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Cytotoxicity of seven bisphenol analogues compared to bisphenol A and relationships with membrane affinity data

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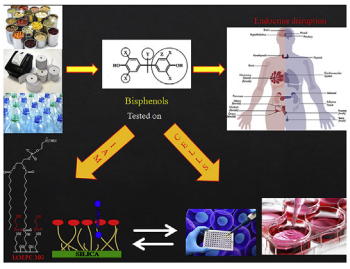
HIGHLIGHTS

- Cell viability data indicate that BPE, BPF and BPS are less toxic than BPA.
- BPAF and BPM were found more toxic than BPA.
- Cell toxicity data related well with (phospho)lipophilicity.
- To surrogate BPA, bisphenols with lower (phospho)lipophilicity should be preferred.

GRAPHICAL ABSTRACT



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ABSTRACT

Bisphenol A (BPA) is a chemical used in numerous industrial applications. Due to its well ascertained toxicity as endocrine disruptor, industries have started to replace it with other bisphenols whose alleged greater safety is scarcely supported by literature studies. In this study, the toxicity of seven BPA analogues was evaluated using both *in silico* and *in vitro* techniques, as compared to BPA toxicity. Furthermore, their affinity indexes for phospholipids (*i.e.* phospholipophilicity) were determined by immobilized artificial membrane liquid chromatography (IAM-LC) and possible relationships with *in vitro* toxic activity were also investigated. The results on four different cell cultures yielded similar ranking of toxicity for the bisphenols considered, with IC_{50} values confirming their poor acute toxicity. As compared to BPA, bisphenol AF, bisphenol B, bisphenol M, and bisphenol A diglycidyl ether resulted more toxic, while bisphenol S, bisphenol F and bisphenol E were found as the less toxic congeners. These results are partly

Abbreviations: ADMET, (Absorption, Distribution, Metabolism, Excretion, Toxicity); AR, androgen receptor; DMEM, Dulbecco's Modified Eagle's Medium; EDCs, endocrine disrupting chemicals; ER, estrogen receptor; FBS, fetal bovine serum; IAM, immobilized artificial membrane; LC, liquid chromatography; WHO, World Health Organization; BPA, bisphenol A; BADGE, bisphenol A diglycidyl ether; BPAF, bisphenol AF; BPB, bisphenol B; BPE, bisphenol E; BPF, bisphenol F; BPM, bisphenol M; BPS, bisphenol S; DMSO, dimethyl sulfoxide.

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Immobilized artificial membrane
ADMET predictor
Cell viability
Toxicity
Endocrine disrupting chemicals

consistent with the scale of phospholipid affinity showing that toxicity increases at increasing membrane affinity. Therefore, phospholipophilicity determination can be assumed as a useful preliminary tool to select less toxic congeners to surrogate BPA in industrial applications.

1. Introduction

Hazardous chemicals escape to the environment in a variety of anthropogenic ways and their release may result in adverse effects both on the human health as well as on the environment (McGrath et al., 2017). Among the environmental pollutants, endocrine disrupting chemicals (EDCs), able to interfere with endocrine system, are increasingly gathering the attention of the scientific community due to their well ascertained toxicity for humans (Le Magueresse-Battistoni et al., 2017). Indeed, the European Commission and World Health Organization (WHO) consider as a priority the broadening of the knowledge about the molecular mechanisms underlying their toxicity. Bisphenol A (BPA) (2,2-Bis(4-hydroxyphenyl)propane) is one of the most widespread EDCs. It is widely used in the production of various polycarbonates and epoxy resins used as food contact materials, internal lining of cans, bottle caps, material of some dental sealants, additive in thermal paper (Vinggaard et al., 2000; Geens et al., 2011; Liao and Kannan, 2011; Mendum et al., 2011; Russo et al., 2017). The primary source of human exposure to BPA is the diet, however air, dust, water are other possible sources of exposure, which may even occur through skin contact.

BPA has been widely investigated with respect to its toxicological hazard and has shown effects also at low-dose (Vandenberg, 2014; Ariemma et al., 2016; Hass et al., 2016). Due to these outcomes, industries have started to replace BPA with other bisphenols (BPs) such as bisphenol A diglycidyl ether (BADGE), bisphenol AF (BPAF), bisphenol B (BPB), bisphenol E (BPE), bisphenol F (BPF), bisphenol M (BPM), and bisphenol S (BPS) (Usman and Ahmad, 2016). There are numerous scientific articles reporting analytical data of bisphenol migration in foods and beverages sold in cans in Europe (Grumetto et al., 2008, 2013; Fattore et al., 2015). However, the safety of BPA analogues is still debated and their effects on cellular metabolism and potential role as endocrine disruptors have not been fully established. Consequently, replacing BPA with its analogues does not necessarily imply an improved safety. Therefore, a more extensive characterization of BPA analogues toxicity, as well as of the structural properties driving it, is highly desirable.

