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Alternative methods *in vitro* for screening of endocrine disruptors

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Abstract

OBJECTIVES: The aim of this study was to compare *in silico* data with results obtained in two alternative *in vitro* methods; and to investigate the potential endocrine activity of bisphenol A analogues. This article contributes to recent findings and brings up-to-date information on development of EU legislation and *in vitro* testing methods of endocrine disruption.

METHODS: *In silico* approach based on the OECD QSAR Toolbox was used for prediction of potential ligands of human estrogen receptor α. Estrogen Receptor Transactivation *in vitro* Assay to Detect Estrogen Receptor Agonists and Antagonists (OECD TG 455/457) using the VM7Luc4E2 (formerly designated BG1Luc4E2) cell line was performed for measurement of transactivation activity of the tested substances. Commercially available yeast-based microplate assay (XenoScreen YES/YAS, Xenometrix, Switzerland) for detection of compounds with estrogenic and androgenic agonistic/antagonistic activity was used as a comparative test to estrogen receptor transactivation assay (OECD TG 455/457) and for screening of the agonistic/antagonistic potential of human estrogen receptor and agonistic/antagonistic activity of tested compounds on human androgen receptor.

RESULTS: The study showed good correlation between the two *in vitro* assays and significant correlation with *in silico* data. All tested substances were identified as agonists for human estrogen receptor α by methods *in silico* and *in vitro*, four substances showed a potentially higher estrogenic activity comparing to bisphenol A, two substances were identified as very weak antagonists of human androgen receptor and one compound showed a potential of agonistic activity to human androgen receptor.

CONCLUSIONS: The study contributes to recent findings and brings new *in silico* and *in vitro* data of bisphenol A analogues, revealing that these analogous substances should be further tested as they may show similar or higher activity *in vivo* comparing to bisphenol A, which has been recently legislatively regulated.

ORIGINAL ARTICLE

Abbreviations:

BPA	- bisphenol A
BPAF	- bisphenol AF
BPAP	- bisphenol AP
BPE	- bisphenol E
BPF	- bisphenol F
BPS	- bisphenol S
BPZ	- bisphenol Z
β-Gal	- β-galactosidase reporter gene
CYP	- cytochrome P450
EC	- European Commission
EDs	- endocrine disruptors
ERTA	- Estrogen Receptor Transactivation Assay
(h)ERα	- (human) estrogen receptor α
(h)AR	- (human) androgen receptor
GC-MS	- Gas Chromatography - Mass Spectrometry
HPLC	- MS - High-Performance Liquid Chromatography with Mass Spectrometry
GST	- glutathione-S-transferase
IPCS	- International Programme on Chemical Safety
JRC	- Joint Research Centre
Luc	- Luciferase Reporter Gene
mRNA	- messenger ribonucleic acid
OD	- Optical Density
OECD	- Organization for Economic Cooperation and Development
NAT	- N-acetyl transferase
SULT	- sulphotransferase
QSAR	- Quantitative Structure-Activity Relationship
REACH	- Registration, Evaluation, Authorisation and Restriction of Chemicals
SVHC	- Substances of Very High Concern
TG	- Test guideline
UGT	- UDP-dependent glucuronosyl transferase
WHO	- World Health Organization
(Z)FET	- (Zebra) Fish Embryo Toxicity
RLU	- Relative Luminiscence Unit

