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MOLECULAR TOXICOLOGY

Minor structural modifications of bisphenol A strongly affect physiological responses of HepG2 cells

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Abstract

Bisphenols represent a large group of structurally similar compounds. In contrast to bisphenol A (BPA) and bisphenol S (BPS), however, toxicological data are usually scarce, thus making bisphenols an ideal candidate for read-across assessments. BPA, bisphenol C (BPC) and a newly synthesized bisphenol A/C (BPA/C) differ only by one methyl group attached to the phenolic ring. Their EC_{50} values for cytotoxicity and $\log P_{OW}$ values are comparable. However, the estrogenic activities of these bisphenols are not comparable and among this group only BPC leads to a decrease of the mitochondrial membrane potential and ATP concentration in HepG2 cells. Conversely, the cell division rate was decreased by BPS, BPA, BPC and BPA/C at 10% toxicity (EC_{10}). At lower concentrations, only BPC significantly affected proliferation. The pro-inflammatory cytokines *TGFB1* and *TNF* were significantly upregulated by BPC only, while *SPP1* was upregulated by BPA, BPA/C and BPS. BPC led to the release of cytochrome c from mitochondria, indicating that this compound is capable of inducing apoptosis. In conclusion, the read-across approach revealed non-applicable in the case of the various structurally and physicochemically comparable bisphenols tested in this study, as the presence of one or two additional methyl group(s) attached at the phenol ring profoundly affected cellular physiology.

Keywords 2,2-*bis*(4-hydroxyphenyl) propane · 2,2-*bis*(4-hydroxy-3-methylphenyl) propane · 4,4′-Sulfonyl-diphenol · Mitochondria · Intrinsic apoptosis · HepG2 cells

Abbreviations

BPA	Bisphenol A
BPC	Bisphenol C
BPS	Bisphenol S
BPA/C	Bisphenol A/C
EC_{50}	Half maximal effective concentration
EC_{10}	10% of the effective concentration
$\Delta\Psi_m$	Mitochondrial membrane potential
CyC	Cytochrome c
BCL2	B cell lymphoma 2
SPP1	Osteopontin
TNF	Tumor necrosis factor α
TGFB1	Transforming growth factor β

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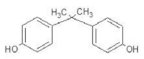
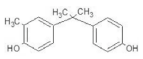
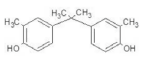
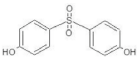
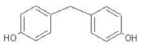
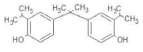
Introduction

Bisphenol A (BPA, see Table 1) is used in different consumer products. At high nanomolar concentrations it exhibits estrogenic activity, as well as liver and kidney toxicity (EFSA 2017). Therefore, BPA is often replaced by bisphenol S (BPS) in its manufacture (Rochester and Bolden 2015). The presence of BPS has been reported in thermal paper, advertised as "BPA-free" (Liao et al. 2012). The presence of bisphenol C (BPC) has been detected in bottled carbonated beverages (Mandrah et al. 2017) as well as in waste water (Cesen et al. 2018). In MCF7 cells BPS and BPA showed a lower estrogenic activity compared to BPC (Kitamura et al. 2005). The human serum levels of BPA are 0.002 µM (Kuroda et al. 2003) and the European Food Safety Authority (EFSA) published a concentration level of up to 1 µg/l (0.004 µM) (EFSA 2015). Interestingly, in the urine of cashiers a twofold increase of BPA (up to 2.76 µg/g creatinine) and a slight increase of BPS (up to 0.54 µg/g creatinine) compared to non-cashiers has been determined, dependent upon the use of thermal paper (Thayer et al. 2016). So far there are no exposure data

Table 1 Structure, IUPAC name, molecular weight (MW), CAS number, distribution coefficient ($\log P_{OW}$), 50% (EC_{50}) and 10% (EC_{10}) of the effective concentration of the bisphenol A (BPA), bisphenol A/C (BPA/C), bisphenol C (BPC), bisphenol S (BPS), bisphenol F (BPF) and bisphenol G (BPG)

		BPA	BPA/C	BPC	BPS	BPF	BPG
Structure							
IUPAC name		2,2-*bis*(4-hydroxyphenyl) propane	2-(4-hydroxy-3-methylphenyl)-2-(4'-hydroxy-phenyl)propane	2,2- *bis*(4-hydroxy-3-methylphenyl) propane	4,4′-Sulfonyl-diphenol	4,4′-Methylene-diphenol	2,2- *bis*(4-hydroxy-3-isopropyl-phenyl)propane
CAS		80-05-7	14151-63-4	79-97-0	80-09-1	620-92-8	127-54-8
$\log P_{OW}$	[-]	3.64	4.06	4.46	1.66	2.89	6.00
MW	[g/mol]	228.29	242.31	256.34	250.27	200.23	312.45
EC_{50}	µM	261	153	196	2061	735	59
EC_{10}	µM	131	60	117	284	224	21

available for BPC. To close this gap, a European human biomonitoring program has been initiated (https://www.hbm4eu.eu/). The presence of different bisphenols in consumer products underlines the need for further risk characterization. The European Chemical Agency (ECHA) has evaluated BPA as substance of very high concern (SVHC) (EU (Nr. 1272/2008) due to its reprotoxic properties (Repr. 1B) and in June 2017 the Member State Committee of ECHA agreed to classify BPA as an endocrine disruptor (ECHA 2018b). Furthermore, it is classified as potential skin sensitizer (Skin Sens. 1), as well as a respiratory (STOT SE 3) and potential eye (Eye Dam. 1) irritant (ECHA 2017c). The EFSA identified several 'likely' target tissues of BPA toxicity, being liver as well as kidney and mammary glands (proliferation). Effects on these organs were used for risk characterization (EFSA 2015). Based on the 'likeliness' of effects the EFSA has derived a temporary tolerable daily intake (tTDI) of 4 µg/kg BW/day. The general toxicity of BPA to liver and kidney as well as reproductive, developmental, neurological, immune, metabolic and cardiovascular toxicity, mammary gland changes, carcinogenicity and genotoxicity are currently re-evaluated (EFSA 2017).

BPC has been registered under REACH, resulting in labels for skin irritation (Skin Irrit. 2), serious eye damage (Eye Irrit. 2) and respiratory irritancy (STOT SE 3) (ECHA 2018d). In contrast, BPS has been fully registered at ECHA, is listed on the Community Rolling Action Plan (CoRAP), and has a self-classification for harmfulness against aquatic life with long-lasting effects (Aquatic Chronic 3), as well as for serious eye irritancy (Eye Irrit. 2) (ECHA 2018a). Structurally, these bisphenols are quite similar: BPC differs from BPA only by two additional methyl groups on either phenolic ring. BPS is a close analog of BPA as well, in which the dimethyl methylene group ($C(CH_3)_2$) is replaced by a sulfonyl functional group (SO_2)).

One accepted method to validate the potential risk, is the read-across assumption, which can be used to substitute for data gaps of a certain target substance using information from analogous substances (ECHA 2017b). Choosing an appropriate model for the route of metabolism is essential to fill these data gaps. The first evidence that described the route of metabolism demonstrated the involvement of the enterohepatic cycle in the metabolism of BPA in rats (Doerge et al. 2010). The enterohepatic cycle is responsible that the same molecule of a potentially toxic substance is metabolized several times in the liver (Malik et al. 2016). Due to this repeated tissue exposure, hepatocytes may be strongly affected by BPA. According to EFSA, liver is one of the main target organs of BPA toxicity (EFSA 2015). Hepatic cell lines are a suitable model for the investigation of potentially adverse effects. Based on a dose-dependent increase of DNA stand breaks observed in hepatoblastoma HepG2 cells, a recent study claimed that BPA and BPS, but not BPC, were genotoxic (Fic et al. 2013). Yet, it is still generally accepted that BPA is not genotoxic (EFSA 2015). One reason might be that different bisphenols can have different effects on various hepatic cell lines.

The aim of this study was to apply a read-across approach using BPA as source substance and the structurally related BPC and BPA/C as target substances. The outcome has been compared with the results obtained in vitro by exposing HepG2 cells to the respective compounds. Mechanistic investigations were performed to evaluate whether a read-across approach is suitable in this case, or not.

Materials and methods

Chemicals and antibodies

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless stated otherwise. Furthermore, Dulbecco's phosphate-buffered saline (DPBS) (PAN-Biotech, Aidenbach, Germany) and Bisphenol A/C (BPA/C) (Angene, London, UK) was used. The following antibodies were used: β-actin (AC-15), α-tubulin, cytochrome C (EPR1327), mitofilin (2E4AD5) (all from Abcam, Cambridge, UK) and ERα (F-10) (Santa Cruz Biotechnology, Heidelberg, Germany).

