Differential Toxicity of Alkylphenols in JEG-3 Human Placental Cells: Alteration of P450 Aromatase and Cell Lipid Composition

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ABSTRACT

Alkylphenols (APs) are a diverse class of chemicals that can cross the placental barrier and interfere with embryonic and fetal development. This work investigates the comparative toxicity, ability to inhibit aromatase activity, and to alter the lipid composition of 10 alkylphenols in the human placenta choriocarcinoma cell line JEG-3. Among the selected APs, 4-dodecylphenol (DP), 4-heptylphenol (HP), and 4-cumylphenol (CP) showed the highest cytotoxicity (EC₅₀: 18–65 μM). Aromatase inhibition was closely related to the hydrophobicity of APs. HP significantly induced the generation of reactive oxygen species (ROS) (43-fold), inhibited placental aromatase activity (IC₅₀: 41 μM), and induced a general dose-dependent depletion of polyunsaturated lipids (10–20 μM), which were attributed to high levels of oxidative stress. In contrast, 2,4,6-tri-tert-butylphenol (TTBP) significantly induced the intracellular accumulation of triacylglycerides (TGs), whereas DP increased the synthesis of phosphatidylcholines (PCs) and TGs at the expense of diacylglycerides (DGs). Overall, this study evidences the different modes of action of alkylphenols in human placental JEG-3 cells, describes differential lipidomic fingerprints, and highlights DP, HP, CP, and TTBP as the ones that caused the most harmful effects.

Key words: JEG-3 cells; alkylphenols; cytotoxicity; P450 aromatase; ROS generation; lipid disruption.

Alkylphenols (APs) are high-production volume chemicals used as nonionic surfactants in a variety of products, including industrial detergents, synthetic leathers, lubricants, polycarbonate and epoxy resins, herbicides, cosmetics, and others (Brooke *et al.*, 2005). The chemical structure of APs is a phenol ring with an alkyl chain tail of 1–12 carbon atoms at *ortho*-, *meta*-, or *para*-position, branched (*tert*- and *sec*-) or lineal. This confers upon APs different physicochemical and toxicological characteristics (eg, water solubility, persistence) that vary with the length of the carbon chain and the position of the substituent (Tollefsen *et al.*, 2008; Xie *et al.*, 2013).

Octylphenol and nonylphenol (OP and NP), the most widely studied and distributed APs, have been thoroughly investigated. Humans are exposed to these compounds through inhalation, ingestion of contaminated food, and dermal absorption. Concentrations up to 200 ng/g NP (approximately 1 μM) and 1.15 ng/ml OP (approximately 0.006 μM) have been reported in blood, umbilical cord, and placenta (Azzouz *et al.*, 2016; Chen *et al.*, 2008; Huang *et al.*, 2014; Tan and Mohd, 2003). Both

compounds can accumulate in lipids, bind to proteins, cause acute cytotoxicity, oxidative stress, and act as endocrine disruptors (Bonefeld-Jørgensen *et al.*, 2007; Kim *et al.*, 2015; Lepretti *et al.*, 2015; Pérez-Albaladejo *et al.*, 2017; Soto *et al.*, 1991; Xie *et al.*, 2013).

4-Dodecylphenol (DP), some isomers of butylphenol (2-*tert*-, 4-*tert*-, 2,4-di-*tert*-), and 4-cumylphenol (CP) are nowadays used as substitutes of NP (Brooke *et al.*, 2005). Among them, CP was reported to increase cell growth in MCF-7 cells, whereas 4-*tert*-butylphenol (4TBP) has been classified as estrogenic and has been reported to cause vitiligo, a cutaneous disease which causes depigmentation in melanocytes, in exposed workers (Hashimoto *et al.*, 2001; Toosi *et al.*, 2012; Yang *et al.*, 2000). In 2000, the Organization for Economic Co-operation and Development (OECD) calculated the daily intake of 4TBP as 9 μg/kg body weight/day. However, despite the increasing production and distribution of short-chain APs, information about their potential toxicological effects is scarce.

The endocrine disruptive properties of APs are of particular concern. Several studies have demonstrated the estrogenic

activity of APs by mimicking 17β -estradiol in PNT1A human prostate cells, inducing cell proliferation and binding to progesterone receptor in human estrogen-sensitive MCF7 cells, triggering mitotic activity in the endometrium of rats, and inducing P450 aromatase activity in JEG-3 human placental cells (Forte et al., 2016; Pérez-Albaladejo et al., 2017; Soto et al., 1991). The human placental choriocarcinoma JEG-3 cell line expresses high levels of P450 aromatase, a key enzyme in steroidogenesis. It has often been used as a model to detect endocrine disrupter compounds, as an alteration of aromatase function may result in disorders of the reproductive system and trigger some types of cancer (Huang and Leung, 2009; Samson et al., 2009; Vinggaard et al., 2000).