INTRODUCTION

Endocrine disruptors (EDs) are hormonally active substances of natural or synthetic origin, able to modulate the endocrine system after binding to receptors, causing their activation or inhibition. Complex reactions may occur also after biotransformation of these compounds into their metabolites. Compounds with hormone disrupting capability may interfere with the organism at different levels – the brain, hypothalamic-pituitary axis, gonads, prostate, endometrium, peripheral target organs, etc. They may mimick the effects of endogenous hormones, acting as agonists or antagonists of specific receptors, i.e. estrogen, androgen, progesterone, thyroid, retinoid, glucocorticoid, aryl hydrocarbon, G protein-coupled estrogen receptors, etc. (Zhao *et al.* 2013; Zoeller *et al.* 2005; Prasanth *et al.* 2010; Boucher *et al.* 2016; Stroheker *et al.* 2003; Manolagas *et al.* 2013). About 50 human nuclear receptors are known and many remain not sufficently described as their ligands have not been exactly specified. Expression of the receptor genes differs in specific tissues (Vrtačnik *et al.* 2014; Schug *et al.* 2011; Luccio-Camelo & Prins 2011; Molina-Molina *et al.* 2013) under specific conditions. Recent evidence shows that exposures to endogenous hormones and EDs during critical periods of development can affect differentiation and organogenesis and may result in adverse effects in later periods of life (Bernal & Jirtle 2010). Developmental exposures to endocrine active substances have been suggested to be able to alter the epigenome (Greally *et al.* 2013), leading to heritable phenotypes with developmental, metabolic, and behavioral disorders. Low dose effects of EDs may have additive or synergistic effects (Silva *et al.* 2002), similar to circulating endogenous hormones (Vandenberg *et al.* 2012). Combination of xenoestrogens at levels below individual no-observed-effect concentrations can enhance steroid hormone activity (Rajapakse *et al.* 2002). However, experience with chemicals evaluated in animal studies for reproductive hazard and risk identification indicate that the body is able to neutralize hazards through homeostatic mechanisms and adaptive physiological changes. Nevertheless, if the homeostatic mechanisms are overwhelmed, adverse effects may occur. *In vivo* studies showed that (chronic) exposure to even low levels of endocrine disrupting compounds including mixtures induces adverse effects, e.g. in zebrafish and rodents (Eladak *et al.* 2015; Ji *et al.* 2013; Richter *et al.* 2007; Stroheker *et al.* 2003; Rider *et al.* 2009; Rider *et al.* 2010; Orton *et al.* 2014). Therefore it seems to be reasonable to hypothesize that chemicals with positive results in both *in vitro* and *in vivo* studies are able to cause adverse effects in humans, EDs with reported low-dose effects in animals (or humans) are polychlorinated biphenyls (e.g. aroclor, present in paints and plastics), bisphenols (e.g. bisphenol A, present in plastics, thermal paper, epoxy resins), nicotine, nonylphenol, octylphenol, sodium fluoride, several fungicides, insecticides, etc. (Wirbisky *et al.* 2016; Vandenberg *et al.* 2012; Nikaido *et al.* 2005; Richter *et al.* 2007). Human exposure to most of the EDs is "multisource and multipathway" (Meeker 2012) and thus, human studies on EDs remain limited. Although human exposure cannot be simply monitored as it is influenced by individual environmental and social factors, some of the studies showed that concentrations of EDs in human body fluids are associated with an increased risk of subfertility, poor sperm quality, alterations in hormone levels, altered thyroid hormone levels and liver function, cardiovascular diseases, insuline resistance, diabetes, hyperglycemia, obesity, neurological and behaviour disorders, endometriosis, breast cancer, low birth weight, preterm birth, anomalies of the male reproductive tract, precocious puberty or earlier menarche in girls (Ranciere *et al.* 2015; Meeker 2012; Den Hond *et al.* 2015; Wolff *et al.* 2015, Trasande *et al.* 2012; Orton *et al.* 2014). Certain compounds with endocrine disrupting effects can be found in various chemical groups, e.g. steroids, cyclic hydrocarbons, phenols, flavonoids, glycosides, phtalates, parabens, toxic metals, and other substances. Compounds with possible endocrine activity (e.g. biocides, plasticizers, surfactants, fire retardants, distinct antimicrobials, UV filters, preservatives) may be released from products intended for consumers, e.g. cosmetics, toys, everyday items,

