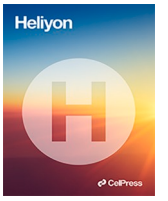
Heliyon 9 (2023) e13003

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

High cytotoxicity of a degraded TBBPA, dibromobisphenol A, through apoptotic and necrosis pathways

Keitaro Suyama [a], Hitoshi Kesamaru [b], Takashi Okubo [b], Kazumi Kasatani [b], Keisuke Tomohara [a], Ayami Matsushima [b], Takeru Nose [a,b,*]

[a] Faculty of Arts and Science, Kyushu University, Fukuoka, 819-0395, Japan
[b] Department of Chemistry, Faculty and Graduate School of Science, Kyushu University, Fukuoka, 819-0395, Japan

ARTICLE INFO

Keywords:
Tetrabromobisphenol A
Halogenation
Cytotoxicity
HeLa cell
LDH release assay
MTT assay

ABSTRACT

Halogenated flame retardants comprising bisphenol A (BPA) derivatives, such as tetrabromobisphenol A (TBBPA), have been studied their adverse effects on human health. However, despite the fact that these halogenated BPAs are easily degraded in the environment, the risks to living organisms due to these degraded products have mostly been overlooked. To evaluate the potential toxicity of degraded TBBPAs and related compounds, we examined the cytotoxicity of halogenated bisphenol A derivatives possessing one to four halogen atoms *in vitro*. The results indicated that the degraded TBBPA derivatives exhibited strong cytotoxicity against HeLa cells than TBBPA. Interestingly, the di-halogenated BPA derivatives possessing two halogen atoms exhibited the strongest cytotoxicity among tested compounds. In addition, a lactate dehydrogenase release assay, fluorescence spectroscopy and flow cytometry results indicated that dibromo-BPA and diiodo-BPA induced both apoptotic and necrotic cell death by damaging the cell membranes of HeLa cells. Moreover, *Escherichia coli* growth was inhibited in the presence of dehalogenated TBBPA and related compounds. These findings suggest that halogenated BPA derivatives that leak from various flame-retardant-containing products require strict monitoring, as not only TBBPA but also its degraded products in environment can exert adverse effects to human health.

1. Introduction

Flame retardants are a class of chemicals widely utilized in various industrial products, including plastics, electronics, building materials, and paints, to prevent the spread of fire [1,2]. As halogenated frame retardants, tetrabromobisphenol A (TBBPA; 2,2′,6,6′-tetrabromo-4,4′-isopropylidenediphenol; CAS registry no. 79-94-7) and tetrachlorobisphenol A (TCBPA; 2,2′,6,6′-tetrachloro-4,4′-isopropylidenediphenol; CAS registry no. 79-95-8) are the most widely produced and used owing to their efficient fire resistance and cost-effectiveness [3-6]. TBBPA and TCBPA have recently attracted considerable attention because of their release into the environment through their persistent use and posed serious threats to living organisms [7–9]. These chemicals have been detected globally in water, soil, and organisms, including human infants [4,10–13]. Owing to their high lipophilicity and thermal stability [14], TBBPA and TCBPA can be readily accumulated in organisms through the food chain [4,14–16], consequently exerting several adverse effects such as hepatotoxicity [9,17–19], endocrine disorders [17], reproductive toxicity [20], and neurotoxicity [21] in human beings. In addition, TBBPA and TCBPA reportedly disrupt signaling pathways via thyroid hormone receptors [22] and peroxisome

* Corresponding author. Faculty of Arts and Science, Kyushu University, Fukuoka, 819-0395, Japan.
E-mail address: nose@artsci.kyushu-u.ac.jp (T. Nose).

https://doi.org/10.1016/j.heliyon.2023.e13003
Received 22 December 2022; Received in revised form 11 January 2023; Accepted 12 January 2023
Available online 16 January 2023

proliferator-activated receptors γ (PPARγ) [23,24]. Despite these concerns, there are currently no good alternatives to TBBPA and its derivatives, and their use is expected to continue for a while [8]. In addition, the risk assessment knowledge of TCBPA, which has been used as an alternative to TBBPA, is currently limited.

There is also growing interest in the toxicity of TBBPA and TCBPA, as well as the chemicals produced when these compounds are broken down. The degradation of TBBPA has been reported as the debromination of TBBPA into lower-brominated derivatives (monoBr-BPA, diBr-BPA, and triBr-BPA) in the environment [25,26]. In addition, commercially available TBBPA originally contained approximately 1% tribromobisphenol A (triBr-BPA) [27]. These brominated derivatives of TBBPA reportedly accumulate at higher levels in human breast milk, exhibit PPARγ activity, and promote adipocyte differentiation [28]. Therefore, the influence of these dibrominated congeners of TBBPA on infant health is of great concern. In addition, dehalogenated compounds derived from TCBPA, monoCl-BPA, diCl-BPA, and triCl-BPA have been reported to exhibit estrogenic activities [22,24,29,30]. Although several uncertainties remain regarding the risk assessment of these degraded chemicals, information regarding the potential toxicity of halogenated BPA-derived compounds possessing halogen substituent(s) on aromatic rings, except for TBBPA, is still lacking. Moreover, when these halogenated flame retardants are completely dehalogenated, their final form is bisphenol A (BPA), a material of polycarbonate and epoxy resins, which has also been suspected to cause endocrine disruption [31,32] and neurodevelopmental disorder [33] at a very low dose in infants. Thus, a comprehensive investigation of the potential toxicity of BPA derivatives is required to assess the safe use of halogenated frame retardants. Previously, we synthesized the 16 of halogenated BPA derivatives possessing one to four halogen atoms as substituents on its aromatic rings and examined their binding affinity and transcriptional activity to ERRγ to assess the latent toxicity of halogenated BPA derivatives [34]. In the present study, to examine the potential toxicity of these halogenated BPA derivatives, their cytotoxic effects on HeLa cell lines were evaluated by methylthiazole tetrazolium (MTT) assay and lactate dehydrogenase (LDH) release assay. Fluorescent spectroscopy was carried out to detect apoptotic and necrotic cells after exposure to halogenated BPA analogs. Furthermore, the effect of halogenated BPA analogs on microbial growth of *E. coli* was also examined.

