (Copenhagen, Denmark) and BPB and BPE were purchased from VWR (Herlev, Denmark). For chemical structures and CAS numbers, see Figure 1.

**H295R steroidogenesis assay.** The H295R steroidogenesis assay (H295R assay) was performed to assess test compound potential to affect steroid hormone synthesis. The assay was conducted as described previously (Rosenmai et al., 2013) using human adrenal cortico-carcinoma cells (ATCC, LGC standards,
Boras, Sweden). Prochloraz (Ehrenstorfer, Augsburg, Germany) and forskolin (Sigma-Aldrich, Brøndby, Denmark) were included as a negative and positive control, respectively. Test compounds were added in seven 2-fold dilutions in triplicates ranging from 0.8 to 50 μM with a constant vehicle concentration in all wells. Cell viability was assessed in the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Mosmann, 1983) as described previously (Rosenmai et al., 2013). For information on criteria for cytotoxicity and exclusion of data due to cytotoxicity for all assays see Supplementary materials 2.

Hormones were extracted from cell supernatants by solid phase extraction (SPE) as previously described (Mortensen and Pedersen, 2007; Vinggaard et al., 2002). Time-resolved fluoroimmunoassays (PerkinElmer, Skovlunde, Denmark) were used for quantification of 17β-estradiol, testosterone, and progesterone in two to three H295R steroidogenesis experiments. Additionally, 10 hormones were analyzed by high pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) including progesterone, 17α-OH progesterone, dehydroepiandrosterone (DHEA), androstenedione, testosterone, dihydrotestosterone, corticosterone, cortisol, 17β-estradiol, and estrone in one experiment. Hormones were separated, identified, and quantified as previously described (Mortensen and Pedersen, 2007) with a few modifications (Rosenmai et al., 2013). The limit of quantification (LOQ) was estimated as described in Rosenmai et al. (2013), and was 1000 pg/ml for DHEA, 500 pg/ml for dihydrotestosterone, 100 pg/ml for androstenedione and 17β-estradiol, and 50 pg/ml for the remaining hormones measured. Both methods were used to quantify testosterone and progesterone, and the results were generally in accordance. DHEA and corticosterone levels at 50 μM HPP exposure as well as all dihydrotestosterone and 17β-estradiol measurements by HPLC-MS/MS were below LOQ.

**Estrogen receptor (ER) reporter gene assay.** Activation of ERs by test compounds were tested in a stably transfected human ovarian adenocarcinoma cell line (BG1Luc4E2) which was provided by Michael Denison (University of California, USA). Experiments were generally conducted according to the Organisation for Economic Co-operation and Development (OECD) test guideline (OECD, 2012). Approximately 72 h before experiment set up, cells were transferred to estrogen-starved stripped medium. Successively cells were seeded (4 × 10^4 cells/well) ~48 h before exposure. After ~22 h exposure, luciferase activity was measured. Positive controls, 17β-estradiol (Sigma-Aldrich), and methoxychlor (ICN biomedicals, Aurora, Ohio, USA) were included in experiments. Two experiments of three to four replicates were conducted with seven 10-fold dilutions of test compounds ranging from 0.0001 to 100 μM. The DMSO concentration was 0.25% in all treatment groups and 0.2% in controls. Cell viability was scored by visual evaluation.

**Androgen receptor (AR) reporter gene assay.** The potential of the chemicals to interfere with the activation of the AR was tested in an AR reporter gene assay as described by Vinggaard et al. (2002). Chinese hamster ovary (CHO) cells were purchased from American Type Culture Collection (ATCC, LGC standards, Boras, Sweden) and plasmids for receptors and reporter gene were gifts from Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). A positive and negative control, R1881 (PerkinElmer) and hydroxyflutamide (OHF) (Toronto Research Chemicals, Toronto, Canada), respectively, was included in experiments. R1881 was added at a concentration of 0.1 nM on antagonism plates. Three experiments in triplicates were conducted with eight 2-fold dilutions of test compound between 0.4 and 100 μM. Vehicle concentrations were constant in all wells.