The chemical structures of the BPs considered in our study are shown in Fig. 1. All BPs feature of a scaffold consisting of two phenol rings, functionalized with different groups, linked by a variously substituted carbon atom. The only exception is BPM, bearing three benzene moieties. The shared core scaffold is believed directly responsible of the endocrine activity. BPs have been demonstrated to interact with a variety of endogenous receptors, e.g. thyroid hormone, glucocorticoid hormone, androgen and estrogen receptors (Chen et al., 2016). Moreover, BPA has been demonstrated able to modulate several serotonin- and dopamine-associated genes (Miller et al., 2009; Castro et al., 2015; Fischer et al., 2016). BPs can act as weak agonist or antagonist on estrogen receptor (ER). Due to its similarity to diethylstilbestrol, a synthetic estrogen known to cause cancer (Kurosawa et al., 2002; Sharma et al., 2017), BPA is suspected to induce carcinogenesis, as in the case of cancer of the hematopoietic system and interstitial-cell tumor of the testis (Keri et al., 2007). Moreover, several studies using *in vitro* yeast-based assays demonstrated the strong anti-androgenic activity of BPA (Sohoni and Sumpter, 1998; Lee et al., 2003).

Several studies have been performed on toxicity of BPA and its analogues, sometimes reporting controversial results (Rochester, 2013; Eladak et al., 2015; Rochester and Bolden, 2015; Chen et al., 2016).

In the present study, to gain a scale of relative toxicity with respect to BPA, the toxicity of BPA and its seven congeners was investigated *in vitro* in terms of interference with cell growth and proliferation, on a restricted panel of well-established mammalian cells, including normal and cancer cell lines. Furthermore, affinity of BPs for membrane phospholipids (phospholipophilicity) was determined by Immobilized Artificial Membrane (IAM)- Liquid Chromatography (LC) (Barbato et al., 2004) and possible relationships between toxicity of BPs and their membrane affinity were investigated. In fact, BP ability to cross biological membranes strongly affects their toxicokinetics, including their capability to access the receptor site(s). Lipophilicity is the reference parameter in describing passive diffusion through the biological barriers and is traditionally expressed by the logarithm of the ratio of analyte concentrations in an organic solvent, usually *n*-octanol, and an aqueous phase (log *P*) (Liu et al., 2011). However, phospholipophilicity measures were found more effective for this purpose (Taillardat-Bertschinger et al., 2003). The occurrence of a significant relationship between phospholipophilicity and toxicity would allow a prediction of toxicity potential of new BPA analogues and contribute to shed light on the molecular features driving toxic effects in bisphenol class.

Finally, some pharmacokinetic and toxicological properties of the eight BPs generated *in silico* by ADMET predictor™ software (Ghosh et al., 2016) were also considered. This software quickly and accurately predicts over 140 properties, including carcinogenicity potential, permeability, and toxicity.

2. Materials and methods

2.1. Chemicals

Methanol (HPLC analytical grade) and acetonitrile (minimum purity ≥95%) were both purchased from Sigma Aldrich (Milan, Italy). BPF (4,4- dihydroxydiphenylmethane, minimum purity ≥ 99.0%), BPS (4,4′-sulfonyldiphenol, minimum purity ≥98%), BPA (2,2-bis(4-hydroxyphenyl)propane, minimum purity ≥ 99%), BADGE (2,2-Bis[4-(glycidyloxy)phenyl]propane, minimum purity ≥ 95%), analytical standards were purchased from Sigma-Aldrich (UK). BPE (4,4′-ethylidenediphenol, minimum purity > 98%), BPB (2,2-Bis(4-hydroxyphenyl)butane, minimum purity ≥ 99%), BPAF (hexafluoroisopropylidene) diphenol, minimum purity > 98%), BPM (4,4-(1,3-Phenylenediisopropylidene)bisphenol, minimum purity > 98%) analytical standards were purchased from TCI Europe (Zwijndrecht, Belgium). L-glutamine, penicillin, streptomycin and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich, (Milan, Italy).