household products, medical devices, packaging materials and other products used in industry or agriculture (Schlumpf *et al.* 2008; Yang *et al.* 2011; Dodson *et al.* 2012; Liao & Kannan 2014; Shannon *et al.* 2016; Wang *et al.* 2011; Rossi *et al.* 2016; Isidori *et al.* 2007; Orton *et al.* 2014). Certain chemicals have already been identified as endocrine disruptors and have been legislatively regulated (e.g. bisphenol A has been regulated in food contact materials in several EU countries; or aminotriazole, use of which within the EU has been banned after September 2017 on the grounds of potential groundwater contamination and risks to aquatic life; there have also been concerns about endocrine-disrupting properties). Effects of structural analogues of already known and legislatively regulated endocrine active substances on human receptors have been described in very recent studies (Roelofs *et al.* 2015; Eladak *et al.* 2015), showing similar order of magnitude, indicating that structural analogues of BPA, i.e. BPF and BPS, are not safer alternatives compared to BPA regarding their endocrine modulating capacity. The use of analogues has not been regulated, however, it has been described that BPS is used as a BPA alternative e.g. in "BPA-free" thermal printing paper (Liao *et al.* 2012a), BPF was found in foodstuff and BPS in urine samples (Liao *et al.* 2012a; Liao *et al.* 2012b; Liao & Kannan 2013; Liao & Kannan 2014). Possible additive effects of various analogous substances, e.g. bisphenols, should be considered as relevant regarding overall human exposure. At the international level, the improvement of testing strategies and new advanced testing methods is highly required and has been discussed in the context of EU and global legislation. Increasing pressure on testing of endocrine disruption potential in the near future is anticipated, along with efforts to reduce the potential exposure of humans, particularly of sensitive populations. This article summarizes the current development of international legislation for the evaluation of chemical substances in terms of endocrine disruption including the available up-to-date *in vitro* testing methods. It also brings *in silico* and *in vitro* data of a pilot study of bisphenol A analogues, showing that analogous substances should be further tested as they may show similar or even higher activity as the compounds recently legislatively regulated.

Regulatory concern and perspective for endocrine disruption in the European Union

The most effective way to reduce risk of human exposure to EDs is at the regulatory level. The regulatory networks in the European Union have been criticised to be ineffective. The WHO/IPCS (2002) scientific definition of endocrine disruptors has reached a consensus of EU public authorities: "*An endocrine disruptor is an exogenous substance or mixture that alters function(s) of endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations.*" (European Chemicals Agency 2015a, 2015b, WHO 2002). According to REACH (Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals, establishing a European Chemicals Agency), potential endocrine disruptors are considered as substances of very high concern (SVHC). The Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products introduced an automatic ban on CMR (carcinogenic, mutagenic, reprotoxic) substances beside exceptions with strict limitations. As a hazardous property of a substance does not necessarily always represent a risk, there is a possibility to allow the use of the substance classified as CMR where, in view of exposure and concentration, it has been found safe for use in cosmetic products by the SCCS (Scientific Committee for Consumer Safety) and is regulated by the European Commission in the Annexes to the Regulation. Validation of tests and test strategies for regulatory purposes have already been performed and set up. In June 2016, the European Commission published science-based criteria for EDs which will have a substantial impact on industrial chemicals, plant protection products and biocides. Joint Research Centre (JRC), the scientific service of the European Commission, presented the developed methodology for the screening of chemicals (Munn *et al.* 2015). Approximately 700 chemical substances have been screened against various ED criteria. The lists of screened chemicals include almost all EU approved pesticides (341 substances), biocides (96 substances) and about 200 other substances regulated by REACH, cosmetic regulations and the water framework regulation. The formal adoption and entry into force of final criteria is planned in 2017. The existing European Commission database of endocrine disruptors and testing methods was published at the EU Commission website. The database covered more than 400 substances with suspected potential of endocrine disruption. 130 reproductive toxicants covering different target cells and toxicological mechanisms, selected according predefined criteria and independently peer reviewed, which were used for the development of *in vitro* tests of ReProTect projects, are listed in a specific list: http://chelist.jrc.ec.europa.eu/?id=2&id_list=16.

OECD Test Guidelines programme

Development of non-animal test methods for the detection of endocrine disruptors has been confirmed as a high priority for regulatory authorities in most OECD countries. In 2012, the "Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption – Series on Testing and Assessment, No. 150" was issued (OECD, 2012a). The extensive document provides the background of each standardised assay, its applicability domain and conceptual framework for testing strategy. In 2012, a "Detailed review Paper on the State of the Science on Novel *in*

vitro and *in vivo* screening and testing methods and endpoints for evaluating endocrine disruptors, No. 178" was published (OECD, 2012b). In 2012, the "Adverse Outcome Pathway Development Programme" was launched as a central concept in future work at OECD on predictive toxicology, covered by the Advisory Group on Molecular Screening and Toxicogenomics (Programme on Chemical Safety). The OECD QSAR Project was initiated for the identification of new methods/profilers for grouping chemicals; with the aim to improve future testing and assessment needs (OECD, 2007).