**Aryl hydrocarbon receptor (AhR) reporter gene assay.** AhR activation leads to transcription of metabolizing enzymes (Ma, 2008). Stably transfected rat hepatoma (H4IIE-CALUX) cells provided by Dr Michael Denison (University of California, USA) were used. The assay was conducted as previously described (Laier et al., 2003) with a few modifications. Cells were incubated for ~22 h in minimum essential medium (MEM) α [1% foetal bovine serum (FBS), 1% penicillin/streptomycin/fungizone (PSF)], chemical exposure was performed for 24 h, and successively luminescence was measured. Cell viability was tested as previously (Laier et al., 2003), but cells were seeded at a concentration of 1.1 × 10^4 cells/well. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was used as a positive control. Two experiments in triplicates were conducted with nine 2-fold dilutions of test compounds ranging from 0.4 to 100 μM with a constant vehicle concentration in all wells.

**Nrf2, retinoic acid receptor (RAR), and p53 reporter gene assays.** The Nrf2, p53, and RAR are involved in responses to oxidative stress (Motohashi and Yamamoto, 2004), responses to DNA damage (Horn and Vousden, 2007), and play a central role during embryonic development (Mark et al., 2009), respectively. The BDS-CALUX reporter gene assays, developed and carried out by BioDetection Systems b.v. (Amsterdam, The Netherlands), are based on the human U2OS osteosarcoma cell line which is stably transfected with a reporter gene and a receptor, RAR assay (Van Vught-Lussenburg et al., unpublished data) or solely a reporter gene, p53-CALUX (Van der Linden et al., 2014) and Nrf2-CALUX (Van Vught-Lussenburg et al., unpublished data).

The U2OS-CALUX cells were maintained in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Breda, Netherlands) with 7.5% fetal calf serum (Invitrogen), 1× nonessential amino acids (Invitrogen) and 10 U/ml penicillin and 10 μg/ml streptomycin (Invitrogen). The assays were conducted in DMEM without phenol-red (Invitrogen) supplemented with 5% dextran coated charcoal (DCC) stripped fetal calf serum,
and amino acids and antibiotics as above. Positive controls, actinomycin D, all-trans-retinoic acid, and curcumin (Sigma-Aldrich, Zwijndrecht, Netherlands) for the p53, RAR, and Nrf2 assays, respectively were included in experiments. Two independent experiments of three to six replicates were conducted with eight 2-fold dilutions of test compounds in concentrations ranging from 0.8 to 100 μM with a constant vehicle concentration in all wells. The number of experiments was determined in preliminary experiments. 3 × 10³ cells/well were seeded in 384-well plates and incubated for 24 h. After incubation, cells were exposed to test compounds for 24 h. Successively the luciferase signal was measured on a luminometer (Berthold Centro, Bad Wildbad, Germany).

Data Handling and Statistical Analysis

In vitro data were normalized to the mean of the control from the same cell plate within each experiment. Normalized residuals to means were assessed for normal distribution (D’Agostino & Pearson’s omnibus test) and variance homogeneity (one-way ANOVA). All variances were homogenous. A one-way ANOVA (Dunn’s post hoc test) was conducted if all data for a specific endpoint were normally distributed. In other cases a Kruskal-Wallis test (Dunn’s post hoc test) was used to assess differences between means. If the test showed significant differences between means and the same qualitative dose-response relationship in the majority of the experiments, the independent experiments and the pooled means of the experiments were fitted to a sigmoidal dose-response fit. Constraints in the curve fits were set at 1 for controls and at the maximum predicted to a sigmoidal dose-response fit. Constraints in the curve fits were set at 1 for controls and at the maximum 80% of the specific maximum change in response compared with control, as well as predictions performed in an array of QSAR models.

Endocrine Interference and Teratogenicity

Steroidogenesis was affected by all test compounds in the human adrenal cortico-carcinoma cells (Fig. 3, Table 3).

Overall, progesterone levels increased with exposure to BPB, BPE, BPF, BPS, and HPP; however, BPA and BPB did not increase progesterone levels significantly in the majority of the experiments. BPF and BPS led to the greatest $E_{\text{max}}$ values on progesterone and 17α-OH progesterone levels, respectively.

Testosterone, androstenedione, and DHEA levels generally decreased with exposure to the test compounds. However, DHEA did not change with HPP exposure and increased in level with BPB exposure. BPA and BPE showed the most potent effect on androgens levels compared with the remaining test compounds. The $E_{\text{50}}$ and $E_{\text{max}}$ values for decreased androgens ranged from 0.3 to 28.0 μM and 44 to 89%, respectively (Table 3).

