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The combined use of biological investigations, bio chromatographic and in silico methods to solve the puzzle of badge and its derivative's toxicity

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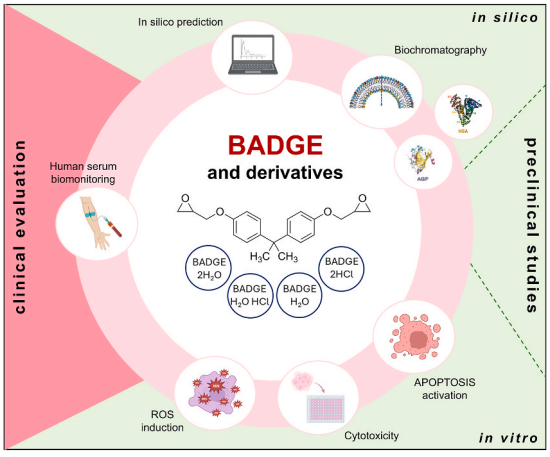
HIGHLIGHTS

- A multiple approach to investigate the BDGEs toxicity.
- BADGE and BADGE·2HCl are the most cytotoxic compounds on MCF-7 cells.
- Di-hydrolysed derivative is the most frequently metabolite detected in human serum.

GRAPHICAL ABSTRACT



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ABSTRACT

Bisphenol A diglycidyl ether (BADGE) is a pre-polymer of BPA widely used in manufacturing of epoxy resins and plastics; due to its high reactivity, unintended by-products, such as chlorinated and hydrolysed products, can reach the human body. This research integrates multiple approaches such as computational predictions, chromatographic experiments, biological assays, and human biomonitoring studies to comprehensively evaluate the toxicological profiles of the parent compound and its derivatives. *In silico* predictions were first utilized to estimate the toxicological properties and interactions of BADGE derivatives, providing insights into their bioactivity. Biomimetic liquid chromatography was then used to simulate membrane permeability and biodistribution, predicting how these chemicals might cross biological membranes and accumulate in tissues. *In vitro* experiments

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assessed cellular toxicity through viability assays, identifying BADGE·2HCl as the most cytotoxic derivative. Reactive Oxygen Species (ROS) production evaluation was performed to assess the oxidative stress induced by these compounds, revealing elevated ROS levels in cells exposed to BADGE and BADGE·2HCl with a consequent significant oxidative damage. Similarly, BADGE and BADGE·2HCl were able to induce cellular death by apoptosis activation. Human serum analysis in a population sample (N = 96), showed BADGE·2H_2O as the most frequently detected metabolite, indicating a considerable human exposure and metabolic processes. The findings highlight a toxicity of BADGE derivatives similar to that of BADGE; BADGE·2HCl resulted particularly cytotoxic and BADGE·2H_2O is the most frequent detected in human serum, underscoring the need for regulatory measures to mitigate potential health risks associated with these compounds.

Abbreviation list:

BADGE	Bisphenol A diglycidyl ether
ROS	Reactive Oxygen Species
EPA	Environmental Protection Agency
PVC	polyvinyl chloride
BADGE·2H2O	Bisphenol A bis (2,3-dihydroxypropyl) ether
BADGE·H2O·HCl	Bisphenol A (3-chloro-2 hydroxypropyl) (2,3-dihydroxypropyl) ether
BADGE·H2O	Bisphenol A (2,3-dihydroxypropyl) glycidyl ether
BADGE·2HCl	Bisphenol A bis (3-chloro-2-hydroxypropyl)
BDGEs	BADGE derivatives
IAM-LC	Immobilized Artificial Membrane Liquid Chromatography
HSA	human serum albumin
AGP	α1-acid glycoprotein
MCF-7	human breast cancer cell line
A2780	human ovarian adenocarcinoma cells
CaCo-2	human colon adenocarcinoma cells
HaCaT	human enterocytes and human keratinocytes
ACN	Acetonitrile
IS	Internal standard IS
BPAF	Bisphenol AF
DMEM	Dulbecco's Modified Eagle Medium
DAPI	4′, 6-diamidino-2-phenylindole
cDDP	cisplatin
SMILES	Simplified Molecular Input Line Entry System
H2DCFDA	dichlorodihydrofluorescein diacetate
HPLC	High Performance Liquid Chromatography
DMSO	dimethyl sulfoxide
IC50	half maximal inhibitory concentration
PBS	phosphate buffer saline

1. Introduction

Bisphenol A diglycidyl ether (2,2-bis(4-(2,3-epoxypropyl) phenyl) propane), commonly known as BADGE, is synthesized by mixing Bisphenol A and epichlorohydrin (ECH) and it is a key component extensively used in the industrial manufacturing of plastics, with an estimated annual production in the United States ranging from 1,000,000 to 20,000,000 pounds in 2015 (Xue et al., 2022). In addition, BADGE as well as other bisphenols, are employed in the manufacturing of coatings and resins to prevent metal corrosion, creating a protective layer between foodstuffs and the metal surfaces of cans.

Unfortunately, such chemicals have been found in various foodstuffs, due to their migration from the packaging such as baby bottles, reusable water bottles, food containers, and cans into food (Sueiro et al., 2003). Data from multiple scientific sources indicate that humans can be exposed to these chemicals, leading to several adverse health effects.

Specifically, BADGE has been shown to have toxic effects. The Environmental Protection Agency (EPA) includes BADGE as an endocrine disruptor in ToxCast, a toxicity database designed for prioritizing the toxicity testing of thousands of chemicals efficiently (USEPA, 2022). ToxCast database: Invitrodb. version 3.5). Due to its chemical structure featuring electrophilic oxirane rings, BADGE is highly reactive and can generate chlorinated and hydrolysed products, particularly mono- and di-hydrolysed compounds, which are more likely to form in aqueous environments.

A significant number of scientific studies covering BADGE's production, applications, analytical methods for determination in food matrices, environmental occurrences, presence in human specimens, abiotic and biotic transformations and both in vitro and *in vivo* toxicity, were conducted (Russo et al., 2018; Marotta et al., 2019). Several studies substantiated mutagenic, genotoxic, and cytotoxic effects exerted by BADGE (Ramilo et al., 2006; Hutler Wolkowicz et al., 2016; Marqueño et al., 2019; Wang et al., 2021a,b). Aside from allergic reaction induced by BADGE among workers involved in manufacturing of BADGE-based epoxy resins (Kanerva et al., 1991), there are evidences of toxic effect of BADGE due to its mutagenic action as bifunctional alkylating agent potentially generating a cross-linking bridge between DNA and proteins. Cell death and apoptosis were observed in human colorectal adenocarcinoma CaCo-2 cell line, human colon carcinoma (HCT-116) cells, and human peripheral blood lymphocytes (Suárez et al., 2000); in addition negative developmental effects in mice, such as a reduced body weight and relative organ weight and testicular toxicity have been observed after a pre-postnatal exposure to BADGE (Xue et al., 2022).