Additionally, APs, as amphipathic molecules, are likely to alter the lipid bilayer of cell membranes, and controversial results have been reported concerning its obesogenic effect in mice and 3T3-L1 cells (Hao et al., 2012; Masuno et al., 2003). The interference of APs with lipid homeostasis is of particular concern since human placenta plays an active role in the release of free fatty acids from maternal serum triacylglycerols and the preferential transfer of long chain polyunsaturated fatty acids to the fetus, as well as the conversion of a certain fraction of arachidonic acid to prostaglandins, the incorporation of some fatty acids into phospholipids, and the synthesis of specific fatty acids, eg, palmitoleic, palmitic, and oleic (Coleman and Haynes, 1987; Herrera, 2002; Jones et al., 2014). Thus, any disruption on the lipid metabolism in placental cells will probably have harmful consequences for embryo development.

Thus, this study investigates the comparative toxicity of a selected number of APs of different chain length and different isomers (4-sec-butylphenol [4SBP], 2-sec-butylphenol [2SBP], 4-tert-butylphenol [4TBP], 2-tert-butylphenol [2TBP], 4-tert-amylphenol [TAP], 2,6-di-tert-butylphenol [DTBP], 2,4,6-tri-tert-butylphenol [TTBP], CP, 4-heptylphenol [HP], DP) in human placental JEG-3 cells by targeting to different endpoints, namely cytotoxicity, generation of reactive oxygen species (ROS), and disruption of aromatase activity. In addition, the ability of 3 of them, namely TTBP, HP, and DP, to alter JEG-3 cells lipidome at non-toxic concentrations was investigated by using high-resolution mass spectrometry.

MATERIAL AND METHODS

Chemicals and solutions

Eagle's minimum essential medium, fetal bovine serum, L-glutamine, sodium pyruvate, nonessential amino acids, penicillin and streptomycin, phosphate-buffered saline (PBS), phosphate-buffered saline with Ca, Mg (DPBS), and trypsin-EDTA 0.25% were from Gibco BRL Life Technologies (Paisley, Scotland, UK). 2',7'-Dihydrodichlorofluorescein diacetate (H_2DCF -DA) and 3-morpholinolinosydnonime (SIN1) were from Sigma (Steinheim, Germany). Alamar Blue (AB) was from Biosource (Solingen, Germany) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) from Molecular Probes (Eugene, Oregon). 1β - 3H -androst-4-ene-3, 17-dione (3H -AD) was purchased from PerkinElmer, Inc. (Boston, Massachusetts).

Stock solutions of APs were prepared in dimethyl sulfoxide (DMSO) (Sigma, Steinheim, Germany). The analytical standards 4SBP, 2SBP, 4TBP, 2TBP, TAP, DTBP, TTBP, CP, and DP were from Sigma-Aldrich (St. Louis, Missouri). HP was from Dr Ehrenstorfer

(LGC Standards). Its properties and chemical structure are indicated in Table 1.

Cell culture

The JEG-3 cell line, derived from a human placental choriocarcinoma, was obtained from the American Type Culture Collection (ATCC-HTB-36). Cells were routinely grown in Eagle's minimum essential medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/l sodium bicarbonate, 50 U/ml penicillin, and 50 μ g/ml streptomycin as described in Pérez-Albaladejo et al. (2017).

Cytotoxicity

Cytotoxic effects of APs in JEG-3 cells after 24 h exposure were measured using two fluorescent dyes, namely AB and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) (Schirmer et al., 1997). Cells were seeded in 96-well plates at a density of 2.5×10^4 cells per well in 100 μ l of medium and left to grow for 24 h. APs were dissolved in DMSO and were added to the medium so that the final solvent concentration was 0.5% (v/v), which was not cytotoxic for cells. Control wells received the culture medium containing 0.5% DMSO. After 24 h exposure, cells were rinsed with PBS and incubated for 1 h 5% v/v AB and 4 μ M CFDA-AM in modified Leibovitz L-15 medium without vitamins or amino acids. Cell viability was measured using a fluorescence plate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelengths of 530/590 nm for AB, and 485/530 nm for CFDA-AM. Fluorescence of blanks (wells without cells, receiving the same treatment than the other ones) was subtracted from that of exposed cells. Cell viability was expressed as a percentage of control cells. Six replicates in three different plates were analyzed.

Reactive oxygen species (ROS) generation

The production of ROS in JEG-3 cells exposed to APs was measured as described in LeBel et al. (1992) with slight modifications. Cells were seeded in 96-well plates 24 h before the assay (4×10^4 cells in 100 μ l of culture medium per well). On the day of the experiment, cells were rinsed with PBS and incubated with 20 μ M H_2DCF -DA in a 1:10 DPBS solution supplemented with 10 mM glucose. After 30 minutes incubation, cells were washed with PBS and exposed to APs dissolved in DMSO and further diluted in DPBS-glucose. The final concentration of DMSO in the assay was 0.5%. 3-Morpholinolinosydnonime (SIN-1) at a concentration of 5 μ M was used as a positive control. Dichlorofluorescein (DCF) was measured after 60 min in a microplate reader (Varioskan, Thermo Electron Corporation) at the excitation/emission wavelength pairs of 485/528 nm. Results were expressed as the ratio of fluorescence in exposed cells to control cells as fold induction.