2. Materials and methods

2.1. Chemicals

2,2-Bis(4-hydroxyphenyl)propane (bisphenol A), tetrachlorobisphenol A (TCBPA or tetraCl-BPA), *N*-fluorobenzenesulfonimide, trichloroisocyanuric acid, *N*-bromosuccinimide, and *N*-iodosuccinimide were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Tetrabromobisphenol A (TBBPA or tetraBr-BPA), selectfluor, and *N*,*N*-dimethylformamide (DMF) were purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide (MTT) and 2-propanol were purchased from Nakalai Tesque, Inc. (Kyoto, Japan). Fetal Bovine Serum (FBS), L-Glutamine, sodium bicarbonate solution, and 0.5% trypsin-EDTA were purchased from Thermo Fisher Scientific Inc. (MA, USA). Other reagents and solvents were purchased from commercial suppliers and used without further purification. Tested halogenated BPA analogs, except for tetraCl-BPA (TCBPA) and tetraBr-BPA (TBBPA), were prepared by the method described in our previous study [34]. Purity and the chemical structures of the tested compounds were summarized in Table S1 and Figure S1 in Supporting Information, respectively.

2.2. MTT assays for HeLa cells

HeLa cells were seeded in a 96-cell plate at a density of 1.00×10^4 cells/well, cultured for 24 h, and then treated with different concentrations of halogenated BPA analogs for 24 h. At the end of treatment, 10.0 μL of MTT (5 mg/mL) was added and incubated for an additional 160 min period. Then formazan crystals were dissolved in a mixed solvent of 4 M HCl and 2-propanol (1: 100, v/v, 200 μl). After dissolution, cell viability was assessed by measuring the absorbance at 590 nm by using ImmunoMini NJ-2300 colorimeter (BIOTEC Co Ltd., Tokyo, Japan). The results represented the average of three independent experiments. The statistical difference between the concentration of the means and standard of the cell viability was determined by the Student's *t*-test.

2.3. LDH release assays for HeLa cells

LDH release assay of HeLa cells was carried out by using Cytotoxicity LDH Assay Kit-WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). HeLa cells were seeded in a 96-cell plate at a density of 1.00×10^4 cells/well in 100 μL of medium and cultured for 24 h. Then, 10 μL of halogenated BPA analogs dissolved in PBS (10^{-4} M in final) was added to the cells and incubated at 37 °C for 24 h in a CO_2 incubator. Working solution (100 μL, included in the kits) was added to each well and the plate was incubated for 30 min under a light-shielded condition at 25 °C. Finally, 50 μL of the stop solution (included in the kits) was added to each well. Then, 50 μL of final mixture was diluted with 150 μL of pure water and measured the absorbance at 490 nm by ImmunoMini NJ-2300 colorimeter (BIOTEC Co Ltd.). For positive control, HeLa cells were added 10 μL of the Lysis Buffer (included in the kits), incubated at 37 °C for 30 min in a CO_2 incubator, and measured the absorbance in the same manner. For negative control, the cells were treated only with medium. The LDH activity was calculated by the following equation

$$\text{LDH activity} = (A - C)/(B - C)$$

where A is the absorbance of the cell culture at 490 nm treated with test compounds, B is the absorbance of positive control, and C is the absorbance of negative control.

2.4. Fluorescent spectroscopy

Apoptotic and necrotic cell detection of HeLa cells was carried out by using Apoptotic/Necrotic Cell Detection Kits (Promocell GmbH, Heidelberg, Germany). HeLa cells were harvested and concentrated to 2.00×10^6 cells/well suspension by centrifugation, and then treated with 10^{-4} M of halogenated BPA analogs. Simultaneously cells were stained by using an assay kit following the manufacturer's instruction to stain suspension cells. The fluorescent observation was performed using BIOREVO BZ-9000 (KEYENCE Co., Osaka, Japan) equipped with PlanApo VC 60 × oil lens (Nikon Co., Tokyo, Japan), and the magnification was 4×.

2.5. Analysis of early apoptosis and necrosis by flow cytometry

Hela cells exposed to DMSO, BPA, diBr-BPA, and diI-BPA were analyzed by flow cytometry using Sony Cell Analyzer EC800 (Sony Corporation, Tokyo, Japan). Hela cells (10^5 cells/mL) were treated with DMSO or BPA analogs (final concentration of 10^{-4} M) and incubated in a 5% CO_2 atmosphere at 37 °C for 12 h. Cells were then washed with PBS, centrifuged, and stained using the Apoptotic/Necrotic Cell Detection Kits (Promocell GmbH). Fluorescence of the cells stained by FITC-Annexin V and ethidium homodimer was monitored at 525 and 620 nm, respectively.