2.2. ADMET predictor calculations

The toxicological profiles of the eight BPs were predicted using

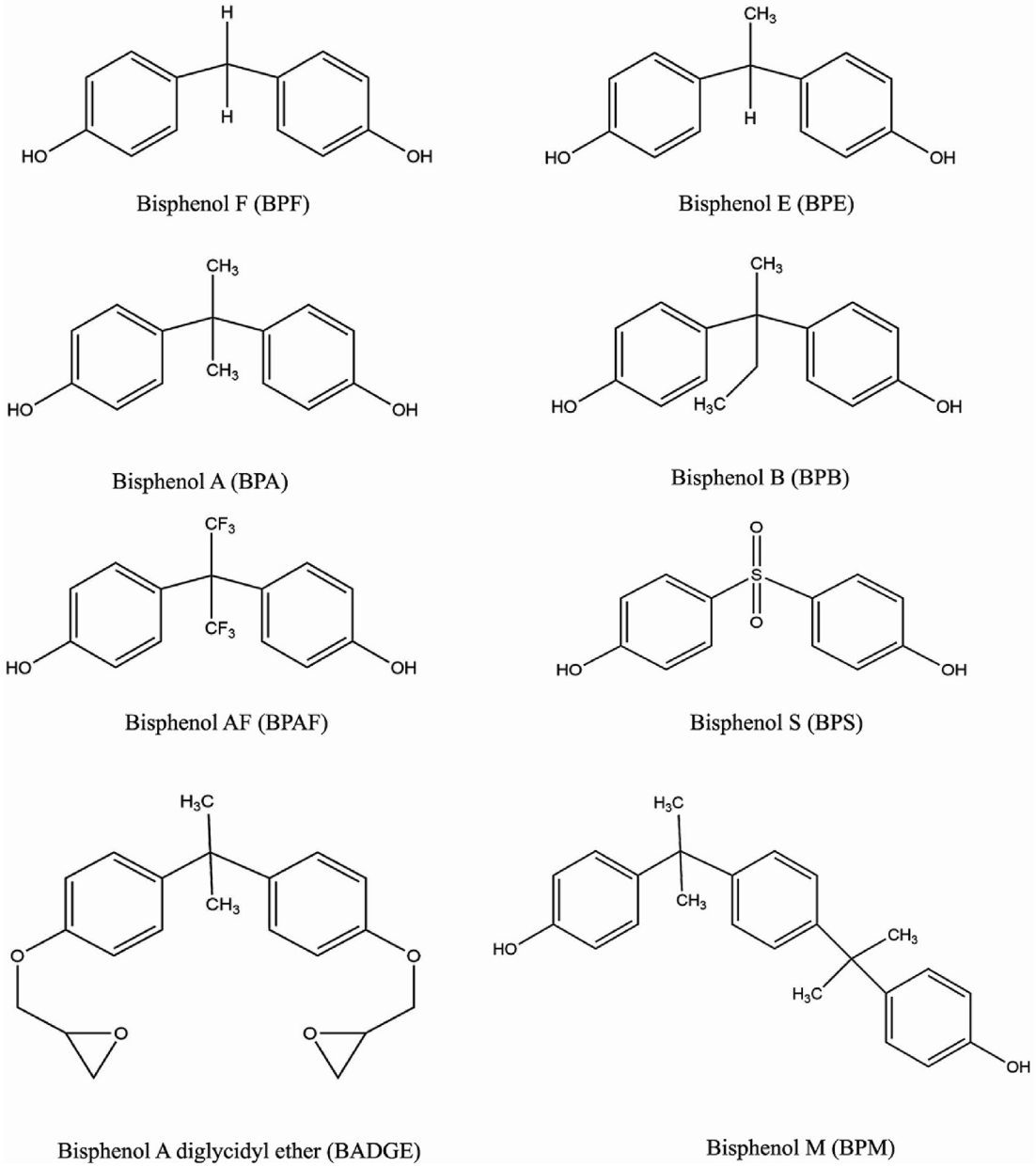

Fig. 1. Common names and chemical structures of the eight bisphenols considered.

Simulations-Plus ADMET Predictor Version 7.1 (SimulationPlus, Lancaster, CA USA) for Windows-based personal computers. ADMET Predictor™ is a computer program that enables researchers to estimate various ADMET (Absorption, Distribution, Metabolism, Excretion, Toxicity) properties of compounds from their molecular structure rapidly and with a high degree of accuracy. It was designed using artificial neural network ensemble (ANNE) models (Paixao et al., 2014) trained with well-defined drugs, and was chosen for its high prediction accuracy, and descriptor sensitivity analysis capabilities. *In silico* predictions were calculated in ADMET Predictor™ (Simulations Plus, Inc.) with default settings at pH 7.4. All predicted properties for each structure, including an out of-scope indicator column for each model are provided as supplementary materials. Among the several ADMET data, toxicity on both androgen (TOX-AR) and estrogen (TOX-ER) receptors were considered.

TOX-AR (and TOX-ER) are dimensionless numbers expressed as the percent ratio: 100% * (50% of the maximum inhibitory concentration (IC_{50}) for R1881/IC_{50} (17 β-estradiol for TOX-ER) for the chemical in question. R1881 is 17R-methyl-[3H]-methyltrienolone, a synthetic substrate that binds androgen receptor more strongly than testosterone. Therefore, this model displays the relative binding affinity of a molecule determined by a competitive binding assay. Higher values indicate greater binding affinity and likelihood for endocrine-related toxicity.

2.3. Phospholipophilicity as measured on IAM stationary phases

Phospholipophilicity was measured in terms of chromatographic retention coefficient on IAM stationary phases, *k*, that is defined as:

$$k = \frac{t_r - t_0}{t_0}$$

in which t_r and t_0 are the retention times of the analyte and a non-retained compound (acetone), respectively. The values referring to 100% aqueous phase (k_w^{IAM}) can be assumed as direct measures of the partition of solutes between phospholipids and buffer aqueous phase (Barbato et al., 2004).

2.4. Chromatographic system

A Shimadzu liquid chromatographic apparatus (LC-10AD), (7725 Rheodyne injection valve 20 μL loop) and a SPD-10AV UV detector (Shimadzu), set at λ 220 nm were used. The chromatograms were recorded and processed by Cromatoplus (2008 software for personal computer (Shimadzu). The stainless-steel column was an IAM.PC.MG (4.6 × 150 mm; Regis Chemical Company, Morton Grove, IL).