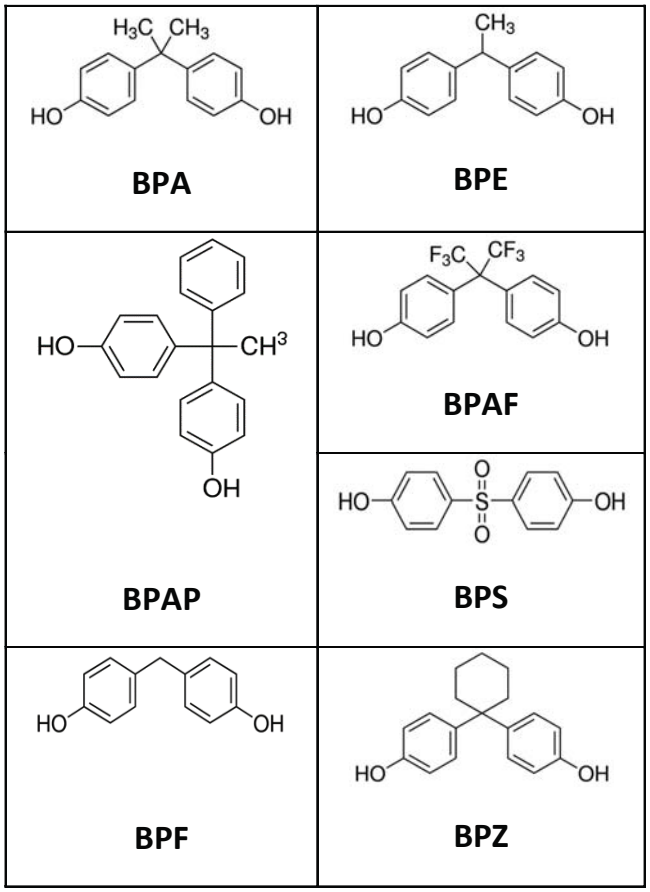
In vitro testing of endocrine disruption

Although *in vitro* models and assays lack full metabolic and homeostatic relevance to human organism *in vivo*, they are indispensable for directing further testing and they are employed. Epigenetic assays using approaches of molecular biology focused on histone and cytosine methylation, transcriptional profiling of mRNAs, microRNAs and mRNA levels of methyltransferases and chromatin immunoprecipitation-based techniques seem suitable for testing complex effects of endocrine disruption (De Felice *et al.* 2015; Kundakovic *et al.* 2013). Cell cultures from phenotypically-affected organs (e.g. sperm, ovary, breast, prostate) are used as suitable substrates for testing of phenotypic effects. In accordance with the 3Rs principles, *in vitro* screening studies should be preferably performed, in order to allow future reduction of numbers of experimental animals used in *in vivo* studies. Available OECD test guidelines that could potentially be adapted for epigenomic studies of endocrine disruption are e.g.:

- OECD TG 455/457 – Performance-Based Test Guideline for Stably Transfected Transactivation *In vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists,
- OECD TG 456 – H295R Steroidogenesis Assay,
- MCF-7 Cell proliferation Assay (agonistic/antagonistic),
- OECD TG 236 – Fish Embryo Acute Toxicity (FET) Test with adaptation to zebrafish embryo epigenetic assay.