2.6. Microbial growth assay with E. coli XL1-blue

The competent *E. coli* XL1-blue strain was purchased from Nippon Gene Co. Ltd. (Tokyo, Japan). Stock cultures were stored at −80 °C in a mixture containing LB media and 20% glycerol. Prior to each inhibition assay, the stock of *E. coli* strains (1 mL) was added in 100 mL of chemically defined mineral media reported by Cuellar et al. [35]. (see supporting information) and incubated aerobically overnight at 200 rpm and 37 °C. Then, 990 μL aliquots from the overnight grown cultures, which were diluted with test medium to an initial OD_{600} of approximately 0.15, were added to measuring cell for spectrophotometry. Then, the solution was mixed with 10 μL of the test solution of BPA, diBr-BPA, or diI-BPA (10 μM in DMSO) and incubated at 37 °C with shaking at 120 rpm. The growth curves were determined by measuring the OD_{600} of each cells every 2 h during 14 h on a spectral photometer JASCO V-600 (JASCO Co., Tokyo, Japan). Then, a final measurement was performed after 24 h. Two independent experiments were carried out. The statistical difference between the concentration of the means and standard errors of the OD_{600} measured at 24 h was determined by the Student's *t*-test.

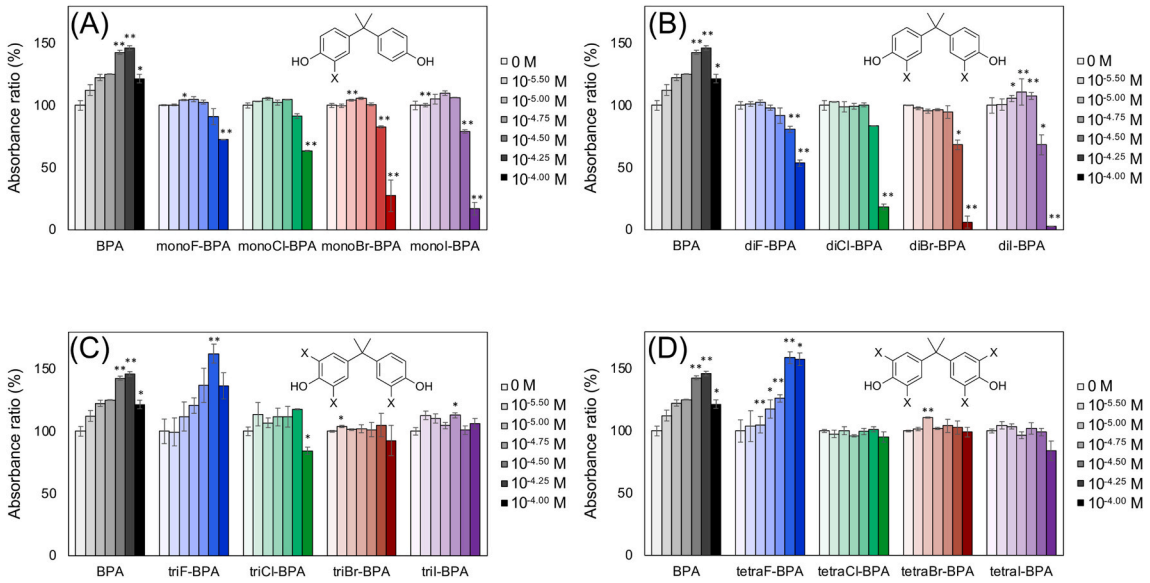


Fig. 1. Effects of BPA analogs on cell viability in HeLa cells. BPA and halogenated BPA compounds were examined their effect on the viability of HeLa cells (1.00×10^4 cells/well in 50 μL of medium) after 24 h of exposure. Cell viability is represented as relative absorbance compared to the control. Duplicate assays were repeated at least three times for each BPA compound. *: $P < 0.05$ in *t*-test between absorbance ratio after treatment with chemicals compared to that of control (0 M). **: $P < 0.01$ in *t*-test between absorbance ratio after treatment with chemicals compared to that of control (0 M).

3. Results

3.1. Cell viability determination by MTT assay

The cytotoxicity of BPA derivatives on cultured HeLa cells was evaluated by MTT assay. BPA and halogenated BPA compounds were examined their effect on the viability of HeLa cells at 24 h after treatment. It was revealed that mono- and di-halogen-substituted BPA demonstrated evident toxicity for HeLa cells in a concentration-dependent manner (Fig. 1). The cell viability treated with 10^{-4} M of monoF-, monoCl-, monoBr-, and monoI-BPA was decreased to 70.0%, 53.2%, 26.8% and 11.8%, respectively (Table 1 and Fig. 1A). Therefore, it was suggested that the cytotoxicity of the halogenated BPA analogs increased with the increment of the size of introduced halogen atom. Furthermore, di-substituted compounds showed more strong toxicity than mono-substituted compounds (Fig. 1B). Notably, treatment with 10^{-4} M of diBr- and diI-BPA greatly diminished the viability of HeLa cells (less than 5%). It was previously reported that a di-chlorinated BPA analog, 3,3-diCl-BPA, was degraded by exposure to ultraviolet-B (UVB) rays to generate 3-OH-BPA, which is an oxidized analog of BPA that exhibits a toxic effect by inducing a DNA double strand break [36]. Thus, we examined whether di-halogenated BPA compounds were degraded to oxidized BPA products during the toxicity assay. However, HPLC analyses revealed that generation of monoI-BPA, 3-OH-BPA, and other degraded products were not observed under the MTT assay conditions in this study (Figure S2 in supporting information). By contrast, tri- and tetra-halogenated compounds showed weak cytotoxicity for HeLa cells (Fig. 1C and D). Thus, these results clearly indicate that the existence of two (not three or four) halogen substituents at ortho-positions of hydroxyl group of each phenolic moieties were important for strong cytotoxicity of the BPA analogs. In this study, when the concentration of BPA was below 10^{-4} M, the viability of HeLa cells was increased in a concentration-dependent manner. It was good agreement with the previous report that BPA also facilitated cell proliferation at the lower concentration [37,38]. Among synthesized halogenated BPA derivatives, it was confirmed that triF-BPA and tetraF-BPA also exhibited evident cell proliferation activities as well as BPA did.

