Project tittle: “*In vitro Approach to Test Estrogen-like Activity of Six Bisphenol A Analogues”*

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IN VITRO APPROACH TO TEST ESTROGEN-LIKE ACTIVITY OF SIX BISPHENOL A ANALOGUES

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March 2016

TOX-80436

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Preamble

For advising and discussing I want to express my gratitude to Assistant Prof. Dr. Ir. Jochem Louisse and Laura de Haan. I would also like to thank Prof. Dr. Ir. Ivonne Rietjens and all the people of the Department of Toxicology of Wageningen UR. Special thanks to my family and friends.

Contents

Abstract

Bisphenol A (BPA) is a synthetic chemical with widespread use as monomer of plastics and hence widespread human exposure. Meanwhile it is one of the most studied chemical compounds with endocrine-disrupting properties. Its presence in food packaging materials but especially in plastic baby bottles increased public concern and scientific warnings which led to the establishment of new regulations for BPA and consequently the introduction of various substitutes in global market. Many of these substitutes have yet to be thoroughly tested for their safety. In order to obtain an overall view of the estrogenic potency of BPA and some of its analogues - namely bisphenol B (BPB), bisphenol C (BPC), bisphenol E (BPE), bisphenol F (BPF), bisphenol S (BPS) and 4-cumylphenol (HPP) - but also to compare their hazards, all the above were tested in CALUX reporter gene assay using two transfected U2-OS estrogen receptor (ER) α and β cell lines. Additionally, BPA together with BPS, one of the most widely used replacement compounds, were further assessed for their potential ERα-mediated proliferative effect in MCF-7 breast cancer cell line. All six analogues showed a clear estrogenic activity acting as agonists for both ER subtypes exhibiting weaker, similar, or higher potencies than BPA's. Compared to estradiol, all tested compounds except BPS appeared to be relatively more estrogenic in the ERβ than in the ERα cell line. With regard to the proliferative potency of BPA and BPS, results revealed that they can both slightly increase the proliferation of MCF-7 cells at the same nanomolar range as in reporter gene assay. In conclusion, "BPA-free" materials do not mean necessarily "(xeno)estrogen-free" materials. The suggested ERβ-mediated beneficial effects versus the adverse ERα-mediated effects make the test of the affinity of these compounds (and especially BPS) towards the two receptors separately, of great importance. Finally, the replacement of BPA with its analogues should be treated wisely and definitely after an extensive risk assessment.

1. INTRODUCTION

1.1 Background information

1.1.1 Endocrine disrupting chemicals (EDCs)

An endocrine disrupting chemical (EDC) is defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" according to the U.S. Environmental Protection Agency (Wetherill et al., 2007). By mimicking the mechanisms of steroid hormones these EDCs exhibit distinct biological activities such as (anti-) androgenic and/ or (anti-) estrogenic effects, among others (Cabaton et al., 2009). Androgens and estrogens are the two main classes of sex steroid hormones.

1.1.2 Estrogens

Estrogens are "…natural steroids (as estradiol) that are formed from androgen precursors, that are secreted chiefly by the ovaries, placenta, adipose tissue, and testes, and that stimulate the development of female secondary sex characteristics and promote the growth and maintenance of the female reproductive system". They can also be either synthetic or semisynthetic which mimic the physiological effect of natural estrogens (Dictionary, 2015). However, their influence on growth, differentiation and functioning of many tissues is not limited to the female reproductive system's but also those of the male. In addition, estrogens play an important role in the bone maintenance and they exert protective effect on the cardiovascular system (Kuiper et al., 1998). Like all steroid hormones, estrogens readily diffuse across the cell membrane and when they enter they bind to and activate estrogen receptors (Nussey & Whitehead, 2013).

1.1.3 Estrogen receptors (ERs) α and β

ERα and ERβ belong to the nuclear hormone receptor (NR) superfamily and function as ligand-inducible transcription factors once estrogens like 17β-estradiol (E_2) bind to them. In other words, the interaction of estrogens with the ERs triggers a series of molecular events that end up to the transcription of target genes (Delfosse et al., 2012). Figure 1 presents the molecular action of estrogens. The two receptors differ not only in their binding capacities for specific ligands but also in their distribution in various tissues. For instance, ERα predominates in uterus, testes and mammary gland and ERβ in prostate whereas both are highly present in ovaries (ter Veld et al.,

2006). It has been proposed that activation of ERβ reduces the ERα-mediated response that results in cell proliferation (Lazennec, Bresson, Lucas, Chauveau, & Vignon, 2001; Ström et al., 2004) but also inhibits cell proliferation through other mechanisms suggesting an ERβ-mediated induction of apoptosis (Acconcia et al., 2005; Paruthiyil et al., 2004). Bisphenol A has been found to weakly bind to both ER subtypes in several *in vitro* studies (Gould et al., 1998; Kuiper et al., 1998; ter Veld et al., 2006).

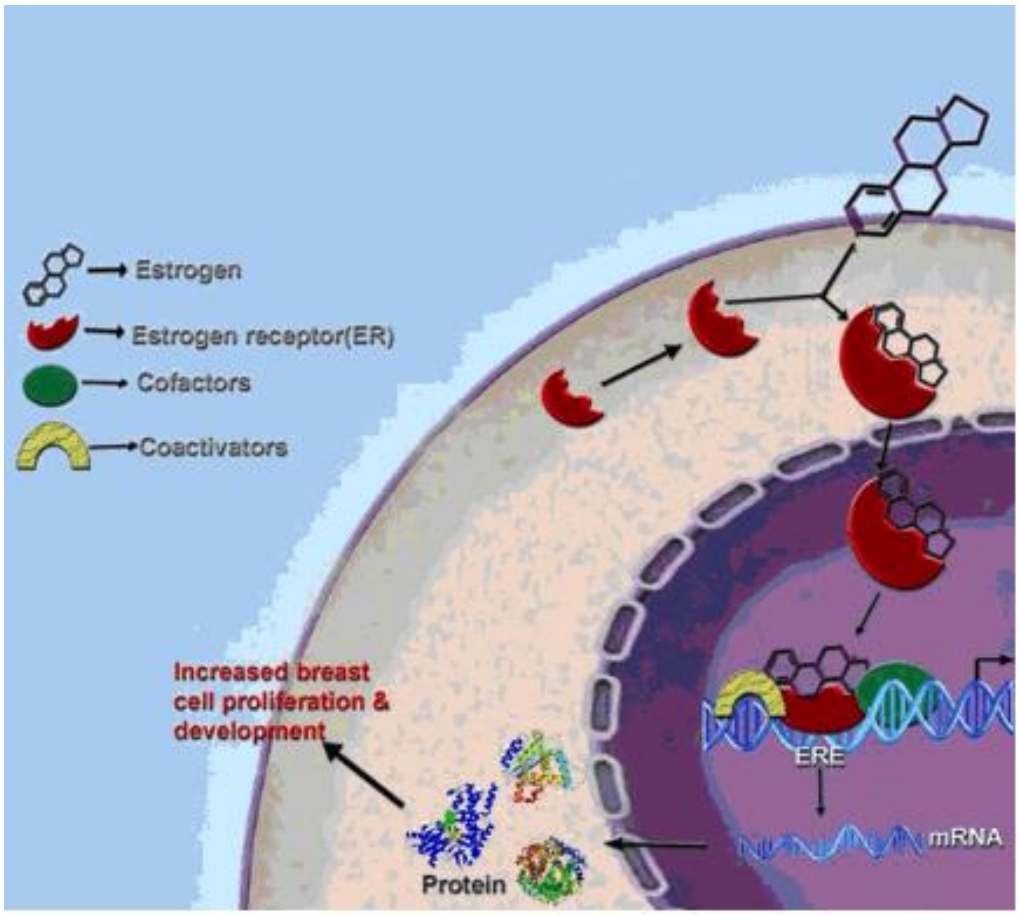


Figure 1. Molecular action of estrogens (Kumar & Kumar, 2008).

1.2 Bisphenol A

1.2.1 General information

Bisphenol A (BPA) is a one of the most studied chemical compounds with endocrine-disrupting properties (Eladak et al., 2015). Figure 2 shows the chemical structure of BPA in comparison to that of estradiol. It was first synthesized in 1891 by the Russian chemist Aleksandr P. Dianin and later on in the 1930s it was examined for its potential commercial use (Rubin, 2011). Nowadays, its annual production exceeds 3.8 million tons (Michałowicz, 2014).

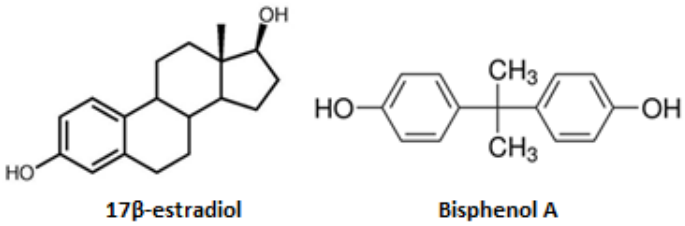


Figure 2. Chemical structures of 17β-estradiol and bisphenol A.

1.2.2 Applications

BPA is the building block of polycarbonate, epoxy, unsaturated polyester, and polysulfone resins which have many applications such as varnishes on the inside of cans and packaging materials for the storage of food products, beverages, and pharmaceuticals. Polycarbonates are widely used in the production of medical equipment as well as feeding bottles for babies and kitchen utensils (Rykowska & Wasiak, 2006). BPA has also been used in the production of vinyl chloride and of thermal paper (Michałowicz, 2014). The unique properties of polycarbonates –light, durable, high tensile strength, high modulus of elasticity, high melting point, and high vitrification temperature– account for their wide use and popularity (Rykowska & Wasiak, 2006).

1.2.3 Sources

BPA is a synthetic compound and as a consequence its presence in the human surrounding originates from anthropogenic activities only (Huang et al., 2012). The production, treatment and processing of BPA as well as the hydrolysis of various polymers including epoxy resins and polycarbonates, release BPA monomers into the environment (Michałowicz, 2014). Apart from its direct release to receiving water bodies and atmosphere, BPA can also be indirectly released into the environment during its processing for manufacturing of several commercial products but also from plastic bottles, packaging materials, landfill leakages and paper (Huang et al., 2012). According to the U.S. Environmental Protection Agency, although conflicting results have been obtained, BPA is not expected to be persistent in the environment since it biodegrades under environmental conditions. Although its hydrolysis is insignificant, the rate of its atmospheric photo-oxidation is rapid (U.S. EPA, 2010). The remaining unreacted BPA in polycarbonate products and epoxy resins may end up into ecosystems and food (Huang et al., 2012). Estimates of BPA exposure based on food, air, dust, and consumer product concentrations and intake rates suggest that diet probably constitutes a major source of exposure for BPA (Rudel et al., 2011).

1.2.4 Levels in foods

The European Commission's Scientific Committee on Food estimated BPA exposure from food sources to be 0.48–1.6 μg/kg b.w./day (Vandenberg, Maffini, Sonnenschein, Rubin, & Soto, 2009). Several studies report BPA levels found in various foods. Indicatively, (Schecter et al., 2010) detected BPA in 63 out of 105 food samples including fresh turkey, canned green beans, and canned infant formula ranging from 0.23 to 65.0 ng/g while it varied with pH of food. BPA levels above the limit of quantification (LOQ, 2 ng/g fresh weight) were measured in fish, meats, potatoes and dairy products according to (Gyllenhammar et al., 2012). BPA was also detected in 69 of 72 canned soft drink products (Cao, Corriveau, & Popovic, 2009) and in all 21 samples of canned liquid infant formula (Cao et al., 2008) at levels ranging from 0.032 to 4.5 μg/l and 2.27 to 10.2 ng/g, respectively (Vandenberg, Hauser, Marcus, Olea, & Welshons, 2007).

1.2.5 Biotransformation

Studies have shown that after oral administration, BPA undergoes a rapid absorption from the human gastrointestinal tract. Next, its efficient first-pass metabolism takes place in the gut wall and the liver where BPA glucuronides and BPA sulfates are mainly formed via UDP-glucuronyltransferase (UGT2B15) and sulfotransferase (SULT1A1) respectively followed by urinary elimination with a terminal half-life of less than 6 h (Nahar, Liao, Kannan, & Dolinoy, 2013; Völkel, Bittner, & Dekant, 2005; Völkel, Colnot, Csanády, Filser, & Dekant, 2002). The BPA glucuronide is the major metabolite of BPA formed *in vivo* which is not hormonally active (World Health Organization, 2009) whereas its other minor metabolites described in the literature are only formed when higher doses of BPA are applied (Völkel et al., 2002). BPA may also be absorbed after inhalation or transdermal contact with BPA-contaminated water while both routes avoid the first-pass conjugation occurring after oral administration (Vandenberg, Hauser, et al., 2007). According to (Snyder et al., 2000) the route of entry decidedly affects the fate of the chemical and thus its estrogenic potential. The bioavailability of the orally administered BPA is low and its intensive biotransformation in the liver deprives it of estrogenicity (Völkel et al., 2002).