Biotransformation by hydration of BADGE in humans produce BADGE·2H_2O, the main metabolite, while BADGE·HCl·H_2O, BADGE·HCl, and BADGE·2HCl, can form through the reaction of BADGE with HCl, particularly when this is used with PVC-based organosol lacquers (Xue et al., 2022). Unfortunately, a gap of knowledge on the toxicological potential of BDGEs subsists, thus motivating us to carry out the present work.

Therefore, this research aims at characterising the toxicity of several BDGEs through a combination of different in vitro and in silico approaches. The derivatives studied include Bisphenol A bis (2,3-dihydroxypropyl) ether (BADGE·2H_2O), Bisphenol A (3-chloro-2 hydroxypropyl) (2,3-dihydroxypropyl) ether (BADGE·H_2O· HCl), Bisphenol A (2,3-dihydroxypropyl) glycidyl ether (BADGE· H_2O), and Bisphenol A bis (3-chloro-2-hydroxypropyl) ether (BADGE· 2HCl), along with their parent compound BADGE as a reference. The chemical structural features of the analytes under investigation are shown in Fig. 1.

To elucidate the affinity of BDGEs and their reference compound to phospholipids (i.e., phospholipophilicity), as an indicator of their potential to go beyond cell membranes, and their binding to serum proteins involved in xenobiotic biodistribution, biochromatography was used. These methods employ chromatographic stationary phases with covalently bound biological structures: a) phosphatidylcholine analogues immobilized on a silica core mimicking biological barrier (Immobilized Artificial Membrane Liquid Chromatography, IAM-LC), b) human serum

albumin (HSA), the main plasma protein binding acidic and neutral xenobiotics, and c) α1-acid glycoprotein (AGP), a plasma protein binding basic and neutral xenobiotics. Fundamental parameters related to toxicokinetics and toxicodynamics were also predicted in silico and discussed.

Biological responses of human breast cancer cell line (MCF-7), human ovarian adenocarcinoma cells (A2780), and human colon adenocarcinoma cells (CaCo-2), human enterocytes and human keratinocytes (HaCaT) to BADGE and its derivatives were examined to assess their toxicity. Finally, an analysis of a repository of human seum samples from healthy volunteers was performed to assess the possible occurrence and baseline concentration levels of BDGEs.

The combination of various methods proposed in this study, such as in silico evaluation, bio and chromatographic techniques, and biological assays can offer a deeper knowledge of BDGEs toxicity.

2. Materials and methods

2.1. Reagents

Acetonitrile (ACN) and Methanol (both HPLC analytical grade) was purchased from Sigma-Aldrich (Milan, Italy) and water (18.2 MΩ cm^{-1}) was purified and deionized in house via a Milli-Q plus instrument from Millipore (Bedford, New Hampshire, USA). Analytical standard of BADGE CAS No. 1675-54-3, BADGE $2H_2O$ CAS No. 5581-32-8, BADGE· H_2O· HCl CAS No. 227947-06-0, BADGE· H_2O CAS No.76002-91-0, BADGE· 2HCl CAS No 4809-35-2, (minimum purity ≥99%) was purchased from Sigma-Aldrich (UK) while 2,2-Bis(4-hydroxyphenyl) hexafluoropropane, used as Internal standard (IS) (BPAF, CAS No. 1478-61-1)(minimum purity >98%) was purchased from TCI Europe (Zwijndrecht, Belgium). 2′,7′-Dichlorodihydrofluorescein diacetate was purchased from Sigma Chemical Co. (St. Louis, MO). IAM.PC.MG column (4.6 mm × 150 mm, 10 μm particle size) from Regis Chemical Company (Morton Grove, IL). Plasma proteins stainless-steel columns (100 × 4 mm i.d. CHIRALPAK® HSA and 100 × 4 mm i.d. CHIRALPAK® AGP) were provided by Chiral Technologies Inc. All the standard stock solutions (2.0 mg mL^{-1}) of each compound were prepared in ACN, as well as those of the mixed standard solution of all the investigated chemicals; the solutions were stored at −20 °C in the dark until the use, the diluted solution were freshly prepared for the daily analyses.

2.2. Biochromatography

Biochromatography was performed using Liquid chromatography (LC-20 VP - Shimadzu Corp., Kyoto, Japan) equipped with a 7725 Rheodyne injection valve fitted with a 20-μl loop and a model RF-20 (Shimadzu Corp., Kyoto, Japan), as Fluorescence detector (FD) set at 276 nm excitation wavelength and 303 nm emission wavelength More information concerning bio chromatographic methods are available in Supplementary Section. Stock solutions of each chemical were made in MeOH, while working solutions (ca. 10^{-3} M) were diluted in the eluents. Analyses were performed at room temperature (20 ± 2 °C) and 60.0 μL of each working solution were injected in the chromatograph. The eluents employed for Human Serum Albumin (HSA) chromatographic experiments were 20%, 25%, and 30% (v/v) of ACN as organic modifier in mixture with 20 mM ammonium acetate pH 7.0 at flow rate of 0.5 mL min^{-1}, while for α1-acid glycoprotein (AGP) chromatographic experiments we used 100% of 20 mM ammonium acetate pH 7.0 For all the tested chemicals but extrapolated values for BADGE and BADGE· 2HCl that required at least 10% of ACN for their elution. 0.9 mL min^{-1} was the flow rate for AGP affinity measurements.