3.2. Effects of halogenated BPA analogs on LDH release of HeLa cells

The release of LDH from HeLa cells was examined as an indicator for cell membrane damage after exposure of the halogenated BPA derivatives to the cells (Fig. 2). The exposure of the mono-halogenated BPAs caused moderately increase in LDH release in a dose-dependent manner (Fig. 2A). Ratio of the LDH release was increased with the size of halogen substituent became larger. Treatment of the di-halogenated BPAs to HeLa cells resulted in a significant dose-dependent increase in LDH release in the concentration range from $10^{-4.5}$ to 10^{-4} M (Fig. 2B). On the other hand, the tri- and the tetra-halogenated BPAs did not obviously affect the LDH release (Fig. 2C and D). These data obtained from the LDH release assay were evidently consistent with the results from MTT assay. Therefore, it was considered that the cell membrane damage induced by the halogenated BPAs were closely related to the viability of HeLa cells.

3.3. Apoptotic and necrotic cell detection by fluorescence microscopy and flow cytometry

To distinguish apoptotic cells from necrotic cells, fluorescence microscopy was carried out to elucidate the mechanism of cell death induced by the BPA compounds (Fig. 3). HeLa cells were exposed to 10^{-4} M of each di-halogenated BPA compound for 12 h and stained with FITC-annexin V (green fluorescent dye) and ethidium homodimer III (red fluorescent dye). As shown in Fig. 3, the apoptotic cells displayed green fluorescence with FITC-annexin V staining and necrotic cells exhibited red fluorescence with ethidium homodimer III.

Table 1
Relationship between hydrophobicity of halogenated BPA derivatives and viability of HeLa cells.

Compound	Molecular weight	log *P*	Relative cell viability (%)		
BPA	228.29	3.32	121.5	±	3.4
monoF-BPA	246.28	4.11	70.0	±	1.6
monoCl-BPA	262.73	4.64	53.2	±	7.5
monoBr-BPA	307.19	4.70	26.8	±	6.8
monoI-BPA	354.19	4.66	11.8	±	4.2
diF-BPA	264.27	4.21	53.0	±	3.8
diCl-BPA	297.18	5.26	17.6	±	6.2
diBr-BPA	386.08	5.39	4.9	±	0.7
diI-BPA	480.08	5.31	3.9	±	0.8
triF-BPA	282.26	4.31	142.8	±	17.3
triCl-BPA	331.62	5.89	76.5	±	6.5
triBr-BPA	464.98	6.08	94.8	±	9.8
triI-BPA	605.98	5.96	94.3	±	10.6
tetraF-BPA	300.25	4.41	171.6	±	7.7
tetraCl-BPA	366.06	6.52	87.7	±	6.1
tetraBr-BPA	543.88	6.77	92.6	±	5.2
tetraI-BPA	731.88	6.61	85.1	±	3.9

The values of log *P* were computed by XLogP3 3.0. Relative cell viability means the ratio of living HeLa cells after treatment with 10^{-4} M of each chemical compared to control (10^{-6} M of each halogenated BPA analog), which was determined by MTT assay. Values of relative cell viability are Mean ± S. E. Duplicate assay were repeated at least three times for each BPA compound.

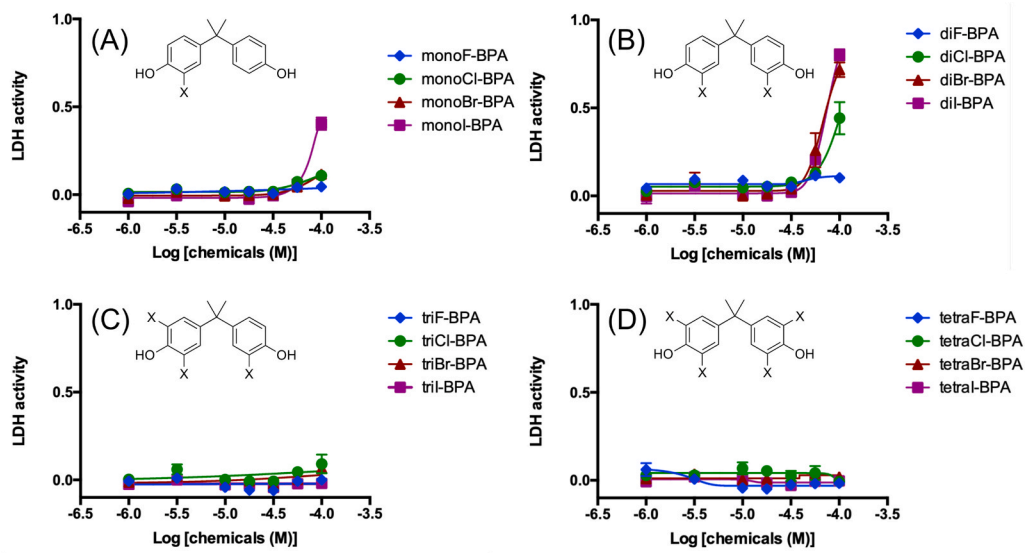


Fig. 2. The effects of the halogenated BPA analogs on the LDH release in the culture medium of HeLa cells after 24 h treatment with each chemical. The effect of the halogenated BPA analogs on the LDH release of HeLa cells (1.00×10^4 cells/well in 50 μL of medium) were examined after 24 h of exposure. Duplicate assays were repeated at least three times for each BPA compound.