P450 aromatase activity

Aromatase activity in JEG-3 human placental cells was determined by measuring the amount of 3H_2O formed during the aromatization of [1β - 3H]androstenedione (3H -AD) as described in Lephart and Simpson (1991), with some modifications. Cells were seeded in 24 well-plates at a density of 10^5 cells in 500 μ l of medium per well and allowed to attach overnight before exposure to APs or 0.5% DMSO (solvent control) in culture medium. After 24 h exposure, cells were rinsed with PBS and incubated

Table 1. Properties of Tested APs (<https://scifinder.cas.org>)

Name	Compound	No CAS	MW	Log K_{OW}	MS (mmol/l)	Formula
4SBP	4-Sec-butylphenol	99-71-8	150.22	3.50	4.80
2SBP	2-Sec-butylphenol	89-72-5	150.22	3.37	5.50
4TBP	4-Tert-butylphenol	98-54-4	150.22	3.40	6.30
2TBP	2-Tert-butylphenol	88-18-6	150.22	3.27	7.20
TAP	4-Tert-amylphenol	80-46-6	164.24	3.91	2.30
DTBP	2,6-Di-tert-butylphenol	128-39-2	206.32	4.48	0.66
TTBP	2,4,6-Tri-tert-butylphenol	732-26-3	262.43	5.30	0.70
CP	4-Cumylphenol	599-64-4	212.29	4.24	0.17
HP	4-Heptylphenol	1987-50-4	192.30	5.12	0.30
DP	4-Dodecylphenol	104-43-8	262.43	7.67	0.04

Note: MW, molecular weight; MS, molar solubility in water.

for 30 min in the presence of 40 nM ^3H -AD in 1 ml of DPBS-glucose. The reaction was stopped by placing the plate on ice and immediately aspirating 0.8 ml of medium that was extracted with 3 ml of dichloromethane. The amount of tritiated water formed was counted in an aliquot of the aqueous phase (Tri-Carb 2100TR, Packard). Aromatase activity was expressed as fmol/min/mg protein or as percentage of activity in solvent control cells.

Lipid analysis

JEG-3 cells were exposed for 24 h to 10 μM DP, 10 and 20 μM HP, and 20 μM TTBP in 24-well plates, as indicated above. Control cells were exposed to culture medium containing 0.1% DMSO. After exposure, the medium was removed and cells were trypsinized, resuspended in PBS, centrifuged at $10000 \times g$ for 10 min and the pellet collected. An aliquot of internal standard mixture containing equal amounts (~ 200 pmol) of different lipids covering each lipid family, namely, 16: 0D31-18: 1 phosphatidylcholine (PC), 16: 0D31-18: 1 phosphatidylethanolamine, 16: 0D31-18: 1 phosphatidylserine, 16: 0D31-18: 1 phosphatidylinositol, 1, 3-17: 0D5 diacylglycerol, and 1, 2, 3-17: 0 triacylglycerol (Avanti Polar Lipids, Inc., Alabama), was added prior to extraction of lipids with a solution of methanol: chloroform (1:2) containing 0.01% of butylated hydroxytoluene (BHT). The mixture was vortexed for 1 min, and after 30 min incubation at room temperature, extracted twice in an ultrasonic bath for 5 min. The solvent was evaporated and the extracts stored at -80°C under an argon atmosphere. The analysis of lipids was performed as described in Vichi *et al.* (2012) with some modifications. Flow injection

analysis of reconstituted lipid extracts (5 μl) were analyzed in an Orbitrap-Exactive (Thermo Fisher Scientific) equipped with an electrospray source (H-ESI II), a Surveyor MS Plus pump and an Accela Open AS autosampler kept at 10°C (Thermo Fisher Scientific, San Jose, California). The mobile phase was methanol: dichloromethane 80:20 at 50 $\mu\text{l}/\text{min}$. Mass spectra were acquired in full scan both in ESI+ and ESI- ionization modes produced by a spray voltage of 3 kV, capillary voltage 37 V, tube lens 150 V, and skimmer voltage 40 V. The sheath gas flow rate was set at 35 a.u. (arbitrary units), and the aux gas flow rate was 5 a.u. Capillary and heater temperature were set to 400°C and 30°C . The acquisition mass range was set to m/z 200–2000 and the total analysis time was 2 min. Ultrahigh resolving power defined as R : 100,000 (m/z 200, FWHM) was set. The mass peaks considered were single positive charged sodium molecular ions, $[\text{M}+\text{Na}^+]$ (TG, DG, PC, PC-plasmany, [PC-O]/PC-plasmalogen [PC-P]) and single negative charge $[\text{M}-\text{H}]$ (phosphatidylethanolamines [PE], PE-plasmany [PE-O]/PE-plasmalogen [PE-P], phosphatidylserines [PS] and phosphatidylinositol [PI]). Data from LIPID MAPS[®], exact mass, isotopic distributions, charge, adducts formed, number of ring plus double bond ($\text{RDB} = 0.5\text{--}15$), and elements in molecular formula, $\text{C} \leq 100$, $\text{H} \leq 150$, $\text{O} \leq 8$, $\text{Na} \leq 1$, $\text{P} \leq 1$, $\text{N} \leq 1$, were used for the identification of the lipid molecules with a maximum permitted mass error fixed at <5 ppm. Mass spectra were processed using Xcalibur 2.1 (Thermo Fisher Scientific, Bremen, Germany) and the lipid species were quantified (pmol/ 10^5 cells) relative to internal standards. Lipid species were annotated as <lipid class> - <total fatty acyl chain>:<total number of double bonds>.