Generally, 17β-estradiol and estrone levels were increased by test compounds though BPS did not cause an effect. BPB and HPP increased estrone levels significantly with efficacies of 1592 and 845%, respectively. $E_{\text{50}}$ values for both estrogens differed by less than a factor of ~3 between the test compounds. BPA, BPB, BPS, and HPP decreased cortisol levels with $E_{\text{max}}$ values in the same range, 72–78%, and $E_{\text{50}}$ values ranging by a factor ~3.5. BPB led to increased cortisol levels. BPB, BPS, and HPP led to decreased corticosterone levels; whereas BPE and BPB led to increased levels (Table 3). BPA exposure did not affect corticosterone levels.

ER activity was increased by all six compounds (Fig. 2). BPB and HPP were equally potent to BPA ($E_{\text{50}}$ ~ 0.1 μM) (Table 2). BPE, BPF, and BPS were less potent; however, the $E_{\text{50}}$ values were all within a factor of ~15 to BPA. BPS was the least potent of the six compounds. $E_{\text{max}}$ values ranged from 156 to 386%. In the QSAR models for estrogenic effects, all six compounds, except BPS, have been experimentally tested positive for ER binding and activation and were part of the training set. BPE was predicted positive for ER binding and activation and was not part of the training set.

BPF and BPS was predicted positive for antiandrogenicity, while BPE showed the most positive effect on androgens levels compared with the remaining test compounds. All test compounds decreased the activity of the AR (Fig. 2). BPS only led to a decreasing trend and was not modeled. BPA, BPB, and BPF had similar $E_{\text{50}}$ values, whereas BPE had higher and HPP lower potency (Table 2). The $E_{\text{50}}$ values differed by less than a factor of ~2.7 and $E_{\text{max}}$ values ranged from 72 to 92%. Of the tested compounds, only BPE led to an agonistic response in the AR assay with an $E_{\text{50}}$ of 16.0 μM (SD = 2.0) and $E_{\text{max}}$ of 169% (SD = 37; data not shown). The QSAR model for AR antagonism included BPA and BPB in the training set, which has previously shown AR antagonism experimentally. BPA and BPE predicted positive for antiandrogenicity, while BPS and HPP were predicted negative. The most relevant biophores in the QSAR model require either substitutions on both benzene rings or one substituted benzene ring combined with an unsubstituted carbon atom in the methylene bridge, which could explain the difference in QSAR prediction and in vitro result for HPP.

Finally, the test compounds were predicted negative in a QSAR model for human teratogenicity (Table 1). This is in accordance with experimental data on RAR activation showing that all test compounds, except BPF, had no effect on this endpoint (data not shown).
**Oxidative Stress, DNA Damage, and Cancer**

BPF and HPP led to reporter gene activation in the Nrf2-CALUX assay (Fig. 2, Table 2), whereas the remaining compounds did not lead to changes in response.

BPA and BPB caused reporter gene activation in the p53-CALUX assay (Fig. 2, Table 2). BPE showed an increasing trend; however, this was not modeled as it was not significant in both experiments. In the QSAR models, BPA had been tested negative for several genotoxicity endpoints and was part of the training sets for these models. BPA was either predicted negative or was out of domain of the genotoxicity models which did not include BPA in the training set. The remaining compounds gave either negative predictions for genotoxicity or were out of domain of the models.

BPA has been tested experimentally and was part of the training sets for the QSAR models for rodent cancer (AF1–AF4) (Table 1). BPA was positive for cancer in male rats, marginally positive in male mice, and negative for female rats and mice. BPB and BPF were predicted positive in male mice and female mice, respectively. However, the prediction for BPB was based on only five chemicals containing the biophore of which two where tested marginally positive including BPA. The overall Research Collaboration Agreement (RCA) QSAR cancer call were negative for all compounds, except BPB which was out of domain. An estimate of the carcinogenic potency in rodents (TD50) indicated very low potency (>1000 mg/kg/day) for all compounds (data not shown).

**Metabolism**

BPA, BPE, and BPF activated the AhR *in vitro* showing potencies ranging from 48.8 to 54.8 μM. BPA led to a lower efficacy than BPE and BPF. BPB, BPS, and HPP had no effect on AhR activity (Table 2).