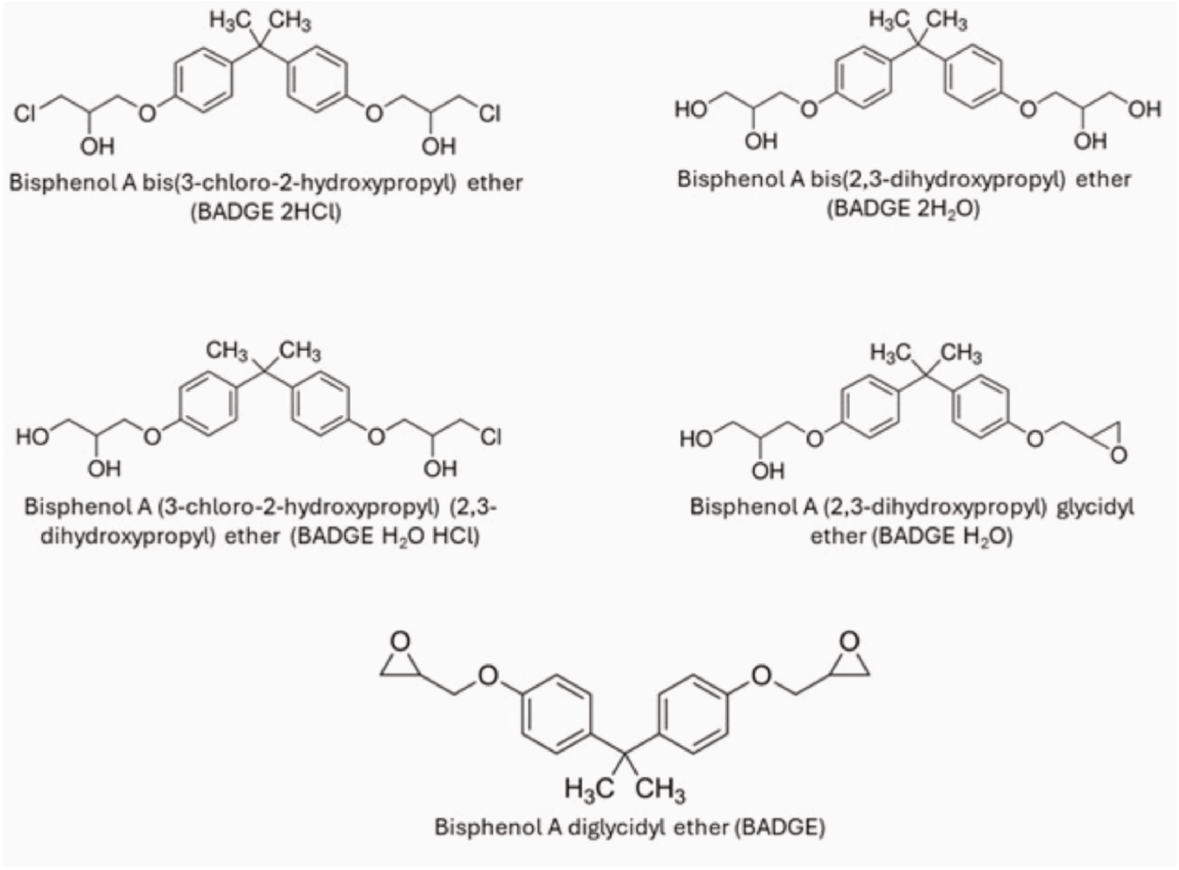

Fig. 1. Chemical structures of analytes under analysis.

2.3. Cell cultures

For the growth of MCF-7 cells (human breast adenocarcinoma), A2780 cells (human ovarian cancer) and HaCaT cells (human keratinocytes) (DMEM) (Invitrogen, Paisley, UK) was used as a culture medium. It was previously supplemented with 10% fetal bovine serum (FBS, Cambrex, Verviers, Belgium), 2 mM of L-glutamine (Sigma, Milan, Italy), 100 units/ml of penicillin (Sigma) and 100 μg/ml of streptomycin (Sigma); while for the growth of human colon adenocarcinoma cells CaCo-2, Eagle's Minimum Essential Medium (EMEM) supplemented with 20% fetal bovine serum (FBS, Cambrex, Verviers, Belgium) was used. Moreover, differentiated CaCo-2 cells were used at post-confluence stage as a model of healthy human enterocytes (Russo et al., 2023; Di Cesare Mannelli et al., 2018). All cell lines were grown and cultured in a humidified 5% carbon dioxide atmosphere at 37 ± 0.5 °C.

2.4. Bioscreens in vitro

Biological responses of BADGE and its derivatives were explored through the study of the "cell survival index". It's a method that results from the combination of the evaluation of cell viability with cellular counting, as we have previously described in detail (Russo et al., 2023). 10^4 cells per well were plated in 96-well culture plates and after 24 h of growth, cells were washed twice and fresh medium was added. All cell lines were treated for 48 h with a range of concentrations (10 → 150 μM) of BADGE, BADGE· 2HCl, BADGE· $2H_2O$, BADGE· H_2O· HCl and BADGE· H_2O. Cell viability was evaluated using the MTT assay, which measures the level of mitochondrial dehydrogenase activity using the yellow 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) as a substrate. At the end of MTT incubation, the absorbance was monitored using a microplate reader (iMark microplate reader, Bio-Rad, Milan, Italy) (550 nm). Instead, total count of cells and a live/dead ratio were revealed by TC20 automated cell counter (Bio-Rad, Milan, Italy). (Russo et al., 2021; Russo et al., 2023)

Ultimately, the IC50 (half maximal inhibitory concentration) values were obtained by means of a concentration response curve by nonlinear regression using the GraphPad Prism 8.0 program, and are expressed as mean values ± SEM (n = 30) of five independent experiments.

2.5. Detection of intracellular reactive oxygen species (ROS)

Intracellular oxidative stress was detected by using a cell permeant reagent dichlorodihydrofluorescein diacetate (H2DCFDA), a fluorogenic molecule that measures hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within cells. H2DCFDA was dissolved in dimethyl sulfoxide (DMSO) and stored as 50 mM stock at −20 °C. MCF-7 were plated in 96-well plates, at a density of 2×10^4 per well, and after 24 h of growth, were washed twice with 1 × PBS and incubated with H2DCFDA. To carry out the intracellular ROS evaluation, cells were loaded with 20 μM of H2DCFDA for 30 min at 37 °C, and then incubated with BADGE, BADGE· 2HCl, BADGE· $2H_2O$, BADGE· H_2O· HCl and BADGE· H_2O at IC_{50} for 48 h. At the end of treatment, cells were lysed with Triton X-100, and fluorescence was measured using a fluorescence microplate reader, at the following settings: excitation at 495 nm, emission at 530 nm. Control samples were: i) untreated cells loaded with H2DCFDA (to detect basal ROS production); ii) untreated cells in culture medium (background negative control); iii) wells containing H2DCFDA without cells; iv) cells exposed to H_2O_2 (150 μM for 1 h). Each experiment was repeated on three separate analyses (n = 3). Relative ROS levels were calculated based on the ratio of H2DCFDA mean fluorescence intensity of untreated control cells vs. bisphenols-treated samples and calculated by subtracting background readings from all measurements.

2.6. Fluorescent detection of apoptosis

Cellular apoptosis was detected by a fluorescent assay. Briefly, apoptotic and healthy cells have been monitored by Apoptosis/Necrosis Detection Kit (Abcam, ab176749). DAPI was used as a DNA stain for nuclear regions (revealed in blue) (DAPI filter, Ex/Em = 350/470 nm), while the PS (phosphatidylserine) sensor for early apoptosis detection has green fluorescence (FITC filter, Ex/Em = 490/525 nm). MCF-7 cells were plated in96-well microplate (with black wall and clear bottom) (Corning Life Sciences, Bedford, U.S.A.) and treated with IC_{50} of BADGE and BADGE 2HCl for 48 h. In the same experimental conditions, cells were treated with cDDP as positive control of apoptosis induction. According to assay kit protocols, cells were incubated with a precise quantity of reagents at room temperature for 30 min, protected from light. At the end of incubation, MCF-7 cells were washed twice with assay buffer, and the fluorescence intensity was verified by using a fluorescent microscopy at 40 × objective (Piccolo et al., 2021).

