Short Communication

Assessing Oestrogenic Effects of Brominated Flame Retardants Hexabromocyclododecane and Tetrabromobisphenol A on MCF-7 Cells

(endocrine disruptors / BFR / MCF-7 cells / *TFF1*)

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Abstract. Tetrabromobisphenol A (TBBPA) is the main flame retardant used in printed circuit boards and laminates. The human population is highly exposed to TBBPA as it is used in consumer electronics as well as office and communication equipment. The main use of hexabromocyclododecane (HBCD) is in insulation foam boards, which are widely used in the construction sector. Brominated flame retardants may possess endocrine disrupting activity and thus represent a threat to the environment, including humans and their reproduction. The aim of this work was to evaluate the oestrogenic effects of TBBPA and HBCD *in vitro* on MCF-7 cells. We used the proliferation test (E-screen assay) in MCF-7 breast cancer cells and reverse transcription quantitative polymerase chain reaction analysis of *TFF1* gene expression to analyse oestrogenicity of the studied compounds. RT-qPCR has proved to be a fast and valuable molecular technique in gene expression quantification. HBCD but not TBBPA increased cell proliferation in MCF-7 cells and up-regulated *TFF1* gene expression in a concentration-dependent manner. Anti-oestrogen ICI 182,780 inhibited up-regulation of *TFF1* by HBCD. We have shown that HBCD displays oestrogen-like effects on MCF-7 cells. TBBPA, on the other hand, has not shown any oestrogenic effect mediated by the oestrogen receptor α.

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Abbreviations: ATCC – American Type Culture Collection, BFR – brominated flame retardant, DC-FBS – dextran charcoal-stripped foetal bovine serum, DeBDE – decabromodiphenyl ether, DMEM – Dulbecco's Minimal Essential Medium, E1 – oestrone, E2 – 17-ß-oestradiol, E3 – oestriol, EE2 – 17-α-ethynyloestradiol, ERα – oestrogen receptor α, FBS – foetal bovine serum, HBCD – hexabromocyclododecane, MTT – 3-(4,5--dimethylthiazolyl-2) 2,5-diphenyltetrazolium bromide, PPIA – peptidyl-prolyl *cis-trans* isomerase A, RT-qPCR – reverse transcription quantitative polymerase chain reaction, SVHC – substance of very high concern, TBBPA – tetrabromobisphenol A, *TFF1* – trefoil factor 1.

Introduction

Brominated flame retardants (BFRs) have proved to be cost-effective and the most efficient flame retardants in plastics and textiles. With a wide use of plastic materials, there are growing concerns about the expansion of BFRs in the environment, their resistance and biodegradation. Due to bromide substituents, BFRs are considered to be toxic, persistent and bioaccumulative in the environment (Birnbaum and Staskal, 2004). BFRs at different concentrations have been measured in indoor and outdoor air and dust samples (Abdallah et al., 2008), in water, sediments, and in sewage sludge (de Wit, 2002). BFRs are detected in plants and wildlife throughout the food chain, in human tissues, blood serum, and in breast milk of the exposed occupational populations and in general population (for review see Jenssen et al., 2007; Lignell et al., 2009).

The highest-volume brominated flame retardant in use today is tetrabromobisphenol A (TBBPA) followed by decabromodiphenyl ether (DeBDE) and hexabromocyclododecane (HBCD). The primary use of TBBPA is as a reactive intermediate in the production of flame-retarded epoxy resins used in printed circuit boards. TBBPA belongs to the aromatic class of reactive BFRs, is chemically bound into plastic, and therefore only a minor part of TBBPA can leach out of the material. HBCD, a major brominated cycloaliphatic flame retardant, is primarily used in polystyrene foam and textiles (www.bsef.com). HBCD, which belongs to the cycloaliphatic class of the additive BFRs, is blended with polymers and hence can more easily diffuse into the environment. Despite the fact that HBCD is a very effective flame retardant and is thus used in lower concentra-

tions than other BFRs, it was found in samples in remote areas. Now it is considered to be persistent and bioaccumulative and was identified as a Substance of Very High Concern (SVHC) in the REACH programme list of the European Union in 2008 (www.echa.europa.eu).