All compounds were predicted positive in at least one of the applied models for cytochrome P450 (CYP) substrate/inhibition (Table 1). Only robust predictions (p ≥ 0.7 or p ≤ 0.3) are mentioned in the following. HPP and BPB were predicted CYP2D6 substrate and inhibitor, respectively. All compounds except BPS were predicted CYP3A4 substrates, and BPS and HPP were predicted CYP2C9 substrates. All compounds except BPB were predicted negative for CYP2C9 inhibition and BPS was furthermore predicted negative for CYP2D6 inhibition. Robust predictions for PXR binding was only obtained for BPB and HPP which were predicted positive.
FIG. 3. Hormone profiles for BPA (dark blue), BPB (green), BPE (light blue), BPF (orange), BPS (pink), and HPP (black) from the H295R steroidogenesis assay. Data shown as fold change compared with control (± SD) as a function of the logarithm to the concentration (log(µM)). Curve fits and data points are based on means of replicates from independent experiments for 17β-estradiol analyzed by immunoassay and one experiment in triplicates for the remaining hormones analyzed by HPLC-MS/MS. Basal production of progesterone, 17β-OH progesterone, corticosterone, cortisol, DHEA, androstenedione, testosterone, and estrone were 494 ± 136, 5732 ± 1009, 185 ± 46, 3202 ± 931, 10,122 ± 4932, 45,347 ± 13,088, 1686 ± 298, and 247 ± 154 pg/ml, respectively, given as means of controls ± SD from six cell plates (n = 18) measured by HPLC-MS/MS.
TABLE 1
QSAR Predictions for Test Co

<table>
<thead>
<tr>
<th>Metabolism</th>
<th>CYP2D6</th>
<th>BPA</th>
<th>BPB</th>
<th>BPE</th>
<th>BPF</th>
<th>BPS</th>
<th>HPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrates</td>
<td>0.69</td>
<td>0.69</td>
<td>0.69</td>
<td>0.69</td>
<td>0.34</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>Inhibitors</td>
<td>0.64</td>
<td>0.73</td>
<td>0.64</td>
<td>0.62</td>
<td>0.16</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.82</td>
<td>0.83</td>
<td>0.80</td>
<td>0.80</td>
<td>0.36</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Inhibitors</td>
<td>0.59</td>
<td>0.62</td>
<td>0.58</td>
<td>0.57</td>
<td>0.37</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.64</td>
<td>0.65</td>
<td>0.64</td>
<td>0.62</td>
<td>0.73</td>
<td>0.70</td>
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<tr>
<td>Substrates</td>
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<td>0.33</td>
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<td>0.25</td>
<td>0.29</td>
<td>0.26</td>
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</tr>
<tr>
<td>Inhibitors</td>
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<td>0.60</td>
<td>0.53</td>
<td>0.74</td>
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</tr>
</tbody>
</table>

Notes. Color code: red, positive; green, negative; white, out of domain. (*) Included in the training set of the model and the experimental result is indicated.

DISCUSSION

Endocrine Interference

BPA and the five analogues showed a clear effect on AR and ER activity as well as on steroid hormone synthesis in the present study, suggesting that these compounds may interfere with the endocrine system through several modes of action. In general, we found similar qualitative effects of the test compounds on endocrine activity.

Estrogenic effects of the test compounds were observed both for ER activation and for estrogen synthesis. In the ER reporter gene assay BPA showed a similar potency as BPB and HPP. BPE, BPF, and BPS were slightly less potent than BPA. BPS showed both low potency and efficacy. Furthermore, BPS was the only compound not leading to an effect on estrogen levels in the H295R assay, suggesting BPS as the overall least estrogenic compound. BPB and HPP showed a higher efficacy on estrone levels than the remaining compounds indicating a greater overall estrogenic potential of these compounds compared with BPA.

Antiandrogenic effects were observed for the six test compounds on AR activity and androgen synthesis. BPS only showed a decreasing trend at higher concentrations on AR activity, indicating a less antiandrogenic potential of BPS compared with the remaining test compounds. Androgen levels in the H295R assay were generally decreased by all test compounds with BPA and BPE being the most potent.
The estrogenic and antiandrogenic potential of BPA and the remaining test compounds have been reported previously (Cabanton et al., 2009; Chen et al., 2002; Grignard et al., 2012; Kitamura et al., 2005; Kuruto-Niwa et al., 2005; Rivas et al., 2002; Xu et al., 2005). Exposure to estrogenic and antiandrogenic chemicals have been associated with breast cancer development (Yue et al., 2013) and effects on the male reproductive system (Sharpe and Skakkebaek, 2008; Skakkebaek et al., 2001), respectively. Potent effects were observed on ER activity (EC50 = 0.08–1.17 μM), AR activity (EC50 = 1.9–5.1 μM) and steroid hormone levels (EC50 = 0.3–28.0 μM) in the present study, which are in the range of high-end urine concentrations of BPA (conjugated and free) in Danish children of ~100 nM (Frederiksen et al., 2013). As the test compounds all exhibited estrogenic and antiandrogenic potential they may contribute to these types of effects.