Cell culture

All single-use plastics were purchased from TPP (Trasadingen, Switzerland) and all cell lines were purchased from DSMZ (Braunschweig, Germany). HepG2 cells were grown in RPMI 1640 (PAN-Biotech, Aidenbach, Germany) containing 10% (v/v) FCS (PAN-Biotech, Aidenbach, Germany), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine at 37 °C and 5% CO_2. THP-1 cells were differentiated under the influence of 100 nM phorbol-12-myristate-13-acetate (PMA) for 24 h. THP-1 cultivation and the co-culture were done according to Wewering et al. (2017). When THP-1 and HepG2 cells were combined, the bisphenol treatment started immediately. MCF7 cells were purchased from American Type culture collection (Manassass, VA, USA) and were grown in DMEM (PAN-Biotech, Aidenbach, Germany) containing 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine at 37 °C and 5% CO_2. Hela9903 cells were purchased from JCRB cell bank (JCRB-No. 1318) (Tokio, Japan) and were cultivated according to Tarnow et al. (2013).

Cytotoxicity testing

The MTT Assay was performed with minor modifications according to Mosmann (1983). All values were corrected for the DMSO solvent controls. All substances were tested in a concentration range using $\log_2$ serial dilutions [BPA (1.75 mM–0.22 µM), BPA/C (1.6 mM–54.75 µM), BPC (1.56 mM–0.22 µM), BPS (6.39 mM–0.88 µM), BPF (1.99 mM–0.44 µM), BPG (1.28 mM–0.22 µM)].

Mitochondrial membrane potential

Cells were seeded at a density of 1.3×10^6 cells per cm^2. After treatment for 24 h, cells were detached using trypsin/EDTA (PAN-Biotech, Aidenbach, Germany). Before staining the cells were incubated for 2 h at 37 °C and 5% CO_2 with 100 µM of the decoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP). The mitochondrial membrane potential ($\Delta\Psi$) was determined via incubation with 30 µM JC-10 (Adipogen, Liestal, Switzerland) for 30 min at 37 °C. Fluorescence intensities were measured with the FACS Aira III (PE channel settings: 585/42 nm, FITC channel settings: 530/30) (BD Biosciences, Heidelberg, Germany) and the cells were analyzed with the software FlowJo v 10 (FlowJo LLC, Ashland, OR, USA).

ATP measurements

Cells were seeded at a density of 1.3×10^6 cells per cm^2. After treatment for 24 h, the ATP concentration was determined using the Bioluminescence Assay Kit HS II (Roche, Basel, Switzerland) according to the manufacturer's protocol.

Cell division analysis

Cells were seeded at a density of 0.75×10^5 cells per cm^2. After 48 h the cells were stained for 30 min at room temperature with 5(6)-carboxyfluorescein *N*-hydroxysuccinimidyl ester (CFSE) (Cayman, Hamburg, Germany). Staining was terminated with 10% FCS in phosphate-buffered saline (PBS) followed by washing with DPBS. Fluorescence was measured with the FACS Aria III (FITC channel settings: 530/30) (BD Biosciences, Heidelberg, Germany) and analyzed with the FlowJo v 10 (FlowJo LLC, Ashland, OR, USA).

PCR analysis

RNA was isolated using the NucleoSpin RNA Kit (Machery-Nagel, Düren, Germany) and the reverse transcriptions were performed with the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed with 7500 Fast Real-Time PCR Instrument using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The primer sequences are listed in Table 2. The $\Delta\Delta C_T$-value was calculated according to Livak and Schmittgen (2001) and normalized to the expression of hypoxanthine–guanine phosphoribosyltransferase (*HPRT*) in control (DMSO treated) cells.

Elisa

HepG2 cells and THP-1 cells were seeded at densities of 1.3×10^6 cells per cm^2 and of 0.65×10^6 cells per cm^2, respectively. TNF-α in the supernatant of cell culture was

Table 2 Primer sequences used

Gene	Forward primer	Reverse primer	Product
HPRT	gttctgtggccatctgcttag	gcccaaagggaactgatagtc	144 bp
BCL2	gaggattgtggccttctttg	acagttccacaaaggcatcc	170 bp
TGFB1	gtggaaacccacaacgaaat	cacgtgctgctccacttttta	165 bp
SPP1	gccgaggtgatagtgtggtt	ctcctcgctttccatgtgtg	119 bp
TNF	cttctgcctgctgcactttggag	ggctacaggcttgtcactcgg	130 bp

The gene symbol, the sequence of the forward and reverse primers and the product size in base pairs (bp) is given

measured using the DuoSet® ELISA Human TNF-α (R&D Systems, Abingdon, UK) according to the manufacturer's protocol.

Western-blot

Cells were lysed at 4 °C with RIPA buffer (50 mM Tris/HCl (pH 7.4), 159 mM NaCl, 1 mM EDTA, 1% Igepal®, 0.25% sodium deoxycholate, and protease inhibitor cocktail). Mitochondrial isolation (Clayton and Shadel 2014a) and purification was performed according to Clayton and Shadel (2014b) including lysis with tight fit douncer, ultracentrifugation using a sucrose step density gradient. The enrichment of the mitochondrial fraction was validated in pooled samples (Figure S1). Protein concentration was measured with the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) and equal amounts of protein were applied to SDS-PAGE transferred onto nitrocellulose membranes according to the manufacturer's instructions. Primary anti-bodies were labelled with the corresponding horseradish peroxidase coupled secondary antibody and visualized with Pierce ECL Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Estrogen activity

Estrogenic activity was measured according to TG 455 (OECD 2016).

Statistics

All shown data contain at least three independent biological replicates. Means, standard deviations and the p values of the ANOVA followed by Bonferroni correction were calculated with GraphPad Prism 6 (Statcon, Witzenhausen, Germany). The effective concentrations (EC_{50}, EC_{10} and EC_{50E}) were calculated under the programming environment R (R Core Team 2018) according to Wewering et al. (2017). The distribution coefficient ($\log P_{ow}$) was calculated with Gastro Plus™ version 9.5 (Simulations Plus, Lancaster, CA, USA).

Results

Physicochemical properties and toxicity of bisphenols in HepG2 cell cultures

BPA/C, BPC and BPS are of comparable molecular weights (MW), that is, between 242.31 g/mol and 256.34 g/mol (Table 1). In contrast, BPA and BPF are of lower MW, being 228.29 g/mol and 200.23 g/mol, respectively. In this series of compounds, only the MW of BPG is greater than 300 g/mol. The oil/water distribution coefficients ($\log P_{ow}$) of BPA, BPA/C and BPC are in the range of 3.64–4.46 (Table 1). BPS and BPF exhibit lower $\log P_{OW}$ values of only 1.66 and 2.89, respectively, while BPG had the highest $\log P_{OW}$ value of 6.0. In summary BPA, BPA/C, BPC have similar molecular structures, similar molecular weights and comparable $\log P_{OW}$ values. A closer look shows that the $\log P_{OW}$ of these three bisphenols increases with increasing numbers of methyl groups (BPA < BPA/C < BPC), making these bisphenols ideal candidates for a read-across approach.

To support the read-across approach further, the cytotoxicity of bisphenols was studied in vitro. HepG2 cells were incubated with different concentrations of BPA, BPA/C, BPC, BPG, BPF and BPS and half maximal effective concentrations (EC_{50}) were determined (Fig. 1). BPA and BPC showed EC_{50} values of 261 ± 27 µM and 196 ± 53 µM, respectively. BPA/C was characterized by an EC_{50} value of 153 ± 8 µM, BPS was found about tenfold less toxic compared to BPC with an EC_{50} value of 2061 ± 1580 µM. The cytotoxicity of BPF was between BPA and BPS with an EC_{50} of 735 ± 412 µM. The most toxic bisphenol investigated in our study was BPG with an EC_{50} of 59 ± 22 µM. In summary, the EC_{50} values of the six bisphenols investigated correlated roughly with the respective $\log P_{OW}$ values. Significant differences ($p \leq 0.01$) were detectable between EC_{50} values of BPA and BPA/C but not for BPA and BPC or BPA/C and BPC. The four compounds BPA, BPA/C, BPC, BPS were selected for further investigation, either based on their relevance as contaminant of food contact materials (BPA, BPS, BPC) or based on their structural and physicochemical similarities (BPA/C). For further experimental studies, the EC_{10} values were calculated, based on the experimental data (Figure S2, Table 1).