2.7. In silico calculations

In silico calculations were performed using two software packages from the SwissDrugDesign suite *i.e.*, SwissADME (Daina et al., 2017) and SwissTargetPrediction (Gfeller et al., 2014). In brief, SMILES for each of the five molecules were input in the applet, and the calculations were run. The output was saved in.csv and converted into formattable tables using Numbers v 14.0 on a Macintosh v 14.2 M2 computer. The structures were submitted individually to SwissTargetPrediction (species selected: *homo sapiens*) and all the targets were displayed in the tables reported in SI. The pie-charts represent instead the classification of top 15 targets that are identified by the engine as those that are most likely to occur.

2.8. In vivo monitoring study

For the monitoring of BADGE and its derivatives we used sera of our repository previously obtained for published research aimed at the determination of Bisphenol A (Marotta et al., 2023). Sera were collected from 96 individuals (30 males and 66 females; median age 51 years, range 19–77), submitted to cytology examination of thyroid nodules, representing a highly prevalent paraphysiological condition (Puzziello et al., 2016). The cohort was heterogeneous in terms of the main factors affecting the risk of environmental chemical contamination (occupational exposure, smoking status, alcohol consumption) and, also, of anthropometric characteristics (Supplementary Material Table S3]. Indeed, the adipose tissue acts as long-term deposit for lipophilic agents (La Merrill et al., 2013), such as BADGE and derivatives, widely affecting their metabolism.

Sample preparation, extraction, and quantification were carried out as follow: thawed sera were mixed up, sampled of 300 μL, added with 150 μL perchloric acid 25% aqueous solution v/v (to precipitate proteins), 150 μL distilled water, 585 μL ethylacetate–n-hexane 50:50 v/v, 100 ± 2 mg (analytically weighed) of NaCl and 25 μL of a BPAF solution in methanol 20 μg mL^{-1} as IS. The vortex-mixed mixture was centrifuged at 3000 rpm for 10 min. The supernatant was collected, dried under N_2, dissolved 100 μL and injected into the HPLC at least in triplicate and results are expressed as averages of at least three determinations. Liquid chromatography (LC-20 VP - Shimadzu Corp., Kyoto, Japan) equipped with a 7725 Rheodyne injection valve fitted with a 20-μl loop and a model RF-20 (Shimadzu Corp., Kyoto, Japan), as Fluorescence detector (FD) set at 276 nm excitation wavelength and 303 nm emission wavelength. The stainless-steel column was a reversed-phase Supelco Ascentis C18 (250 × 4.6 mm, 5.0 μm i.d.) with a Supelguard Ascentis C18 guard column (both from Supelco, Bellefonte, PA, USA) for the monitoring method validation. The mobile phase consisted of acetonitrile:water (60:40, v/v). The analyses were carried out at room temperature (20 ± 2 °C) at a flow rate of 0.6 mL min^{-1} in isocratic mode.

PC software Chromatoplus from Shimadzu Corp processed the signals from the fluorescence detector. All mobile phases were vacuum filtered through 0.45 μm nylon membranes and previously analytically verified as free of the target compounds to avoid any possible background contamination; plasticware was treated with a solution of 50:50 n-hexane: tetrahydrofuran (Olivieri et al., 2012). Serum samples, previously verified not having any signals interfered with the analyses, were used as a blank.

2.9. Statistical analysis

All results are reported as mean values ± SEM. Graph-Pad Prism (version 8.0, Graph-Pad software Inc., San Diego, CA) was employed for statistical analysis and ANOVA test for multiple comparisons was performed followed by Bonferroni's test.

3. Results

3.1. Biochromatography

A closer look to Table 1 and Table S1 reveals that the relationships between computed lipophilicity of BADGE and its derivatives and their chromatographic affinities are weak. Indeed, computed lipophilicity value of BADGE·2HCl are the highest according to the calculations methods Xlog P, Wlog P, Mlog P, Silicos-IT log P and Consensus log P but it is the fourth to last according to the iLog P engine which accounts for solvent accessible surface areas (Daina et al., 2014). The presence of a chlorine introduces two contrasting forces: from one side there's an increase in the molecular volume of the solute, resulting in a specular rise in the compound's hydrophobicity, on the other side the chlorine is responsible of a polarity rise due to the electronegative nature of this element, leading to more polarised bonds. This polarity contributions seems to be particularly disruptive on the analytical retention on both IAM.MG and HSA, certainly to an extent that seems to be greater than that reflected in the *n*-octanol/water lipophilicity calculations. On the other hand, the AGP phase does not seem to be particularly selective for this dataset given that the logarithms of the chromatographic retention factors are in a 0.72–0.75 range, which is minute.

3.2. Biological effects on cell viability

BADGE and its derivatives were tested in vitro to characterize their toxicity at cellular level. Biological effects on a selected panel of healthy and cancer cell lines (HaCaT, enterocytes, MCF-7, A2780 and CaCo-2) were explored after 48 h of BADGE and derivatives exposure. As depicted by graphs in Fig. 2, BADGE represents the most cytotoxic compound and the effect on cellular proliferation is dose-dependent, decreasing cell viability in a significant way. Among the panel of cell lines tested, MCF-7 cells represent the most sensitive ones to the effect of BADGE and its derivative BADGE·2HCl, showing low IC_{50} values (37 μM for BADGE; 65 μM for BADGE· 2HCl), as reported in Table 1. All other BADGE derivatives, such as BADGE·H_2O·HCl, BADGE·H_2O and BADGE·$2H_2O$, have a similar effect on all analysed cells, significantly reducing cell viability at the highest concentration used (150 μM). Noteworthy, the effects of BADGE and BADGE·2HCl on the healthy tested cells. As shown by the graphs in Fig. 2 and the IC_{50} values in Table 2, these two compounds showed a strong cytotoxic activity against HaCaT and enterocytes, in which BADGE reduced cell viability by 50% already at the concentration of 25 μM.

Table 1
Logarithms of retention coefficients of analytes on IAM.PC.MG, HSA, AGP stationary phases. The values refer to a 100 % (v/v) aqueous eluent and are achieved by an extrapolation method.