The effects on progestagen levels differed between BPA and the remaining test compounds as BPA had little effect on these hormones whereas the remaining compounds generally led to increased progestagen levels. BPS as well as BPF led to the greatest changes in efficacy on 17α-OH progesterone and progestosterone levels, respectively. Administration of a synthetic progestagen in utero has been associated with virilization of female mice and feminization in male mice (Willingham et al., 2006). Thus, these data indicate that the five analogues, especially BPS and BPF, may have effects which are not prominent for BPA on this endpoint.

Finally, the effect on the corticosteroids differed between the six compounds. Generally BPE and BPF led to increased corticosteroid levels, whereas the remaining test compounds led to decreased levels, suggesting a compound-specific mechanism of effect which could be caused by dissimilar upstream or downstream effects. The increased corticosteroid levels observed for BPE and BPF may be of concern as such increases in utero have been associated with development of effects involved in the metabolic syndrome, as well as changes in behavior later in life (Drake et al., 2007), and thus these compounds may be of specific concern for these endpoints.

In summary, the six test compounds generally led to increased progestagen and estrogen levels, and decreased androgen levels in the H295R assay (Fig. 3, Table 3). This suggests that the observed effects were caused by specific interactions, and were
Table 3
Te Maximum Efficacy ($E_{\text{max}}$) and Values for Concentrations Causing 50% of the Maximum Response (EC_{50}) for Test Compounds in the H295R Steroidogenesis Assay

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$E_{\text{max}}$ (%)</th>
<th>CI (%)</th>
<th>EC_{50} (µM)</th>
<th>CI (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>689</td>
<td>1493</td>
<td>744</td>
<td>387</td>
</tr>
<tr>
<td>17α-OH progesterone</td>
<td>22</td>
<td>74</td>
<td>198</td>
<td>510</td>
</tr>
<tr>
<td>Cortisol</td>
<td>73</td>
<td>72</td>
<td>43</td>
<td>74</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>44</td>
<td>22</td>
<td>68</td>
<td>86</td>
</tr>
<tr>
<td>Dehydroandrosterone</td>
<td>89</td>
<td>77</td>
<td>73</td>
<td>64</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>3.1</td>
<td>16.0</td>
<td>0.3</td>
<td>5.6</td>
</tr>
<tr>
<td>Testosterone</td>
<td>83</td>
<td>78</td>
<td>58</td>
<td>47</td>
</tr>
<tr>
<td>Estrone</td>
<td>205</td>
<td>1592</td>
<td>226</td>
<td>248</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>209</td>
<td>326</td>
<td>212</td>
<td>481</td>
</tr>
</tbody>
</table>

Notes. $E_{\text{max}}$ and EC_{50} values (bold) based on means of predicted values from dose-response curve fits for independent experiments. Standard deviations (SD) are shown if more than one experiment was modeled and 95% confidence intervals (CI) if one experiment was modeled. Color code: green, activation; red, deactivation; white, no significant change. VW: very wide; —: the constraints of the model was reached and thus no confidence interval could be predicted.

Normalized to means of controls from all cell plates within experiment.

**not a result of a general down- or upregulation of steroidogenesis.** The specific interactions within steroidogenesis have previously been investigated for BPA in the H295R assay (Zhang et al., 2011a). BPA exposure was suggested to cause an increase in progesterone and decrease in androgen levels through inhibition of the CYP17 lyase reaction and to increase estrogen levels through inhibition of metabolism of estrogens (Zhang et al., 2011a). Overall, the present study is in accordance with these findings (Table 3), suggesting that one or both of the specific interactions of BPA suggested by Zhang et al. (2011a) may be...
evident for the test compounds. Differences in hormone profiles for BPA between the two studies may reflect temporal or compensatory effects as a result of differential exposure times, 24 h versus 48 h.