Bisphenol treatment decreases cell division rate

BPA affects the cell division rate in vitro, depending on substance concentration and the cell line. Previously, a BPA-mediated increase of cell proliferation has been

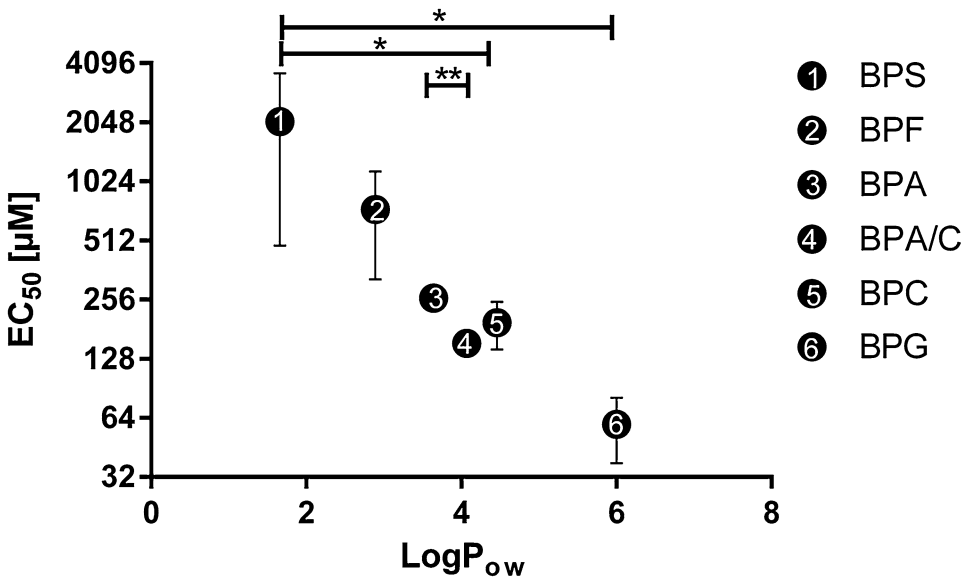


Fig. 1 Correlation between the logP_{OW} and the EC_{50}. HepG2 cells were treated with bisphenol S (BPS), bisphenol F (BPF), bisphenol A (BPA), bisphenol A/C (BPA/C), bisphenol C (BPC) and bisphenol G (BPG) for 24 h ($n=3$). The mean and the SD of the half maximal effective concentration (EC_{50}) were calculated under the program environment R and the oil/water distribution coefficient (logP_{OW}) with Gastro Plus™ version 9.5. *$p<0.05$, **$p<0.01$

observed in MCF7 cells due to estrogen receptor (ER) activation (Potratz et al. 2017). Conversely a BPA-mediated decrease in cell proliferation was observed in murine osteosarcoma cells (Kidani et al. 2017). In our study, we looked into the effects of bisphenols on HepG2 cell division rates. Low, non-toxic concentrations (EC_{10}) were used for cell treatments (see Table 1). At EC_{10} the cell division rates were significantly ($p \leq 0.01$) reduced by 0.37-, 0.63- and 0.41-fold for BPA, BPA/C and BPC, respectively (Fig. 2b). In contrast, BPS did not change the cell division rate of HepG2 cells. At the dose of one-third of the EC_{10}, that is, 44 µM of BPA, 19 µM of BPA/C, 39 µM of BPC, and 95 µM of BPS, only BPC decreased the cell division rate significantly ($p \leq 0.01$) 0.65-fold, compared to the vehicle control (Fig. 2a).

Bisphenol C decouples the mitochondrial membrane potential $\Delta\Psi_m$

There is evidence that the mitochondrial membrane potential and the oxygen consumption changes in different cell types (Jurkat, HeLa and HEK-293T) during early and late phase of the cell cycle (Schieke et al. 2008). Therefore, we were interested to determine whether changes in the cell division rate were linked to the mitochondrial membrane potential ($\Delta\Psi_m$) in HepG2 cells. The EC_{10} and one-third of EC_{10} were used to study the effect on the $\Delta\Psi_m$. Only the BPC-treated

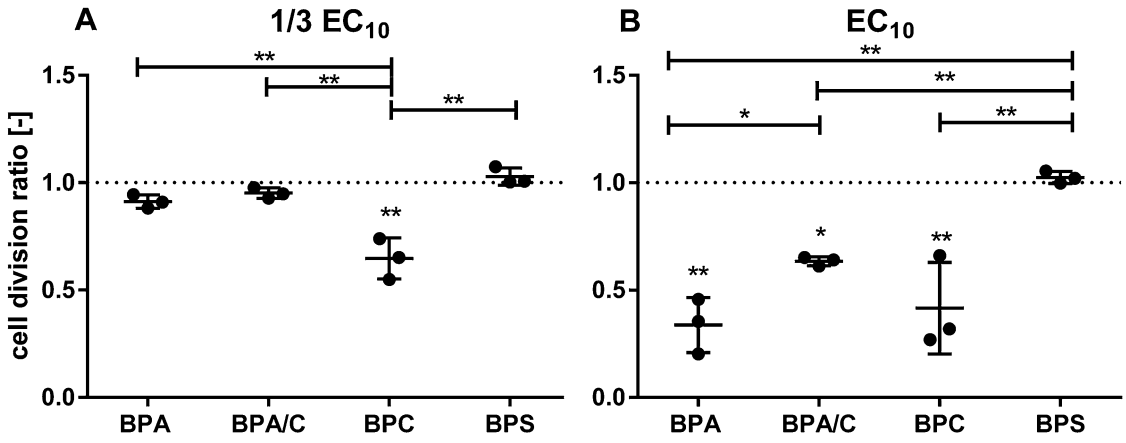


Fig. 2 Cell division rate is affected by bisphenols. Effects of different bisphenols on the cell division rate of HepG2 cells after 48 h. HepG2 cells were treated with bisphenol A (BPA), bisphenol A/C (BPA/C), bisphenol C (BPC) and bisphenol S (BPS) for 48 h ($n=3$). Measurements (•), mean (——) and SD (−) of the cell division related to solvent control (•••) are shown. Treatment concentrations: **a** 1/3 of 10% of the effective concentration (EC_{10}) and **b** the EC_{10}. *$p<0.05$, **$p<0.01$

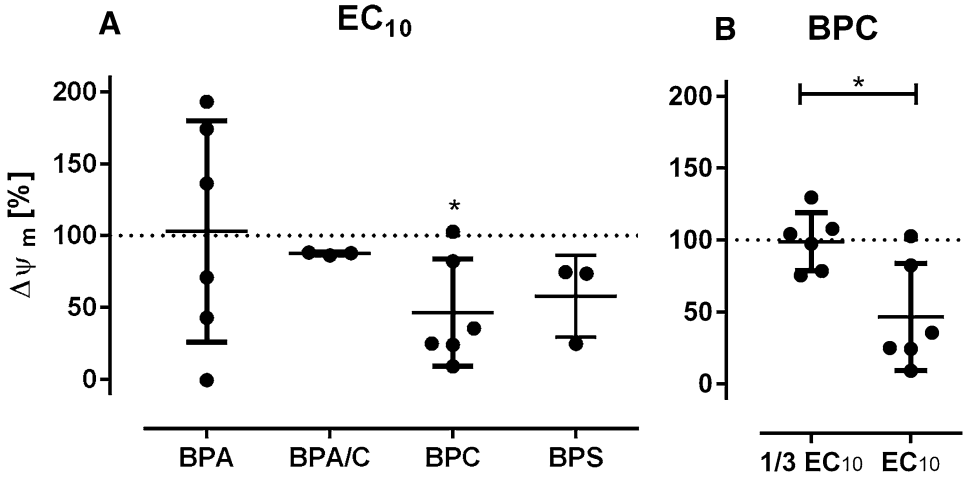


Fig. 3 Effects of different bisphenols on mitochondrial membrane potential $\Delta\Psi$. HepG2 cells were treated with bisphenol A (BPA), bisphenol A/C (BPA/C), bisphenol C (BPC) and bisphenol S (BPS) for 24 h ($n=3$). Measurements (•), mean (—) and SD (−) of the mitochondrial membrane potential ($\Delta\Psi_m$) related to solvent control (•••) and based to the positive control (carbonyl cyanide 3-chlorophenylhydrazone, CCCP) are shown. **a** Shown are the 10% of effective concentration (EC_{10}) and **b** one-third of EC_{10} and the EC_{10} of BPC. $^*p<0.05$

cells showed a significant decrease in $\Delta\Psi_m$ (Fig. 3a). This decrease of $\Delta\Psi_m$ was dependent on the BPC concentration applied. The $\Delta\Psi_m$ value at the EC_{10} of BPC significantly decreased to $46\pm37\%$ ($p\leq0.05$) in HepG2 cells when compared to the treatment with one-third of EC_{10} (Fig. 3b). In summary, BPA, BPA/C and BPS did not reduce the $\Delta\Psi_m$, while BPC had a strong effect on this cellular parameter.