Substances	Log k_w $^{IAM.\ MG}$	Log k_w ^{HSA}	Log k_w ^{AGP}
BADGE	2.58	3.46	0.75
BADGE·2HCl	1.86	0.50	0.72
BADGE· H_2O· HCl	3.06	2.79	0.74
BADGE·H_2O	3.14	1.87	0.73
BADGE·$2H_2O$	3.04	2.93	0.74

3.3. Apoptosis activation by BADGE and BADGE· 2HCl in MCF-7 cells

After cell viability assays, the next step was to explore the possible mechanism of action of the two most in vitro cytotoxic compounds, *i.e.* BADGE and BADGE· 2HCl. Moving in this direction, we studied the activation of apoptosis, because one of programmed cell death pathways. In this context, fluorescence analyses, carried out by specific fluorescent probes and fluorescent microscopy as reported in the experimental section, revealed a significant activation of apoptotic cell death pathway after exposure of MCF-7 cells to the IC_{50} values of BADGE and BADGE·2HCl for 48 h. As depicted in fluorescence microphotographs (Fig. 3), treatment of MCF-7 cells with BADGE and BADGE· 2HCl induced a marked activation of apoptosis. In fact, many more green fluorescent cells (derived from the probe that selectively recognizes the membrane PS) can be visualized in the treated samples than in the untreated control ones. Moreover, the analysis of positive green fluorescence cells shows results rather similar to those obtained after in vitro cisplatin (cDDP) exposure, used as cytotoxic positive control.

3.4. Effect of BADGE and BADGE· 2HCL on ROS generation in MCF-7 cells

Since apoptosis activation is also complemented by reactive oxygen species (ROS) intracellular production, and ROS induction is an effective mechanism to kill cells for many compounds, we have explored cellular ROS production after BADGE and BADGE· 2HCl exposure in MCF-7 cells. The effect of BADGE and BADGE· 2HCl on ROS generation were examined after 48 h of incubation in vitro. As depicted in Fig. 4, treatments with IC_{50} values of both tested compounds had significant effects on ROS production in MCF-7 cells, revealing much higher intracellular ROS levels than untreated control cells. As we can see by bar chart (Fig. 4), ROS production (indicated as RFU) in cells treated with BADGE and BADGE· 2HCl was significantly increased, showing doubled values of ROS levels following treatment with BADGE (red bar), comparable with those produced in H_2O_2-treated cells (grey bar), here used as positive control. Treatment with BADGE· 2HCl, on the other hand, produced cellular ROS levels significantly higher than those in untreated control cells (white bar), and slightly lower than those in cells treated with the most cytotoxic bisphenol (BADGE).

3.5. Human serum monitoring

We developed and validated a method to identify and quantify BADGE and its derivatives in human sera from a heterogeneous study cohort with recovery values calculated on BPAF as IS, ranging from 54.30 to 105.0 ng mL^{-1} This resulted simple and easy to use, after adjustment of various factors such as the stationary phase and the size of column used, mobile phase composition (using acetonitrile instead of methanol for better peak symmetry) and the flow rate. These adjustments allowed us to obtain an acceptable resolution for all the five investigated chemicals. All the performed method validation parameters are reported in Supplementary Section (Table S2), as well as a chromatogram showing the analytical separation of the standards (a), and (b) a real sample (Fig. S7). Fig. 5 shows the detection frequency (DF) % of all investigated analytes. The mean concentration values found were: 616.6 ± 309.7 ng mL^{-1} for BADGE·$2H_2O$ ($n = 7$), the most frequent detected metabolite and, 166.69.1 ± 38.75 ng mL^{-1} for BADGE H_2O·HCl ($n = 5$). BADGE·2HCl was detected in only one sample, at the concentration level of 602.6 ± 0.0 ng mL^{-1} while BADGE·H_2O values have been found < LOQ ($n = 3$).

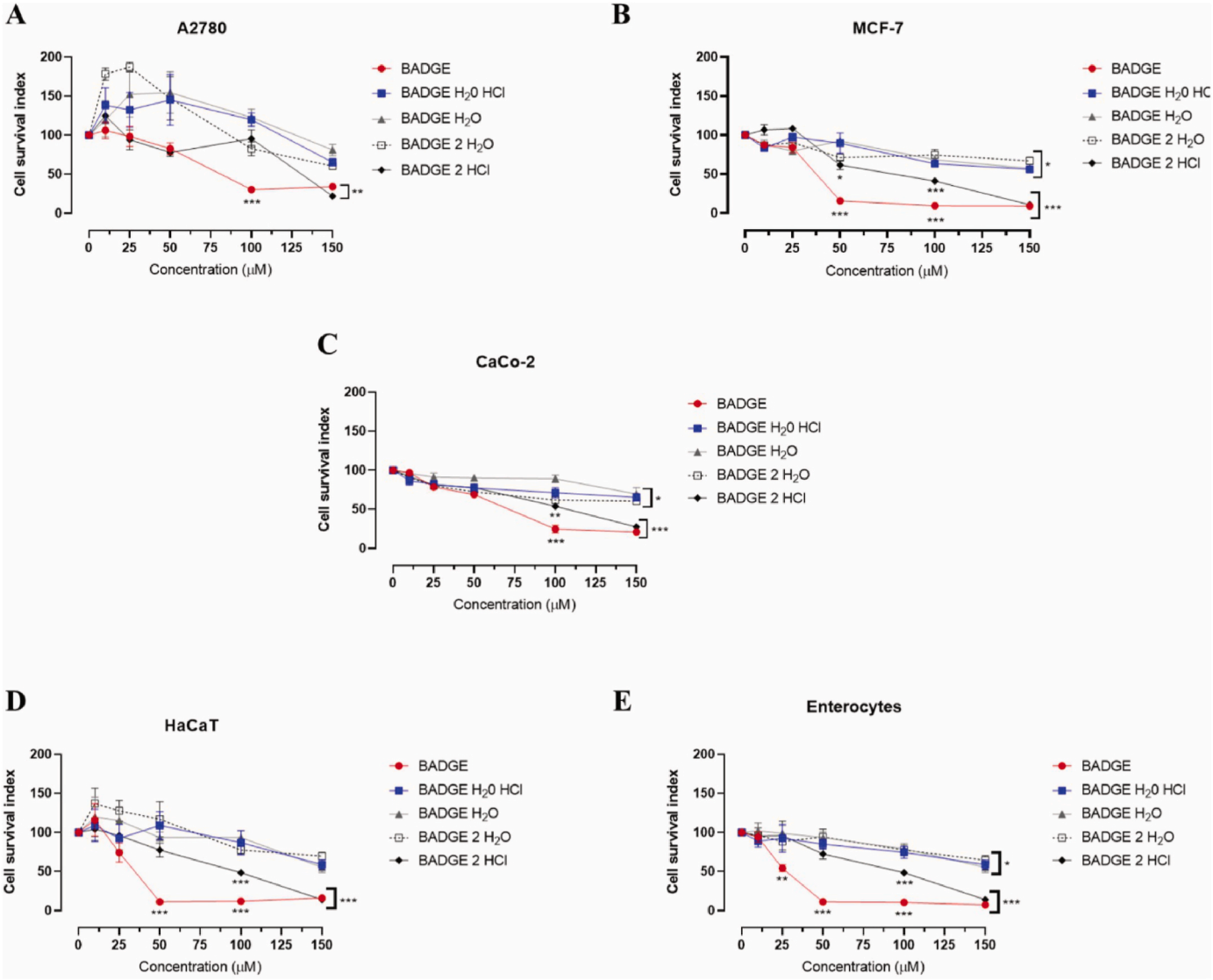

Fig. 2. Biological effects of BADGE and its derivatives. Cell survival index of cancer cells (i.e. A2780, MCF-7 and CaCo-2 cells) (A–C) and healthy cell lines (i.e. HaCaT and enterocytes) (D and E) treated or not for 48 h with the indicated concentration (10, 25, 50, 100 and 150 μM) of BADGE and other four derivatives (BADGE·2HCl, BADGE·H_2O·HCl, BADGE·H_2O, BADGE· $2H_2O$), as indicated in the legend. Data are expressed as percentage of untreated control cells and are reported as mean of 5 independent experiments ± SEM (n = 30). *p < 0.05 vs. untreated cells; **p < 0.01 vs. untreated cells; ***p < 0.001 vs. untreated cells.