1.2.6 Levels in human tissues and fluids

In a number of studies BPA was detected and/or measured in human serum, urine, amniotic fluid, follicular fluid, placental tissue and umbilical cord blood (Vandenberg, Hauser, et al., 2007). (Calafat et al., 2005) reported that 95% of the urine samples examined in the U.S.A. (n=394) had detectable levels of BPA (median, 1.28 μg/l) whereas in the 2003-4 National Health and Nutrition Examination Survey (NHANES)

the total concentration of BPA (free + conjugated) was measured in 92.6% of the urine samples tested with a median value that of 2.7 μg/l (Calafat, Ye, Wong, Reidy, & Needham, 2008). In an Asian study the detection of BPA (conjugated) amounted to 97.5% of the urine samples analyzed (median, 7.86 μg/l) (n=172; (Yang, Kim, Chang, Lee, & Kawamoto, 2006)). In serum, BPA was detectable with a rate of 17% in the Chinese population (median, < limit of detection (LOD)) (n=886; (He et al., 2009)) and 98% in Swedish elderly population (median, 3.76 ng/ml) (n=1016; (Olsén, Lampa, Birkholz, Lind, & Lind, 2012)). According to (Engel, Levy, Liu, Kaplan, & Wolff, 2006) BPA was also detected although at very low concentrations, in amniotic fluid of pregnant women in the U.S.A. In another study in Korea, 84% and 40% of maternal and fetal samples (blood and umbilical cord blood) respectively, contained detectable levels of BPA. Maternal blood BPA levels were found to range from <LOD to 66.48 μg/l while umbilical cord blood levels from <LOD to 8.86 μg/l (n=300; (Y. J. Lee et al., 2008)). All the above studies suggest that human exposure to BPA is significant.

1.2.7 Receptor binding potency/ cell proliferation activity *in vitro*

BPA may affect the endocrine system by mimicking or antagonizing endogenous hormones. Several *in vitro* studies have shown that BPA can weakly bind to both ERs α and β (Gould et al., 1998; Kuiper et al., 1998; ter Veld et al., 2006), the androgen receptor (AR) (H. J. Lee, Chattopadhyay, Gong, Ahn, & Lee, 2003; Wang, Rijk, et al., 2014; Xu et al., 2005) and the thyroid hormone receptor (TR) (Moriyama et al., 2002; Zoeller, Bansal, & Parris, 2005) acting as agonist for ERα/β and antagonist for AR and TR. In addition, BPA was found to strongly bind to the transmembrane estrogen receptor, G protein-coupled receptor 30 (GPR30) (Thomas & Dong, 2006) and the orphan nuclear receptor called estrogen-related receptor gamma (ERRγ) (Takayanagi et al., 2006) but also to activate transcription factors such as the human pregnane x receptor (hPXR) (Molina-Molina et al., 2013; Sui et al., 2012) and the aryl hydrocarbon receptor (AhR). Other studies evaluating the cell proliferation effect of BPA have shown that cells display proliferative response to it depending on the cell line (Varandas, 2014). The proliferative effects of BPA have been observed in the human breast cancer cells MCF-7 (Krishnan, Stathis, Permuth, Tokes, & Feldman, 1993; Molina-Molina et al., 2013; Perez et al., 1998; Samuelsen et al., 2001; Vivacqua et al., 2003), in the human ovarian cancer cells OVCAR-3 (Ptak, Wróbel, & Gregoraszczuk, 2011) and BG-1 (Park et al., 2009) but also in HeLa cells (Bolli et al., 2008) and in JKT-1 cells (Bouskine, Nebout, Brucker-Davis, Benahmed, & Fenichel, 2009).

1.2.8 Effects *in vivo*

Several review articles provide detailed summaries of findings from *in vivo* studies of BPA exposure. Indicatively, (Richter et al., 2007) based on existing evidence concluded that low doses of BPA (< 50 mg/kg b.w./day) administered to rodents (either orally or via injection) during development phase have persistent effects on brain structure, function and behavior, on the male reproductive tract (both developmental and adult effects) and on enzymes' activity in tissues and thus metabolic processes and growth. (Rubin & Soto, 2009) summarized the proposed actions by current data of perinatal BPA exposure that may influence body weight including adipocyte deposition, glucose uptake and homeostasis, and the development and maturation of crucial pathways for energy homeostasis. In an animal study, a dose of BPA that falls within the range of estimated human exposure (250 ng/kg b.w./day) administered to mouse dams (via osmotic pumps) from 8th to 18th day of gestation, was found to alter the development of the fetal mouse mammary gland (Vandenberg, Maffini, et al., 2007). In another study, the exposure of female rats to low dose of BPA (0.1 mg/kg b.w./day) via mini pumps accelerated the growth of mammary glands and perturbed epithelial cell cycle kinetics in the mammary gland (Colerangle & Roy, 1997). In human studies, the higher concentrations of BPA in urine were positively associated with increased incidence of liver enzyme abnormalities, cardiovascular disease (CVD), and diabetes (Erler & Novak, 2010) but also with peripheral arterial disease (PAD) (Shankar, Teppala, & Sabanayagam, 2012). Similar findings that started to emerge in the past led to an increasing concern about BPA and its use as packaging material for foodstuffs and thus new measures were started to be taken.

1.2.9 Ban

In 2006, the European Food Safety Authority (EFSA) based on the No-Observed-Adverse-Effect-Level (NOAEL) of 5 mg/kg b.w./day derived in two multi-generation reproductive toxicity studies in rodents, set a Tolerable Daily Intake (TDI) of 50 μg/kg b.w./day which was reaffirmed later in 2008 and 2010 (Grignard, Lapenna, & Bremer, 2012). In October 2008, the Canadian government proposed to ban the importation, sale and advertising of polycarbonate baby bottles made with BPA monomer as well as to adopt the As Low As Reasonably Achievable (ALARA) principle for this compound in food packaging for products intended for newborns and infants (Government of Canada, 2008). The ban entered into force in September 2010. The same year, France and Denmark taking into account the controversial issue of BPA and its low-dose effects prohibited its use in baby bottles for reasons of precautionary

consumer protection. In turn, the European Commission banned the use of BPA in the production of baby bottles and the placing on the market of such baby bottles as for a uniform legal situation to be created within the EU (BfR, 2015). In 2012, the FDA amended its regulations to no longer provide for the use of BPA-based polycarbonate resins in baby bottles and sippy cups and of BPA-based epoxy resins as coatings in packaging for infant formula (U.S. FDA, 2010). Recently, EFSA changed TDI of BPA to (temporary-) tTDI and reduced it to 4 µg/kg b.w./day (EFSA, 2015) whereas in France its use was forbidden by law in any food packaging from January 2015 (USDA, 2013). Consequently, in order to comply with the new regulations on BPA, several functional alternatives were introduced into the global market.

1.3 BPA analogues

1.3.1 Applications

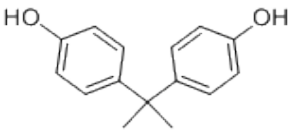
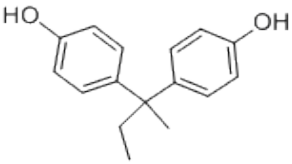
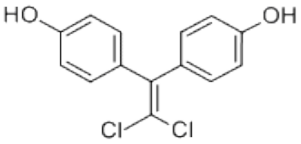
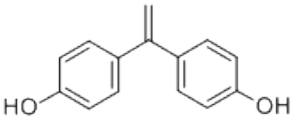
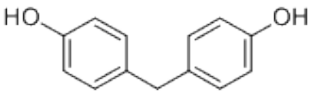
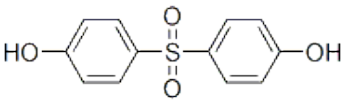
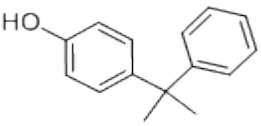
The ban imposed on BPA prompted industry to search for alternative chemicals in order to substitute it. As manufacturers started to abandon the use of BPA in their products due to public concern, a gradual shift to using bisphenol analogues has begun (Rochester & Bolden, 2015). Such chemicals known as BPA analogues bear similar structure to BPA consisting of two phenolic rings joined by a bridging carbon or other chemical structures (Table 1). Some of them are now used in the production of plastics and resins and are considered candidates for the partial replacement of BPA in the industrial applications (Molina-Molina et al., 2013). For example BPS is used as a wash fastening agent in cleaning products or as a developer in thermal paper, including products marketed as “BPA-free paper” while BPF is used in the production of durable epoxy resins and coatings that end up in several consumer products including food packaging (Rochester & Bolden, 2015).

1.3.2 Levels in foods

So far not much data is available on the amounts of BPA analogues in food. Nonetheless, BPS has been found in various canned foodstuffs at concentrations from 11.5 to 175 µg/l in the supernatant and below the LOD to 36.1 µg/kg in the food (Viñas, Campillo, Martínez-Castillo, & Hernández-Córdoba, 2010). BPB was detected in 21.4% of the canned products tested in a concentration range from 27.1 to 85.7 µg/kg (Grumetto, Montesano, Seccia, Albrizio, & Barbato, 2008). Although there is hardly any information for human exposure to BPF, the latter has been detected in surface water, sewage and sediments at concentration levels ranging from 0.0001 to

0.180 µg/l, 0.022 to 0.123 µg/l and 1.2 to 7.2 µg/kg, respectively (Liao et al., 2012; Molina-Molina et al., 2013).

Table 1. Chemical names and structures, CAS numbers and molecular weights of BPA and analogues.

Chemical name		CAS number	Molecular weight (g/mol)	Chemical structure
Bisphenol A (BPA)	2,2-Bis(4-hydroxyphenyl) propane	80-05-7	228.29	
Bisphenol B (BPB)	2,2-Bis(4-hydroxyphenyl) butane	77-40-7	242.31	
Bisphenol C (BPC)	Bis(4-hydroxyphenyl)-2,2-dichlorethylene	14868-03-2	281.13	
Bisphenol E (BPE)	1,1-Bis(4-hydroxyphenyl) ethane	2081-08-5	212.24	
Bisphenol F (BPF)	Bis(4-hydroxyphenyl) methane	620-92-8	200.23	
Bisphenol S (BPS)	Bis(4-hydroxyphenyl) sulfone	80-09-1	250.27	
4-Cumylphenol (HPP)	4-(2-phenyl-2-propanyl)phenol	599-64-4	212.29	

1.3.3 Levels in human tissues and fluids

Eighty one per cent of the urine samples collected from the U.S.A. and seven Asian countries were found to contain BPS in a concentration range from below the LOQ (0.02 ng/ml) to 21.0 ng/ml, with a mean value of 0.654 ng/ml (n=315; (Liao et al., 2012). In another study, BPS and BPF were detected in 78% and 55% of the urine samples analyzed with median concentrations these of 0.13 ng/ml and 0.08 ng/ml, respectively (n=100; (Zhou, Kramer, Calafat, & Ye, 2014). (Cobellis, Colacurci, Trabucco, Carpentiero, & Grumetto, 2009) analyzed blood serum from both healthy

and endometriotic women and found detectable levels of BPB (mean concentration 5.15±4.16 ng/ml) in 27.6% of the samples coming from the women with endometriosis.

1.3.4 Receptor binding potency/ cell proliferation activity *in vitro*

Similarly to BPA, both BPF and BPS have been found to exert estrogenic activity by binding to ERs α and β in a number of *in vitro* studies (Cabaton et al., 2009; Grignard et al., 2012; Molina-Molina et al., 2013). Other BPA analogues (including the former), namely BPB, BPE, BPS and HPP, have also been found to bind to ERα (Kitamura et al., 2005; Rosenmai et al., 2014) as well as BPC tested by (Delfosse et al., 2012; Wang, Rijk, et al., 2014). Moreover, all the above compounds have shown a clear anti-androgenic activity upon binding to AR (Kitamura et al., 2005; Wang, Rijk, et al., 2014) whereas BPS has also showed weak androgenic activity (Molina-Molina et al., 2013). In addition, BPE and BPF were reported to activate the transcription factor AhR (Rosenmai et al., 2014) while BPB and HPP have been identified as hPXR agonists (Sui et al., 2012). Finally, BPA and BPB were found to increase the p53 activity indicating potential to cause DNA damage while BPF and HPP increased the Nrf2 activity indicating potential for oxidative stress (Rosenmai et al., 2014). Other studies that tested the proliferation potential of various BPA analogues using MCF-7 cell line found that BPF, BPS, BPB and BPE did induce cell proliferation (Y Hashimoto et al., 2001; Molina-Molina et al., 2013; Perez et al., 1998; Rivas et al., 2002) and also BPC (Delfosse et al., 2012).

1.3.5 Effects *in vivo*

A number of *in vivo* studies have revealed that some BPA analogues can cause adverse effects to the animals. Developmental exposure of zebrafish to BPS (0, 0.1, 1, 10 and 100 μg/l) for 75 days resulted in decrease of body length, weight and testosterone in males, alteration of sex ratio in favor of females and increase of liver weight and E_2 in both males and females (Naderi, Wong, & Gholami, 2014). Moreover, BPS was found to disrupt the reproduction process by decreasing the egg production and their hatching rate as well as the sperm count (Naderi et al., 2014) but also to alter the gene expression of several genes in the brain and gonads, involved in the hypothalamic-pituitary-gonadal axis in females and males (Ji, Hong, Kho, & Choi, 2013). (Yamasaki, Noda, Imatanaka, & Yakabe, 2004) and (Stroheker, Chagnon, Pinnert, Berges, & Canivenc-Lavier, 2003) found BPS and BPF to exert their estrogenic activity via induction of uterine growth in immature rats. Oral administration of BPF to adult rats at doses 0, 20, 100, 500 mg/kg b.w./day for 28 days led to alteration of various hematological and biochemical parameters and

thyroid hormones levels, decrease of body weight and food consumption in both females and males and increase of testes, liver, thyroid, brain, and kidney weights (Higashihara et al., 2007).

1.4 Aim of the study

Ideally, chemicals that are used as substitutes of a chemical of concern should be at least less toxic than the original. However, many substitutes are not thoroughly tested before being placed on the market and in some cases are very much alike to the original and it may therefore be likely that they exert the same effect. These chemicals have been described as “regrettable substitutions” (Rochester & Bolden, 2015). The aim of the present study is to obtain a better characterization of the estrogenic potency of BPA and its analogues and to try to translate this information into possible differences in their hazards. To achieve this, a series of bioassays were performed: 1) CALUX bioassay for ERα mediated effects, 2) CALUX bioassay for ERβ mediated effects and 3) cell proliferation of MCF-7 wild type cells.