BPC reduces the intracellular ATP concentration

The $\Delta\Psi_m$ is used for the generation of ATP. Therefore, we asked whether changes in $\Delta\Psi_m$ would affect the ATP levels in the cells. Neither BPA, BPA/C nor BPS treatment led to a significant reduction of the intracellular ATP concentration compared to the solvent control (DMSO). In contrast a significant 5-fold ($p\leq0.01$) reduction occurred after treatment with BPC at EC_{10} after 24 h (Fig. 4).

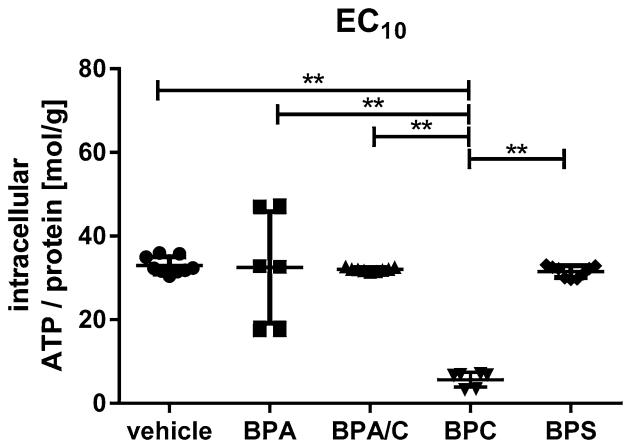


Fig. 4 Effects of bisphenols on intracellular ATP levels, normalized to the total protein. HepG2 cells were treated with 10% of the effective concentration (EC_{10}) of bisphenol A (BPA), bisphenol A/C (BPA/C), bisphenol C (BPC), bisphenol S (BPS) and dimethyl sulfoxid (vehicle control) for 24 h ($n=3$). Measurements (•), mean (—) and SD (−) of the ATP concentration per g total protein are shown from 3 biological replicates. $^{**}p<0.01$

Cytokine gene expression differs after exposure to different bisphenols

The intracellular ATP concentration might be associated with inflammation. It has already been shown that treatment of neutrophils with nonsteroidal anti-inflammatory drugs, like flufenamic acid, reduces the intracellular ATP levels (Manica et al. 2018). Therefore, the expression profiles of the cytokines *TNF*, *TGFB1* and *SPP1* (osteopontin) were investigated to study the pro- or anti-inflammatory effects of the bisphenols in focus. The pro-inflammatory cytokine osteopontin participates in several adaptive and innate immune responses, including the migration of macrophages and T-helper (Th) cells and the proliferation and survival of Th cells (Clemente et al. 2016). TGF-β (*TGFB1*) represents a cytokine with both pro- and anti-inflammatory effects depending on the physiological conditions (Worthington et al. 2012). HepG2 cells were treated at the EC_{10} of bisphenols and the gene expression was quantified 24 h later (Fig. 5). The *TGFB1* expression of HepG2 cells exposed to BPA, BPC and BPS was significantly ($p\leq0.01$) upregulated (between 1.4- and 6-fold) compared to the vehicle control. By contrast, treatment with BPA/C did not affect the expression of *TGFB1* compared to solvent control (Fig. 5a). In

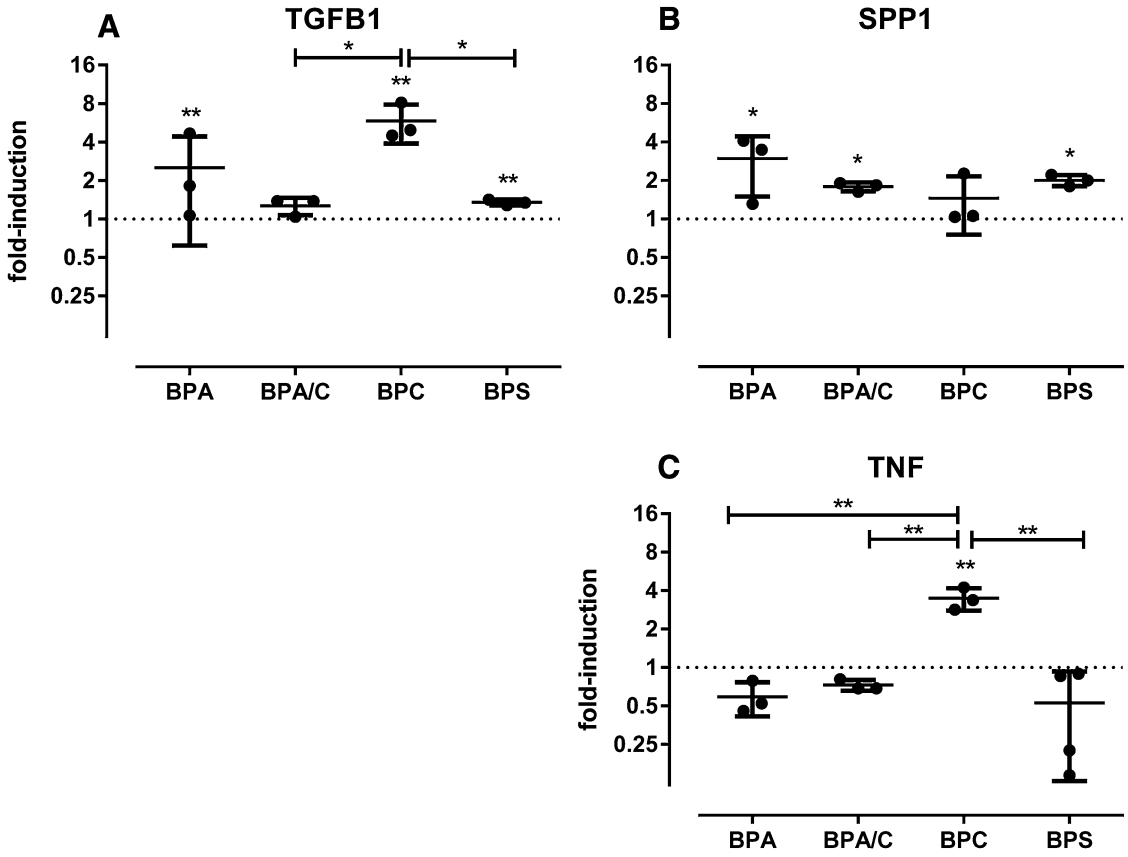


Fig. 5 Expression of cytokines in HepG2 under the influence of bisphenols. HepG2 cells were treated with 10% of the effective concentration of bisphenol A (BPA), bisphenol A/C (BPA/C), bisphenol C (BPC) and bisphenol S (BPS) for 24 h ($n=3$). Measurements (•), mean (——) and SD (−) of the expression of **a** the transforming growth factor β 1 (*TGFB1*), **b** osteopontin (*SPP1*) and **c** tumor necrosis factor (*TNF*) α related to solvent-control (•••) and *HPRT* ($\Delta\Delta C_T$) are shown. $^*p<0.05$, $^{**}p<0.01$

addition, treatment of HepG2 cells with BPA, BPA/C or BPS resulted in a comparable up-regulation of *SPP1* (two and threefold), while BPC had no effect (Fig. 5b). Interestingly, BPC caused a significant ($p\leq0.01$) threefold up-regulation of *TNF*, the gene encoding for TNF-α. In contrast, BPA, BPA/C and BPS had no significant effect (Fig. 5c).

At pro-inflammatory cellular conditions bisphenols alter the expression of BCL2

Under single cell culture conditions treatment of HepG2 cells with bisphenols changed the expression of selected cytokines (Fig. 5). Here, we wanted to investigate cytokine expression in a co-culture system under pro-inflammatory conditions. It has been shown before, that this co-cultivation resulted in the release of chemokines and cytokines such as CCL3, IL-1α, and CXCL8 (Wewering et al. 2017).

The protein BCL-2 (*BCL2*) is commonly known as inhibitor of the intrinsic apoptotic pathway (Czabotar et al. 2014). The expression levels of *BCL2* in single cell cultures of HepG2 cells were not affected by any of the bisphenols applied (data not shown). However, this expression profile changed when cells were cultivated in a pro-inflammatory environment (Fig. 6a). BPA (EC_{10}) led to a significant ($p\leq0.05$) up-regulation (1.8-fold) of *BCL2* in co-cultivated HepG2 cells compared to the solvent control (Fig. 6a). BPS and BPA/C showed no effect on the expression of *BCL2* compared to cells treated with the vehicle, while HepG2 cells treated with BPC (EC_{10}) showed a fourfold down-regulation of *BCL2*. In summary, an inflammatory environment in combination with bisphenol treatment seems to affect the up- or down-regulation of key genes of the intrinsic apoptotic pathway.