Under these conditions, relative standard deviation (RSD) of the internal standards ranged from 17–18% (PSs, PCs) to 32% (PEs). RSD of 6 repeated no consecutive injections of pure external standards was satisfactory for TGs, DGs, PCs, and PEs (7–14%), but high for PSs (40%). The matrix effect, calculated as the difference in response between the internal standard of each sample and the external standard, divided by the external standard response, was of 17% and 19% for PCs and PSs, respectively; whereas it increased for PEs (32%), TGs (41%), and DGs (45%).

Statistical methods

Dose-response curves and concentrations causing 50% decline on cell viability (EC_{50}) and inhibition of aromatase activity (IC_{50}) were performed with Sigmaplot 11.0 software. Significant differences from controls were determined using one-way ANOVA with Dunnett's or Turkey's post hoc test, with SPSS Statistics v.23 software. Level of significance was set at $p < .05$.

Lipid profiles were analyzed by multivariate and univariate approaches using Metaboanalyst software (version 3.0; [Xia and Wishart, 2016](#)). Data were autoscaled by mean-centering and dividing by standard deviation of each variable. Partial least square-discriminant analysis (PLS-DA) was performed as multivariate approach to compare the lipid profile of control and exposed cells, and to identify discriminant lipids responsible for lipidome alteration based on the variable influence on the projection (VIP) parameter, which provided a score value for each variable. VIP values > 1 were selected ([Checa et al., 2015](#)). The goodness of fit (R^2) and the predictive ability of the model (Q^2) were calculated in order to assess the quality of the multivariate approach. Volcano plots based on fold-change values > 1.5 and a significance threshold of $p < .05$ (Student *t* test) were used to visualize the significance and magnitude of the changes.

RESULTS

Cytotoxicity and Generation of Reactive Oxygen Species

Low or no significant cytotoxicity was detected for butylphenols 2SBP, 4TBP, 2TBP, and TTBP ($EC_{50} > 385 \mu M$), as well as for 4SBP, TAP, and DTBP (EC_{50} : 120–280 μM) after 24 h of exposure. On the contrary, the long-chain DP showed the highest cytotoxicity (EC_{50} : 18 μM), followed by HP and CP ($EC_{50} \sim 50$ and 65 μM , respectively) (Table 2). No significant differences between AB and CFDA probes were observed.

Regarding generation of ROS, the short-chain APs substituted in position 4, namely 4SBP, 4TBP, and TAP, significantly induced ROS in JEG-3 cells (3.4–6.5-fold induction at 100–200 μM), whereas butylphenol isomers substituted in position 2, and those di- and tri-substituted, generated lower (2.5-fold; 2SBP) or no ROS (2TBP, DTBP, TTBP). The long-chain DP, and CP slightly induced the production of ROS (2–3-fold at 50 μM) (Figure 1). Surprisingly, HP had the highest response in the H_2DCF -DA assay (43-fold induction at 40 μM). SIN-1, which was used as positive control, generated an induction of ROS of 15.4 ± 3.3 fold over control cells.

P450 Aromatase Activity

The tri-substituted TTBP, which showed no cytotoxicity at the highest concentration tested, HP and DP, significantly inhibited aromatase activity, with IC_{50} of 58, 41, and 13 μM , respectively. Interestingly, a significant inhibition of aromatase activity

Table 2. EC_{50} for AB and CFDA-AM Assays in JEG-3 Cells Exposed for 24 h to APs and IC_{50} for P450 Aromatase Inhibition

Name	Alamar Blue	CFDA-AM	P450 Aromatase Inhibition
4SBP	249 $\pm$ 36	279 $\pm$ 36	268 $\pm$ 12
2SBP	454 $\pm$ 60	392 $\pm$ 27	317 $\pm$ 20
4TBP	> 500	> 500	283 $\pm$ 6.2 ^a
2TBP	385 $\pm$ 52	433 $\pm$ 90	299 $\pm$ 17
TAP	211 $\pm$ 15	229 $\pm$ 16	209 $\pm$ 5.2
DTBP	119 $\pm$ 16	126 $\pm$ 20	99 $\pm$ 4.0
TTBP	> 500	> 500	58 $\pm$ 7.0 ^a
CP	64 $\pm$ 2.1	67 $\pm$ 1.7	62 $\pm$ 7.6
HP	50 $\pm$ 1.2	51 $\pm$ 1.9	41 $\pm$ 4.6 ^a
DP	18 $\pm$ 0.5	18 $\pm$ 0.4	14 $\pm$ 0.8

Note: Values are in μM as mean $\pm$ SEM of at least 3 assays.

^aStatistically significant differences from EC_{50} ; in that case, inhibition of aromatase activity is not due to cytotoxicity.