2.5. Chromatographic conditions

Eluent: 0.1 M phosphate buffer at pH 7.0 and acetonitrile at various percentages; the flow rate was from 1.0 to 2.3 mL min^{-1}. The analyses were carried out at room temperature. Samples were dissolved in acetonitrile (*ca.* 10^{-4} M) and injected. Chromatographic retention data are expressed by the logarithm of the retention factor, log *k*. All the compounds required the addition of acetonitrile to the eluent to elute within 20 min. The log *k* values relative to 100% aqueous eluent ($\log k_w^{IAM}$) were calculated by performing a polycratic method of extrapolation (Braumann et al., 1983): at least four different mobile phases containing acetonitrile in percentages (φ) ranging from 10% to 30% (v/v) were employed. Linear relationships between log k and φ values were found for all compounds in the range of eluent composition examined ($r^2 = 0.99$). All values of log *k* are the average of at least three measurements; the 95% confidence interval associated with each value never exceeded 0.04 for each log *k* value. Possible occurrence of retention changes due to column aging was monitored by checking the retention times of five test compounds (amlodipine, *p*-nitroaniline, toluene, isradipine, and ketoprofen). During the study, no retention value of test compounds changed more than 4% and no correction was done to the retention values experimentally determined for the analytes.

2.6. Lipophilicity

Log P values, i.e. partition coefficients *n*-octanol/aqueous phase of the analytes, were calculated *in silico* by clogP program (clog P for Windows version 2.0, Biobyte Corp., Claremont, CA).

2.7. Cell cultures and treatments

The *in vitro* experiments were carried out in human breast adenocarcinoma cells (MCF-7), human cervical epithelial cancer cells (HeLa), mouse fibroblasts (3T3-L1) and rat glioma cells (C6). MCF-7, HeLa and C6 cell lines were obtained from the American Type Culture Collection (ATCC), whereas 3T3-L1 cell line was from the European Collection of Cell Cultures (ECACC). 3T3-L1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, UK), C6 cells were grown in DMEM containing high glucose (4.5 g/l), while MCF-7 and HeLa cells were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK). Media were supplemented with 10% fetal bovine serum (FBS) (Cambrex, Verviers, Belgium), L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. For bioscreens *in vitro*, cells were washed, collected by trypsin and then inoculated in 96-microwell culture plates at a density of 10^4 cells/well. Cultures were allowed to grow for 24 h reaching sub-confluence, then the medium was replaced with fresh medium and the cells were treated for further 48 h with a range of micromolar concentrations (10, 25, 50, 100, 200, 300 μM) of BPA, BADGE, BPS, BPF, BPB, BPE, BPM, and BPAF. All BPs were dissolved in DMSO. More in detail, 1 or 2 μL of DMSO solutions containing the test compounds were added to the cell culture medium to give final concentrations ranging from 10 to 300 μM; 1 or 2 μL of DMSO alone (vehicle) were added to control cells (0.5% and 1% v/v final concentrations, respectively).

2.8. Assessment of cell toxicity

The cytotoxic effects of the bisphenols were determined using the MTT colorimetric assay based on the redox ability of the active mitochondrial dehydrogenases of living cells to convert a soluble tetrazolium salt, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), into insoluble formazan. The MTT assay was performed as previously described (Miniaci et al., 2016). The calculation of the concentration required to inhibit the cell viability by 50% (IC_{50}) is based on plots of data carried out in triplicates and repeated three times. IC_{50} values were obtained using a concentration-effect curve by nonlinear regression using a curve fitting program, GraphPad Prism 5.0, and are expressed as mean ± SEM.

2.9. Statistical analysis

All data were presented as mean ± SEM. The statistical analysis was performed using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA) and ANOVA test for multiple comparisons was performed followed by Bonferroni's test.

3. Results and discussion

3.1. Lipophilicity and phospholipophilicity

Lipophilicity and phospholipophilicity values of the eight BPs considered were expressed as clogP values (calculated *in silico*) and log k_w^{IAM} values (experimentally measured on IAM stationary phase), respectively. The determination of log P by the "*shake-flask*" method (Andres et al., 2015) revealed as scarcely reproducible, probably due to the very high lipophilicity values (in some cases > 4) of the considered BPs. Moreover, BADGE is poorly stable in solution, as it can undergo acid or basic hydrolysis yielding various degradation products. For these reasons, an LC approach was chosen as it allows higher reproducibility; LC was performed on an IAM stationary phase, yielding log k_w^{IAM} values which are a direct measure of the analyte affinity for phosphatidylcholine, the main constituent of biological membranes. It should be outlined that, as reported in our previous studies, log k_w^{IAM} values for neutral, even structurally unrelated, compounds relate unambiguously with the respective *n*-octanol/water partition coefficients (log P) (Grumetto et al., 2016). Therefore, being BPs non-ionizable compounds, the scales of log k_w^{IAM} and log P values are expected to be collinear. IAM-LC should be considered as the technique of choice for determination of the interactions analyte/biomembrane actually realizing *in vivo* mainly when ionizable analytes are also taken into account (Grumetto et al., 2015, Russo et al., 2017). This arises from at least two reasons. First, the IAM stationary phase is anisotropic implying that the analyte partition is strongly affected by the topological orientation of the analyte moieties as well as by their three-dimensional arrangement. Therefore, IAM indexes

encode directional recognition forces, occurring also *in vivo*, which are not accounted for by isotropic partition systems, such as *n*-octanol/water. In addition, differently from *n*-octanol which is electrically neutral, IAM phases are zwitterionic at the experimental (and physiological) pH 7.4. Consequently, the intermolecular interaction forces of electrostatic and polar nature differently affect partition in phospholipids with respect to that in *n*-octanol.