1.4.1 Introduction into bioassays

It has been suggested that ERβ reduces the ERα-mediated response resulting in positive effects such as the inhibition of proliferation of breast cancer cells (Lazennec et al., 2001; Ström et al., 2004). Therefore, it is of importance to test the affinity of BPA and its analogues for the two receptors separately. The ER-CALUX (Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression) is a very sensitive and rapid *in vitro* bioassay for assessing estrogenic activity where the luciferase gene is introduced into a cell line under the transcriptional control of response elements for activated estrogen receptors (Hamers et al., 2008). On the other hand, MCF-7 cell line has contributed a lot to the study of the ERα and thus to breast cancer research as it can express considerable levels of ER mimicking in this way most of the human breast cancers that express ER (A. V. Lee, Oesterreich, & Davidson, 2015). Hence, by combining the more mechanistic ER-CALUX assay with an assay that simulates the physiological response of ERα activation in wild type cells, it is expected to better understand the potential endocrine-disrupting properties of BPA and some of its analogues.

2. MATERIALS & METHODS

2.1 Chemicals

Test compounds: Bisphenol A (BPA), bisphenol E (BPE), bisphenol F (BPF), bisphenol S (BPS), 4-Cumylphenol (HPP) and 17β-estradiol (E_2) were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). Bisphenol B (BPB) and bisphenol C (BPC) were obtained from TCI Tokyo Chemical Industry Co. (Zwijndrecht, Belgium).

Other: Fetal calf serum (FCS) and 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12), phenol red-free DMEM/F12, phosphate buffered saline (PBS) and dextran coated charcoal-treated FCS (DCC-FCS) were obtained from Gibco (Paisley, UK). Non-essential amino acids (NEAA), geneticine G418, penicillin/streptomycin solution and trypsin-EDTA solution were obtained from Invitrogen (Breda, the Netherlands) whereas hygromycin from Duchefa Biochemie B.V. (Haarlem, the Netherlands). Dimethyl sulfoxide (DMSO) and resazurin reagent were obtained from Merck (Darmstadt, Germany). 5-bromo-2'-deoxy-uridine (BrdU) was obtained by Roche Diagnostics Nederland B.V. (Almere; the Netherlands). The low salt buffer (LSB) (10 mM Tris, 2 mM DDT and 2 mM CDTA; pH: 7.8) and the flash mix [100 mM Tricine, 1.07 mM $(MgCO_3)4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 2.0 mM dithiothreitol, 470 µM luciferine and 5.0 mM ATP; pH: 7.8] were prepared in the lab.

2.2 Cell culture

U2-OS ERα/ERβ cell lines (BioDetection Systems B.V.; Amsterdam, the Netherlands): This is a human osteosarcoma cell line stably co-transfected with an expression construct for the human ERα or ERβ (pSG5-neo-hERα/β) and a pGL3 (Promega)-based reporter construct containing 3 EREs and a TATA box in front of a *luc* + luciferase reporter gene (pGL3-3xERE*tata*Luc) as described by (Wang, Aarts, et al., 2014). The cells were sub-cultured every 3-4 days when reaching about 80% of confluence. In short, after removing the medium the cells were washed with 5 ml PBS and then treated with 1 ml of trypsin-EDTA solution (cond.: 37°C, 5% CO_2, 100% humidity) until they detach from the bottom (around 10 min). Afterwards, 9 ml of fresh medium were transferred into the flask (VWR; Breda, the Netherlands) and then the cell suspension was re-suspended several times to ensure that cells are

evenly distributed into the medium. Finally, 2 ml of the cell suspension were transferred to one new flask (or more depending on the needs) and fresh medium was added to each flask until the total volume is 10 ml. Two different media were used:

Culture medium: DMEM/F12 supplemented with 10% FCS, 2.5 ml of NEAA and 4 µl/ml geneticine G418 (50mg/ml). Medium for ERα cells contained also 1 µl/ml hygromycine (400mg/ml).

Assay medium: phenol red-free DMEM/F12 supplemented with 5% DCC-FCS and 2.5 ml NEAA.

MCF-7 cell line (ATCC American Type Culture Collection; Manassas, VA, U.S.A): This is a human breast cancer cell line that expresses endogenously the ERα. The cells were sub-cultured every 6-7 days when reaching about 80% of confluence. The procedure that was followed is described above. Two different media were used:

Culture medium: DMEM/F12 supplemented with 10% FCS and 5 ml penicillin/streptomycin solution (10,000 U/mL).

Assay medium: phenol red-free DMEM/F12 supplemented with 5% DCC-FCS.

2.3 MTT cytotoxicity assay

The effects of BPA, BPB, BPC, BPE, BPF and HPP on the viability of U2-OS ERα/ERβ cell lines were evaluated with the MTT assay. First, the cells were counted using a Cellometer Auto T4 (Nexcelom Biosciences; Lawrence, USA) and then were seeded in transparent 96-well plates (Greiner bio-one; Kremsmünster, Austria) at a density of 10^4 and 7.5×10^3 cells/well for ERα and ERβ cells/well respectively with culture medium (100 µl) and incubated for 1 day to attach (cond.: 37°C, 5% CO_2, 100% humidity). The next day, the culture medium was replaced by assay medium and the cells were incubated for a further 24 h. On the third day, the medium was renewed with fresh medium containing test compounds dissolved in DMSO (max. final DMSO concentration: 0.5%). After 24 h of exposure, 10 µl of MTT solution (10% of medium volume) were added to each well and the plates were placed back in the incubator. After 1 h of incubation the metabolically active cells have converted the MTT tetrazolium ring into blue-violet formazan crystals, whereas dead cells remained colorless. The MTT-containing medium was gently removed, and DMSO (100 µl) was added to each well to dissolve the formazan crystals. Finally, after 15 min of shaking the absorbance was measured at 562 nm and 620 nm (background) using

SpectraMax M2 spectrophotometer (Molecular Devices; Sunnyvale, USA). One experiment of three replicates was conducted for each test compound at seven concentrations ranging from 33.3 to 33300 nM. E_2 served as positive control in five different concentrations ranging from 10^{-5} to 1 nM. Solvent control (0.5% DMSO) served as negative control.

2.4 ERα/β CALUX reporter gene assays

Activation of ERs by all seven test compounds was assessed using the U2-OS ERα/β cell lines. In short, after counting, the cells were plated in white opaque 96-well plates (PerkinElmer; Groningen, the Netherlands) at a density of 10^4 and 7.5×10^3 cells/well for ERα and ERβ cells respectively with culture medium (100 µl). One day later, the medium was removed and replaced by assay medium and the cells were incubated for another 24 h. The next day, the medium was renewed with fresh medium containing test compounds dissolved in DMSO (max. final DMSO concentration: 0.5%). After 24 h of exposure, the medium was removed and the cells were washed with 0.5×PBS and lysed with hypotonic LSB. Plates were put on ice for 15 min and subsequently placed in -80°C for at least 24 h to lyse the cells. Finally, the plates were thawed at room temperature while shaking and the luciferase activity was measured using GloMax 96 Microplate luminometer (Promega; Leiden, the Netherlands) by adding 100 µl of flash mix per well. At least three independent experiments of three to six replicates were conducted for each test compound at seven concentrations ranging from 0.333 to 33300 nM. E_2 served as positive control in five different concentrations ranging from 10^{-5} to 1 nM. Solvent control (0.5% DMSO) served as negative control.

2.5 Cell proliferation assay

ERα-mediated proliferation of cells caused by BPA and BPS was assessed using the MCF-7 cell line.

Pilot: To check the best exposure time and cell density needed to test the cell proliferation, a pilot experiment was carried out. Cells were plated in different densities (5×10^2, 10^3, 5×10^3, 10^4 and 5×10^4 per well) to test whether the increase in cell number could be detected reliable. In brief, after counting, cells were seeded in transparent 96-well plates with assay medium (100 µl) and incubated for 1 day to attach. The next day, the medium was renewed with fresh medium in half of the plates whereas the other half was exposed to E_2 (10^{-2} nM) (max. final DMSO concentration: 0.5%). Three plates were prepared to test for 24 h, 48 h and 72 h response. After 1, 2 and 3 days respectively the proliferation behavior of the cells

was measured using the resazurin reagent. Five µl (10% w/v in PBS) were added directly to each well and the plates were incubated for 1 h to allow for conversion of resazurin to resorufin by the mitochondrial enzymes of the cells. Finally the fluorescence was measured at 530 nm excitation and 590 emission wavelengths using the SpectraMax M2 spectrophotometer. One experiment of six replicates was conducted for each cell concentration and time. Medium without cells served as control to measure the background resorufin fluorescence. Exposure medium without cells served as a second control to test if E_2 interferes with the reaction.

Main experiment (ELISA, BrdU): The concentration of cells and the time of exposure were decided to be 5×10^3 per well and 72 h, respectively. The criteria were that the next lower concentration (10^3) gave a 3-times lower response (quite close to 5) whereas the next higher (10^4) gave the double response than that of selected concentration. As for the time of exposure, 3 days (72 h) were thought to be more appropriate to measure the proliferative effect. After counting, the cells were plated in white opaque 96-well plates with assay medium (100 µl) and incubated for 1 day to attach. On the second day, the medium was renewed with fresh medium containing test compounds dissolved in DMSO (max. final DMSO concentration: 0.5%). After around 72 h of exposure cell proliferation was determined by measuring the BrdU incorporated into the DNA following company's chemiluminescent BrdU protocol. Briefly, the BrdU-labeling solution was added to the cells 4 h prior to the end of the exposure so as to be incorporated into the DNA of proliferating cells. After removing the labeling solution, FixDenat was added (30 min at room temperature) to denature the DNA of the cells. Then, the anti-BrdU-POD antibody was added after removing FixDenat and plates were left for 90 min at room temperature. The immune complexes were detected by the subsequent substrate reaction which in turn was quantified by measuring the light emission using GloMax 96 Microplate luminometer. Two independent experiments of three replicates were conducted for each test compound at seven concentrations ranging from 33.3 to 33300 nM. E_2 served as positive control in eight different concentrations ranging from 10^{-4} to 1 nM. Solvent control (0.5% DMSO) served as negative control.

2.6 Calculations and statistics

MTT cytotoxicity assay: The formazan extinction was calculated by subtracting the signal at 620 nm from that of 562 nm. The average of the negative control (solvent control; 0.5% DMSO) was set at 100% while the exposed wells were expressed as %

of the negative control. Averages and standard deviations were calculated in excel to plot a graph (viability %-concentration in nM).

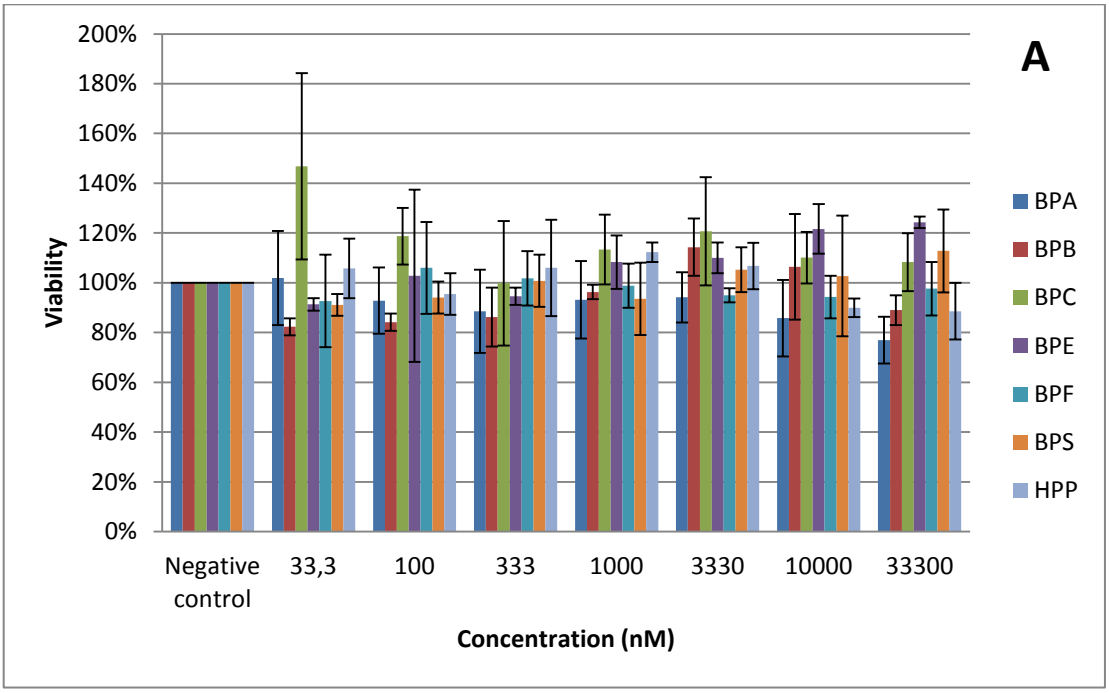
ERα/β CALUX reporter gene assays: The luciferase activity was measured as relative light units (RLU). Fold induction was calculated by dividing the mean value of light units from exposed and nonexposed (solvent control) wells. The EC_{50} value which is the concentration of the test compound needed to achieve 50% of maximal response was derived from each fitted dose-response curve (three parameter sigmoidal dose-response curve, GraphPad Prism software version 5.04). In general, dose-response curves that didn't appear to have data points along the whole curve or didn't show saturation at the maximum concentration were excluded from this report. The average EC_{50} values and their S.D. were calculated from taking the average of the EC_{50} values of three independent experiments. Estradiol equivalency factors (EEF_{50} = EC_{50} E_2/EC_{50} test compound) were also calculated for the responses in ERα and ERβ cell lines.