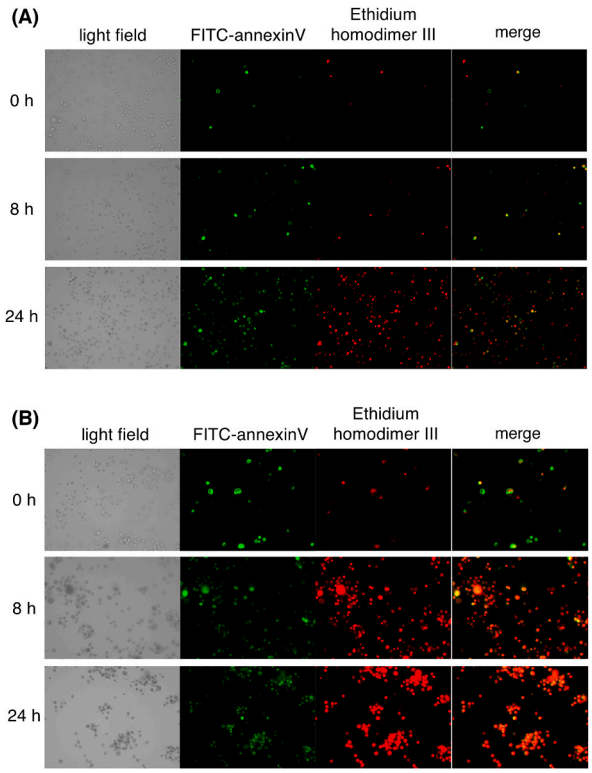


Fig. 3. Fluorescence staining of HeLa cells after treatment of halogenated BPA analogs. HeLa cells treated with the halogenated diBr-BPA (A) and diI-BPA (B) were co-stained with FITC-annexin V (green) and ethidium homodimer III (red). The figures at rightmost column were the merged views of stained cells.

As depicted in Fig. 3A, Hela cells treated with diBr-BPA showed at earlier stages green fluorescence with FITC-annexin V and at later stages red fluorescence with ethidium homodimer III, respectively. This feature indicated that exposure to diBr-BPA led to cell death via the apoptosis pathway. The cells stained by green fluorescent dye gradually increased 8 h after exposure; subsequently, cells

stained with the red dye emerged from 8 to 24 h after exposure. This result suggested that diBr-BPA induced cell death via apoptotic pathway. The results of treatment with diF-BPA or diCl-BPA were similar to those with diBr-BPA, whereas fewer number of stained cells were observed when Hela cells were treated with DMSO (control) or unsubstituted BPA in the fluorescence microscopy experiments (Figure S3 in supporting information). Among the tested compounds, diI-BPA showed the strongest toxicity against Hela cells. As shown in Fig. 3B, cells treated with diI-BPA were stained immediately by green and red fluorescent dyes. The cells showed very strong green and red fluorescence simultaneously by 8 h after treatment with diI-BPA. Thus, diI-BPA was considered to induce rapid cell death via both the apoptotic and necrotic pathways. Notably, the number of living cells observed by right field microscopy was lower when treated with diBr-BPA or diI-BPA than with BPA, diF-BPA, and diCl-BPA; the morphologically intact cells were observed only sparsely and non-intact cells were increased after exposure to BPA analogs possessing large halogen substituents. This result supported the result obtained from the LDH release assay, thus suggesting that the cell membrane damage of HeLa cells was induced by treatment with halogenated BPAs.

To further investigate the pathway of cell death induced by BPA derivatives, flow cytometry analyses of Hela cells exposed to BPA analogs were carried out (Fig. 4). After treatment with DMSO (as control), BPA, diBr-BPA, and diI-BPA, cells were co-stained employing FITC-Annexin V and ethidium homodimer III for analysis by flow cytometry. The ratio of apoptotic and necrotic HeLa cells treated with DMSO and 100 μM of BPA showed similar profiles (Fig. 4A and B). This result was consistent with a previous study, which reported that BPA induced evident apoptotic cell death when the concentration of BPA was greater than 100 μM [37,39]. By contrast, exposure to diBr-BPA apparently induced apoptotic cell death of HeLa cells at 100 μM of concentration (Fig. 4C). Furthermore, treatment with 100 μM of diI-BPA significantly increased necrotic cells compared to that with BPA and diBr-BPA (Fig. 4D). Taken together, the results of fluorescence spectroscopy indicated that BPA analogs possessing large halogen substituents induced not only apoptotic cell death, but also necrotic cell death by damaging cell membranes of HeLa cells.