(30–40%) was observed at low HP concentrations (2–30 μM). 4TBP also inhibited aromatase activity, but at higher concentrations (IC_{50} : 283 μM). For the other compounds (4SBP, 2SBP, 2TBP, TAP, DTBP, and CP), dose-response curves for inhibition of aromatase activity and cytotoxicity were closely related, and the apparent inhibition was mostly attributed to cytotoxicity (Figure 2, Table 2).

Additionally, P450 aromatase activity was significantly increased in cells exposed to the long-chain DP (up to 1.4 fold at 10 μM), the 2-benzene rings CP (1.3-fold at 20 μM), and 2TBP (1.3-fold at 200 μM) (Figure 2).

Figure 3 shows a linear regression model where $\log EC_{50}$ (AB assay) and $\log IC_{50}$ (aromatase activity) are related with $\log K_{ow}$ of the different APs investigated. OP and NP data from a previous study were included in the model ([Pérez-Albaladejo et al., 2017](#)).

Analysis of Lipids

A total of 156 lipid species subdivided in 9 classes were identified in JEG-3 cells. After preprocessing, the data matrix was restricted to 136 lipids. The composition by subclass and amount of individual glycerolipid and glycerophospholipid species, as well as fatty acid profiles, are reported in Supplementary Tables 1 and 2. Free fatty acid profiles were obtained by dividing the intensity of the peak by the sum of the intensities of all fatty acids in the sample. Glycerolipids encompassed 30% of the identified lipids in JEG-3 cells. Among them, TG 50:2, 52:2, 52:3 and 54:3 were the most abundant species (48% of total TGs). DGs were at substantially lower concentration, the most abundant species were DG 34:1 and 36:2. Among glycerophospholipids, PCs (28%) and PEs (17%) were the most abundant. These two classes contained substantial amounts of plasmalogen/plasmenyl analogues that were named as PC-Ps/PC-Os (12%) and PE-Ps/PE-Os (5.5%). PIs (5.7%) and PSs (2.5%) were also identified. Regarding fatty acids, palmitic acid (16:0) was the major constituent (47%) followed by stearic acid (18:0; 33%) and oleic acid (18:1; 11%) (Supplementary Table 2). These 3 molecules comprise about 91% of all free fatty acids detected in JEG-3 cells. PUFAs represented about 4% of total free fatty acids, being 18:2 (linoleic acid) the most abundant ($\sim 1\%$).

PLS-DA analysis revealed changes in the lipidome of cells exposed to HP (10, 20 μM), DP (10 μM), and TTBP (20 μM) when compared with unexposed cells (Figure 4). The score plots showed a clear separation between the lipidome of cells exposed to 10 μM HP

Figure 1. Generation of ROS in JEG-3 cells after 60 min of exposure to APs (μM) or $5\text{ }\mu\text{M}$ SIN-1 (positive control). Values are expressed as mean fold induction $\pm$ SD of at least 6 replicates in 3 different plates. *Statistically significant differences from control ($p < .05$).

($R^2 = 0.697$, $Q^2 = 0.276$) and $20\text{ }\mu\text{M}$ HP (50.8% of variance explained, $R^2 = 0.975$, $Q^2 = 0.879$) and control cells. Nonetheless, the quality parameters of the model for $10\text{ }\mu\text{M}$ HP were low (Figs. 4A and B). Up to 57 lipids yielded VIP scores > 1.0 in the PLS-DA models (Supplementary Table 3). A selection of the highest 25 VIPs are indicated in Figures 4A and B. The lipidome of HP-exposed cells showed a relative enrichment of (a) DG 32:0, TG 48:0, and PC 32:0, probably formed by 2 and 3 molecules of palmitic acid (16:0), and (b) monounsaturated species, namely, DG (32:1, 34:1), TG (46:1, 48:1), and PC (34:1, 36:1). In contrast, a general decline of polyunsaturated PCs (34:3, 36:3, 38:4, 40:7), PC-Ps (36:3, 36:4, 38:4, 38:5), DGs (32:2, 34:2, 36:2, 36:3, 38:4), a large number of TGs (eg. 52:4, 52:5, 54:3, 54:4, 56:4, 56:5, 58:5, 58:7, 58:8), and PSs (34:2, 36:2, 38:2, 38:3) was observed.

Univariate analysis (volcano plots) identified up to 50 lipids that differed significantly between control and HP-exposed cells (fold change > 1.5 , $p < .05$) (Figs. 5A and B). Only lipid species that fulfilled the VIP > 1.0 and volcano criteria were considered as potential biomarkers of HP exposure. Namely, PC-P 36:4, PE (34:3, 36:4), PE-P 34:2, PS (36:2, 38:2, 38:3), PI 38:3, and TG (48:3, 52:4, 52:5, 54:4, 56:4, 58:5) significantly decreased; whereas DG 32:0 and PC 36:1 increased. Moreover, at the highest concentration ($20\text{ }\mu\text{M}$ HP), additional polyunsaturated lipids declined, including PC 34:3 and PC-Ps 36:3, 38:3, 38:4, 38:5, whereas TG 48:0 increased (Figure 5B).