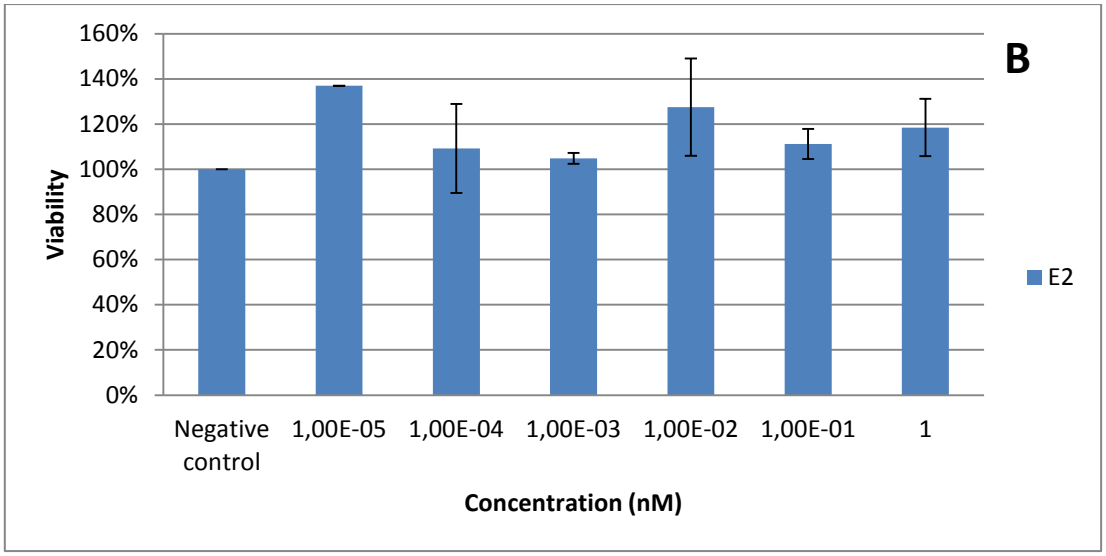
Cell proliferation assay: Pilot: The average of the background resorufin fluorescence (control) was first subtracted from each well. Averages and standard deviations were calculated in excel to plot a graph (fluorescence-N of cells). Main experiment: The cell proliferation was measured as RLU. Proliferative effect (PE) (or fold induction) was calculated by dividing the mean value of light units from exposed and nonexposed (solvent control) wells. The EC_{50} value was derived from each fitted dose-response curve using GraphPad Prism software.

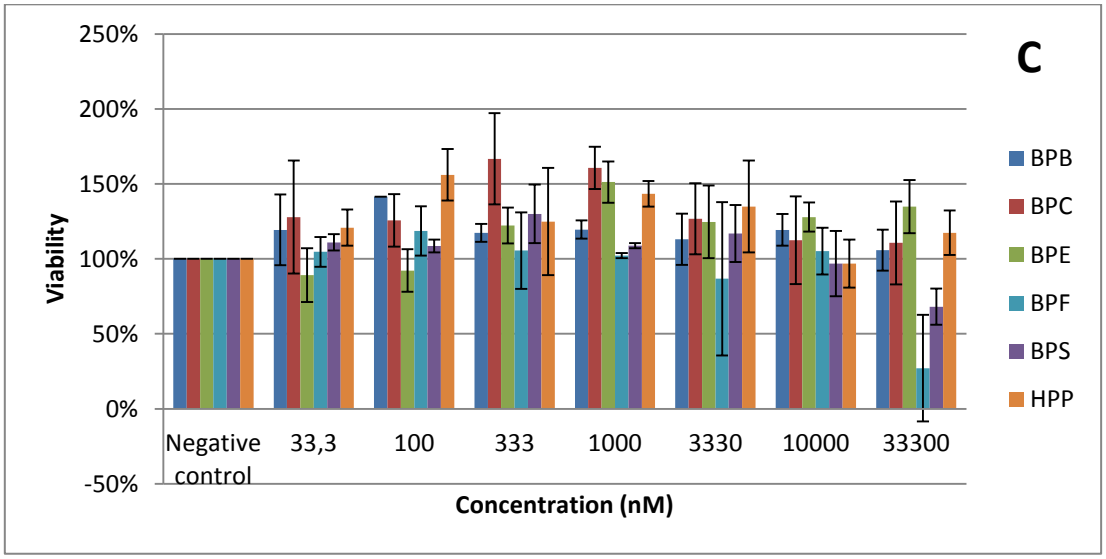
3. RESULTS

3.1 Cell viability

The concentrations of test chemicals were determined by MTT cytotoxicity assay before performing the reporter gene assay and the highest concentration was initially set at 10^5 nM. The first results revealed that the highest concentration (10^5 nM) could cause a cytotoxic effect (data not shown). Hence, the highest concentration tested in all assays was set at 33300 nM. Figure 3 shows the results of the effects of all the test compounds (E_2, BPA, BPB, BPC, BPE, BPF and HPP) on the viability of U2-OS ERα/ERβ cell lines using MTT cytotoxicity assay. The effects of BPA initially tested with resazurin assay were considered unreliable because of inconsistency between experiments and high S.D. Due to limitation of ERβ cells BPA could not be finally tested with MTT cytotoxicity assay. Almost in all cases, the tested compounds were devoid of any cytotoxicity (avg. cell viability ranging from 82 to 167%) in the concentration range that were tested (33.3-33300 nM). Only BPF and BPS in ERβ cells seem to cause an important decrease in cell viability at the highest concentration (cell viability 27% (± 35.5) and 68% (±12) respectively). This test could not be repeated due to limited number of ERβ cells.







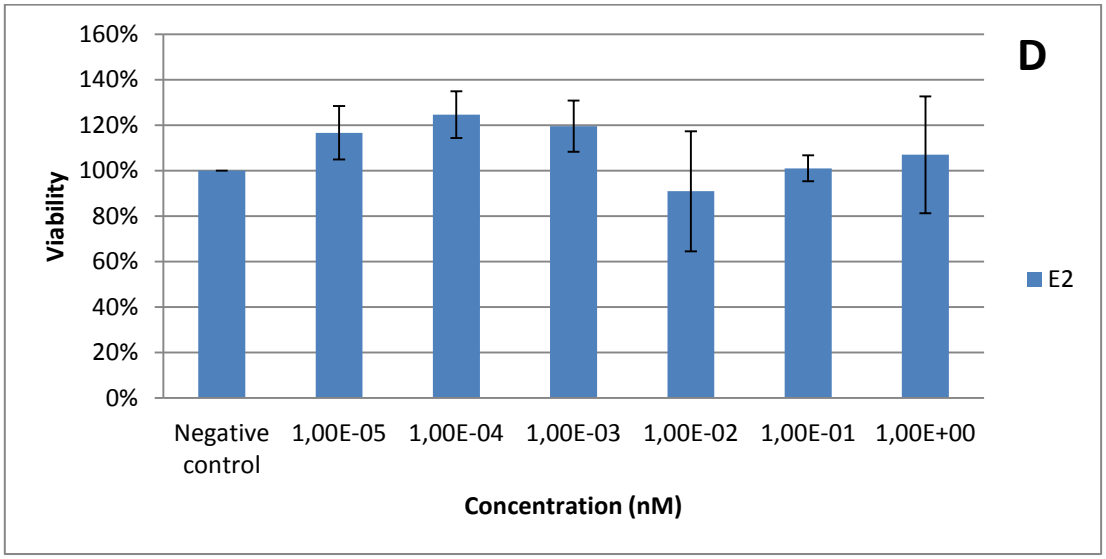


Figure 3. Cell viability of U2-OS cell lines assessed by MTT cytotoxicity assay. U2-OS ERα exposed to indicated test compounds (A) and positive control, E_2 (B); U2-OS ERβ exposed to indicated test compounds (C) and positive control, E_2 (D).

3.2 Estrogenic activity

BPA and its six analogues BPB, BPC, BPE, BPF, BPS, HPP were tested in the ERα/β CALUX bioassay for their estrogenic activity. Figure 4 presents typical dose-response curves of all the test compounds compared to E_2. For the two graphs, datasets of the best results were used out of the three independent experiments conducted for each test compound, based on the R^2 and the EC_{50} 95% confidence intervals of all dose-response curves. More specifically, those dose-response curves with R^2 >0.6 and with the smallest possible range of 95% confidence intervals were chosen for this purpose. Dose-response curves of all three independent experiments of each compound are provided in the Appendix 1. Table 2 summarizes the EC_{50} values obtained from the fitted dose-response curves of the three independent experiments as well as the EEF_{50} values calculated from the avg. EC_{50}s. Table 2 also presents the ratio EEF_{50}α/EEF_{50}β so as to compare the results obtained in the ERα and ERβ cell lines. If the ratio is <1 means that the compound is relatively more estrogenic in the ERβ cell line showing a relatively higher EEF_{50} in the ERβ than in the ERα cell line, compared to E_2 (ter Veld et al., 2006). All compounds except BPS are relatively more estrogenic as compared to E_2 in the ERβ than in the ERα cell line. Due to limitation of ERβ cells BPB, BPC and HPP could not be finally retested for third time in this assay. As expected, the endogenous hormone E_2 which served as positive control showed the highest potency as ER agonist compared to all test compounds with EC_{50} values those of 0.0019 (±0.0016) and 0.036 (±0.016) nM for ERα and ERβ, respectively. All seven test compounds showed a clear estrogenic activity acting as agonists for both ER subtypes. More specifically, for ERα the ranking order of the compounds related to their estrogenic potency is: BPC > BPB > HPP > BPA > BPE > BPF > BPS while in ERβ is: BPC > BPB > BPA > HPP > BPE > BPF > BPS. It is worth noting that all test compounds (except E_2) were initially tested in the concentration range of 33.3-33300 nM. However, the range of BPB, HPP and BPC was lowered after obtaing the first results, to 3.33-3330 nM for the first two and 0.333-333 nM for the latter so as to enable proper concentration-response curves.

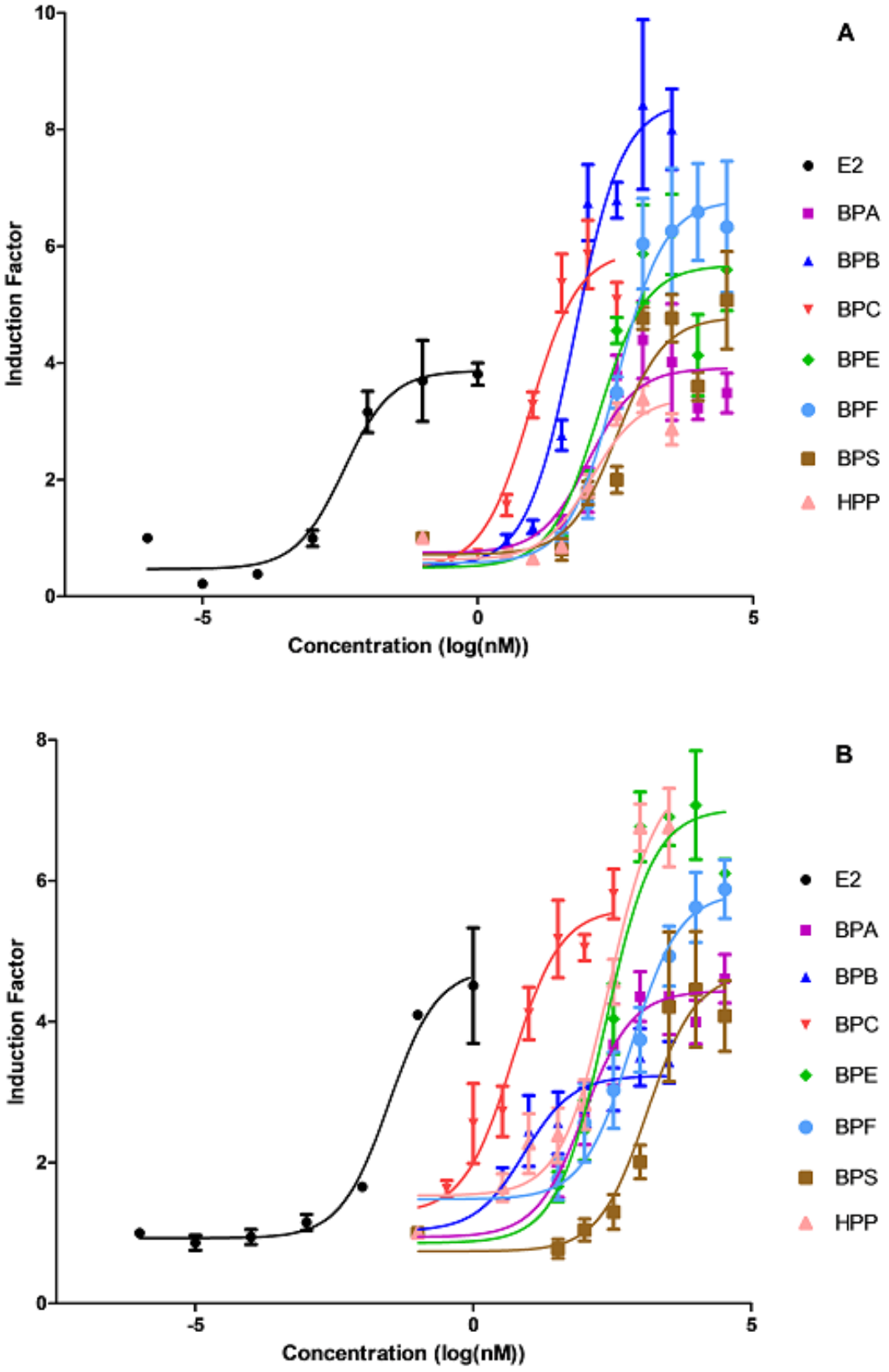


Figure 4. Dose-response curves of the indicated test compounds in the *in vitro* ERα CALUX bioassay (A) and ERβ CALUX bioassay (B). Data shown as fold change compared to control.