Table 2
IC_{50} values (μM) relative to BADGE and its derivatives determined in the indicated cell lines, after 48 h of incubation in vitro. IC_{50} values are reported as mean ± SEM (n = 30).

CELL LINES	BADGE	IC_{50} (μm)			
		BADGE·2HCl	**BADGE·H_2O· HCl**	**BADGE·H_2O**	**BADGE·2 H_2O**
A2780	79 ± 4	130 ± 8	150 ± 9	>150	>150
MCF-7	37 ± 3	65 ± 4	>150	>150	>150
CACO-2	66 ± 5	100 ± 7	>150	>150	>150
HACAT	32 ± 3	85 ± 5	>150	>150	>150
ENTEROCYTES	24 ± 2	88 ± 4	>150	>150	>150

3.6. In silico results

The in silico calculations indicate that all the tested compounds are predicted to have a good human intestinal absorption, however only BADGE and BADGE·H_2O are those expected to achieve a significant exposure at the level of the central nervous system (CNS) based on their lipophilicity (WLOGP) and topological polar surface area (TPSA). However, BADGE·2HCl, despite having calculated physico-chemical properties that would in theory support its gaining access to the brain is identified to be a substrate of the p-glycoprotein and therefore effluated from the CNS, potentially reducing neurotoxicity via a detoxifying mechanism (Fig. 6).

4. Discussion

Despite the widespread use of BADGE to protect PVC from

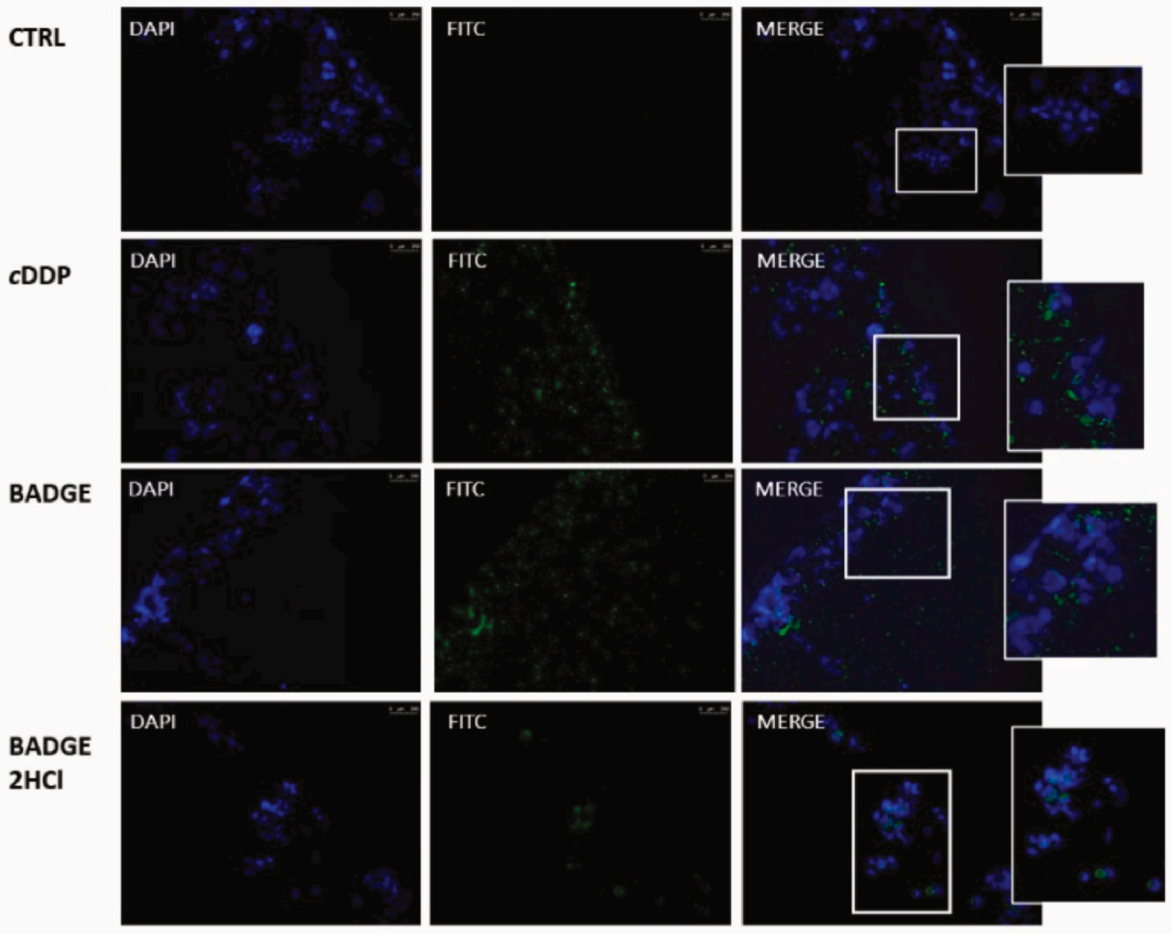

Fig. 3. Apoptosis activation in MCF-7 cells by fluorescent microscopy. Apoptotic and healthy cells have been monitored by fluorescent microscopy after incubation for 48 h with IC_{50} concentrations of BADGE, BADGE·2HCl and cisplatin (cDDP) (cytotoxic positive control). Cellular nuclei were revealed in blue fluorescence (DAPI filter, Ex/Em = 350/470 nm). Apoptotic cells were revealed in green fluorescence (FITC filter, Ex/Em = 490/525 nm) upon binding to membrane PS (phosphatidylserine). In merged images (MERGE), the fluorescent patterns from cell monolayers are overlapped. Fluorescent microphotographs are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