TNF-α secretion is affected by bisphenols

While BCL2 is a known inhibitor of the intrinsic apoptotic pathway, the cytokine TNF-α is known as pro-apoptotic (van Horssen et al. 2006) and essential pro-inflammatory mediator (Sedger and McDermott 2014). Consequently, the TNF-α secretion of bisphenol-treated HepG2 and PMA-differentiated THP-1 kept in co- and single-culture (Fig. 6b) was

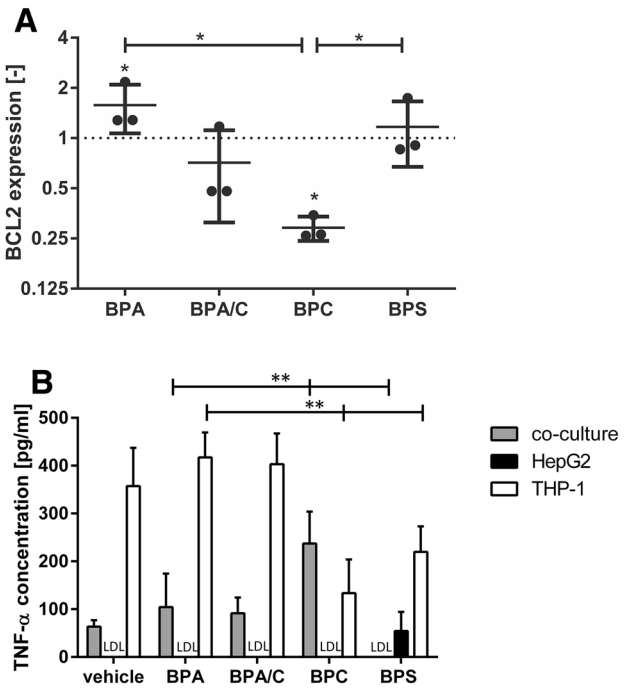


Fig. 6 Pro-inflammation model under influence of bisphenols. HepG2 cells were co-cultivated with PMA-activated THP-1 cells and treated with the EC_{10} of bisphenol A (BPA), bisphenol A/C (BPA/C), bisphenol C (BPC) and bisphenol S (BPS) for 24 h. **a** Measurements (•), mean (——) and SD (−) of the expression of the B-cell lymphoma 2 (*BCL2*) related to solvent-control (•••) and *HPRT* (ΔΔCT) are shown ($n=3$). **b** Concentration of tumor necrosis factor α (TNF-α) in the supernatant of single- and co-cultivated HepG2 and PMA differentiated THP-1 cells ($n=4$). LDL indicates values below the limit of detection of 7.8 pg/ml. *$p<0.05$, **$p<0.01$

determined. To exclude combinatory effects of bisphenols and cytokines already secreted due to the co-culture conditions, the bisphenols were added at the onset of co-cultivation conditions. PMA-differentiated THP-1 cells secrete 404 ± 59 pg/ml TNF-α. After BPC or BPS treatment the secretion is significantly ($p<0.01$) reduced to 133 ± 70 pg/ml and 220 ± 53 pg/ml, respectively. In contrast, no TNF-α was detectable in the single culture of HepG2 cells, except for BPS treatment (TNF-α: 54 ± 40 pg/ml). In general, TNF-α secretion at co-cultivation conditions was higher 64 ± 13 pg/ml to 237 ± 67 pg/ml when compared to the single cultivation conditions of HepG2 and lower as in the single cultivation of PMA-differentiated THP-1. However, under the influence of BPC, the concentration of TNF-α in co-cultivation are comparable to PMA-differentiated THP-1 cells alone (237 ± 67 pg/ml and 133 ± 70 pg/ml). TNF-α levels in the supernatant of co-cultures was not detectable after treatment with BPS (Fig. 6b). In brief, the TNF-α amount is thus dependent on the cultivation conditions and on the kind of bisphenol treatment. BPC increases the secretion of the pro-apoptotic TNF-α in co-cultivated HepG2 cells thereby promoting apoptosis. This coincides with the concomitant decrease in the expression level of the anti-apoptotic BCL2 protein.

Bisphenol C changes intracellular cytochrome c localization

The activation of the intrinsic apoptotic pathway can result in a cytochrome c (CyC) release from mitochondria (Ichim and Tait 2016). This results in a decreased mitochondrial CyC concentration, while the total cellular amount of CyC remains constant. An activation of the intrinsic apoptotic pathway was studied by comparison of the CyC concentration in the mitochondrial fraction (Figure S1) and the total cell lysate (Fig. 7a). Only BPC treatment resulted in a decrease of the CyC concentration, while BPA, BPA/C and BPS had no effect. Therefore, these data support the results of the cytokine determinations and it can be concluded that

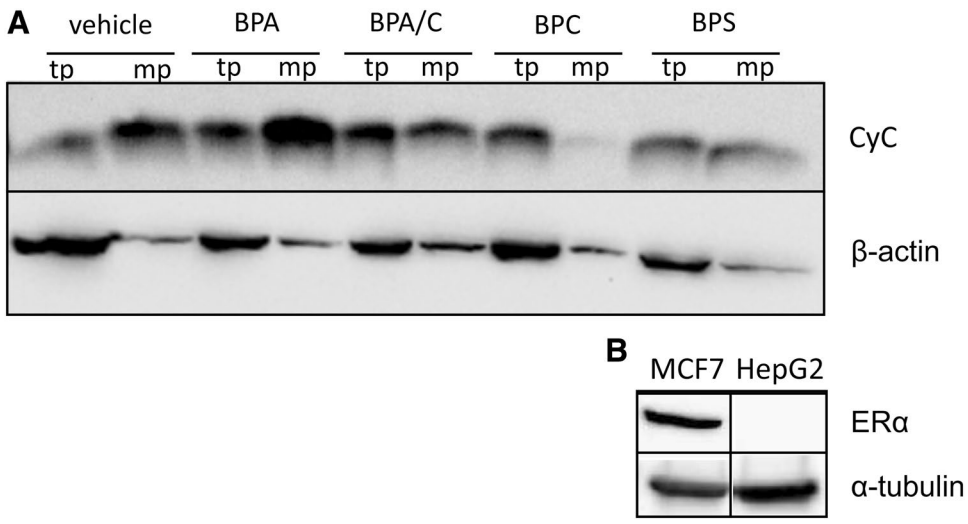


Fig. 7 Qualitative analysis of the estrogen receptor (ER) and intracellular distribution of the protein cytochrome C. **a** HepG2 were treated with the EC_{10} of bisphenol A (BPA), bisphenol A/C (BPA/C), bisphenol C (BPC) and bisphenol S (BPS) for 24 h. The total protein (tp) and the mitochondrial protein fraction (mp) were analyzed for cytochrome C (CyC) content, with β-actin as loading control. **b** Determination of the expression of ERα in HepG2 and MCF7 cells. The loading control α-tubulin was used

only BPC activates the intrinsic apoptotic pathway, which resulted in a release of CyC.

The estrogen receptor is not detectable in HepG2 cells

BPA is a known xenoestrogen (Kitamura et al. 2005). Since MCF7 cells undergo apoptosis after long-term estrogen deprivation and subsequent estrogen treatment (Song et al. 2001), we determined the total estrogen receptor α (ERα) levels in HepG2 cells to clarify whether estrogenic activity plays a crucial role for the cellular endpoints investigated in our study. Yet, Fig. 7b shows that HepG2 cells do not express any detectable levels of the ERα protein. Therefore, the described effects of bisphenols in HepG2 cells are likely independent from any estrogen receptor signaling pathways.

To validate this conclusion, the activation of the ERα signalling by the different bisphenols was investigated according to TG 455 (OECD 2016). The half maximal effective concentration for estrogen activity (EC_{50E}) was 0.7 ± 0.2 µM, 1.2 ± 0.2 µM and 0.2 ± 0.02 µM for BPA, BPA/C and BPC, respectively (Fig. 8). BPS and BPF had an EC_{50E} of 1.5 ± 0.4 µM and 1.1 ± 0.3 µM. The highly toxic BPG had an EC_{50E} of 0.89 ± 0.23 µM, similar to BPA. This shows that the increase in the number of methyl groups (BPA → BPA/C → BPC) has no comparable linear effect on the ERα activation. The EC_{50E} of the ERα activation does not follow the same sequence, since BPC has the lowest and BPA/C the highest EC_{50E} (Fig. 8).

ERα is expressed in healthy liver tissue. Therefore, we asked, whether the results of the presented work might be affected by the absence of estrogen receptor signalling in HepG2 cells. The ERα signalling was activated by BPA, BPC and BPF at 10 µM, 3.16 µM and 10 µM, respectively. BPA/C activated the ERα to 73.4 ± 8.8% at 10 µM. This result clearly demonstrates that the EC_{50} and EC_{10} concentrations used in the present study are sufficient to activate ERα in healthy liver.