Table 2. Estrogenic activities of the indicated test compounds in the *in vitro* ERα/β CALUX bioassays. Highlighted EC_{50} values correspond to dose-response curves of Figure 4 whereas black, blue and red values correspond to dose-response curves 1, 2 and 3, respectively (see Appendix 1)

	U2-OS ERα			U2-OS ERβ			
Compound	**EC_{50} (nM)**	**Avg. EC_{50}**	**EEF_{50}**	**EC_{50} (nM)**	**Avg. EC_{50}**	**EEF_{50}**	**$EEF_{50}\alpha$/ $EEF_{50}\beta$**
E_2	0,0037 0,0011 0,0009	0,0019 (±0,0016)	1	0,0303 0,0545 0,0237	0,036 (±0,016)	1	1
BPA	42,65 454,1 102,6	199,78 (±222,26)	9,51E-06	63,85 98,15 56,7	72,9 (±22,16)	4,94E-04	0,0193
BPB	55,76 57,22 21,29	44,76 (±20,34)	4,24E-05	0,18 8,16 -	4,17 (±5,64)	8,63E-03	0,0049
BPC	4,6 8,97 10,71	8,09 (±3,15)	2,35E-04	4,99 0,26 -	2,62 (±3,34)	1,37E-02	0,0171
BPE	340 148 151,9	213,3 (±109,74)	8,91E-06	767,3 194,2 233,3	398,27 (±320,19)	9,04E-05	0,0985
BPF	220 213,1 329	254,03 (±65)	7,48E-06	134,7 434,3 688,6	419,2 (±277,26)	8,59E-05	0,0871
BPS	295,7 349,4 335,7	326,93 (±27,9)	5,81E-04	133,7 1334 1801	1089,57 (±860,11)	3,30E-05	17,59
HPP	88,72 114,8 178,1	127,21 (±45,96)	1,49E-05	279 173,3 -	226,15 (±74,74)	1,59E-04	0,0938

3.3 Cell proliferation of MCF-7 cell line

The estrogenic potential of BPA and BPS was further characterized by using the BrdU ELISA kit to investigate their ability to stimulate cell proliferation in MCF-7 cells. Figure 5 presents the proliferative response of MCF-7 cell line incubated with or without E_2 (10^{-2} nM) for 72 h. Results of the proliferative response after 24 and 48 h of incubation are provided in the Appendix 2. Figure 6 shows dose-response curves of BPA and BPS compared to E_2. For the graph, datasets of the best results were used out of the two independent experiments conducted for each test compound.

Dose-response curves of the second experiment are not provided due to inconsistent results. In this cell line, E_2 induced cell proliferation up to 4.1 (±0.25)-fold higher than control-treated cells at 1 nM and with an EC_{50} value of 0.0064 nM. BPA and BPS also increased cell proliferation with max. proliferative effects (PEs) those of 1.8 (±0.41) at 10^3 nM and 1.7 (±0.17) at 10^4 nM, respectively. EC_{50} values for the two bisphenols were found to be 58.7 and 772.6 nM respectively, the 95% confidence intervals of which ranged from 2 to 3 orders of magnitude and the R^2s of the dose-response curves were considerably <0.6.

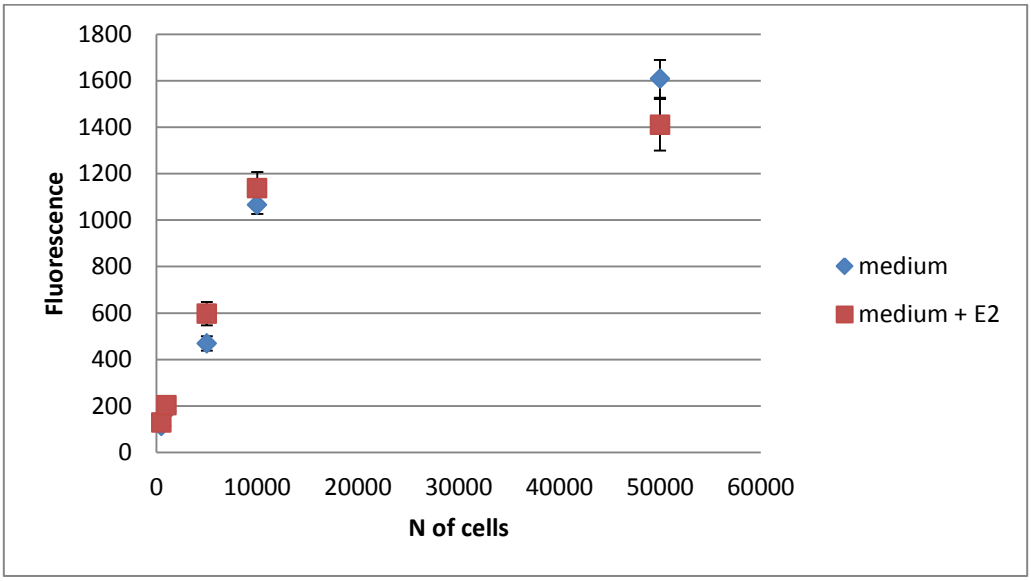


Figure 5. Proliferative response of MCF-7 cell line incubated with the absence or presence of E_2 (10^{-2} nM) for 72 h.

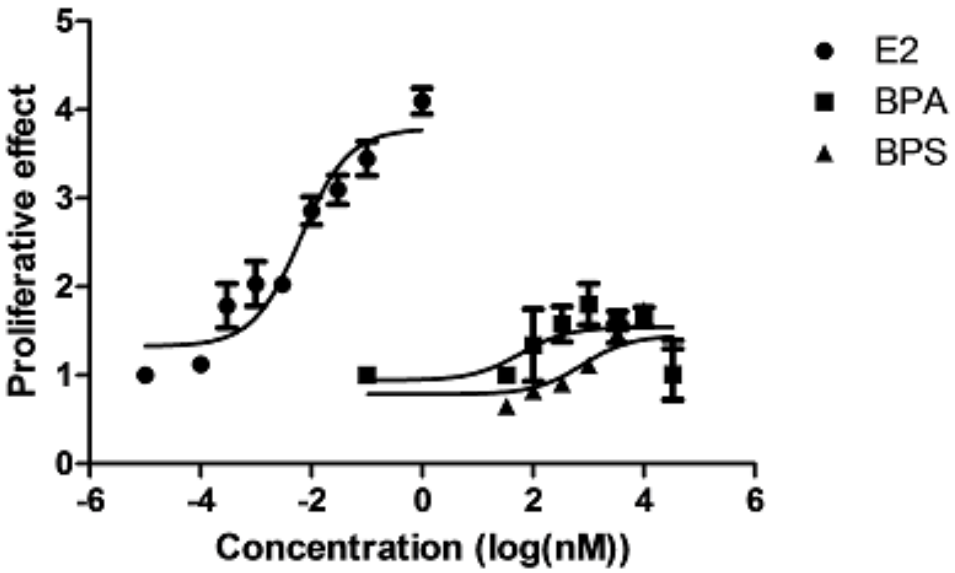


Figure 6. Dose-response curves of E_2, BPA and BPS in the *in vitro* MCF-7 cell proliferation assay. Data shown as fold change compared to control.

4. DISCUSSION

The present study was conducted in order to obtain a better characterization of the estrogenic potency of BPA and its analogues through investigating their ER subtype specific potency using U2-OS ERα/β reporter gene cell lines. In addition, BPA together with BPS, an analogue that is already used in the market as substitute of BPA, were further tested for their potential ERα-mediated proliferative effect in MCF-7 breast cancer cell line. All seven test compounds showed a clear estrogenic activity acting as agonists for both ERs. Results showed that all tested bisphenols differ from each other in their affinities for the two receptors as well as that each bisphenol exhibits different estrogenic activity towards the two receptors separately. It has been shown using various reporter gene cell lines that E_2 has higher affinity for ERα than for ERβ (Kuiper et al., 1998; Matthews, Twomey, & Zacharewski, 2001; Molina-Molina et al., 2013; ter Veld et al., 2006; van der Woude et al., 2005). However, the EC_{50}s and the fold differences between the different cell lines vary quite a lot. In this study, the EC_{50} of E_2 for ERβ was found to be almost 19-fold higher than that of ERα, which is in agreement with the findings of (ter Veld et al., 2006) and (van der Woude et al., 2005) who used the same cell lines. Table 3 presents an indicative overview of EC_{50} values of all the test compounds for both ER subtypes, as reported in the literature.

Table 3. Overview of EC_{50} values of all tested bisphenols using various reporter gene cell lines as reported in the literature.

Compound	Reporter gene cell line	EC_{50} (nM)		Reference
		ERα	ERβ	
BPA	MELN	2320	–	Grignard et al., 2012
	MCF-7	630	–	Kitamura et al., 2005
	MCF-7	1090	–	Kuruto-Niwa et al., 2005
	MCF-7	710	450	Matthews et al., 2001
	HELN	410	520	Molina-Molina et al., 2013
	BG1Luc4E2	80	–	Rosenmai et al., 2014
	CV-1	272	–	Teng et al., 2013
	U2-OS	216	234	ter Veld et al., 2006
	U2-OS	270	–	Wang et al., 2014
BPB	MCF-7	70	–	Kitamura et al., 2005
	BG1Luc4E2	120	–	Rosenmai et al., 2014
	U2-OS	120	–	Wang et al., 2014
BPC	U2-OS	27	–	Wang et al., 2014
BPE	BG1Luc4E2	470	–	Rosenmai et al., 2014

BPF	HepG2	2390	6040	Cabaton et al., 2009
	MCF-7	1000	–	Kitamura et al., 2005
	HELN	1730	1430	Molina-Molina et al., 2013
	BG1Luc4E2	820	–	Rosenmai et al., 2014
BPS	MELN	4240	–	Grignard et al., 2012
	MCF-7	1100	–	Kitamura et al., 2005
	MCF-7	1750	–	Kuruto-Niwa et al., 2005
	HELN	3960	1720	Molina-Molina et al., 2013
	BG1Luc4E2	1170	–	Rosenmai et al., 2014
	CV-1	2200	–	Teng et al., 2013
HPP	MCF-7	150	–	Kitamura et al., 2005
	BG1Luc4E2	100	–	Rosenmai et al., 2014

As it can be seen from Table 3 not all bisphenols have been tested in the same extent nor for their ERβ specific potency. However, the fact that BPC was found to be the most potent of all seven compounds for ERα (and ERβ) followed by BPB is in line with the findings of (Wang, Rijk, et al., 2014) although in the present study their EC_{50}s were found to be around 3-fold lower than what they reported. (Rosenmai et al., 2014) who tested all bisphenols except BPC for their ERα potency found BPA to be the most potent followed by HPP, BPB, BPE, BPF and BPS in decreasing order. In this study, BPB appeared to be the next most potent bisphenol for both ER subtypes and especially for ERβ, which contradicts findings of (Rosenmai et al., 2014) since BPB and HPP, although less potent, had similar EC_{50}s compared to that of BPA. Results of (Kitamura et al., 2005) are in agreement with the ranking order of bisphenols given in this study for ERα since they found the following order of potency for the compounds of interest: BPB > HPP > BPA > BPF > BPS. Noteworthy is that EC_{50}s of BPA, BPF and BPS obtained by (Kitamura et al., 2005) were 3-4 fold higher than EC_{50}s in the present study. Likewise, (Molina-Molina et al., 2013) found BPA to be the most potent for ERα followed by BPF and BPS. (Teng et al., 2013) who tested BPA and BPS found BPA to be 8-fold more potent for ERα than BPS in contrast with (Kuruto-Niwa, Nozawa, Miyakoshi, Shiozawa, & Terao, 2005) who found them to have similar potencies. With regard to ERβ, (Molina-Molina et al., 2013) came to the same ranking order as ERα (BPA > BPF > BPS) which is in consonance with what was found in the present study however BPS appeared to be more potent for ERβ which is not true in the present study. (ter Veld et al., 2006) found BPA to exhibit similar potencies for both ER subtypes whereas (Matthews et al., 2001) found it to be around 1.5-fold more potent for ERβ. In this study BPA appeared to have higher affinity (~3-fold) for ERβ than for ERα. (Cabaton et al., 2009) who tested BPF alone

reported higher potency for ERα than for ERβ which is in line with the findings of this study. In summary, taking into account findings of all the above studies (Table 3) it can be stated that BPF and BPS are the least potent compounds of all seven tested bisphenols for both ERs. It could also be stated that BPC and BPB are more potent than BPA for ERα. However, given the scarcity of the studies that tested some specific bisphenols (e.g. BPC) but also of the studies that assessed ERβ specific potency of bisphenols, a confident conclusion cannot be drawn. Finally, such differences in the EC_{50} values that obtained from all these studies including the present are probably due to the fact that some of these cell lines also express other endogenous steroid receptors; hence interactions between the different receptors may have not been avoided (ter Veld et al., 2006).