In this study, we examined the oestrogenic effect of TBBPA and HBCD on human breast cancer cell line MCF-7. In addition to the analysis with widely used cell proliferation assay, we performed a very sensitive and fast protocol for the measurement of expression of the endogenous oestrogen-dependent *TFF1* (trefoil factor 1) gene.

Material and Methods

Chemicals

Oestrone (E1), 17-ß-oestradiol (E2), oestriol (E3), 17-α-ethynyloestradiol (EE2), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA) and fulvestrant (ICI 182,780) were all from Sigma-Aldrich (Prague, Czech Republic).

Cell culture

Human oestradiol-dependent breast cancer cell-line MCF-7 (American Type Culture Collection, ATCC) was maintained in Dulbecco's Minimal Essential Medium (DMEM, Sigma-Aldrich, Prague, Czech Republic), supplemented with 10% foetal bovine serum (FBS, Sigma-Aldrich, 1% penicillin-streptomycin (Sigma-Aldrich) in T-75 cm^2 flasks at 37 °C, in an atmosphere of 5% CO_2/95% air under saturating humidity. Stock culture was passaged every 3–4 days using a trypsin 0.25%-EDTA 0.02% solution (Sigma-Aldrich).

MTT proliferation assay

The cell proliferation rate was estimated by a modification of the MTT [3-(4,5-dimethylthiazolyl-2) 2.5--diphenyltetrazolium bromide] assay (Denizot and Lang, 1986). Briefly, cells were maintained in 24-well plates with oestrogen exhausted for five days in phenol red-free DMEM supplemented with 10% dextran charcoal-stripped FBS (DC-FBS, either commercial from Sigma-Aldrich or prepared in our laboratory according to Jørgensen et al. (2000)) before treatment. The cells were then exposed to the tested compounds for six days, the medium was exchanged every second day. For a negative control, cells were treated with 10 nM anti-oestrogen ICI 182,780. Then the cells were incubated in tetrazolium MTT (Sigma-Aldrich), dissolved at a final concentration of 1 mg/ml in serum-free, phenol-red-free medium, for a further 4 h. MTT-formazan was solubilized in isopropanol and the optical density was measured at a wavelength of 570 nm and a reference wavelength of 690 nm.

RNA isolation and RT-qPCR

Cells were plated in 24-well plates at a density of 5×10^4 cells/well. The day after plating, culture medium was changed to phenol red-free DMEM supplemented with 10% DC-FBS. Plastic ware was chosen carefully to minimize medium contamination with xenoestrogenic compounds (Ishikawa et al., 2001). Starting on day 5, after changing to oestrogen-free medium, the tested compounds were added at a range of concentrations and cells were treated for a total of 36 h (Jorgensen et al., 2000). Total RNA samples were isolated from cell cultures using an RNeasy mini-kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. The concentration and purity of the purified RNA was determined by UV spectrophotometry and confirmed by agarose gel electrophoresis. cDNA was synthesized from 1 µg of total RNA using SuperScript® III Reverse Transcriptase (Invitrogen, Prague, Czech Republic) or RevertAid™ Reverse Transcriptase (Fermentas, Burmington, Canada) and used for qPCR amplification of the *TFF1* gene using specific primers (forward: 5'-GAATTGTGGTTTTCCTGGTGTC-3';reverse:5'-AGCAGCCCTTATTTGCACACT-3') in a Mastercycler ep realplex real-time PCR system. The qPCR conditions were: initial denaturation 15 min, followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s. After PCR a melting curve was constructed by increasing the temperature from 72 to 95 °C to ensure that the correct product was amplified in the reaction. PCR was repeated three times in doublets for each gene, and the average Ct and standard deviations were calculated. *PPIA* was used as a reference gene and the primers were: forward: 5'-TTCATCTGCACTGCCAAGAC-3' reverse: 5'-TCGAGTTGTCCACAGTCAGC-3'.