Regarding the lipidome of cells exposed to $10\text{ }\mu\text{M}$ DP, PLS-DA explained approximately 46% of the variance with two components ($R^2 = 0.931$; $Q^2 = 0.792$) (Figure 4C). The analysis revealed 47 lipids with VIP > 1 as responsible for the discrimination between DP-exposed and unexposed cells (Figure 4C; Supplementary Table 4). DP-exposed cells showed reduced levels of DG and a concomitant increase of TGs and PCs. The volcano plot highlighted 19 lipid species that were significantly altered, all of them with VIP > 1 (Figure 5C). A relative decrease of DGs (32:0, 32:1, 34:1, 34:2, 36:2, 36:3, 38:4), PE 38:6, and PI 36:3 was observed together with a significant increase of saturated phospholipids (PC 32:0, PC-O 32:0) and TGs (50:4, 54:6, 56:6, 56:8).

The PLS-DA model clearly discriminated between lipid profiles of cells exposed to $20\text{ }\mu\text{M}$ TTBP and unexposed cells

(explained variance = 39%, $R^2 = 0.840$, $Q^2 = 0.726$) (Figure 4D). Multivariate analysis showed 46 lipids with VIP > 1 , most of them TGs with different number of double bonds (Supplementary Table 5). When volcano plot was performed, the list of the disrupted lipids was reduced to 19, being most of them TGs (46:1, 46:2, 48:1, 48:2, 50:3, 54:4, 56:8, 58:7) that increased in exposed cells, and the depletion of a compound that was tentatively identified as PC-O 36:1 (Figure 5D).

DISCUSSION

The most hydrophobic and long-chain APs (DP and HP), the 2-benzene rings (CP), and the tri-substituted butylphenol (TTBP) induced the highest toxicological response in JEG-3 cells in terms of cytotoxicity and/or alteration of aromatase activity. Thus, DP was the most cytotoxic compound (EC_{50} : $18\text{ }\mu\text{M}$), followed by HP ($\sim 50\text{ }\mu\text{M}$) and CP ($\sim 65\text{ }\mu\text{M}$). Similarly, DP was the strongest inhibitor of P450 aromatase (IC_{50} : $14\text{ }\mu\text{M}$), followed by HP ($\sim 41\text{ }\mu\text{M}$), TTBP ($\sim 58\text{ }\mu\text{M}$), and CP ($\sim 62\text{ }\mu\text{M}$). On the contrary, short-chain APs (2SBP, 2TBP, 4SBP, 4TBP, TAP, and DTBP) showed low or no cytotoxicity ($\text{EC}_{50} > 119\text{ }\mu\text{M}$) and did not alter aromatase activity in JEG-3 cells. Generally, cytotoxicity increased with the hydrophobicity of the APs regardless of the length, structure, and position of the alkyl chain, except for TTBP that was not cytotoxic despite having a $\log K_{OW} > 5$; and OP and HP that had EC_{50} lower than expected (Figure 3A). Thus, cytotoxicity of APs in JEG-3 cells cannot be predicted based solely on their K_{OW} , since the lineal regression model ($R^2 = 0.647$) did not guarantee the accuracy of the expected EC_{50} value. Similarly, Tollefsen et al. (2008) reported that the cytotoxicity of APs ($\log K_{OW} < 4.9$) in rainbow trout primary hepatocytes increased with hydrophobicity, whereas APs with higher $\log K_{OW}$ deviated from this relationship. Interestingly, both rainbow trout hepatocytes and placenta cells, responded similarly to HP, DP, and TTBP in terms of cytotoxicity, but higher cytotoxicity was reported for the short-chain 2TBP, 4TBP, 4SBP, and DTBP in rainbow trout hepatocytes (EC_{50} between 28 and $217\text{ }\mu\text{M}$) than in placenta cells (EC_{50} : 120 to $> 500\text{ }\mu\text{M}$), which suggest stronger

Figure 2. Aromatase activity (solid line) and viability-AB assay (dotted line) in JEG-3 cells after 24 h exposure to APs. Aromatase activity in control cells was 336 ± 22 fmol/min/mg protein. Values expressed as percentage of activity in control cells (mean $\pm$ SEM) of at least 3 assays. ^{a,b}Significant differences from control ($p < .05$) for aromatase activity and cytotoxicity, respectively.

metabolism and detoxification mechanisms in placenta cells. When $\log K_{OW}$ was plotted against aromatase inhibition ($\log IC_{50}$), a good linear relationship was observed ($R^2 = 0.9367$; Figure 3B), which indicates that aromatase inhibition is closely related to the hydrophobicity of APs. However, it should be noted that the inhibitory response for some of the compounds

is closely associated to cytotoxicity, since the response curves for aromatase and cytotoxicity overlap (Figure 2).

Among the investigated compounds, the activation (DP: 44% at 10–20 μ M) and inhibition (HP, TTBP) of placental P450 aromatase are of particular concern, since this enzyme is responsible for estrogen production and the sexual steroid balance

Figure 3. Relationship between APs hydrophobicity (log K_{ow}) and A, cytotoxicity-AB assay (log EC_{50}); B, inhibition of aromatase activity (log IC_{50}) in JEG-3 cells.

necessary for normal embryonic and fetal development in pregnant women. Likewise, NP and OP increased aromatase activity in JEG-3 cells in a similar range of concentrations (20–30 μ M); just prior a cytotoxic effect was observed (Pérez-Albaladejo *et al.*, 2017). The mechanisms through which DP and other long-chain APs induce aromatase activity in JEG-3 cells in a narrow range of concentrations may be various (up-regulation of CYP19, regulation at the post-translational level, and/or changes in signaling pathways) and should be further investigated.