As regards the proliferative potency of BPA and BPS (tested as the most widely used congener), results revealed that these two compounds slightly increased the proliferation of MCF-7 cells although their potency was found to be very low compared to E_2 (Figure 5). Despite that EC_{50} values of the two test compounds are not considered very reliable, it is true that BPA was found to be around 13-fold more potent than BPS which is in line with the fact that it caused a similar max. PE to BPS's but at a 10-fold lower concentration. Comparing these EC_{50} values to the ones obtained from the ERα-reporter gene assay, both E_2 and BPS were found to be less potent in the cell proliferation assay in contrast to BPA which showed the opposite. These findings are partly in agreement with the findings of (Molina-Molina et al., 2013) who also found lower potency for BPS (1.2×10^4 nM) in the cell proliferation assay but similar potencies for E_2 and BPA in the cell proliferation (0.018 and 470 nM, respectively) and in the reporter gene assay (0.019 and 410 nM, respectively). (Y Hashimoto et al., 2001) who also tested BPA and BPS ($1\text{-}10^5$ nM) among other bisphenols with E-screen bioassay found that both compounds caused a max. PE of almost 4.0 at 10^3 and 10^4 nM, respectively whereas in an older study they found for the same compounds somewhat lower max. PEs (~3.0 and 3.5 respectively) at 10^4 nM (Y. Hashimoto & Nakamura, 2000). EC_{50} values were not given in any of the two studies. (Molina-Molina et al., 2013) using the E-screen bioassay too, reported that the max. PE of BPA reached 7.0 whereas BPS's was only 4.0 at 10^4 nM. Considering the findings of all the above studies, it seems that both bisphenols have the ability to stimulate cell proliferation in MCF-7 cells in a concentration range between $1\text{-}10^4$ nM with BPA to be around 10-30-fold more potent than BPS. In another study evaluating the proliferative potency of other analogues it was found that their max. PEs ranged from 6.0 to 7.0 while their Relative Proliferative Potency (RPP) which is defined as

the ratio between the concentration of E_2 and that of compound both needed to produce maximal cell yield × 100, appeared to be 10^4-10^5-fold lower than that of E_2 with the following order: BPA=BPB > BPF=BPE (Perez et al., 1998). (Y Hashimoto et al., 2001) found that BPB, BPF and HPP had also a proliferative effect on MCF-7 cells up to 4.5 between 1-10^5 nM. Finally, (Molina-Molina et al., 2013) based on experimental data reported that both the nature of the bridging carbon substituent and the distance between *para* hydroxyl groups determine the estrogenicity of the compound.

All together the results of the present study show that all BPA analogues exert estrogenic activity in both U2-OS ERα/β reporter gene cell lines and that at least BPS can slightly stimulate cell proliferation of MCF-7 cells in a similar way as BPA. BPB and BPC appeared to have higher affinity for ERβ than ERα in contrast with BPE, BPF, BPS and HPP which showed the opposite. However, BPE, BPF and BPS were found to be less potent than BPA in both reporter gene assays whereas HPP was less potent only in the ERβ assay. As regards the estrogenicity of all bisphenols in comparison to the endogenous estrogen E_2, it is obvious from the ratio $EEF_{50}α$/ $EEF_{50}β$ provided in Table 2 that all bisphenols apart from BPS are around 10-5000 more estrogenic in ERβ cell line than in the ERα. Taking into consideration findings of (Lazennec et al., 2001) and (Ström et al., 2004) which support the idea that ERβ may reduce proliferation of ER-positive breast cancer cells, the above mentioned suggest that all bisphenols except BPS could perhaps moderate the ERα-mediated negative effects through creating relatively larger ERα/ERβ heterodimers than what E_2 can achieve. Interestingly, BPS was found to be relatively 20 times more estrogenic in ERα cell line, compared to E_2, which indicates that this compound which is already used as substitute of BPA could theoretically abet the negative effects.

5. CONCLUSION

In conclusion, none of the tested BPA analogues seems to be free of estrogenic activity showing weaker, similar, or higher potency than BPA. BPF and BPS which is known that are already used as replacement compounds of BPA, were found to be the least potent analogues of all. However, the fact that BPS appeared to be relatively more estrogenic in U2-OS ERα cell line compared to E_2, unlike findings of (Molina-Molina et al., 2013), makes it definitely to be considered suspicious. In addition, its much lower biodegradation under aerobic conditions compared to BPA (Ike, Chen, Danzl, Sei, & Fujita, 2006) and its ongoing release to the environment due to replacement of BPA, should be also taken seriously into account. In several *in vitro* and fewer *in vivo* studies the disrupting effects of some analogues have already been observed. Although the ERα specific binding potency of various bisphenols have been tested in various assays so far, not much attention has been paid yet to ERβ specific potency, which needs to be changed since both ER subtypes are expressed in various tissues and it is important to know their responses separately. In general, it is really urgent to assess in depth the toxicity of all BPA analogues and their metabolites giving higher priority to those that are already used, like BPF and BPS, but also those that have been shown to have comparable or even higher estrogenic potency as BPA, like BPB and BPC. In the present study, all bisphenols were found to be active in nanomolar concentrations which at least for the most potent compounds could be much lower than those that have been detected in food products and human fluids. BPB for instance has been found at around 350 μM in food and at around 20 μM in blood serum of women something that rings the bell for an urgent risk assessment of BPA substitutes. Finally, one must consider that man is exposed to environment and from this point of view the possibility of additive effects due to exposure to all different analogues is much closer to reality than being exposed to one and only analogue.

References

Acconcia, F., Totta, P., Ogawa, S., Cardillo, I., Inoue, S., Leone, S., . . . Marino, M. (2005). Survival versus apoptotic 17β-estradiol effect: Role of ERα and ERβ activated non-genomic signaling. *Journal of cellular physiology, 203*(1), 193-201.

BfR. (2015, 19 February). FAQs on bisphenol A in consumer products. Retrieved 5 January, 2016, from http://www.bfr.bund.de/en/faqs_on_bisphenol_a_in_consumer_products-60837.html

Bolli, A., Galluzzo, P., Ascenzi, P., Del Pozzo, G., Manco, I., Vietri, M. T., . . . Marino, M. (2008). Laccase treatment impairs bisphenol A-induced cancer cell proliferation affecting estrogen receptor α-dependent rapid signals. *IUBMB life, 60*(12), 843-852.

Bouskine, A., Nebout, M., Brucker-Davis, F., Benahmed, M., & Fenichel, P. (2009). Low doses of bisphenol A promote human seminoma cell proliferation by activating PKA and PKG via a membrane G-protein-coupled estrogen receptor. *Environ Health Perspect, 117*(7), 1053-1058.

Cabaton, N., Dumont, C., Severin, I., Perdu, E., Zalko, D., Cherkaoui-Malki, M., & Chagnon, M.-C. (2009). Genotoxic and endocrine activities of bis (hydroxyphenyl) methane (bisphenol F) and its derivatives in the HepG2 cell line. *Toxicology, 255*(1), 15-24.

Calafat, A. M., Kuklenyik, Z., Reidy, J. A., Caudill, S. P., Ekong, J., & Needham, L. L. (2005). Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. *Environmental health perspectives*, 391-395.

Calafat, A. M., Ye, X., Wong, L.-Y., Reidy, J. A., & Needham, L. L. (2008). Exposure of the US population to Bisphenol A and 4-tertiary-Octylphenol: 2003-2004. *Environmental health perspectives*, 39-44.

Cao, X.-L., Corriveau, J., & Popovic, S. (2009). Levels of bisphenol A in canned soft drink products in Canadian markets. *Journal of agricultural and food chemistry, 57*(4), 1307-1311.

Cao, X.-L., Dufresne, G., Belisle, S., Clement, G., Falicki, M., Beraldin, F., & Rulibikiye, A. (2008). Levels of bisphenol A in canned liquid infant formula products in Canada and dietary intake estimates. *Journal of agricultural and food chemistry, 56*(17), 7919-7924.

Cobellis, L., Colacurci, N., Trabucco, E., Carpentiero, C., & Grumetto, L. (2009). Measurement of bisphenol A and bisphenol B levels in human blood sera from healthy and endometriotic women. *Biomedical Chromatography, 23*(11), 1186-1190.

Colerangle, J. B., & Roy, D. (1997). Profound effects of the weak environmental estrogen-like chemical bisphenol A on the growth of the mammary gland of Noble rats. *The Journal of steroid biochemistry and molecular biology, 60*(1), 153-160.

Delfosse, V., Grimaldi, M., Pons, J.-L., Boulahtouf, A., Le Maire, A., Cavailles, V., . . . Balaguer, P. (2012). Structural and mechanistic insights into bisphenols action provide guidelines for risk assessment and discovery of bisphenol A substitutes. *Proceedings of the National Academy of Sciences, 109*(37), 14930-14935.

Dictionary, M.-W. M. (2015). Medical Definition of estrogen. Retrieved 28 February, 2016, from http://www.merriam-webster.com/medical/estrogen

EFSA. (2015, 21 January). No consumer health risk from bisphenol A exposure. Retrieved 17 February, 2016, from http://www.efsa.europa.eu/en/press/news/150121

Eladak, S., Grisin, T., Moison, D., Guerquin, M.-J., N'Tumba-Byn, T., Pozzi-Gaudin, S., . . . Habert, R. (2015). A new chapter in the bisphenol A story: bisphenol S and bisphenol F are not safe alternatives to this compound. *Fertility and sterility, 103*(1), 11-21.

Engel, S. M., Levy, B., Liu, Z., Kaplan, D., & Wolff, M. S. (2006). Xenobiotic phenols in early pregnancy amniotic fluid. *Reproductive toxicology, 21*(1), 110-112.

Erler, C., & Novak, J. (2010). Bisphenol a exposure: human risk and health policy. *Journal of pediatric nursing, 25*(5), 400-407.

Gould, J. C., Leonard, L. S., Maness, S. C., Wagner, B. L., Conner, K., Zacharewski, T., . . . Gaido, K. W. (1998). Bisphenol A interacts with the estrogen receptor α in a distinct manner from estradiol. *Molecular and cellular endocrinology, 142*(1), 203-214.

Government of Canada. (2008, 17 March). Proposed Risk Management Approach for Phenol, 4,4'-(1-methylethylidene) bis (Bisphenol A) Chemical Abstract Service Registry Number (CAS RN): 80-05-7 Retrieved 5 January, 2016, from http://www.ec.gc.ca/ese-ees/default.asp?lang=En&n=6FA54372-1

Grignard, E., Lapenna, S., & Bremer, S. (2012). Weak estrogenic transcriptional activities of Bisphenol A and Bisphenol S. *Toxicology in Vitro, 26*(5), 727-731.

Grumetto, L., Montesano, D., Seccia, S., Albrizio, S., & Barbato, F. (2008). Determination of bisphenol A and bisphenol B residues in canned peeled tomatoes by reversed-phase liquid chromatography. *Journal of agricultural and food chemistry, 56*(22), 10633-10637.

Gyllenhammar, I., Glynn, A., Darnerud, P. O., Lignell, S., van Delft, R., & Aune, M. (2012). 4-Nonylphenol and bisphenol A in Swedish food and exposure in Swedish nursing women. *Environment international, 43*, 21-28.

Hamers, T., Kamstra, J. H., Sonneveld, E., Murk, A. J., Visser, T. J., Van Velzen, M. J., . . . Bergman, Å. (2008). Biotransformation of brominated flame retardants into potentially endocrine-disrupting metabolites, with special attention to 2, 2', 4, 4'-tetrabromodiphenyl ether (BDE-47). *Molecular nutrition & food research, 52*(2), 284-298.

Hashimoto, Y., Moriguchi, Y., Oshima, H., Kawaguchi, M., Miyazaki, K., & Nakamura, M. (2001). Measurement of estrogenic activity of chemicals for the development of new dental polymers. *Toxicology in Vitro, 15*(4), 421-425.

Hashimoto, Y., & Nakamura, M. (2000). Estrogenic activity of dental materials and bisphenol-A related chemicals in vitro. *Dental materials journal, 19*(3), 245-262.

He, Y., Miao, M., Herrinton, L. J., Wu, C., Yuan, W., Zhou, Z., & Li, D.-K. (2009). Bisphenol A levels in blood and urine in a Chinese population and the personal factors affecting the levels. *Environmental research, 109*(5), 629-633.

Higashihara, N., Shiraishi, K., Miyata, K., Oshima, Y., Minobe, Y., & Yamasaki, K. (2007). Subacute oral toxicity study of bisphenol F based on the draft protocol for the "Enhanced OECD Test Guideline no. 407". *Archives of toxicology, 81*(12), 825-832.

Huang, Y., Wong, C., Zheng, J., Bouwman, H., Barra, R., Wahlström, B., . . . Wong, M. (2012). Bisphenol A (BPA) in China: a review of sources, environmental levels, and potential human health impacts. *Environment international, 42*, 91-99.

Ike, M., Chen, M., Danzl, E., Sei, K., & Fujita, M. (2006). Biodegradation of a variety of bisphenois under aerobic and anaerobic conditions. *Water science and technology, 53*(6), 153-160.

Ji, K., Hong, S., Kho, Y., & Choi, K. (2013). Effects of bisphenol s exposure on endocrine functions and reproduction of zebrafish. *Environmental science & technology, 47*(15), 8793-8800.

Kitamura, S., Suzuki, T., Sanoh, S., Kohta, R., Jinno, N., Sugihara, K., . . . Ohta, S. (2005). Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. *Toxicological Sciences, 84*(2), 249-259.

Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L., & Feldman, D. (1993). Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology, 132*(6), 2279-2286.

Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., . . . Gustafsson, J.-A. k. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology, 139*(10), 4252-4263.

Kumar, K. S., & Kumar, M. M. (2008). Anti-estrogen therapy for breast cancer: an overview. *Cancer Therapy, 6*(2).

Kuruto-Niwa, R., Nozawa, R., Miyakoshi, T., Shiozawa, T., & Terao, Y. (2005). Estrogenic activity of alkylphenols, bisphenol S, and their chlorinated derivatives using a GFP expression system. *Environmental toxicology and pharmacology, 19*(1), 121-130.

Lazennec, G., Bresson, D., Lucas, A., Chauveau, C., & Vignon, F. (2001). ERβ inhibits proliferation and invasion of breast cancer cells. *Endocrinology, 142*(9), 4120-4130.