In vitro bioassays, e.g. Yeast Estrogen Screen (YES), Yeast Androgen Screen (YAS), Estrogen Receptor Transactivation Assays (ER TA), Androgen Receptor Transactivation Assays (AR TA), CALUX assays (Chemical Activated LUciferase gene eXpression), e.g. ER CALUX (Estrogen Receptor Chemical Activated LUciferase gene eXpression), AR CALUX (Androgen Receptor Chemical Activated LUciferase gene eXpression), are suitable for screening of endocrine activity. *In vitro* assays focused on activity of CYP family enzymes (e.g. CYP19), transferases (NAT, UGT, SULT, GST) and aromatases, and the use of relevant cell lines (e.g. H295R, a human steroid-producing cell line, isolated from adrenocortical carcinoma, used for OECD TG 456 – H295R Steroidogenesis Assay, MA-10, a Leydig cell line isolated from *Mus musculus,* CV-1 cell line derived from *Cercopithecus aethiops* monkey kidneys) are suitable for detecting the effects of xenobiotics on metabolism (OECD 2008). Chromatographic analyses (by GC-MS, HPLC-MS) are very reliable tools for detection of EDs in various types of samples (e.g. biological fluids, extracts, water) or their migration from e.g. packaging materials (Piecha *et al.* 2016; Mráz *et al.* 2016; Grafnetterová *et al.* 2015). Human relevant cell cultures which could be potentially used for *in vitro* screening of endocrine activity include e.g. MCF-7 cell line derived from a human breast adenocarcinoma, hERalpha-HeLa-9903 cell line derived from human cervical tumor, yeast cells (e.g. *S. cerevisiae*) with stably transfected human estrogen or androgen receptors (Greally & Jacobs 2013), Leydig cells, ovarian carcinoma cells. Reporter gene assays, H295R steroidogenesis assay and $Ca^{(2+)}$ fluorimetry based assays using human sperm cells, optional primary cell cultures and organ explants, human embryonic stem cells as well as 2D or 3D model systems, human mammospheres and human microsomes may be promising (Zalko *et al.* 2011; Calderon-Gierszal and Prins 2015; Shannon *et al.* 2016). Biological fluids, e.g. urine, semen, serum, breast milk, colostrum, saliva, blood, biological samples of hair and adipose tissue, and tissues and cells of breast, ovaries, testes or skin have been used in studies relevant to humans (Müllerová & Kopecký 2007; Hampl

Tab. 1. Chemical structure of BPA and analogues.



et al. 2013; Hampl *et al.* 2016; Boucher *et al.* 2016; Teeguarden *et al.* 2015; Tzatzarakis *et al.* 2015; Thayer *et al.* 2015; Goldstone *et al.* 2015; Den Hond *et al.* 2015; Mráz *et al.* 2016; Hormann *et al.* 2014; Dekant & Völkel 2008; Vandenberg *et al.* 2010; Trasande *et al.* 2012).

MATERIAL AND METHODS

Tested chemicals

Chemicals analogous to bisphenol A (BPA), i.e. BPAF, BPF, BPS, BPZ, BPE, BPAP (Sigma Aldrich) were tested in a pilot study for endocrine activity, comparing to BPA and relevant analytical standards (Methoxychlor, 17β-estradiol, Sigma Aldrich). Chemical structure of the tested compounds is indicated in Table 1.

OECD QSAR Toolbox

In silico approach, using the OECD QSAR Toolbox (Toolbox 3.3.2 Release Notes) was used for prediction of potential ligands and their binding affinity to the estrogen receptor α based on the chemical structure, molecular weight and partition coefficient octanol-water of the screened compounds. OECD QSAR Toolbox, v. 3.3.2. database allows to categorize the screened compounds according to their binding affinity into groups, e.g. very strong binder, binder, non binder.

Estrogen Receptor Transactivation Assay (OECD TG 455/457)

A continuous human cell line VM7Luc4E2 (formerly designated BG1Luc4E2) with endogenous ERα, suitable for luciferase reporter gene assay (OECD TG 455/457) was kindly provided by Prof. Michael Denison, UC Davis, California, USA, for research purposes (Figure 1). The functional assay is based on binding of a tested substance to ERα. The culture and assay procedure was performed according to Rogers and Denison, with minor modifications (Rogers & Denison, 2000). In brief, cells were cultured in MEMα medium (Gibco), containing 10% fetal bovine serum and 1% penicilin/streptomycin. Five days prior analysis, cells were cultured in Dulbecco´s modified eagle medium, estrogen stripped and phenol red free (Sigma Aldrich) with 8% charcoal stripped fetal bovine serum and 1.9% supplement of L-glutamine with daily media change. Cells were plated in 96-well plates (100 µl per well) at a concentration of 500.000 cells/ml and incubated for 24 h (37 °C, 5% CO_2). The next day the plated cells were treated with tested compounds in triplicates in selected concentrations (from 1.0*10E-07 to 1.0*10E-02 mg/ml) for 24 h (37 °C, 5% CO_2). Luciferase Assay System (Promega) in combination with Promega Injector Luminometer was used for luminiscence measurement of activation the ERα by the tested substances.