Although two IAM stationary phases, *i.e.* IAM.PC.MG and IAM.PC.DD2, differing from each other in the end-capping of free propylamino residues, are commercially available, the affinity values of BPs for phospholipids were measured on the former, because highly lipophilic neutral molecules, such as BPs, were too strongly retained on IAM.PC.DD2 producing retention measures unsuitable as indexes related to lipophilicity (Taillardat-Bertschinger et al., 2003).

The values of clogP and log k_w^{IAM} for the eight BPs considered are summarized in Table 1.

As evident in Fig. 2, the values of the two scales strongly relate ($r^2 = 0.98$), but only after the exclusion of BPAF, since its log k_w^{IAM} is much higher than the expected value on the basis of clogP. Indeed, relatively important gaps between calculated and measured values are often observed. They depend on parameters and increments used by the software and caution is required when using clog P values, even inside homogeneous series. In the present case this may occur because clogP program sometimes struggles to properly manage the role of the fluorine atoms (Jacobs et al., 1994). They account for a decrease of BPAF lipophilicity (clogP 2.49) with respect to BPA (clogP 3.67). In contrast, BPAF was experimentally found as more lipophilic than BPA being stronger retained than the latter not only on IAM but also on C18 stationary phases (Russo et al., 2016).

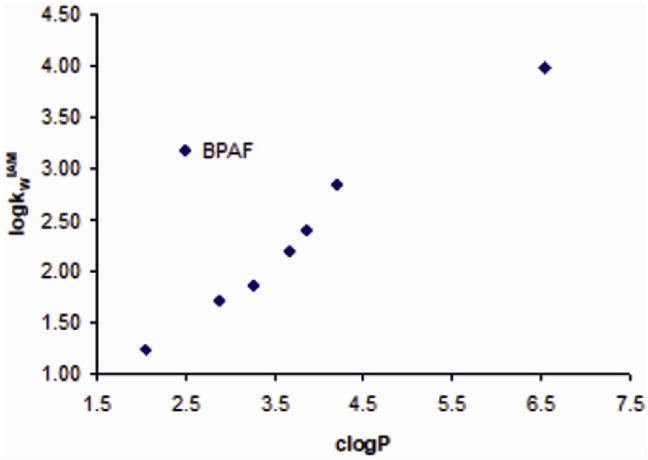

Fig. 2. - Relationship between $logk_w^{IAM}$ and clogP values.

Table 2
IC_{50} values (μM) reported as mean ± SEM (n = 9) relative to the various BPs in the indicated cell lines following 48 h of incubation.

Compound	3T3-L1	MCF-7	C6	HeLa
BPA	>100	50.0 ± 5.0	160.0 ± 0.9	209.1 ± 1.6
BPS	>100	>100	168.4 ± 6.9	299.3 ± 5.6
BADGE	71.0 ± 4.2	20.2 ± 6.0	91.6 ± 4.8	105.5 ± 1.1
BPB	53.8 ± 6.6	64.4 ± 9.5	118.5 ± 6.1	130.5 ± 3.1
BPF	110.6 ± 7.6	>100	239.4 ± 3.9	274.4 ± 4.6
BPM	53.2 ± 15.0	23.0 ± 5.0	46.9 ± 7.8	52.8 ± 5.7
BPE	112.5 ± 8.7	>100	144.2 ± 7.7	200.4 ± 29.6
BPAF	11.5 ± 9.8	36.4 ± 9.6	44.5 ± 0.2	58.3 + 4.1