**Oxidative Stress, DNA Damage, and Cancer**

BPA and BPB increased p53 activity and BPE caused an increasing trend indicating potential to cause DNA damage. BPF and HPP led to increased Nrf2 activity indicating potential for oxidative stress (Table 2). Furthermore, BPB and BPF had positive predictions in one of the QSAR models for cancer (Table 1) in which BPA was tested experimentally positive.

In previous studies BPA has been reported to have genotoxic potential (Atkinson and Roy, 1995; Iso et al., 2006; Tsutsui et al., 1998), but other studies have reported negative findings for genotoxicity (Ibuki et al., 2008; Lee et al., 2003b). Furthermore, BPF has shown genotoxic potential in the HepG2 cells using the Comet assay (Cabaton et al., 2009) and when assessing the ability to cause histone H2AX phosphorylation (Audebert et al., 2011), but BPB showed no effect in the micronucleus assay (Cabaton et al., 2009). These contradicting data for BPA and BPB from previous studies are in accordance with the data from the present study. To our knowledge, no data exists on the potential of the remaining test compounds to cause DNA damage or cancer. The *in vitro* assays were conducted without a metabolizing system and thus bioactivated metabolites are not assessed in this study. The authors suggest that further investigations of these compounds into their genotoxic and carcinogenic potential as well as potential to cause oxidative stress should be performed.

**Metabolism**

Interactions with several CYP enzymes as well as two receptors, AhR and PXR, associated with metabolism were investigated either *in silico* or *in vitro*. In the AhR reporter gene assay effects were observed at high concentrations for BPA, BPE, and BPB. All compounds were predicted positive in at least one of the QSAR models for CYP substrate recognition and inhibition, and BPB and HPP were predicted PXR binders (Table 1).

All the test compounds have previously been reported to induce activation of the human PXR receptor *in vitro* (Sui et al., 2012). Thus, these data indicate that at least BPB and HPP may lead to PXR activation though binding directly to the receptor. PXR as well as AhR activation induce expression of enzymes involved not only in the metabolism of xenobiotics but also of endogenous hormones (Arlotto et al., 1991; Ma, 2008; You, 2004). PXR activation has been associated with decreased androgen levels (Zhang et al., 2010) and increased corticosterone and aldosterone levels (Zhai et al., 2007). Thus, activation of these receptors by the test compounds may add to the overall endocrine potential by increasing or decreasing the removal of endogenous hormones *in vivo* causing disruption of homeostasis.

Metabolism of the bisphenol analogues have to our knowledge not been investigated *in vivo* previously. However, several BPA analogues were studied in river water (Ike et al., 2006), pond sediments (Ike et al., 2006), and seawater (Danzl et al., 2009) showing that the degradation varied with BPS having the least efficient degradation compared with the remaining analogues tested. Furthermore, ToxCast data for BPA, BPB, BPS, and HPP have been released by the U.S. Environmental Protection Agency (EPA) for effects on various CYP enzymes (U.S. EPA, 2014). Comparing these data with the QSAR predictions in the present study there were some deviations in CYP enzyme inhibition and activation data. The deviations may be due to reasons related to the basis and/or the performance of the models/assays. The CYP QSAR predictions are based on human clinical data and various human *in vitro* endpoints (Jonsdottir et al., 2012), whereas the ToxCast data origin from *in vitro* experiments in human cell lines. Furthermore, the criteria for classification as active/inactive for ToxCast as well as QSAR data may explain differential outcomes.

The differences in the QSAR profiles for the test compounds indicate that the metabolism of the tested compounds differs. However, further studies have to be conducted with respect to metabolism of the BPA analogues to obtain firm knowledge regarding this issue.

**CONCLUSIONS**

In the present study, the most marked effects were on endocrine interference. Potent effects were observed on ER activity, AR activity, and steroid hormone levels, suggesting that the compounds may act by several modes of action within the endocrine system. When comparing the compounds, BPS had the lowest estrogenic and antiandrogenic activity, but had high efficacy on progesteragen levels. The remaining BPA analogues had toxicological profiles that were generally similar to BPA. Finally, several of the compounds were flagged for genotoxicity, carcinogenicity, effects on metabolism and oxidative stress. Considering these findings, substituting BPA with any of the tested analogues should be carried out with caution.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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