Results and Discussion

In this study we investigated the oestrogenic activity of two brominated flame retardants: TBBPA and HBCD, comparing them with oestrogenic activity of known oestrogens and an anti-oestrogen.

MTT assay

To confirm the oestrogen responsiveness of the MCF-7 cell line, we started experiments with the MTT proliferation assay. Both E2 and EE2 induced cell growth at 10 pM concentration. Anti-oestrogen ICI 182,780 (10 nM) was used as a negative control and completely inhibited cell growth. TBBPA had no effect on cell growth even at a maximum concentration of 20 µM. On the other hand, HBCD at higher concentrations 10–20 µM induced cell growth in an oestrogen-dependent manner and ICI 182,780 inhibited the HBCD effect (Fig. 1).

TFF1 gene expression

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) has become an established and powerful technique for gene expression studies. Relative quantification is a crucial and frequently used method to assess RT-qPCR data, while target gene ex-

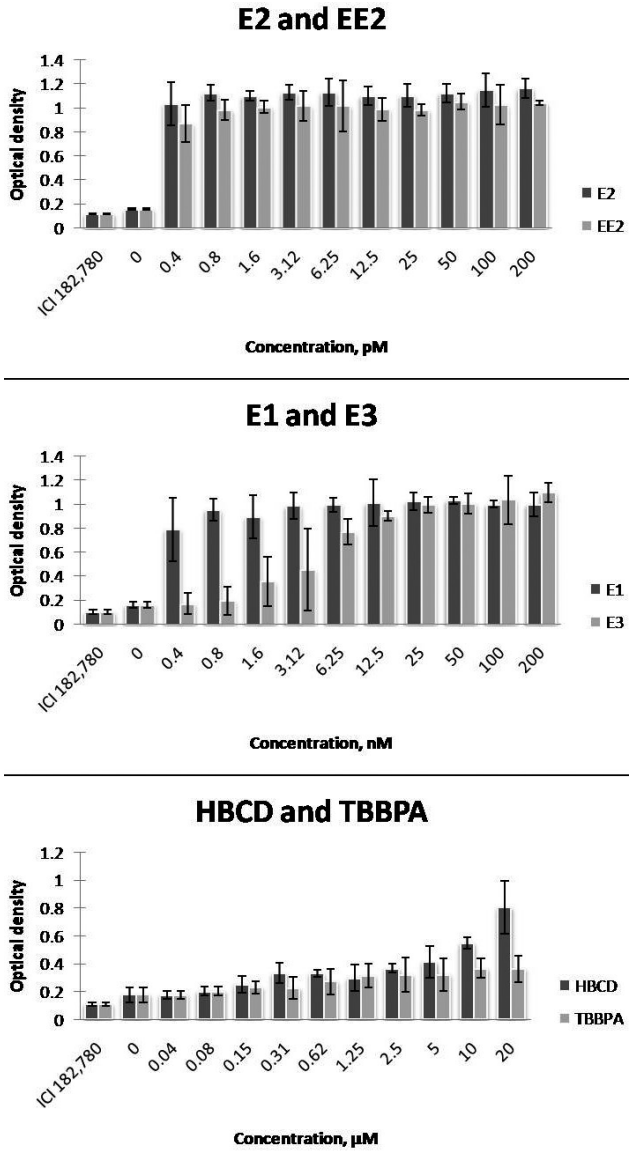

Fig. 1. Effect of E2, TBBPA and HBCD on MCF-7 cell proliferation. Cells were untreated (control), treated with 10 nM anti-oestrogen ICI 182,780, and treated with E2 in the concentration range 0.4 to 200 nM and with TBBPA and HBCD in the concentration range 40 nM to 20 µM. Numbers show optical density at 570 nm of solubilized MTT formazan.

pression levels are associated with a stably expressed internal reference gene determined in the same biological sample at the same time.