3.4. Microbial growth of *E. coli* in the presence of halogenated BPA analogs

Trends of the microbial growth of *E. coli* in the presence of 10^{-4} M of BPA, diBr-BPA, and diI-BPA were monitored by estimating the optical density at 600 nm (OD_{600}) of each culture. OD_{600} of all the cultures gradually increased by 10 h and reached equilibrium after 12 h. Increment of the OD_{600} values of cultures in the presence of BPA analogs was reduced compared to that of a control culture (Fig. 5). Statistical analysis showed that growth of the all the strains was significantly affected ($P < 0.05$ for BPA and diBr-BPA, and $P < 0.01$ for diI-BPA) by the chemicals used at a concentration of 10^{-4} M.

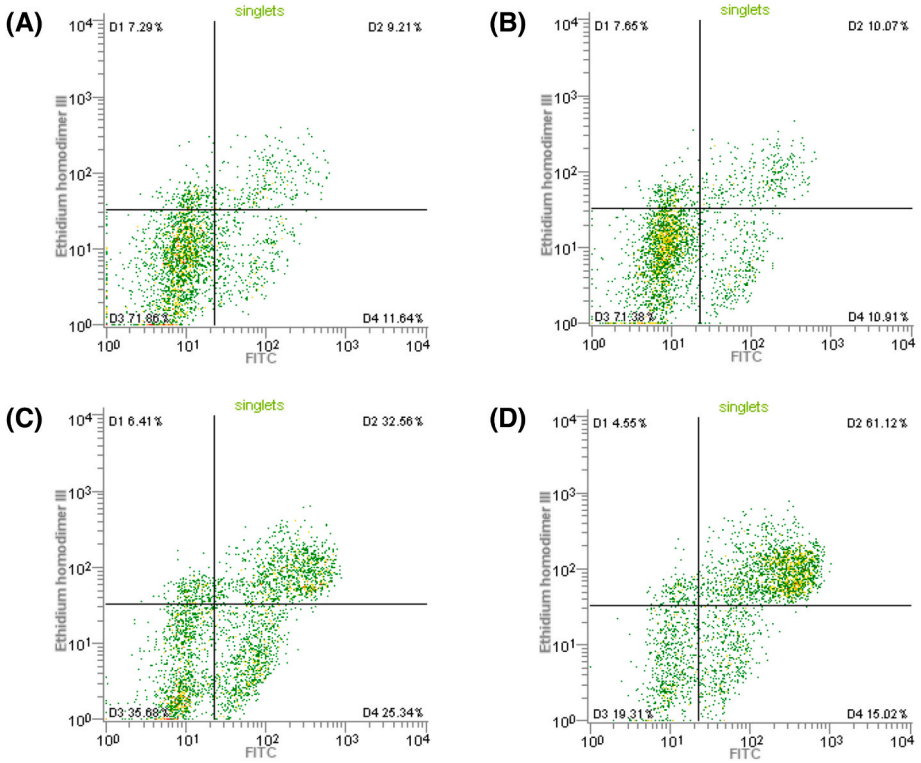


Fig. 4. Determination of early apoptosis and necrosis by flow cytometry. Flow cytometry measurement of HeLa cells (1×10^5 cells/mL) were carried out after treatment with (A) DMSO, (B) BPA, (C) diBr-BPA, and (D) diI-BPA for 12 h. Live cells are in the lower left quadrant. The upper right quadrant represents necrotic and late apoptotic cells. The lower right quadrant represents early apoptotic cells. Similar results were observed in three independent experiments.

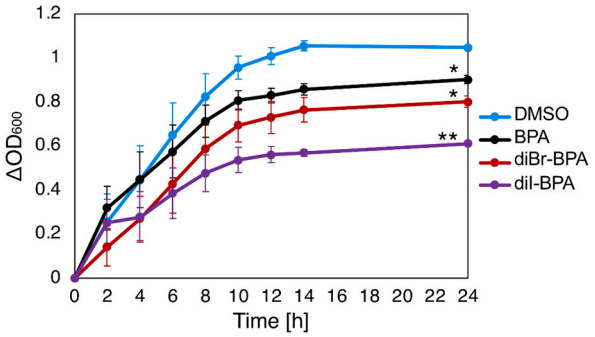


Fig. 5. Microbial growth assay with *E. coli* XL1-blue. The growth curves of *E. coli* were determined by measuring the increment of OD_{600} from initial value of each culture containing DMSO as control (red line), 10^{-4} M of BPA (black line), diBr-BPA (orange line), and diI-BPA (purple line) every 2 h during 14 h on a spectral photometer. All growth curves are averages of two independent assays. Error bars show standard deviation from the mean. *: $P < 0.05$ in *t*-test between ΔOD_{600} values at 24 h compared to that of control. **: $P < 0.01$ in *t*-test between ΔOD_{600} values at 24 h compared to that of control.

4. Discussion

TBBPA (tetraBr-BPA) and TCBPA (tetraCl-BPA) have been developed and are used worldwide to produce flame-retardant materials for commodity products. Although the toxicity of both chemicals has been well analyzed, the toxicity of degraded compounds is yet to be systematically studied. We first analyzed the cytotoxicity of a series of possible dehalogenated compounds: TBBPA and TCBPA. In addition, we focused on fluorine-or iodine-substituted compounds for the bromine of TBBPA, because halogen-containing chemicals could be used as future engineering materials. The results indicated that di-substituted compounds (diF-BPA, diCl-BPA, diBr-BPA, and diI-BPA) had the highest cell cytotoxicity compared to the other corresponding halogen-containing compounds analyzed under both apoptotic and necrotic pathways owing to their biological and physical properties.