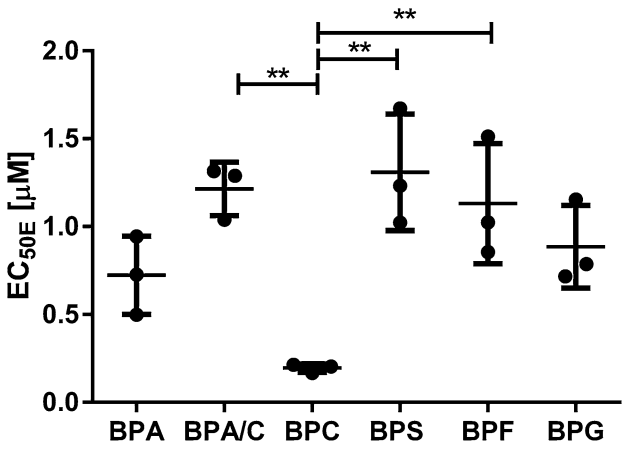


Fig. 8 Concentration of half maximal estrogen receptor activation (EC_{50E}). Hela9903 cells were treated with bisphenol A (BPA), bisphenol A/C (BPA/C), bisphenol C (BPC), bisphenol S (BPS), bisphenol F (BPF) and bisphenol G (BPG) or dimethyl sulfoxide (vehicle control) and 2 nM Estrogen (positive control) for 24 h ($n = 3$). The EC_{50E} were calculated under the program environment R (•) and shown are mean (——) and SD (−). $^{**}p < 0.01$

Discussion

The read-across approach allows the prediction of missing toxicological data for a target substance, based on data from similar substances (source substance). The starting point of the read-across approach could be the grouping of several source substances. This grouping is based on common functional groups, precursors, and a reportedly consistent pattern in terms of the changes in physicochemical and/or biological properties (ECHA 2017a). Based on this, BPA and BPA/C (source substance) were grouped to predict the toxicological properties of BPC (target substance). These three bisphenols had comparable EC_{50}, EC_{10} and $\log P_{OW}$ values (Table 1). Structurally, the BPA, BPA/C and BPC differed only with respect to the number of additional methyl groups (Table 1). Experimental data were generated to validate the predictions for BPC and will be discussed in the following section.

We showed that the in vitro toxicity (EC_{50}) correlates well with the $\log P_{OW}$ (Fig. 1); the latter represents the distribution of a substance between water and the lipophilic solvent octanol (Sangster 1989). Our data showed that the toxicity increased with decreasing water solubility (Fig. 1).

Bisphenols with a higher hydrophobicity had previously been shown to be located in the hydrophobic layer of the cell membrane thereby disturbing the membrane fluidity (Maczak et al. 2017). Recent computer-based simulations underlined that BPA accumulates in the membrane, causes potential cluster generation and increased numbers of membrane pores, and leads to an enhanced water influx into the cell (Chen et al. 2016). Based on this computer-based simulation, it is likely that the cell membrane of HepG2 cells becomes disturbed by BPA, which might result in the generation of membrane pores.

BPA/C produced a significant increase of the cell division rate at the EC_{10} (compared to BPA) in concert with an increase in one methyl group. Therefore, one would predict that BPC with two additional methyl groups would show a further increase in the cell division rate. However, experimental data using BPC showed that a decrease in cell division was detectable (Fig. 2b). At a lower dose of 1/3 EC_{10} there was no change in the cell division rate between BPA, BPA/C and BPS, despite differences in the $\log P_{OW}$. Even at this 1/3 EC_{10} concentration, BPC resulted in a significantly lower number of cell divisions (Fig. 2a).

Using a read-across approach one would predict different effects of BPC on the cell division rate: at the EC_{10} the cell division rate of BPC-treated cells should be above the rate found in cells treated with BPA or BPA/C, since BPA and BPA/C induced increasing cell division ratios corresponding to the increasing number of additional methyl groups (Fig. 2b). In summary, an application of read-across in terms of cell division rates is not possible for BPA, BPA/C and BPC.

In general, the initiation of cell division depends not only on cyclins and the corresponding kinases but also on factors like ATP and oxygen (Gelfant 1960). Therefore, the effect of the bisphenols on the mitochondrial membrane potential $\Delta\Psi_m$ was studied (Fig. 3). Uncoupling of $\Delta\Psi_m$ led to an opening of the mitochondrial permeability transition pores (Petronilli et al. 1993). In contrast to BPA, BPA/C and BPS, only BPC decreased $\Delta\Psi_m$ significantly at EC_{10} concentrations (Fig. 3a). At lower concentrations (1/3 EC_{10}), no effect was detectable. Therefore, a read across from BPA and BPA/C to BPC is not possible.

A recent study has shown that the intracellular ATP concentration is a sensitive endpoint for mitotoxicity (i.e., mitosis toxicity) in the absence of cell death (Kamalian et al. 2015). In our study, the determination of the intracellular ATP concentration clearly showed that only BPC had an effect. The other investigated bisphenols (BPA, BPA/C, and BPS) had no mitotoxic effects, regardless of the chemical structure. Macczak et al. (2017) showed that derivatives of BPA exerted different effects on erythrocyte membranes, depending on its hydrophobicity; this also suggests the potential for mitochondrial membrane disturbance. It has been reported that 1000-fold lower concentrations of BPA can induce a reduction in the $\Delta\Psi_m$ in HepG2 cells after 12 h of incubation (Moon et al. 2012), indicating a short-term effect (12 h) of BPA. In the present study, no change in $\Delta\Psi_m$ was detected after 24 h, except for BPC, for which a decreased $\Delta\Psi_m$ was detectable after 24 h (Fig. 3). The only structural differences between BPA and BPC are the two additional methyl groups attached to the phenol rings, which might be essential for the decoupling effect. To validate a potential structure–activity relationship (SAR), the effects of an intermediate structure, BPA/C, were investigated (Table 1). Ultimately, BPA/C was shown to lack any effect on $\Delta\Psi_m$ (Fig. 3). Therefore, it can be concluded that the membrane potential related effects of BPC are dependent on the presence of both methyl groups. Interestingly, the effect of $\Delta\Psi_m$ seemed to be a reliable indicator for the intracellular ATP concentration. In summary, the decrease of the intracellular ATP concentration is supported by the $\Delta\Psi_m$ determinations. Yet, a prediction of the effects of BPC, based on data from BPA and BPA/C, seemed to be limited.

The effects of BPC on HepG2 cells differ, when compared to BPA, BPA/C and BPS. The differences are based not only on the cell division rate but also on $\Delta\Psi_m$ (Fig. 3) and the cellular ATP concentration (Fig. 4). It has been reported that decoupling of $\Delta\Psi_m$ can result in the mitochondrial release of CyC (Li et al. 2010). CyC is part of the mitochondrial electron transport chain. A release of CyC into the cytosol subsequently results in an activation of apoptosis-related caspases, which then may trigger the intrinsic apoptosis pathway (Tait and Green 2010). Since only BPC-treated cells revealed a reduced amount of CyC in the mitochondrial fraction (Fig. 7a), it was assumed that only BPC might be capable to activate the intrinsic apoptotic pathway by mitochondrial depolarization and translocation of CyC into the cytosol. The activation of this apoptotic pathway could be enhanced by the recently established co-cultivation system (Wewering et al. 2017), reflecting a pro-inflammatory cell state. BCL-2 is an inhibitor of apoptosis pathway (Kluck et al. 1997). It was significantly down-regulated by BPC during conditions of pro-inflammation in HepG2 and PMA-activated THP-1 cells while growing in co-culture (Fig. 6a). Especially the increased secretion of TNF-α during co-cultivation enhanced the effect on BPC treated cells (Fig. 6b). It has been already shown, that intracellular TNF-α occurs in HepG2-cells (Zhang et al. 2013) and we could show that BPS treatment led to secretion of TNF-α (Fig. 6b).