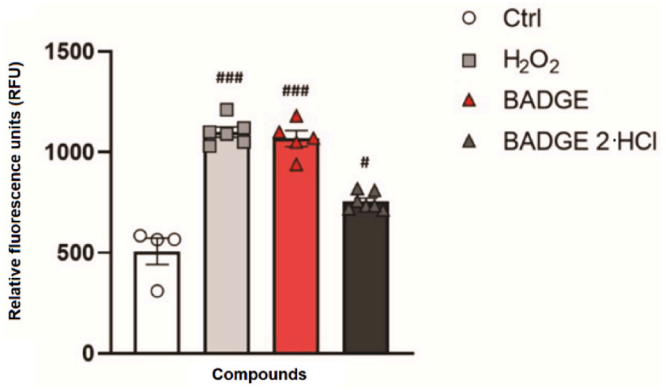

Fig. 4. ROS levels determination in MCF-7 cells after 48h of incubation with IC_{50} values of BADGE and BADGE·2HCl. H_2O_2 was used as positive inductor of intracellular ROS. #p < 0.05 vs. untreated cells; ###p < 0.001 vs. untreated cells.

hydrochloric acid during the curing process and its employment in the thermal coating process, only few studies can be retrieved in literature concerning BADGE chlorinated derivatives, as well as mono- and di-hydrolysed products generated by its contact with aqueous foodstuffs. In fact, BDGEs toxicity is yet to be thoroughly investigated even if their structures look like very similar to those of their parent compound, therefore they can be reasonably considered likewise hazardous. In fact, Regulation (EU Commission Regulation) 1895/2005 sets a maximum specific migration limit (SML) of 9 mg kg^{-1} for the total of BADGE and its hydrolysed derivatives (BADGE·H_2O, BADGE·$2H_2O$), and an SML of 1 mg kg^{-1} for the total of their hydrochloric derivatives (BADGE·H_2O·HCl, BADGE·HCl, BADGE·2HCl). To deeper investigate the behaviour of BADGE and its derivatives in their partitioning into a biological membrane, we employed IAM.PC.MG stationary phase because it correctly can reproduce the asset of natural phospholipids, in we except the glycerol moiety in the phospholipid analogues immobilized on a simplified biomimetic stationary phase. The partitioning is indeed the crucial step of interactions with phosphatidylcholine-like structures. However, we did not find a relationship between the predicted in silico lipophilicity values and experimental Log $k_w^{IAM.MG}$. The glycerol residue of the IAM analogues could support secondary interaction of polar nature as this can engage hydrogen-bonding specifically for the BDGEs supporting H_2O molecules in their structures.

The molecular target predictions are reported in SI. The presence of a chlorine atom seems to reduce the affinity of these ligands for the androgen receptor, which is estimated to be likely for BADGE and its hydrolysed derivatives, and increase their affinity for several kinases, whose role in homeostasis is anyhow difficult to unravel. This would probably support that interaction with the androgen receptor requires H-bonding from the moieties in 4 of the aromatic rings that is not

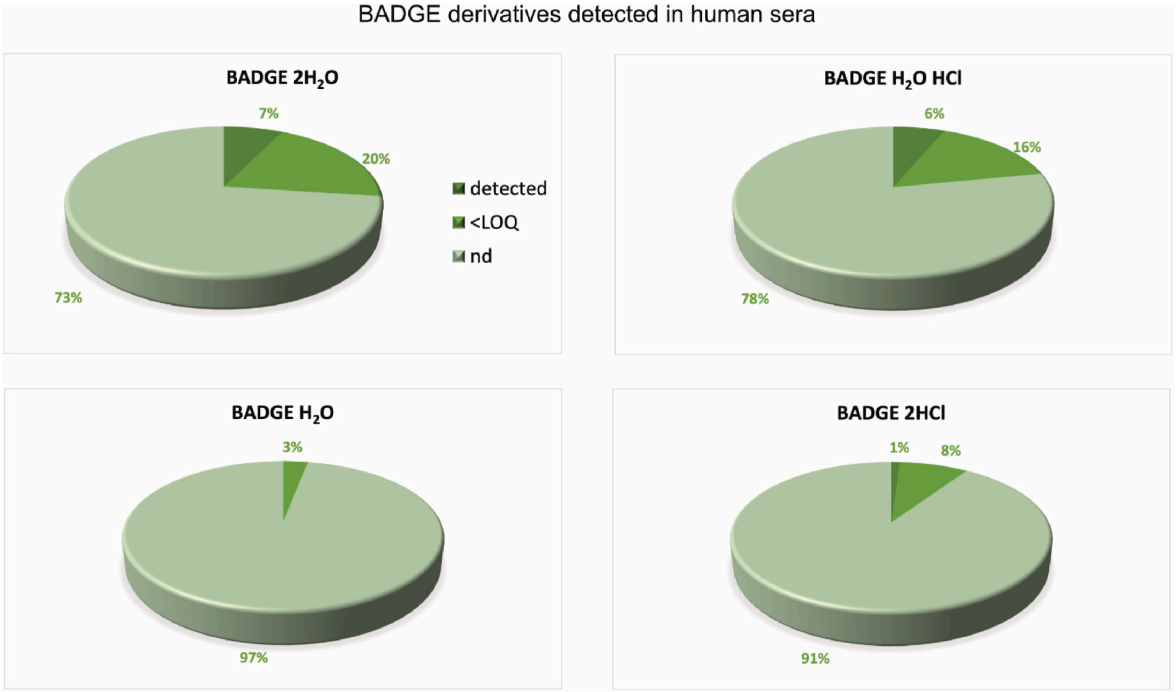

Fig. 5. Percentages of BDGEs detected in human sera; a) BADGE·2H_2O, b) BADGE·H_2O·HCl, c) BADGE·2HCl, d) BADGE·H_2O.