3.2. *In vitro* bioscreenings for cytotoxic activity

The biological effects of bisphenols were evaluated *in vitro* using a selected panel of well-established healthy and cancer cells (3T3-L1, MCF-7, HeLa, and C6 cell lines). Specifically, 3T3-L1 are mouse embryo fibroblasts, a useful model to assess the *in vitro* cytotoxic effects of a substance on healthy tissues (Santamaria et al., 2006). MCF-7, an estrogen receptor (ER) positive cell line, is a useful cell model to analyze the effects of xenobiotics that can interact with estrogen receptors (Irace et al., 2017). HeLa is the oldest immortal cell line derived from human cervical cancer, widely used to investigate xenobiotic interference with cell growth and proliferation (Masters, 2002). Rat C6 glioma is an experimental model for the study of glioblastoma growth and invasion. Gliomas have so far shown poor sensitivity to cytotoxic agents including xenobiotics, being very resistant to many types of *in vitro* treatments (Grobben et al., 2002). The bioactivity values of BPs are reported in Table 2 as IC_{50} values. Within the panel of compounds under investigation, only BPAF and BPM can induce acute toxic effects in all the used *in vitro* models, showing IC_{50} values in the low micromolar range. The most apparent cytotoxic effects are those of BPAF on 3T3-L1 fibroblasts (IC_{50} of about 11 μM). For other BPs, acute toxicity is not apparent on all cell lines, considering that BPA exclusively interferes with the viability of MCF-7 cells. BADGE is moderately toxic on HeLa cells and definitely toxic on the other cell lines, while BPB is clearly toxic on both 3T3-L1 and MCF-7 cells. Moderate toxicity was found for BPE, BPF and BPS on all cell lines considered. Furthermore, IC_{50} values indicate that MCF-7 cells were the most sensitive cell lines to the action of bisphenols, mainly BADGE, BPM and BPAF, as well as 3T3-L1 cells, on which BPAF is primarily bioactive. It's worth to note that MCF-7 breast adenocarcinoma cells are an ER-positive model *in vitro* that endogenously expresses ERα and ERβ (Brooks et al., 1973; Levenson and Jordan, 1997; Chen et al., 2016). Analogously, concerning 3T3-L1 preadipocytes, investigation on estrogenic receptors have shown that ERα and ERβ are expressed in both rat and human preadipocytes and mature adipocytes (Price and O'Brien, 1993; Crandall et al., 1998; Dieudonne et al., 2004). On the contrary, HeLa cells do not express endogenous estrogenic receptors (Zhang et al., 1999; Binai et al., 2010; Szafran et al., 2017), whilst the presence of ER in C6 cells is controversial. Indeed, some authors did not detect ERs in C6 cells, whereas others showed the presence of ERα and the absence of ERβ, or the expression of both ERs (Yague et al., 2004; Kim et al., 2005; Sribnick et al., 2006). These differences in estrogenic receptor expression in C6 cells may in part depend on the varied cell culture conditions (Su et al., 2012). In the light of these considerations, it is interesting to note that in our experimental conditions the only BPs reducing cell viability and showing cytotoxic activity on HeLa and C6 cells are BPM and BPAF. Based on these data, we can hypothesize that BPM and BPAF biological effects might be ER-

Table 1
Calculated lipophilicity (clogP) and experimentally determined phospholipophilicity ($logk_w^{IAM}$) values for the considered BPs.

Compound	clogP	logk_w^{IAM}
BPA	3.67	2.193
BPS	2.06	1.244
BADGE	3.87	2.405
BPB	4.20	2.846
BPF	2.88	1.713
BPM	6.54	3.991
BPE	3.27	1.870
BPAF	2.49	3.170