Up-regulation of the *TFF1* gene expression was used for the measurement of oestrogenicity because its expression is oestrogen receptor-mediated and its transcription is induced in MCF-7 cells by oestrogenic compounds. We detected an increase in the *TFF1* gene expression in MCF-7 cells for three natural oestrogens E1, E2 and E3 and one synthetic EE2. The highest levels of *TFF1* expression were observed at the concentrations of 20 pM and 200 pM for E2 and EE2, respectively. Both E1 and E3 have weaker oestrogenic activity and induced *TFF1* expression at the 1000-fold higher concentration of 200 nM. As expected based on the MTT proliferation assay, TBBPA had no effect on *TFF1* expression at the studied concentrations, whereas HBCD strongly up-regulated the expression of *TFF1* starting from 200 nM, reaching its peak at the concentration of 20 µM. In addition, ICI 182,780 (10 nM) abolished the oestrogenic effect of all studied compounds (Fig. 2).

TFF1, also known as *pS2*, is well recognized as a marker and control gene in the studies where the oestrogen-dependent gene expression is involved.

As TBBPA is produced at highest rates and HBCD is considered to be a persistent bioaccumulative, and as both of them are found ubiquitously in the environment, their potential effect on live organisms, including humans, has attracted a great deal of attention in recent years. The agonistic activity of TBBPA and HBCD on the thyroid hormonal activity has already been reported (Veldhoen et al., 2006; Saegusa et al., 2009; Sun et al., 2009). However, the data on their oestrogenic effect are still contradictory. It was shown that neither TBBPA nor HBCD display an oestrogenic effect on the vitellogenin serum level in rainbow trout (Ronisz et al., 2004). In another complex and systematic work on endocrine-disrupting potency of BFR, TBBPA and HBCD showed no oestrogenic activity in ER-CALUX assay (Hamers et al., 2006). On the other hand, TBBPA was shown to have weak affinity to the oestrogen receptor as well as a low estrogenic effect on MCF-7 cell proliferation, progesterone receptor and pS2 protein expression at higher concentrations of 30 µM (Olsen et al., 2003), but there is no further information on the potential oestrogenic effect of HBCD.

The structure of the ligand-binding site of the oestrogen receptors can precisely distinguish between oestrogens and androgens (Nahoum et al., 2003). However, due to its hydrophobic pocket nature, it often becomes the target for the synthetic endocrine disrupting compounds. Therefore, we studied whether HBCD and TBBPA can influence the oestrogen-responsive *TFF1* gene expression.

Our results show that TBBPA does not induce *TFF1* gene expression *in vitro.* On the other hand, HBCD induced both *TFF1* expression and MCF-7 cell proliferation at 20 and 200 nM concentrations. These concentrations are 10,000-fold higher than for natural oestrogen, E2, and at least 100-fold higher than the average concentration in the environment. Nevertheless, it shows that HBCD can influence live organisms by inducing expression of oestrogen-regulated genes. On the other hand, our results do not exclude the effect of TBBPA on live organisms. There are *in vivo* studies showing reproductive toxicity of TBBPA (Van der Ven et al., 2008), possibly by TBBPA binding to other nuclear receptors or by oestrogenic activity of its metabolites. In addition, it was also shown that hydroxylated products of TBBPA inhibit oestrogen sulphotransferase and can thus increase the oestrogen level in the organism (Kester et al., 2002).