The adverse effects of diBr-BPA, in which two bromine atoms are removed from TBBPA, have been reported in aquatic organisms, such as bacteria, algae, micro-invertebrates, and fish [40]. This study revealed that diBr-BPA suppressed the growth rate of bacteria, and our results are consistent with the results of previous studies. We also demonstrated the adverse effects of diCl-BPA and diI-BPA on cultured cells. These results are the first to indicate that di-halogenated compounds are more harmful than their parent compounds, such as TBBPA, TCBPA, and tetraI-BPA. Although it is unclear why di-halogenated BPA analogs exhibit the strongest toxicity among the synthesized analogs, it is possible that the hydrophobicity or water solubility of the molecules may have been related to its cytotoxicity. When the log *P* value of each di-halogenated compound was compared, it was observed that the di-halogenated compounds possessing log *P* between 4.6 and 5.4 induced strong cytotoxicity in HeLa cells; the log *P* values of diCl-BPA, diBr-BPA, and diI-BPA were 5.26, 5.39, and 5.31, respectively (Table 1). In contrast, our results indicated that three tetra-halogenated compounds, namely tetraCl-BPA(TCBPA), tetraBr-BPA(TBBPA), and tetraI-BPA, did not affect cell viability in the MTT assay. Compounds with a high log *P* value (approximately 6.00 or more) exhibited extremely high hydrophobicity. During the experiments, the tri- and tetra-halogenated BPA compounds were less water-soluble than the di-halogenated BPA compounds. Therefore, they may not be sufficiently distributed in the solution to exert their effects in physiological saline. In addition, the molecular weights of the di-halogenated BPAs were <500, whereas those of some tri- and tetra-substituted BPAs were >500 (Table 1). The log *P* value and molecular weight of chemicals are important criteria of Lipinski's "rule of 5," which correlates the physicochemical properties of a chemical with its membrane permeability and bioavailability [41,42]. Based on this theory, it is difficult to induce biological effects in compounds with high log *P* values and large molecular weights. Tri- and tetra-halogenated BPA derivatives with log *P* values well above 5.00 and molecular weights above 500 did not exhibit sufficient cytotoxicity. Our results indicated that these features of TBBPA and tetraI-BPA lead to no visible adverse effects on cultured cells in some experiments. Thus, these results imply that the appropriate hydrophobicity of di-halogenated compounds could be an important cause of cell death. Currently, TBBPA shows no neurotoxicity in humans, experimental animals, or fish [43,44], except for some early stages of neural development [45]. However, low-substituted BPA compounds, including di-substituted BPA, could be generated by the decomposition of the high-substituted derivatives, and the strong cell toxicity of the di-substituted BPA compounds could be a health problem.

TBBPA and its dehalogenated compounds have chemical structures similar to BPA; therefore, these compounds show adverse effects via the same molecular mechanism as BPA. The exposure of human cells to high concentrations of BPA may lead to apoptosis [37,46–48]. It was hypothesized that dehalogenated compounds of TBBPA and TCBPA could be involved in cell viability by inducing an increase in intracellular Ca^{2+} concentration, followed by the generation of reactive oxygen species or by activation of the MAPK family, such as extracellular signal-regulated kinase or c-Jun *N*-terminal kinase [37]. To investigate the mechanism of cell death by BPA compounds involved in apoptosis, the LDA release assay of HeLa cells was conducted after treatment with di-halogenated BPA compounds. The LDH release assay results showed that treatment of HeLa cells with di-halogenated derivatives resulted in a significant dose-dependent increase in LDH release, suggesting that the cell membrane was damaged after exposure to highly cytotoxic halogenated BPA analogs. The mechanisms of cell death by dehalogenated compounds were accurately analyzed via fluorescence spectroscopy and flow cytometry. As shown in the image of HeLa cells co-stained with FITC-annexin V and ethidium homodimer III, the

cells treated with diF-BPA, diCl-BPA, and diBr-BPA exhibited green fluorescence approximately 8 h after exposure. Afterward, the number of co-stained cells by red and green fluorescent dyes was gradually increased with the lapse of time, indicating that these BPA derivatives induced the apoptosis of HeLa cells and led to cell death. In contrast, cells exposed to diI-BPA immediately showed strong red fluorescence, implying that diI-BPA induced apoptotic death as well as necrotic death in HeLa cells. Furthermore, flow cytometric analyses demonstrated that diBr-BPA increased the ratio of early apoptotic HeLa cells, whereas diI-BPA strongly induced necrotic and late apoptotic cell death rather than early apoptosis in HeLa cells. These results indicate that diI-BPA exhibits rapid apoptosis and necrosis owing to cell membrane damage, suggesting that diI-BPA may have adverse effects on organisms and the environment, similar to TCBPA and TBBPA. Furthermore, the mechanism of cell death induced by dehalogenated compounds was different from that of other halogenated BPA compounds, although all the dehalogenated compounds exhibited stronger cytotoxicity than BPA and caused cell membrane damage in HeLa cells.

We demonstrated that the dehalogenated compounds could exhibit strong cytotoxicity in the present study. Furthermore, diBr- and diI-BPA displayed not only cytotoxic effects in HeLa cells, but also showed inhibitory effects on proliferation of the *E. coli* XL1-blue strain. As *E. coli* is known to be a well-established host for genetic engineering, the exposure of laboratory strains of *E. coli* to the dehalogenated compounds present in the environment could adversely affect the production of a broad range of bio-products, including proteins, biofuels, and so on. Therefore, it was concerned that leakage of these BPA-related chemicals from various consumer products with dehalogenation reaction could cause serious problems on the health of the living organisms including human as well as on genetic engineering using *E. coli*.