Lee, A. V., Oesterreich, S., & Davidson, N. E. (2015). MCF-7 Cells—Changing the Course of Breast Cancer Research and Care for 45 Years. *Journal of the National Cancer Institute, 107*(7), djv073.

Lee, H. J., Chattopadhyay, S., Gong, E.-Y., Ahn, R. S., & Lee, K. (2003). Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. *Toxicological Sciences, 75*(1), 40-46.

Lee, Y. J., Ryu, H.-Y., Kim, H.-K., Min, C. S., Lee, J. H., Kim, E., . . . Jang, D. D. (2008). Maternal and fetal exposure to bisphenol A in Korea. *Reproductive toxicology, 25*(4), 413-419.

Liao, C., Liu, F., Alomirah, H., Loi, V. D., Mohd, M. A., Moon, H.-B., . . . Kannan, K. (2012). Bisphenol S in urine from the United States and seven Asian countries: occurrence and human exposures. *Environmental science & technology, 46*(12), 6860-6866.

Matthews, J. B., Twomey, K., & Zacharewski, T. R. (2001). In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors α and β. *Chemical research in toxicology, 14*(2), 149-157.

Michałowicz, J. (2014). Bisphenol A–Sources, toxicity and biotransformation. *Environmental toxicology and pharmacology, 37*(2), 738-758.

Molina-Molina, J.-M., Amaya, E., Grimaldi, M., Sáenz, J.-M., Real, M., Fernández, M. F., . . . Olea, N. (2013). In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors. *Toxicology and applied pharmacology, 272*(1), 127-136.

Moriyama, K., Tagami, T., Akamizu, T., Usui, T., Saijo, M., Kanamoto, N., . . . Nakao, K. (2002). Thyroid hormone action is disrupted by bisphenol A as an antagonist. *The Journal of Clinical Endocrinology & Metabolism, 87*(11), 5185-5190.

Naderi, M., Wong, M. Y., & Gholami, F. (2014). Developmental exposure of zebrafish (Danio rerio) to bisphenol-S impairs subsequent reproduction potential and hormonal balance in adults. *Aquatic Toxicology, 148*, 195-203.

Nahar, M. S., Liao, C., Kannan, K., & Dolinoy, D. C. (2013). Fetal liver bisphenol A concentrations and biotransformation gene expression reveal variable exposure and altered capacity for metabolism in humans. *Journal of biochemical and molecular toxicology, 27*(2), 116-123.

Nussey, S. S., & Whitehead, S. A. (2013). *Endocrinology: an integrated approach*: CRC Press.

Olsén, L., Lampa, E., Birkholz, D. A., Lind, L., & Lind, P. M. (2012). Circulating levels of bisphenol A (BPA) and phthalates in an elderly population in Sweden, based on the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS). *Ecotoxicology and environmental safety, 75*, 242-248.

Park, S.-H., Kim, K.-Y., An, B.-S., Choi, J.-H., Jeung, E.-B., Leung, P. C., & Choi, K.-C. (2009). Cell growth of ovarian cancer cells is stimulated by xenoestrogens through an estrogen-dependent pathway, but their stimulation of cell growth appears not to be involved in the activation of the mitogen-activated protein kinases ERK-1 and p38. *Journal of Reproduction and Development, 55*(1), 23-29.

Paruthiyil, S., Parmar, H., Kerekatte, V., Cunha, G. R., Firestone, G. L., & Leitman, D. C. (2004). Estrogen receptor β inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer research, 64*(1), 423-428.

Perez, P., Pulgar, R., Olea-Serrano, F., Villalobos, M., Rivas, A., Metzler, M., . . . Olea, N. (1998). The estrogenicity of bisphenol A-related diphenylalkanes with various substituents at the central carbon and the hydroxy groups. *Environmental health perspectives, 106*(3), 167.

Ptak, A., Wróbel, A., & Gregoraszczuk, E. L. (2011). Effect of bisphenol-A on the expression of selected genes involved in cell cycle and apoptosis in the OVCAR-3 cell line. *Toxicology letters, 202*(1), 30-35.

Richter, C. A., Birnbaum, L. S., Farabollini, F., Newbold, R. R., Rubin, B. S., Talsness, C. E., . . . vom Saal, F. S. (2007). In vivo effects of bisphenol A in laboratory rodent studies. *Reproductive toxicology, 24*(2), 199-224.

Rivas, A., Lacroix, M., Olea-Serrano, F., Laïos, I., Leclercq, G., & Olea, N. (2002). Estrogenic effect of a series of bisphenol analogues on gene and protein expression in MCF-7 breast cancer cells. *The Journal of steroid biochemistry and molecular biology, 82*(1), 45-53.

Rochester, J. R., & Bolden, A. L. (2015). Bisphenol S and F: a systematic review and comparison of the hormonal activity of bisphenol A substitutes. *Environ Health Perspect, 10*.

Rosenmai, A. K., Dybdahl, M., Pedersen, M., van Vugt-Lussenburg, B. M. A., Wedebye, E. B., Taxvig, C., & Vinggaard, A. M. (2014). Are structural analogues to bisphenol a safe alternatives? *Toxicological Sciences*, kfu030.

Rubin, B. S. (2011). Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. *The Journal of steroid biochemistry and molecular biology, 127*(1), 27-34.

Rubin, B. S., & Soto, A. M. (2009). Bisphenol A: perinatal exposure and body weight. *Molecular and cellular endocrinology, 304*(1), 55-62.

Rudel, R. A., Gray, J. M., Engel, C. L., Rawsthorne, T. W., Dodson, R. E., Ackerman, J. M., . . . Brody, J. G. (2011). Food packaging and bisphenol A and bis (2-ethyhexyl) phthalate exposure: findings from a dietary intervention. *Environmental health perspectives, 119*(7), 914-920.

Rykowska, I., & Wasiak, W. (2006). Properties, threats, and methods of analysis of bisphenol A and its derivatives. *Acta chromatographica, 16*, 7.

Samuelsen, M., Olsen, C., Holme, J., Meussen-Elholm, E., Bergmann, A., & Hongslo, J. (2001). Estrogen-like properties of brominated analogs of bisphenol A in the MCF-7 human breast cancer cell line. *Cell biology and toxicology, 17*(3), 139-151.

Schecter, A., Malik, N., Haffner, D., Smith, S., Harris, T. R., Paepke, O., & Birnbaum, L. (2010). Bisphenol A (BPA) in US Food. *Environmental science & technology, 44*(24), 9425-9430.

Shankar, A., Teppala, S., & Sabanayagam, C. (2012). Bisphenol A and peripheral arterial disease: results from the NHANES. *Environmental health perspectives, 120*(9), 1297.

Snyder, R. W., Maness, S. C., Gaido, K. W., Welsch, F., Sumner, S. C., & Fennell, T. R. (2000). Metabolism and disposition of bisphenol A in female rats. *Toxicology and applied pharmacology, 168*(3), 225-234.

Stroheker, T., Chagnon, M.-C., Pinnert, M.-F., Berges, R., & Canivenc-Lavier, M.-C. (2003). Estrogenic effects of food wrap packaging xenoestrogens and flavonoids in female Wistar rats: a comparative study. *Reproductive toxicology, 17*(4), 421-432.

Ström, A., Hartman, J., Foster, J. S., Kietz, S., Wimalasena, J., & Gustafsson, J.-Å. (2004). Estrogen receptor β inhibits 17β-estradiol-stimulated proliferation of the breast

cancer cell line T47D. *Proceedings of the National Academy of Sciences of the United States of America, 101*(6), 1566-1571.

Sui, Y., Ai, N., Park, S.-H., Rios-Pilier, J., Perkins, J. T., Welsh, W. J., & Zhou, C. (2012). Bisphenol A and its analogues activate human pregnane X receptor. *Environmental health perspectives, 120*(3), 399-405.

Takayanagi, S., Tokunaga, T., Liu, X., Okada, H., Matsushima, A., & Shimohigashi, Y. (2006). Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERRγ) with high constitutive activity. *Toxicology letters, 167*(2), 95-105.

Teng, C., Goodwin, B., Shockley, K., Xia, M., Huang, R., Norris, J., . . . Tice, R. R. (2013). Bisphenol A affects androgen receptor function via multiple mechanisms. *Chemico-biological interactions, 203*(3), 556-564.

ter Veld, M. G., Schouten, B., Louisse, J., van Es, D. S., van der Saag, P. T., Rietjens, I. M., & Murk, A. J. (2006). Estrogenic potency of food-packaging-associated plasticizers and antioxidants as detected in ERα and ERβ reporter gene cell lines. *Journal of agricultural and food chemistry, 54*(12), 4407-4416.

Thomas, P., & Dong, J. (2006). Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *The Journal of steroid biochemistry and molecular biology, 102*(1), 175-179.

U.S. EPA. (2010, 29 March). Bisphenol A Action Plan (CASRN 80-05-7) [CA Index Name: Phenol, 4,4'-(1-methylethylidene)bis-] Retrieved 5 January, 2016, from http://www.epa.gov/sites/production/files/2015-09/documents/bpa_action_plan.pdf

U.S. FDA. (2010, 1 June). Bisphenol A (BPA): Use in Food Contact Application. Retrieved 5 January, 2016, from http://www.fda.gov/NewsEvents/PublicHealthFocus/ucm064437.htm#regulations

USDA. (2013, 2 June). French Law Banning Bisphenol A in Food Containers Enacted. Retrieved 17 February, 2016, from http://gain.fas.usda.gov/Recent%20GAIN%20Publications/French%20Law%20Banning%20Bisphenol%20A%20in%20Food%20Containers%20Enacted_Paris_France_2-5-2013.pdf

van der Woude, H., ter Veld, M. G., Jacobs, N., van der Saag, P. T., Murk, A. J., & Rietjens, I. M. (2005). The stimulation of cell proliferation by quercetin is mediated by the estrogen receptor. *Molecular nutrition & food research, 49*(8), 763-771.

Vandenberg, L. N., Hauser, R., Marcus, M., Olea, N., & Welshons, W. V. (2007). Human exposure to bisphenol A (BPA). *Reproductive toxicology, 24*(2), 139-177.

Vandenberg, L. N., Maffini, M. V., Sonnenschein, C., Rubin, B. S., & Soto, A. M. (2009). Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocrine reviews, 30*(1), 75-95.

Vandenberg, L. N., Maffini, M. V., Wadia, P. R., Sonnenschein, C., Rubin, B. S., & Soto, A. M. (2007). Exposure to environmentally relevant doses of the xenoestrogen bisphenol-A alters development of the fetal mouse mammary gland. *Endocrinology, 148*(1), 116-127.

Varandas, E. S. G. R. V. (2014). Low-dose effects of Bisphenol A on human primary vascular endothelial cells and colon cancer cells.

Viñas, P., Campillo, N., Martínez-Castillo, N., & Hernández-Córdoba, M. (2010). Comparison of two derivatization-based methods for solid-phase microextraction–gas chromatography–mass spectrometric determination of bisphenol A, bisphenol S and biphenol migrated from food cans. *Analytical and bioanalytical chemistry, 397*(1), 115-125.

Vivacqua, A., Recchia, A. G., Fasanella, G., Gabriele, S., Carpino, A., Rago, V., . . . Liguori, A. (2003). The food contaminants bisphenol A and 4-nonylphenol act as agonists for estrogen receptor α in MCF7 breast cancer cells. *Endocrine, 22*(3), 275-284.

Völkel, W., Bittner, N., & Dekant, W. (2005). Quantitation of bisphenol A and bisphenol A glucuronide in biological samples by high performance liquid chromatography-tandem mass spectrometry. *Drug Metabolism and Disposition, 33*(11), 1748-1757.

Völkel, W., Colnot, T., Csanády, G. A., Filser, J. G., & Dekant, W. (2002). Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. *Chemical research in toxicology, 15*(10), 1281-1287.

Wang, S., Aarts, J. M., Haan, L. H., Argyriou, D., Peijnenburg, A. A., Rietjens, I. M., & Bovee, T. F. (2014). Towards an integrated in vitro strategy for estrogenicity testing. *Journal of Applied Toxicology, 34*(9), 1031-1040.

Wang, S., Rijk, J. C., Besselink, H. T., Houtman, R., Peijnenburg, A. A., Brouwer, A., . . . Bovee, T. F. (2014). Extending an in vitro panel for estrogenicity testing: the added value of bioassays for measuring antiandrogenic activities and effects on steroidogenesis. *Toxicological Sciences, 141*(1), 78-89.

Wetherill, Y. B., Akingbemi, B. T., Kanno, J., McLachlan, J. A., Nadal, A., Sonnenschein, C., . . . Belcher, S. M. (2007). In vitro molecular mechanisms of bisphenol A action. *Reproductive toxicology, 24*(2), 178-198.

World Health Organization, W. (2009). Food and Agriculture Organization of the United Nations. Bisphenol A (BPA)—Current State of Knowledge and Future Actions by WHO and FAO: INFOSAN Information Note.

Xu, L.-C., Sun, H., Chen, J.-F., Bian, Q., Qian, J., Song, L., & Wang, X.-R. (2005). Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol in vitro. *Toxicology, 216*(2), 197-203.

Yamasaki, K., Noda, S., Imatanaka, N., & Yakabe, Y. (2004). Comparative study of the uterotrophic potency of 14 chemicals in a uterotrophic assay and their receptor-binding affinity. *Toxicology letters, 146*(2), 111-120.

Yang, M., Kim, S. Y., Chang, S. S., Lee, I. S., & Kawamoto, T. (2006). Urinary concentrations of bisphenol A in relation to biomarkers of sensitivity and effect and endocrine-related health effects. *Environmental and molecular mutagenesis, 47*(8), 571-578.