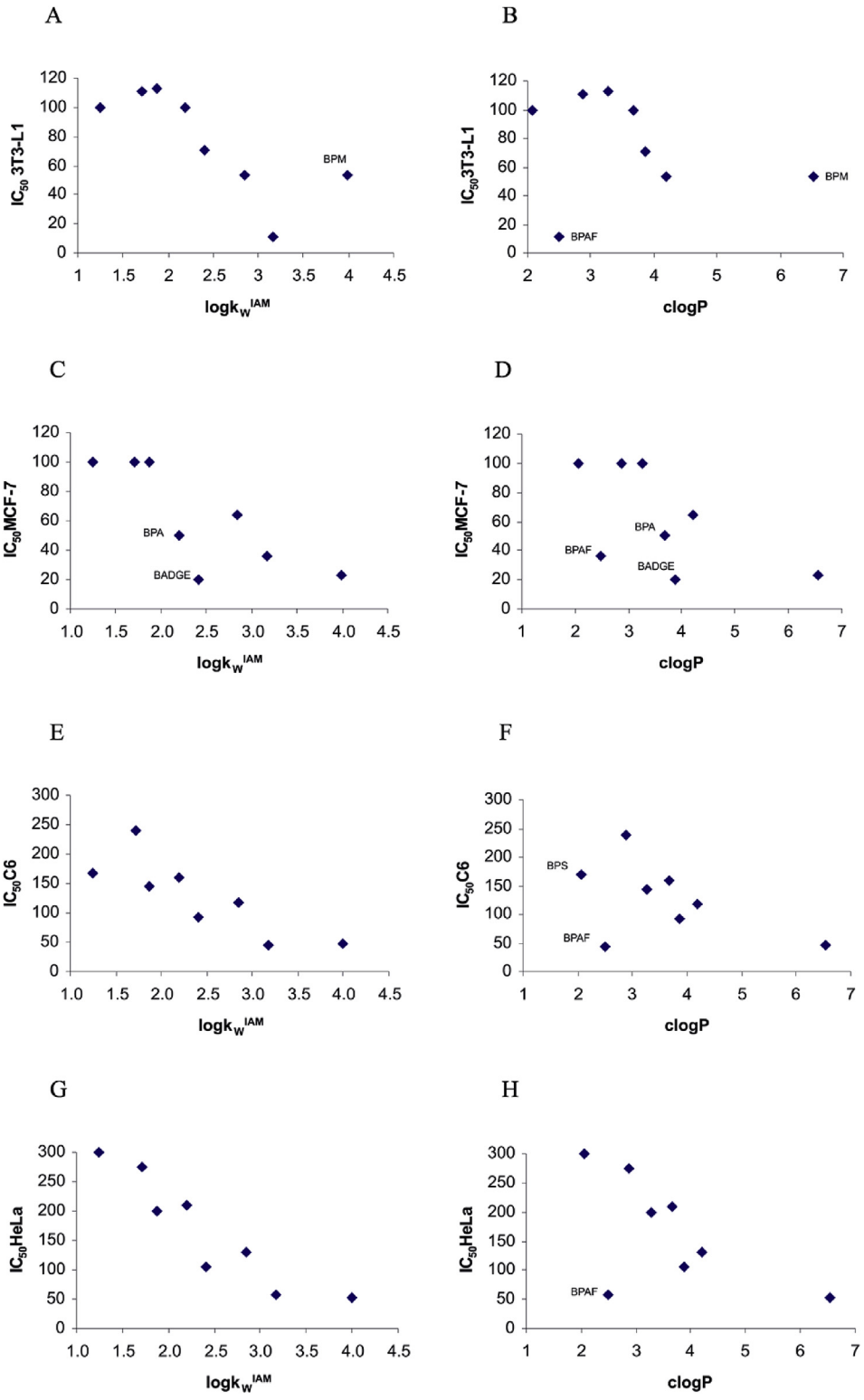

Fig. 3. Relationships between $logk_w^{IAM}$ and clogP values and IC_{50} values determined on the selected cultured cell lines 3T3-L1 (A and B), MCF-7 (C and D), C6 (E and F) and HeLa (G and H).

independent, and other molecular mechanisms of action could be considered, as the binding to other nuclear or transmembrane receptors. Overall, the results on the four cell lines are coherent in indicating that bisphenols have increasing toxicity in the order BPF and/or BPS < BPA and/or BPE < BPB and/or BADGE < BPM and/or BPAF.

3.3. Relationships between log k_w^{IAM} and cell toxicity data

The toxicity data on 3T3-L1 cells relate quite well with

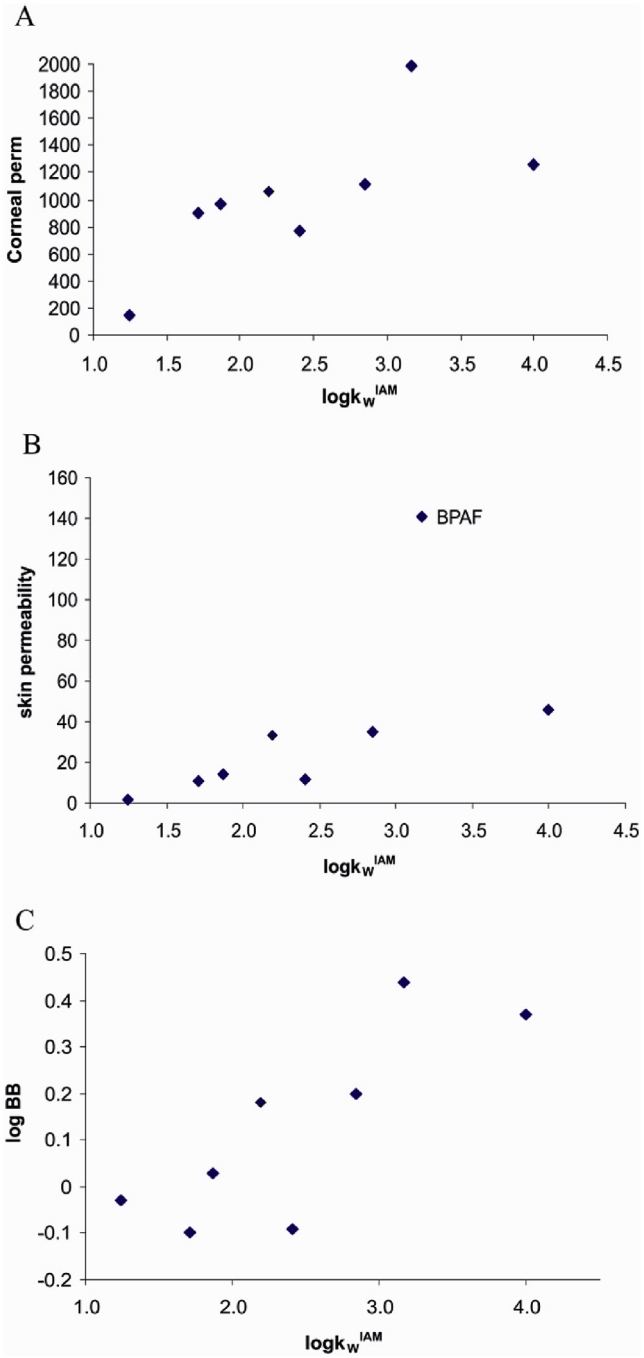

Fig. 4. Relationships between corneal permeability (A), skin permeability (B), log BB (C) and $logk_w^{IAM}$ values.

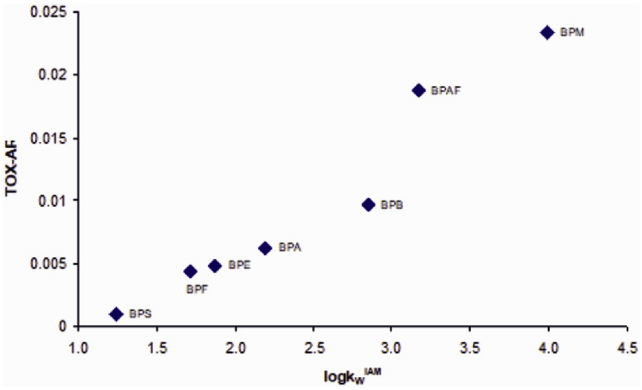

Fig. 5. Relationship between toxicity on androgen receptor (TOX-AR) and $logk_w^{IAM}$ values.

phospholipophilicity (log k_w^{IAM}) (Fig. 3A) and lipophilicity (clogP) (Fig. 3B) values. A general trend can be observed indicating that, in the phospholipophilicity range 2–3.5 and lipophilicity range 3–5, toxicity increases at increasing (phospho)lipophilicity. Toxicity is almost constant below this range. BPM, the most lipophilic compound, behaves as an outlier. More interestingly, BPAF, the only BP whose clogP was lower than expected on the basis of measured phospholipophilicity values, only fits the general trend when log k_w^{IAM}, but not clogP, is taken into account.