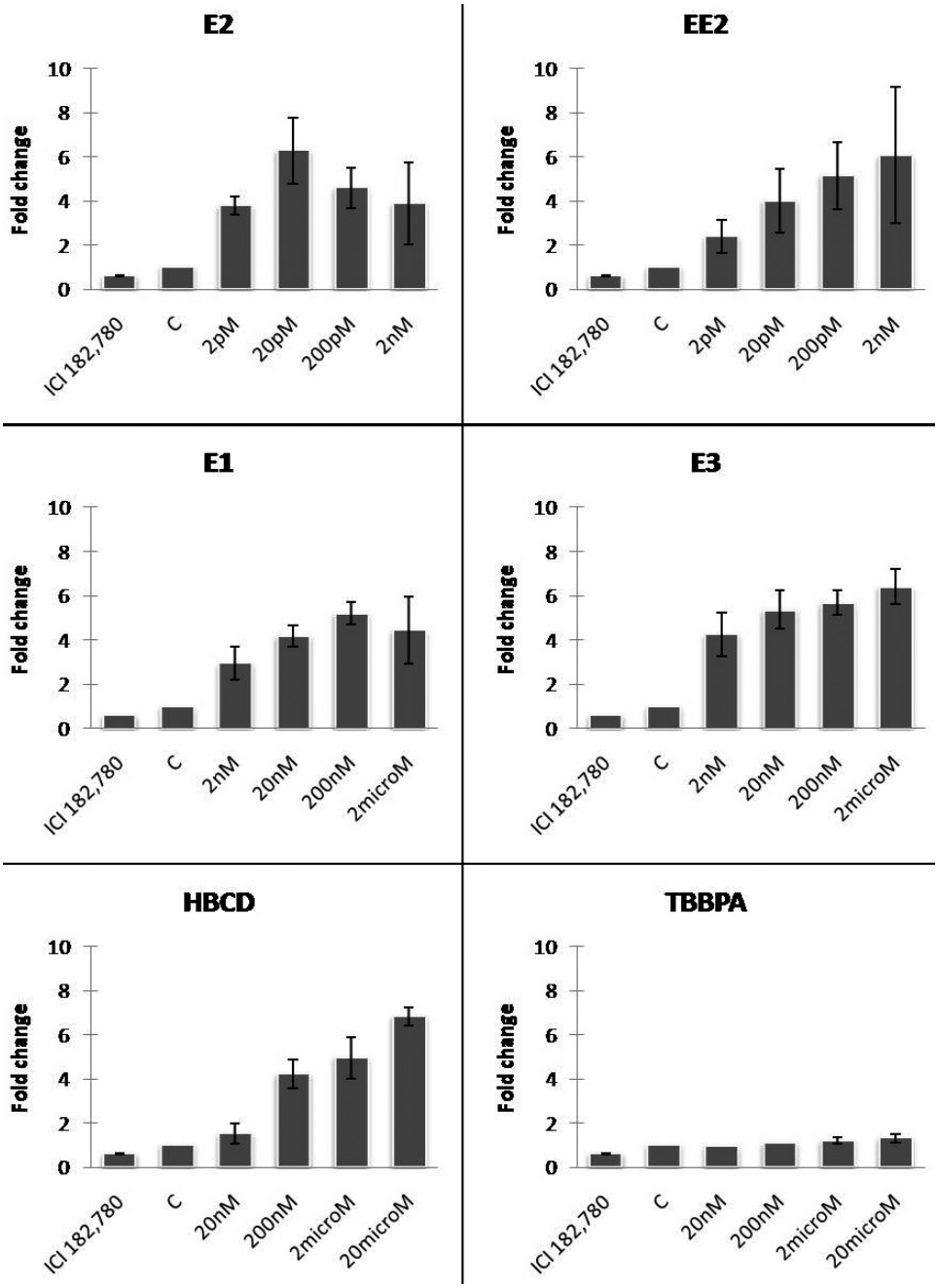

Fig. 2. Expression of the *TFF1* gene. Relative gene expression in untreated cells, cells treated with ICI 182,780 and a range of concentrations of the studied compounds. The values are the means of double determinations on each of the three replicate exposures, and are presented as the mean ± SEM.

To sum up, our current study demonstrated that *TFF1* gene expression data were in agreement with the MTT proliferation assay and that HBCD at higher concentrations induced both proliferation of MCF-7 cells and expression of the *TFF1* gene, thus displaying oestrogenic properties. Therefore, HBCD has the potential to disrupt the endocrine system. TBBPA, on the other hand, has not shown any oestrogenic effect at any measured concentration.

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The authors declare that they have no competing financial interests.

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