In general, HepG2 cells were also capable of expressing pro-inflammatory cytokines under the influence of external stimuli (Gutierrez-Ruiz et al. 1999). The investigated bisphenols were known to carry intrinsic estrogenic activity (Kitamura et al. 2005). It has been shown that pro-inflammatory cytokines, like TNFα, were upregulated in liver after treatment with estrogens (Colantoni et al. 2003). Due to the lack of ERα protein in HepG2 cells, all effects on cytokine expression were independent from the estrogenic properties of the investigated bisphenols though (Fig. 7b). However, one has to keep in mind that the ERα is expressed in normal liver tissue (Zhao and Li 2015) and that differences in the activation of ERα by bisphenols might alter the reported effects. BPA and BPC activated the ERα signaling completely and BPA/C up to $73.8 \pm 8.8\%$ at concentrations lower than the ones used in the present study (Figure S3). Furthermore, the EC_{50E} of ERα activation differs between BPA, BPA/C and BPC, and no correlation between the number of methyl-groups and the EC_{50E} was detected (Fig. 8). The EC_{50E} values of BPA, BPC, BPS and BPF were similar to the values reported by Kitamura et al. (2005). Moreover, the data support the scheme proposed by the authors only partly. It remains to be elucidated why the single methyl group of BPA/C reveals with highest EC_{50E} of ERα activation, while one less (BPA) or an additional methyl group (BPC) lowers this value.

These results show that there is no constant increase in ERα activation by the addition of single methyl groups. Therefore, a read across from BPA and BPA/C to BPC

is not possible. In addition, the data show that ERα is already activated at concentrations below the ones used in the present study. It is likely that this situation occurs in healthy liver. Effects based on differences in the activation are unlikely.

The read-across approach was used to predict the expression of cytokines in treated HepG2 cell culture. BPA, BPA/C were used as source substances and BPC as target substance. The expression of the pro-inflammatory cytokine TGF-β (Worthington et al. 2012) was significantly up-regulated in the presence of BPC and BPA (Fig. 5a) but not by BPA/C. Since both former substances differed only by the number of methyl-groups and its logP_{OW} values, it should be expected that the addition of a single methyl group to BPA/C would result in the down-regulation or at least in the lack of any expression changes of this pro-inflammatory cytokine. However, our experimental data show that BPC increases *TGFB1* expression even more than BPA. Therefore, it seems that BPA and BPA/C cannot be used as reliable source substances to predict the expression of BPC. To summarize, the prediction of the read-across approach was not found to be reliable for the set of bisphenols investigated in our study due to the fact that the expression of *TGFB1* was significantly up-regulated compared to the expression produced by a structurally similar BPA/C.

We next studied additional pro-inflammatory cytokines. The expression profiles of *SPP1* and *TNF* differed between BPC-treated HepG2 cells when compared to the other bisphenol treatments. The treatment with BPA, BPA/C and BPS led to an up-regulation of *SPP1*, but not of *TNF*. BPC did not change the expression of *SPP1*, but up-regulated the expression of *TNF* (Fig. 5b, c). Actually, applying the read-across approach one would expect an up-regulated *SPP1* expression, similar to BPA and BPA/C, and an unchanged expression of this cytokine in BPC-treated cells. However, the strong up-regulation of *TNF* contradicted this prediction.

Generally TNF-α secretion can be also used for the read-across approach. One would expect that the TNF-α secretion levels in co-cultures treated with BPA and BPA/C (Fig. 6b) would be comparable to the BPC-treated co-culture, based on the structurally similarities. But our data clearly show that only BPC significantly enhances the TNF-α secretion in co-culture (Fig. 6b). However, no TNF-α was secreted in BPS-treated co-culture (Fig. 6b). In brief, the read-across approach could be not applied to the current set of substances.

In theory, a read-across approach for the prediction of the effects of BPC seemed feasible using data from BPA- and BPA/C-treated HepG2 cells. The more so as these bisphenols differed only by one or two methyl groups. Yet our data clearly showed that the application of read-across was impossible within this group, despite the high structural similarity. At non-toxic concentrations (EC_{10}), different biochemical effects of BPA, BPA/C, BPC and BPS became obvious in HepG2 cell cultures.

The in vitro data on bisphenol derivatives indicate that a single methyl group can have a profound effect on cellular toxicity. Similar effects have been shown with other chemicals as well, such as, 1-amino-2-propanol (CAS 78-96-6) and 2-aminoethanol (CAS 141-43-5). Both chemicals differ only in a single methyl group. Here, this small structural change caused significantly different in vivo toxicity in rats, with 1-amino-2-propanol resulting in an increased liver weight, generally, and an increased thymus weight in females only (ECHA 2018c). In contrast, oral exposure to 1-aminoethanol resulted in decreased weights of prostate, as well as corpus and cauda epididymis (ECHA 2016). Our study adds to these observations and prompts us to suggest caution in cases where the read-across approach is thought to be applicable due to small structural alterations of the compounds under investigation, such as the adding or loss of a single methyl side chain.

Conclusion

In this study, we showed that the cytotoxicity of a selected set of bisphenols depended largely on the respective logP_{ow} values. Furthermore, methylation of both phenolic moieties is required to trigger the intrinsic apoptotic pathway in HepG2 cells. This is mediated by the compound's decoupling effect on the mitochondrial membrane or the activation of the TNF pathway. BPS, used as an alternative for BPA in consumer products, revealed with a similar expression pattern for *TGFB1*, *SPP1* and *TNF*. BPS was able to evoke the expression of similar cytokines (*SPP1* and *TGFB1*) as BPA suppressed TNF-α secretion in a pro-inflammatory co-culture system without affecting cell division rates. In conclusion, the read-across method should be used carefully with regard to bisphenols, since the addition of a single methyl group can trigger large differences in terms of potentially adverse biological effects.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Cesen M, Lenarcic K, Mislej V et al (2018) The occurrence and source identification of bisphenol compounds in wastewaters. Sci Total Environ 616–617:744–752. https://doi.org/10.1016/j.scitotenv.2017.10.252

Chen L, Chen J, Zhou G, Wang Y, Xu C, Wang X (2016) Molecular dynamics simulations of the permeation of bisphenol A and pore formation in a lipid membrane. Sci Rep 6:33399. https://doi.org/10.1038/srep33399

Clayton DA, Shadel GS (2014a) Isolation of mitochondria from tissue culture cells. Cold Spring Harb Protoc 20(10):pdb.prot080002. https://doi.org/10.1101/pdb.prot080002

Clayton DA, Shadel GS (2014b) Purification of mitochondria by sucrose step density gradient centrifugation. Cold Spring Harb Protoc 10:pdb.prot080028. https://doi.org/10.1101/pdb.prot080028

Clemente N, Raineri D, Cappellano G et al (2016) Osteopontin bridging innate and adaptive immunity in autoimmune diseases. J Immunol Res 2016:7675437. https://doi.org/10.1155/2016/7675437

Colantoni A, Idilman R, De Maria N et al (2003) Hepatic apoptosis and proliferation in male and female rats fed alcohol: role of cytokines. Alcohol Clin Exp Res 27(7):1184–1189. https://doi.org/10.1097/01.ALC.0000075834.52279.F9

Czabotar PE, Lessene G, Strasser A, Adams JM (2014) Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol 15(1):49–63. https://doi.org/10.1038/nrm3722

Doerge DR, Twaddle NC, Vanlandingham M, Fisher JW (2010) Pharmacokinetics of bisphenol A in neonatal and adult Sprague-Dawley rats. Toxicol Appl Pharmacol 247(2):158–165. https://doi.org/10.1016/j.taap.2010.06.008

ECHA (2016) Substance evaluation report for 2-aminoethanol EC No 205-483-3 CAS No 141-43-5. http://echa.europa.eu/web/guest/information-on-chemicals/registered-substances

ECHA (2017a) Read-across assessment framework (RAAF). https://doi.org/10.2823/619212

ECHA (2017b) Read-across assessment framework (RAAF)—considerations on multi-constituent substances and UVCBs. https://doi.org/10.2823/794394

ECHA (2017c) Substance evaluation conclusion as required by reach Article 48 and evaluation report for 4,4′-Isopropylidenediphenol EC No 201-245-8 CAS No 80-05-7. https://echa.europa.eu/documents/10162/7971ab80-03c9-4d87-e117-e4dbc9cc54d2

ECHA (2018a) Decision on substance evaluation pursuant to article 46(1) of regulation (EC) No. 1907/2006 for 4,4'-sulfonyldiphenol, CAS No 80-09-1 (EC No201-250-5). https://echa.europa.eu/documents/10162/776a7a2e-1526-430a-8630-70163473dfc0

ECHA (2018b) Annex XV report – proposal for identification of a substance of very high concern on the basis of the criteria set out in reach article 57, Substance Name(s): 4,4'-isopropylidenediphenol (Bisphenol A). https://echa.europa.eu/documents/10162/f19ec5b1-dfae-107a-44d2-6e74979d68f1

ECHA (2018c) Registration dossier (Regulation (EC) No 1907/2006) of 1-aminopropan-2-ol. https://echa.europa.eu/registration-dossier/-/registered-dossier/13935/7/6/2

ECHA (2018d) Registration dossier (Regulation (EC) No 1907/2006) of 4,4′-isopropylidenedi-o-cresol. https://echa.europa.eu/registration-dossier/-/registered-dossier/24781