In Fig. 3C and D the IC_{50} values measured on MCF-7 cells are plotted *versus* phospholipophilicity and lipophilicity values, respectively. The general trend already observed on 3T3-L1 cells is only partly confirmed since both BPA and BADGE show higher activity than that expected based on their (phospho)lipophilicity. Again, as already observed in 3T3-L1 cell line, the activity of BPAF is predicted only by log k_w^{IAM} and not by clogP value. A possible explanation about the high toxicity of BPA and BADGE on MCF-7 cell line could be related to the differential ERα and ERβ expression in various cell lines or to a cell-type specific expression of different ER isoforms with a greater expression of estrogen receptors in this breast cancer cell line (Huang et al., 2015). This could enhance the final biological effect observed for BPA (and its derivative BADGE) with respect to the other BPs. Therefore, the results obtained on this cell line do not fully relate with (phospho)lipophilicity, being the latter able to govern the phenomena mainly depending upon penetration rate in the cell.

The relationships observed between toxicity data measured on C6 and HeLa cells show that toxicity increases at increasing (phospho)lipophilicity. Toxicity data excellently relate with phospholipophilicity data (log k_w^{IAM}) (Fig. 3E and G), whereas the relationships with clogP are not so good since BPAF and, only on C6 cells BPS, are outliers (Fig. 3F and H).

These results indicate that the lipophilicity indexes related to membrane affinity, such as log k_w^{IAM}, can be useful to predict cell toxicity, suggesting that membrane affinity modulates toxicity of bisphenols in a remarkable extent. Indeed, the observed effects on the cell viability arise from bisphenol activity on surface and/or intracellular targets, as well as from their capability to cross and/or interact with cell membranes. However, the relationships we found between phospholipophilicity and toxicity would suggest the membrane affinity playing a pivotal role in modulating bisphenol bioactivity.

3.4. Relationships between in silico calculated properties, cell toxicity data, and (phospho)lipophilicity

Among the over 140 properties calculated *in silico* by ADMET predictor™ software, we selected corneal and skin permeability, passage capability of Blood-Brain Barrier (log BB), as pharmacokinetic properties, as well as rat and mouse toxicity, toxicity on estrogen and androgen receptor, as toxic properties.

No relationship was found between pharmacokinetic properties and cell toxicity data (data not shown). In contrast, corneal and skin permeability data, as well as log BB, relate quite well with phospholipophilicity (Fig. 4). As to the ADMET toxicity data, rat and mouse toxicity, as well as toxicity on estrogen receptor, poorly related with either (phospho)lipophilicity or toxicity data on cell cultures (data not shown). In contrast, an excellent relationship was

found between toxicity data on androgen receptor (TOX-AR) and phospholipophilicity (Fig. 5), but only after the exclusion of BADGE whose TOX-AR value is not provided by ADMET predictor software.

Since TOX-AR values are a measure of the relative binding affinity of a molecule determined by a competitive binding assay, this relationship suggests that increasing lipophilicity values promote the binding of bisphenols to androgen receptor, but not necessarily their activity.

Since cell viability data were also found related to lipophilicity, probably because of a modulation of their passage through cell membrane, some relationship can be expected between TOX-AR and IC_{50} values on cell lines, as actually found (Fig. S1). Indeed, C6 glial cells were found to express the AR (Gatson et al., 2006) as well as MCF7 breast cancer cells (Molina-Molina et al., 2013; Chen et al., 2016), 3T3-L1 preadipocytes (Morooka et al., 2016) and HeLa cells (De Vos et al., 1994; Szafran et al., 2008). However, this would indicate that the two data sets, TOX-AR and cell viability, are substantially modulated by a same physico-chemical property, *i.e.* (phospho)lipophilicity, and not that they interrelate in a mechanistic way.

4. Conclusions

Toxicity data of the eight bisphenols on four mammalian cell lines, which resulted sensitive to bisphenols to a different extent, were consistent in indicating that, as compared to BPA, only BPE, BPF, and BPS are less toxic congeners. BADGE and BPB resulted moderately more toxic than BPA while BPAF and BPM are the more toxic ones.

The cell toxicity data relate quite well with phospholipophilicity values experimentally measured by IAM-LC method. Furthermore, androgen receptor affinity data also related with phospholipophilicity values.

The present study suggests that the use of bisphenol analogues to surrogate BPA in various industrial uses should be regarded carefully. Indeed, some congeners could be endowed with greater toxicity and a higher risk to human health. In this context, the IAM-LC technique can be a useful tool for a preliminary screening in order to select the less phospholipophilic and dangerous bisphenols.

Conflicts of interest

The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.03.014.

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