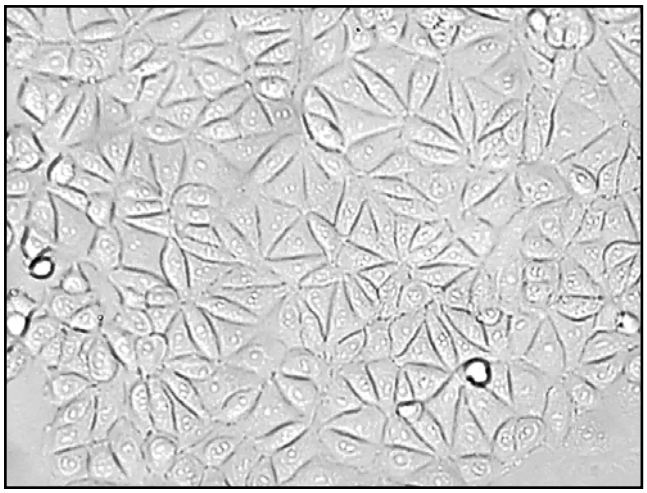


Fig. 1. VM7Luc4E2 (BG1Luc4E2) cell line, magnification 200×.

Yeast based reporter gene assay (XenoScreen YES/YAS)

A commercially available yeast based microplate assay (XenoScreen YES/YAS, Xenometrix®, Switzerland) designed for detection of compounds with estrogenic and androgenic agonistic/antagonistic activities of chemicals, water samples and biological fluids, using recombinant *Saccharomyces cerevisiae* strains with human estrogen (hERα) and androgen (hAR) receptors was used as a comparative test to Estrogen Receptor Transactivation Assay (OECD TG 455/457). The Yeast based reporter gene assay was performed according to the provided standard operating procedure, using the supplied standardized material and chemicals (in concentrations from 3.16*10E-08 to 1.0*10E-04 mol/L). The OD of the red product resulting from conversion of the yellow substrate after secretion of β-galactosidase was measured on Biotec Eon™ High Performance Microplate Spectrophotometer at 570 nm. The OD_{570} of the end product in comparison with controls provides direct correlation with the endocrine activity of the tested substances.

RESULTS

In silico results obtained with the use of OECD QSAR Toolbox predicted all the screened compounds as very strong binders to ERα based on their chemical structure, molecular weight and partition coefficient

Tab. 2. QSAR prediction of ERα binding affinity.

Compound	CAS No.	ERα binding affinity
BPA	80-05-7	Very strong
BPAF	1478-61-1	Very strong
BPAP	1571-75-1	Very strong
BPE	2081-08-5	Very strong
BPF	620-92-8	Very strong
BPS	80-09-1	Very strong
BPZ	843-55-0	Very strong

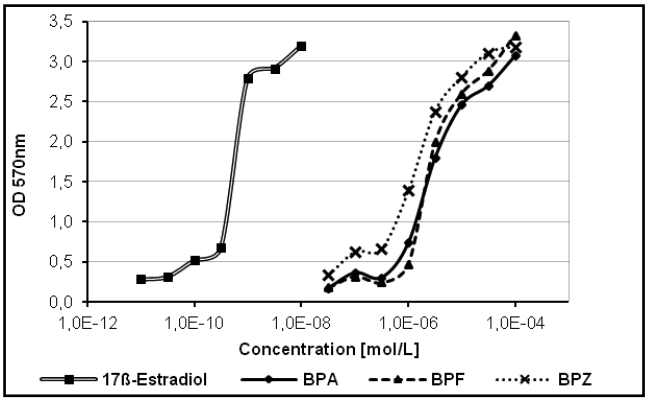


Fig. 2. Agonistic activity to human estrogen receptor α in XenoScreen® YES/YAS assay (β-Gal expression).

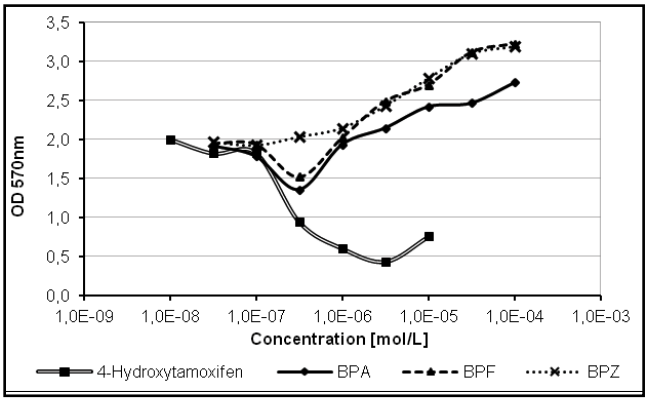


Fig. 3. Antagonistic activity to human estrogen receptor α in XenoScreen® YES/YAS assay (β-Gal expression).