Zhou, X., Kramer, J. P., Calafat, A. M., & Ye, X. (2014). Automated on-line column-switching high performance liquid chromatography isotope dilution tandem mass spectrometry method for the quantification of bisphenol A, bisphenol F, bisphenol S, and 11 other phenols in urine. *Journal of Chromatography B, 944*, 152-156.

Zoeller, R. T., Bansal, R., & Parris, C. (2005). Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. *Endocrinology, 146*(2), 607-612.

Appendix 1

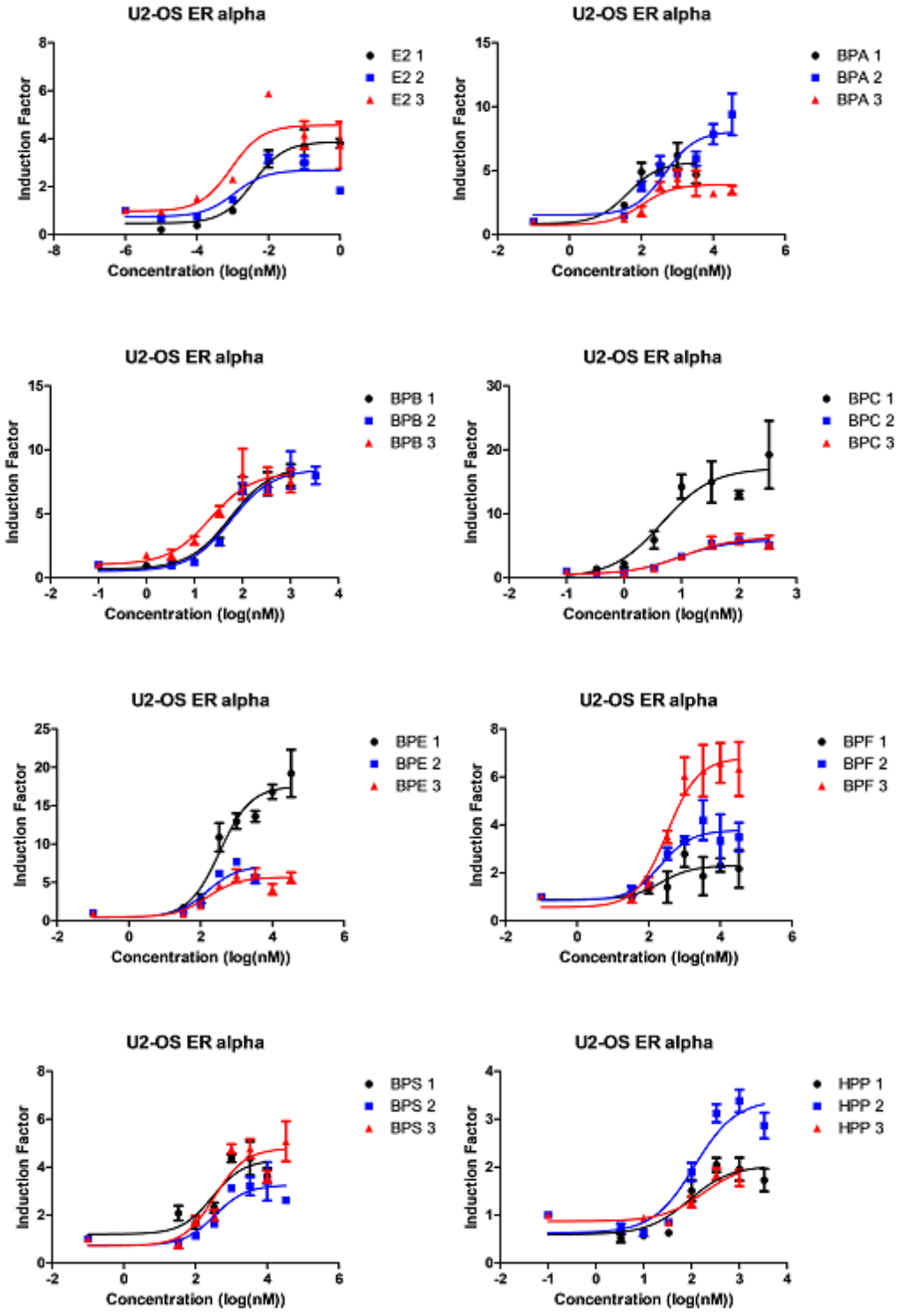


Figure A1. Dose-response curves of the indicated test compounds in the *in vitro* ERα CALUX bioassay. Data shown as fold change compared to control.

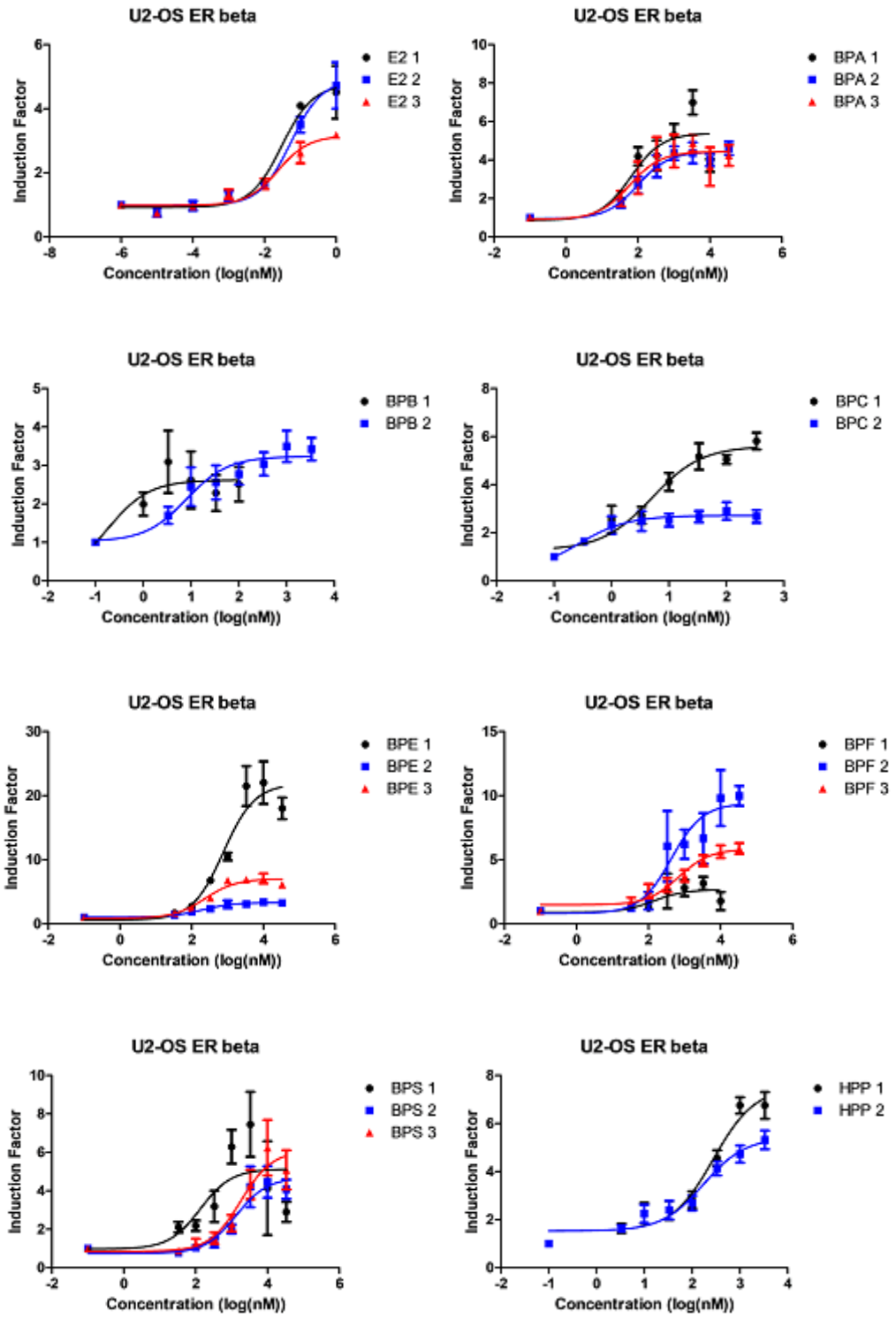
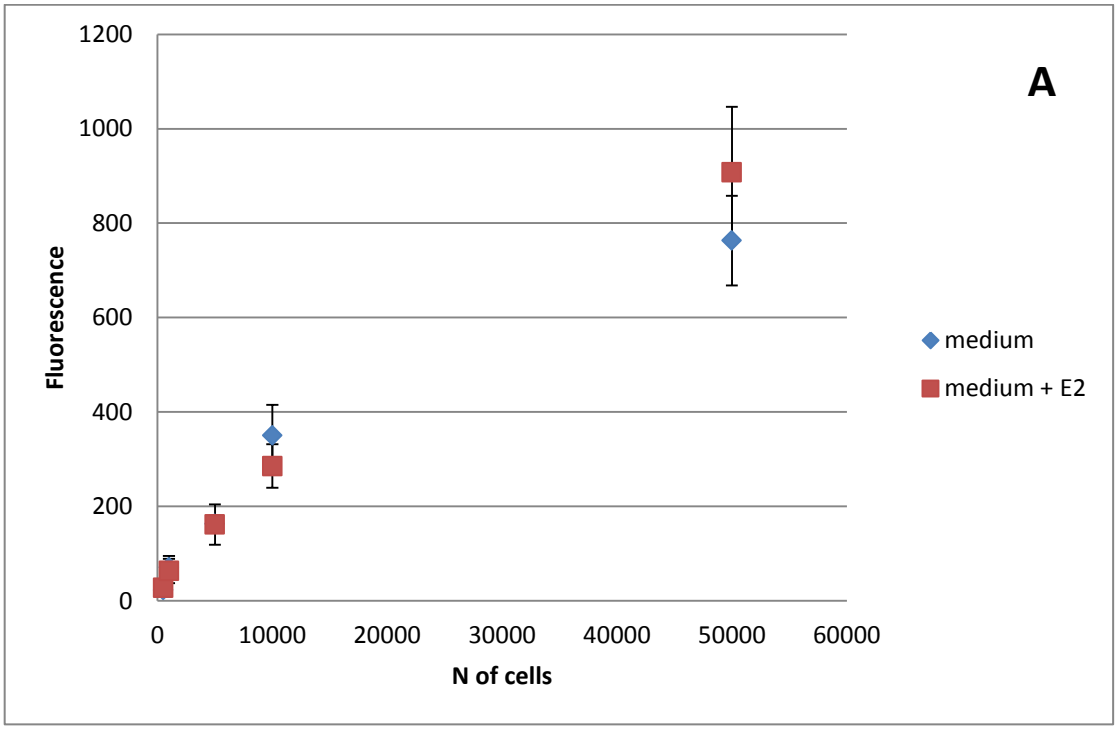


Figure A2. Dose-response curves of the indicated test compounds in the *in vitro* ERβ CALUX bioassay. Data shown as fold change compared to control.

Appendix 2



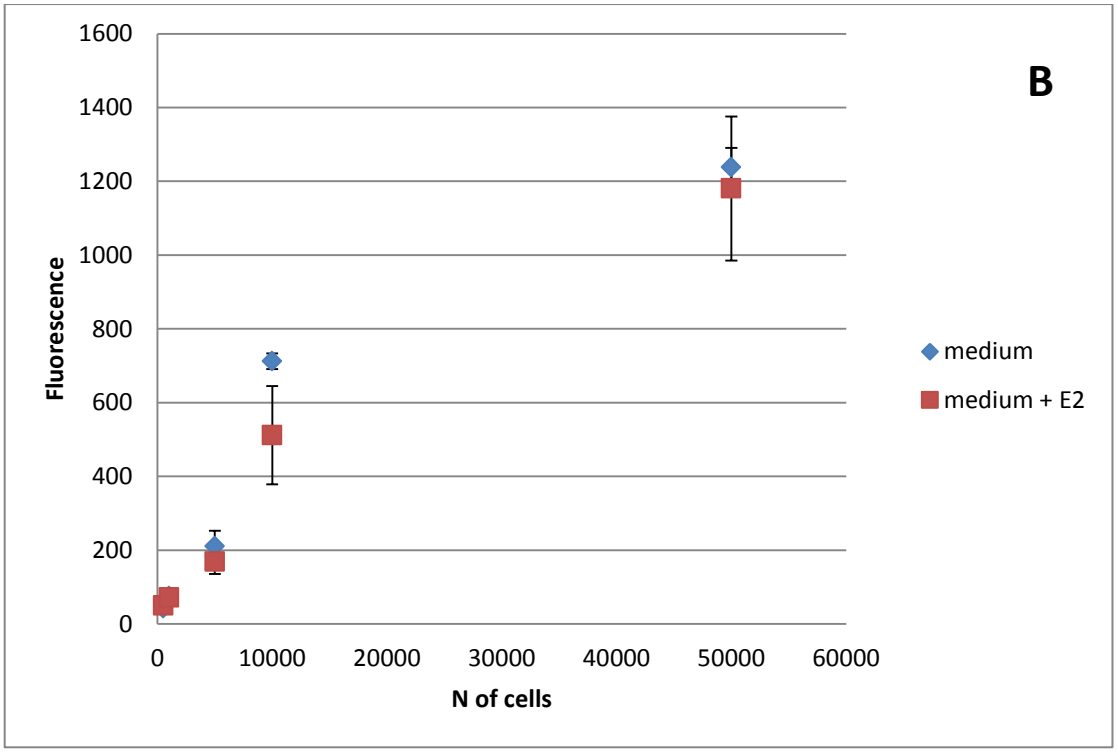


Figure A3. Proliferative response of MCF-7 cell line incubated with the absence or presence of E_2 (10^{-2} nM) for 24 h (A) and 48 h (B).