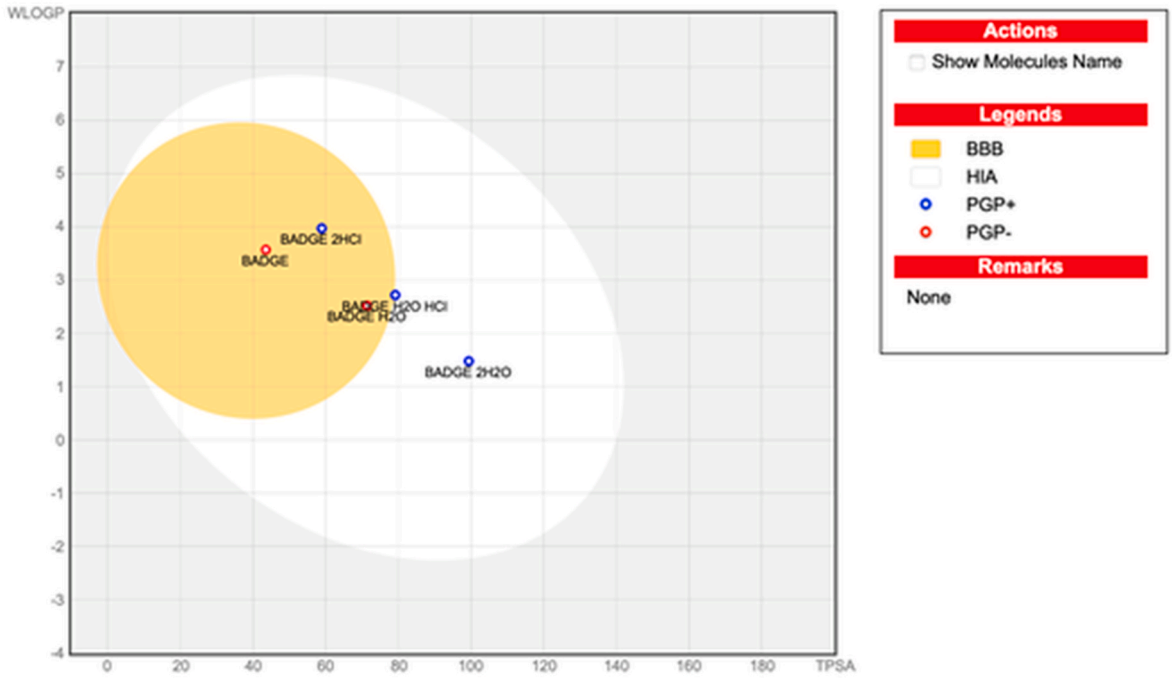

Fig. 6. Boiled-egg plot for all the BDGEs studied. BBB = Blood-Brain barrier passively permeable; HIA = Human intestinal absorption; PGP+: predicted to be effluated from the central nervous system by the p-glycoprotein; PGP-: predicted NOT to be effluated from the central nervous system by the p-glycoprotein.

afforded by when even only one chlorine atom is present. The fact that no compounds is outside the boiled-egg area indicates that their absorption potential is overall good and may represent a concern particularly if exposure occurs through the diet. However, the likelihood values reported in the SI are from 9 to 12%, which is overall modest.

Multiple scientific data indicate that the amount of bisphenols to which humans are exposed are very high and that cause many adverse health effects (Kim et al., 2020), among which male and female reproductive system diseases (Rehfeld et al., 2020; Czarnywojtek et al., 2023), as well as the autoimmune system (Huang et al., 2023) and neurological system (Costa and Cairrao, 2024). In this context, we have tested BADGE and its derivatives in interfering with cell viability and proliferation, as well as ROS production and apoptosis activation. First, according to our previous data (Russo et al., 2018), in vitro biological effects on healthy and tumour cell lines supported that BADGE was the most toxic compound, revealing a strong toxicity at cellular level. In particular, ER positive breast cancer cells MCF-7 represent the most sensitive cells to the effect of BADGE and to one of its derivate BADGE·2HCl, showing low

IC_{50} values (37 μM for BADGE; 65 μM for BADGE· 2HCl).

Considerable data report that bisphenols can cause numerous harmful effects because of their involvement with ROS production, lipid peroxidation, mitochondrial dysfunction, and alterations in cell signalling (Ďurovcová et al., 2021). So that, we analysed ROS formation and apoptosis activation in endocrine responsive breast cancer cells MCF-7, as the most sensitive cells to BADGE and BADGE·2HCl exposure. A significant level of ROS was detected in treated cells. Moreover, concerning mechanism of action in vitro and according to studies by Fehlberg and co-workers (FEHLBERG et al., 2002), we revealed pro apoptotic effects in MCF-7 cancer cells following exposure to BADGE and BADGE·2HCl. Overall, our data suggests that these two analytes can induce cell death through oxidative stress increase and apoptosis induction.

This empowers the hypothesis of a detrimental impact on human health. Indeed, owing to these biological properties, human exposure to BADGE and BADGE·2HCl may trigger or contribute to progression of a variety of human diseases, such as atherosclerosis, neurodegenerative diseases, and cancer (Forman and Zhang, 2021).The developed analytical method offered a reliable response in the analysis of serum samples allowing for the simultaneous analysis of BADGE and its derivatives in a relatively short time with good resolution on a serum sample matrix. Indeed, the analysis of blood serum is an important strategy to assess the human exposure to different contaminants.

Several studies have been performed on various matrices to monitor BDGEs content (Guo et al., 2020; Lestido-Cardama et al., 2022), even if to the best of our knowledge, the studies performed on human biological fluids regard most frequently urine samples instead of blood samples and not all the BDGEs under our investigation (Chang et al., 2014). Indeed, Chang and coworkers found concentration levels of BADGE·H_2O ranging from 0.108 to 0.222 ng ml^{-1}, observing only four venous blood samples, while concentrations of BADGE·$2H_2O$ varying from 0.660 to 303.593 ng ml^{-1} in ten venous blood samples. Yang and coworkers (Yang et al., 2022) analysed urine and sera collected from 181 children and adolescents, assessing that the derivatives have a higher enrichment capacity in human blood. In a study conducted by Kuwamura et al. (2024) only BADGE, BADGE·H_2O, BADGE·$2H_2O$ were monitored and only on 10 infants. Wang et al. (2015) performed a study on BDGEs on human blood in USA, but to best of our knowledge no studies were performed yet in Europe assessing the concentration levels of BADGE derivatives in human serum.

5. Conclusion

There's a knowledge gap on the effects on the human health of BDGEs, even if their structural features would suggest a toxicity similar to that of BADGE, this has not yet been thoroughly investigated. Our preclinical in vitro research indicates that BADGE and its derivative BADGE· 2HCl represent the most toxic compounds in human endocrine responsive breast cancer cells (MCF-7). In addition, these compounds have been shown to be effective in inducing intracellular oxidative stress, causing cell death by activation of the apoptotic pathway.

Currently, the European Commission established migration limits into food of 9 mg kg^{-1} for the total of BADGE and its hydrolysed derivatives (BADGE· H_2O, BADGE· $2H_2O$) and 1 mg kg^{-1} for the total of BADGE reaction products with HCl (BADGE· HCl, BADGE· 2HCl, BADGE· H_2O· HCl) ((EU), 2005). Indeed, biomonitoring studies may add some crucial information on human exposure also to BDGEs, as demonstrated by the high frequency detection of BADGE· $2H_2O$, therefore the developed analytical method may represent a preliminary pilot assessment to shed light on the human serum circulating levels of these compounds.

CRediT authorship contribution statement

Ilaria Neri: Methodology, Investigation, Formal analysis, Data curation. **Marialuisa Piccolo:** Methodology, Investigation, Formal analysis, Data curation. **Giacomo Russo:** Investigation, Data curation. **Maria Grazia Ferraro:** Formal analysis. **Vincenzo Marotta:** Visualization, Resources, Data curation. **Rita Santamaria:** Writing – review & editing, Writing – original draft. **Lucia Grumetto:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143640.

Data availability

Data will be made available on request.

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