Despite the low cytotoxicity and ability to disrupt aromatase activity, the short-chain APs substituted in position 4, namely, TAP, 4SBP, and 4TBP, significantly induced the generation of ROS (up to 7-fold). HP caused the highest induction of ROS (up to a 43-fold) in exposed cells. Such a high induction has never been reported, but it should be interpreted with caution due to the complex redox chemistry of H_2DCF in the cell. H_2DCF -DA, the probe used to assess intracellular ROS generation, is deacetylated by intracellular esterases into a nonfluorescent compound (H_2DCF), which is subsequently oxidized by ROS into 2',7'-dichloro-fluorescein (DCF). Some compounds (viz. tetrabromobisphenol

A) can stimulate the conversion of H_2DCF to DCF in cell-free models (Szychowski *et al.*, 2016; Tetz *et al.*, 2013). Nevertheless, this was not the case for HP, as no fluorescence was detected in the assays performed with NaOH-activated H_2DCF -DA in the absence of cells. Peroxidases may also induce the oxidation of H_2DCF and generate DCF semiquinone-free radical, DCF phenoxyl radical, and $O_2^{\cdot-}$, which would cause an amplification of the fluorescence (Tarpey and Fridovich, 2001). Interestingly, when ROS generation was measured after 24 h exposure to the APs, HP was the only compound that induced the generation of ROS (up to 40-fold) (Supplementary Figure 1). Previous works have related oxidative stress to inhibition of placental aromatase and pregnancy complications, including spontaneous abortion, recurrent pregnancy loss, and preeclampsia (Agarwal *et al.*, 2012; Hempstock *et al.*, 2003; Milczarek *et al.*, 2008). Thus, the general decline of aromatase activity in JEG-3 cells after 24 h exposure to HP (1–30 μ M) could be due to the high generation of ROS and the consequent oxidative stress.

Oxidative stress can result in peroxidative damage to PUFA in cellular lipids; the sensitivity of PUFA to ROS-induced damage

Figure 4. PLS-DA scores plot shows the differentiation between the lipidome of exposed cells ($n=6$) and control cells ($n=10$). Left panel shows the 25 most important lipids ranked by VIP scores. A, 10 μ M HP; B, 20 μ M HP; C, 10 μ M DP; D, 20 μ M TTBP. Lipid species are annotated as lipid class followed by 3 digits that indicate the number of carbons (2 digits) and number of double bonds (1 digit).

increases exponentially as a function of the number of double bonds per molecule (De la Haba et al., 2013). Accordingly, significant changes in the lipidome of JEG-3 cells exposed to HP were observed. These changes consisted on a depletion of polyunsaturated lipid species, mainly membrane lipids involved in the protection against oxidative stress (eg, PC-Ps 36:3, 36:4, 38:3, 38:4; PE 34:3, PS34:2, 38:2), second messengers (eg, DGs 32:2, 36:3, 38:3, 38:4), and storage lipids (eg, TGs 52:4, 56:5, 58:5), and a relative enrichment of saturated and monounsaturated lipids (PC 36:1, DG 32:0, TG 48:0). Nonetheless, despite of the significant changes in the cell lipidome, most PCs and their fatty acid composition were maintained, whereas TGs significantly

decreased and their fatty acid composition changed toward a decrease in the PUFA content after exposure to HP. It has been reported that damaged PUFA can be rapidly replaced with undamaged ones by deacylation/reacylation cycles in cells, and TGs provide the undamaged PUFA in this process (Girón-Calle et al., 1997). This observation was also consistent with the decline of putative PC-Ps. The analytical technique used does not allow discriminating between plasmalogens (ether lipids with an O-(1-alkenyl) or vinyl ether bond, also called plasmenyl lipids) and plasmalogen lipids (ether lipids with an O-(1-alkyl)) by exact mass. Nonetheless, due to the relatively high abundance of plasmalogens and the fact that at the sn-2 position, plasmalogens are enriched in PUFAs, specifically docosahexaenoic (FA 22:6) or arachidonic acid (FA 20:4), some of the compounds have been tentatively identified as plasmalogens (for details, see Supplementary Table 1) (Braverman and Moser, 2012). Interestingly, the hydrogen atoms adjacent to the vinyl ether bond of PC-Ps have relatively low disassociation energies and are preferentially oxidized over PCs when exposed to oxidative stress. Moreover, the oxidative products of plasmalogens are unable to further propagate lipid peroxidation. This has been proposed to reduce the oxidation of PUFAs and other susceptible lipids, highlighting the crucial role of PC-Ps in protecting the cell against oxidative stress (Braverman and Moser, 2012). In addition, the significant accumulation of fully saturated glycerolipids (DG 32:0, TG 48:0) and PC 36:1, and the significant depletion of highly unsaturated PEs, PIs, and PSs elevated the overall lipid saturation in the cells and may result in a decrease of cell membrane fluidity (Lin et al., 2017). The depletion of PEs, PIs, and PSs (inner membrane lipids) may also result in lipid packaging defects and indirect modulation of membrane properties, as curvature and permeability (Bigay and Antonny, 2012).