EFSA (2015) Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. EFSA J 13(1):3978. https://doi.org/10.2903/j.efsa.2015.3978

EFSA, Gundert-Remy U, Bodin J, Bosetti C, FitzGerald RE, Hanberg A, Hass U, Hooijmans C, Rooney AA, Rousselle C, van Loveren H, Wölfle D, Barizzone F, Croera C, Putzu C, Castoldi AF (2017) Bisphenol A (BPA) hazard assessment protocol. EFSA Support Publ 14(12):EN-1354. https://doi.org/10.2903/sp.efsa.2017.en-1354

Fic A, Žegura B, Sollner Dolenc M, Filipič M, Peterlin Mašič L (2013) Mutagenicity and DNA damage of bisphenol A and its structural analogues in HepG2 cells. Arch Ind Hyg Toxicol 64(2):189–200. https://doi.org/10.2478/10004-1254-64-2013-2319

Gelfant S (1960) The energy requirements for mitosis. Ann N Y Acad Sci 90(2):536–549. https://doi.org/10.1111/j.1749-6632.1960.tb23271.x

Gutierrez-Ruiz MC, Quiroz SC, Souza V et al (1999) Cytokines, growth factors, and oxidative stress in HepG2 cells treated with ethanol, acetaldehyde, and LPS. Toxicology 134(2–3):197–207

Ichim G, Tait SW (2016) A fate worse than death: apoptosis as an oncogenic process. Nat Rev Cancer 16(8):539–548. https://doi.org/10.1038/nrc.2016.58

Kamalian L, Chadwick AE, Bayliss M et al (2015) The utility of HepG2 cells to identify direct mitochondrial dysfunction in the absence of cell death. Toxicol In Vitro 29(4):732–740. https://doi.org/10.1016/j.tiv.2015.02.011

Kidani T, Yasuda R, Miyawaki J, Oshima Y, Miura H, Masuno H (2017) Bisphenol A inhibits cell proliferation and reduces the motile potential of murine LM8 osteosarcoma cells. Anticancer Res 37(4):1711–1722. https://doi.org/10.21873/anticanres.11503

Kitamura S, Suzuki T, Sanoh S et al (2005) Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci 84(2):249–259. https://doi.org/10.1093/toxsci/kfi074

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275(5303):1132–1136

Kuroda N, Kinoshita Y, Sun Y et al (2003) Measurement of bisphenol A levels in human blood serum and ascitic fluid by HPLC using a fluorescent labeling reagent. J Pharm Biomed Anal 30(6):1743–1749. https://doi.org/10.1016/s0731-7085(02)00516-2

Li T, Brustovetsky T, Antonsson B, Brustovetsky N (2010) Dissimilar mechanisms of cytochrome c release induced by octyl glucoside-activated BAX and by BAX activated with truncated BID. Biochem Biophys Acta 1797(1):52–62. https://doi.org/10.1016/j.bbabio.2009.07.012

Liao CY, Liu F, Kannan K, Bisphenol S (2012) A new bisphenol analogue, in paper products and currency bills and its association with bisphenol A residues. Environ Sci Technol 46(12):6515–6522. https://doi.org/10.1021/es300876n

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(− ΔΔC) method. Methods 25(4):402–408. https://doi.org/10.1006/meth.2001.1262

Macczak A, Duchnowicz P, Sicinska P, Koter-Michalak M, Bukowska B, Michalowicz J (2017) The in vitro comparative study of the effect of BPA, BPS, BPF and BPAF on human erythrocyte membrane; perturbations in membrane fluidity, alterations in conformational state and damage to proteins, changes in ATP level and Na(+)/K(+) ATPase and AChE activities. Food Chem Toxicol 110:351–359. https://doi.org/10.1016/j.fct.2017.10.028

Malik MY, Jaiswal S, Sharma A, Shukla M, Lal J (2016) Role of enterohepatic recirculation in drug disposition: cooperation and complications. Drug Metab Rev 48(2):281–327. https://doi.org/10.3109/03602532.2016.1157600

Mandrah K, Satyanarayana GNV, Roy SK (2017) A dispersive liquid-liquid microextraction based on solidification of floating organic droplet followed by injector port silylation coupled with gas

chromatography-tandem mass spectrometry for the determination of nine bisphenols in bottled carbonated beverages. J Chromatogr A 1528:10–17. https://doi.org/10.1016/j.chroma.2017.10.071

Manica A, Da Silva AM, Cardoso AM et al (2018) High levels of extracellular ATP lead to chronic inflammatory response in melanoma patients. J Cell Biochem 119(5):3980–3988. https://doi.org/10.1002/jcb.26551

Moon MK, Kim MJ, Jung IK et al (2012) Bisphenol A impairs mitochondrial function in the liver at doses below the no observed adverse effect level. J Korean Med Sci 27(6):644–652. https://doi.org/10.3346/jkms.2012.27.6.644

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65(1–2):55–63

OECD (2016) Test No. 455: performance-based test guideline for stably transfected transactivation in vitro assays to detect estrogen receptor agonists and antagonists

Petronilli V, Cola C, Massari S, Colonna R, Bernardi P (1993) Physiological effectors modify voltage sensing by the cyclosporin A-sensitive permeability transition pore of mitochondria. J Biol Chem 268(29):21939–21945

Potratz S, Tarnow P, Jungnickel H et al (2017) Combination of metabolomics with cellular assays reveals new biomarkers and mechanistic insights on xenoestrogenic exposures in MCF-7 cells. Chem Res Toxicol 30(4):883–892. https://doi.org/10.1021/acs.chemrestox.6b00106

Rochester JR, Bolden AL (2015) Bisphenol S and F: a systematic review and comparison of the hormonal activity of bisphenol A substitutes. Environ Health Perspect 123(7):643–650. https://doi.org/10.1289/ehp.1408989

Sangster J (1989) Octanol-water partition-coefficients of simple organic-compounds. J Phys Chem Ref Data 18(3):1111–1229. https://doi.org/10.1063/1.555833

Schieke SM, McCoy JP, Finkel T (2008) Coordination of mitochondrial bioenergetics with G(1) phase cell cycle progression. Cell Cycle 7(12):1782–1787

Sedger LM, McDermott MF (2014) TNF and TNF-receptors: from mediators of cell death and inflammation to therapeutic giants—past, present and future. Cytokine Growth Factor Rev 25(4):453–472. https://doi.org/10.1016/j.cytogfr.2014.07.016

Song RX, Mor G, Naftolin F et al (2001) Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 93(22):1714–1723

Tait SW, Green DR (2010) Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol 11(9):621–632. https://doi.org/10.1038/nrm2952

Tarnow P, Tralau T, Hunecke D, Luch A (2013) Effects of triclocarban on the transcription of estrogen, androgen and aryl hydrocarbon receptor responsive genes in human breast cancer cells. Toxicol In Vitro 27(5):1467–1475. https://doi.org/10.1016/j.tiv.2013.03.003

R Core Team (2018) R: a language and environment for statistical computing. https://www.R-project.org/, Vienna, Austria. R Foundation for Statistical Computing

Thayer KA, Taylor KW, Garantziotis S et al (2016) Bisphenol A, bisphenol S, and 4-hydroxyphenyl 4-isoprooxyphenylsulfone (BPSIP) in urine and blood of cashiers. Environ Health Perspect 124(4):437–444. https://doi.org/10.1289/ehp.1409427

van Horssen R, Ten Hagen TL, Eggermont AM (2006) TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility. Oncologist 11(4):397–408. https://doi.org/10.1634/theoncologist.11-4-397

Wewering F, Jouy F, Wissenbach DK et al (2017) Characterization of chemical-induced sterile inflammation in vitro: application of the model compound ketoconazole in a human hepatic co-culture system. Arch Toxicol 91(2):799–810. https://doi.org/10.1007/s00204-016-1686-y

Worthington JJ, Fenton TM, Czajkowska BI, Klementowicz JE, Travis MA (2012) Regulation of TGFβ in the immune system: an emerging role for integrins and dendritic cells. Immunobiology 217(12):1259–1265. https://doi.org/10.1016/j.imbio.2012.06.009

Zhang C, Wang C, Tang S et al (2013) TNFR1/TNF-alpha and mitochondria interrelated signaling pathway mediates quinocetone-induced apoptosis in HepG2 cells. Food Chem Toxicol 62:825–838. https://doi.org/10.1016/j.fct.2013.10.022

Zhao Y, Li Z (2015) Interplay of estrogen receptors and FOXA factors in the liver cancer. Mol Cell Endocrinol 418(Pt 3):334–339. https://doi.org/10.1016/j.mce.2015.01.043