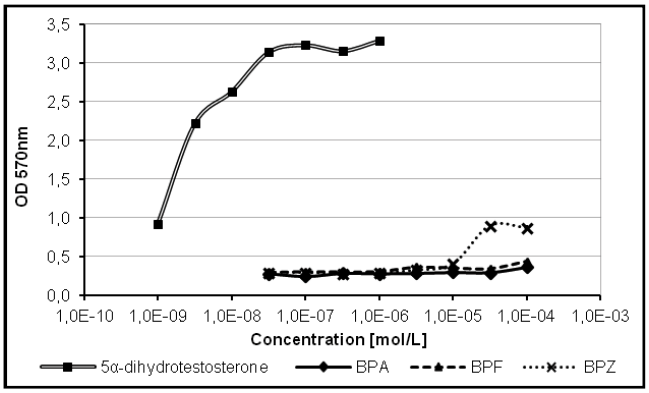


Fig. 4. Agonistic activity to human androgen receptor in XenoScreen® YES/YAS assay (β-Gal expression).

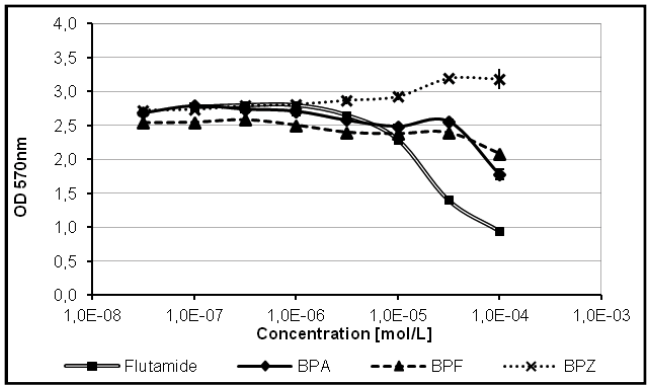


Fig. 5. Antagonistic activity to human androgen receptor in XenoScreen® YES/YAS assay (β-Gal expression).

octanol–water as indicated in Table 2. In two novel *in vitro* bioanalytical tests – Estrogen Receptor Transactivation Assay (ER TA, OECD TG 455/457), and the Yeast based reporter gene assay (XenoScreen YES/YAS), BPA and its analogues (BPA, BPF, BPAF, BPS, BPZ, BPE, BPAP) showed similar estrogenic activity comparing to 17β-estradiol (Figures 2, 6, 7). BPZ and BPF showed a potential of even higher estrogenic activity comparing to BPA in both *in vitro* assays (Figures 2, 6). BPAF in low concentrations showed a potential of even higher estrogenic activity comparing to BPA in ER TA (OECD TG 455/457) reporter gene assay (Figure 6). Significant antagonistic activity to human ERα was not clearly confirmed for any of the tested compounds in the Yeast based reporter gene assay (XenoScreen YES/YAS) comparing to 4-hydroxytamoxifen used as a positive control (Figure 3). BPZ showed a potential of weak agonistic activity to human AR while BPA and BPF were not identified as agonists of human AR in the Yeast based reporter gene assay comparing to 5α-dihydrotestosterone as a positive control (Figure 4). BPA and BPF showed a potential of antagonistic activity to human AR comparing to flutamide as a positive control in the Yeast based reporter gene assay, and BPZ was not detected as an antagonist of human AR (Figure 5).

DISCUSSION

Using *in silico* approach and two novel *in vitro* toxicological methods alternative to animal experiments seven analogues of BPA (BPAP, BPAF, BPE, BPF, BPS, BPZ) were tested. In this limited study all of the tested analogous bisphenols showed similar dose-response estrogenic activity curve as BPA and similar activity as 17-β estradiol in very low concentrations. The data support recent findings that BPS, BPAF and BPF may display BPA-like activity in cell lines and tissues with weaker, equal or higher potency than BPA, depending on the employed *in vitro* model and selected endpoints (Eladak *et al.* 2015; Rochester & Bolden 2015; Goldinger *et al.* 2015; Molina-Molina *et al.* 2013; Cano-Nicolau, *et al.* 2016). Previous studies have shown that BPA exhibits multiple effects, e.g. both estrogen receptor agonistic activity and androgen receptor antagonistic activity *in vitro* and *in vivo* (Luccio-Camelo & Prins 2011; Rubin BS 2011; Paris *et al.* 2002). Results of this study indicate that BPA and BPF are agonists of human estrogen receptor α and to certain extent may antagonize human androgen receptor. Using the OECD QSAR Toolbox the tested BPA analogues, i.e. BPF, BPAF, BPS, BPZ, BPE, BPAP, were classified as very strong binders of human