Moreover, a significant decrease of free long-chain PUFAs (20:2, 20:3, 20:4, 22:3, 22:4 and 22:6) were observed in HP-exposed JEG-3 cells (Supplementary Figure 2). These long-chain PUFAs are derivatives of essential fatty acids (linoleic [18:2] and α -linolenic [18:3] acids) and are critical for normal development. During embryo growth, large amounts of docosahexaenoic and arachidonic acids released from placenta are deposited in the fetal retina and brain and these fatty acids play a key role involved in neuronal and visual function (Dunstan et al., 2004). Arachidonic acid can be metabolized by several enzymes to form eicosanoid lipid mediators, such as prostaglandins, leukotrienes, lipoxins, epoxyeicosatrienoic acids, and thromboxanes (Watson, 2006), which corroborates the important function of the long-chain PUFAs in placenta cells. In the same line, the general decrease of polyunsaturated TGs in presence of HP is of particular concern since the fetus compartment is provided with these PUFAs from maternal TGs, from which are released by lipoprotein lipase activity of placental cells (Coleman and Haynes, 1987; Gude et al., 2004). Also, alterations of membrane composition may result in imperfect packing of the lipid bilayer, affect fluidity or dynamism and thereby influence membrane potential, vesicle and/or ion transport, signal transduction, and loss of cell integrity (Wolfgang and Lane, 2006).

In contrast exposure to DP, which did not induce ROS generation in placenta cells, lead to a lipidomic profile characterized by a depletion of DGs (32:0, 32:1, 32:2, 34:1, 34:2, 34:3) and a concomitant increase of the corresponding PCs (32:0, 32:1, 32:2, 34:1, 34:2, 34:3) and some highly unsaturated TGs (eg, TGs 54:5, 56:6, 54:6, 58:5, 58:6), suggesting that cells are producing PCs and TGs at the expense of DGs. DP exposure also led to a significant increase of PC 32:0 (3-fold), PC-P/PC-O 32:0, and PE 36:1 and a

Figure 5. Volcano plots of the lipid of exposed and control cells, revealing those species with a p-value < .05 and a fold change > 1.5 (red dots). A, 10 μ M HP; B, 20 μ M HP; C, 10 μ M DP; D, 20 μ M TTBP.

decrease of PC-P 38:4 and PE 38:7, which increases the overall saturation index of membrane lipids. Nonetheless, this effect was not associated to significant changes in free fatty acids, as observed in HP-exposed JEG-3 cells. The fluidity of placenta cell membranes modulates transport processes between fetus and mother, and membrane lipids are modified during

pregnancy, according to the needs of the growing fetus (Damiano, 2011). Thus, exposure to DP might alter membrane fluidity and the functionality of membranes. Actually, an induction of P450 aromatase activity, an enzyme closely linked to endoplasmic reticulum membranes, was observed after exposure to 10 μ M of DP.

Finally, a significant accumulation of TGs was evidenced when JEG-3 cells were exposed to 20 μ M of TTBP. The formation of TGs from DGs is catalyzed by diacylglycerol acyltransferase (DGAT), whose activity is high in those organs that need more energy (adipose tissue, liver, lactating mammary gland) and consequently, they have high rates of TGs synthesis (Coleman and Lee, 2004). In placenta and other tissues, the rates of TGs synthesis are low, and the primary function of TGs is to provide phospholipids for cellular needs. Previous studies have demonstrated the ability of some xenobiotics to induce the intracellular accumulation of TGs through diverse mechanisms (Blumberg, 2011). The placenta is a site for fat storage during pregnancy, and lipid droplets have been observed in a rodent model for preeclampsia using histochemical staining (Sun et al., 2012). Moreover, TGs (eg, 54:5, 56:6) that accumulated in JEG-3 cells after exposure to DP and TTBP were reported to accumulate in placentas with preeclampsia (Brown et al., 2016) and elevated placental triglyceride storage have been reported in maternal diabetes and obesity. These metabolic changes affect placental nutrient handling and may induce pro-adipogenic changes in the fetus (Lewis and Desoye, 2017). Overall, this work highlighted DP, HP, CP, and TTBP as the most harmful compounds that caused endocrine and/or lipid disruption, oxidative stress, and cytotoxicity in human placental cells. The work evidences for the first time the potential of HP to generate oxidative stress and to consequently inhibit aromatase activity and disrupt cellular lipids. TTBP acted as an inhibitor of placental aromatase and induced the intracellular accumulation of TGs, whereas the long-chain DP induced aromatase activity and stimulated the synthesis of membrane and storage lipids. All three compounds acted at concentrations at the low micromolar range. In contrast, short-chain APs (4TBP, 2TBP, 2SBP, and 4SBP) were less cytotoxic and did not act as endocrine disruptors in placental cells.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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