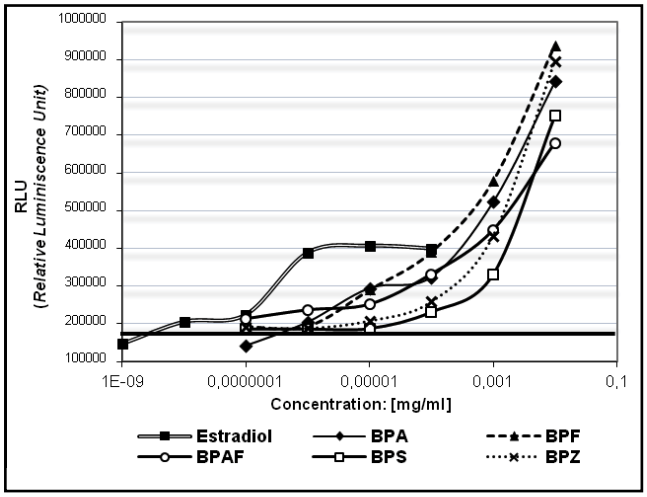


Fig. 6. Estrogenic activity of BPA and analogues in ER TA assay, OECD TG 455/457 (Luc gene expression).

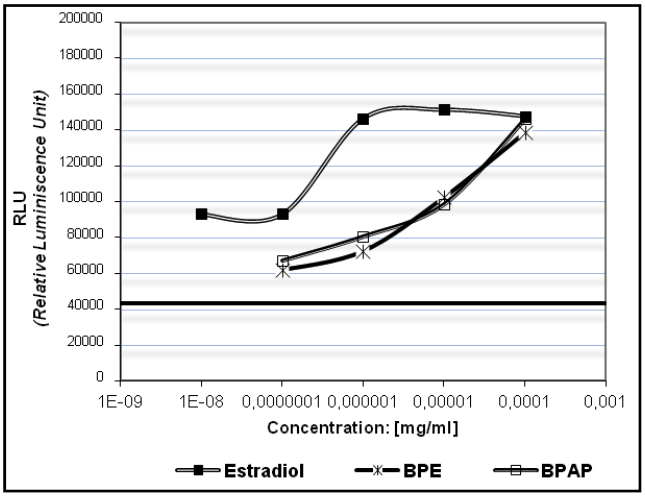


Fig. 7. Estrogenic activity of BPA and analogues in ER TA assay, OECD TG 455/457 (Luc gene expression).

estrogen receptor α, which is in accordance with previous observations that compounds with endocrine activity contain phenolic groups in para orientation with strongly reactive hydroxyl groups. Replacement of hydrogens in the methyl group by halogens (e.g. BPAF) may lead to enhancement of endocrine activity. With the use of long term knowledge about chemical structure activities it may be possible to develop useful analogous monomers of BPA with reduced endocrine activity (Coleman *et al.* 2003). Further testing of bisphenols containing multiple reactive alkyl substituents (e.g. bisphenol G, M, C, P, TMC) could contribute to clarification of their mechanisms of action in the endocrine system. Analogous chemicals, which are produced and available on the market, are suspected to be replacing those already regulated, e.g. BPA in thermal paper (Goldinger *et al.* 2015, Liao *et al.* 2012a), and thus should be of considerable interest for testing and evaluation. Chemicals with positive results in *in vitro* systems are suspected to cause adverse effects *in vivo* to living organisms in the environment and also to humans. Sensitive human populations should be instructed to avoid exposure to these pollutants, as many of them may be found in consumer products and in the environment (Liao *et al.* 2012a; Liao & Kannan 2013; Chen *et al.* 2016). Further testing of the presence of endocrine active substances and their metabolites with the use of human biological fluids should be performed and suitable strategies to monitor human exposure developed.

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