5. Conclusion

In this study, we performed a comprehensive evaluation of the potential toxicity of the halogenated BPA compounds against HeLa cells. It was revealed that the halogenated BPA compounds exhibited stronger cell toxicity than the original BPA. Cytotoxicity of the BPA compounds was increased with the increment of the size of introduced halogen substituent. Among them, the di-substituted BPA analogs showed stronger toxicity than other halogenated compounds. The results of LDH release assay and fluorescence microscopy experiments revealed that cell membrane damage induced by the halogenated BPAs was closely related to the viability of HeLa cells. These di-halogenated BPA analogs also displayed an inhibition effect on the growth of *E. coli* strains in a chemically controlled growth medium. It was suggested that the mechanisms of cytotoxicity were different for different halogen substituents; iodinated BPA analogs could cause necrotic cell death, whereas other halogenated BPAs could induce apoptosis of HeLa cells. Our finding demonstrated that the halogenated BPAs (especially di-halogenated compounds) exhibited strong cytotoxic effects in HeLa cells, thereby suggesting that endocrine disrupting effects and potential health problems induced by the BPAs with substituted aromatic rings might pose serious concerns. Since these lower-substituted BPA compounds could be generated by decomposition of TBBPA and TCBPA, which have been used to produce high-functional plastic materials, it is necessary to carefully study the influence of such BPA analogs on living organisms. Further studies to clarify the mechanisms by which these halogenated BPA analogs exhibit potent cytotoxicity are urgently required.

Author contribution statement

Keitaro Suyama: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Hitoshi Kesamaru: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Takashi Okubo; Kazumi Kasatani: Performed the experiments.

Keisuke Tomohara: Analyzed and interpreted the data.

Ayami Matsushima: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Takeru Nose: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

Professor Takeru Nose was supported by Japan Society for the Promotion of Science (JSPS), KAKENHI Grant Number [JP15H02827 & JP19H04303].

Data availability statement

The datasets used during the current study are available from the corresponding author upon reasonable request.

Additional information

Supplementary content related to this article has been published online.

Declaration of competing interest

The authors declare no competing financial interests.

Acknowledgements

We gratefully thank Center of Advanced Instrumental Analysis, Kyushu University for the flow cytometry measurement.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e13003.

References

[1] A. Covaci, S. Harrad, M.A.-E. Abdallah, N. Ali, R.J. Law, D. Herzke, C.A. de Wit, Novel brominated flame retardants: a review of their analysis, environmental fate and behavior, Environ. Int. 37 (2011) 532–556.
[2] Z. Shi, L. Zhang, J. Li, Y. Wu, Legacy and emerging brominated flame retardants in China: a review on food and human milk contamination, human dietary exposure and risk assessment, Chemosphere 198 (2018) 522–536.
[3] L.S. Birnbaum, D.F. Staskal, Brominated flame retardants: cause for concern? Environ. Health Perspect. 112 (2004) 9–17.
[4] R.J. Law, C.R. Allchin, J. de Boer, A. Covaci, D. Herzke, P. Lepom, S. Morris, J. Tronczynski, C.A. de Wit, Levels and trends of brominated flame retardants in the European environment, Chemosphere 64 (2006) 187–208.
[5] S. Song, M. Song, L. Zeng, T. Wang, R. Liu, T. Ruan, G. Jiang, Occurrence and profiles of bisphenol analogues in municipal sewage sludge in China, Environ. Pollut. 186 (2014) 14–19.
[6] M. Jarosiewicz, B. Bukowska, Tetrabromobisphenol A - toxicity, environmental and occupational exposures, Med. Pr. 68 (2017) 121–134.
[7] H. Zhou, N. Yin, F. Faiola, Tetrabromobisphenol A (TBBPA): a controversial environmental pollutant, J. Environ. Sci. 97 (2020) 54–66.
[8] E.S. Okeke, B. Huang, G. Mao, Y. Chen, Z. Zeng, X. Qian, X. Wu, W. Feng, Review of the environmental occurrence/analytical techniques, degradation and toxicity of TBBPA and its derivatives, Environ. Res. 206 (2021), 112594.
[9] X. Jia, R. Yan, H. Lin, Z. Liu, L. Shen, H. Yang, H. Wu, X. Shan, H. Zhang, TBBPA and its alternative TCBPA induced ROS-dependent mitochondria-mediated apoptosis in the liver of Rana nigromaculata, Environ. Pollut. 297 (2022), 118791.
[10] J.W. Voordeckers, D.E. Fennell, K. Jones, M.M. Häggblom, Anaerobic biotransformation of tetrabromobisphenol A, tetrachlorobisphenol A, and bisphenol A in estuarine sediments, Environ. Sci. Technol. 36 (2002) 696–701.
[11] S. Chu, G.D. Haffner, R.J. Letcher, Simultaneous determination of tetrabromobisphenol A, tetrachlorobisphenol A, bisphenol A and other halogenated analogues in sediment and sludge by high performance liquid chromatography-electrospray tandem mass spectrometry, J. Chromatogr. A 1097 (2005) 25–32.
[12] Z. Fan, J. Hu, W. An, M. Yang, Detection and occurrence of chlorinated byproducts of bisphenol a, nonylphenol, and estrogens in drinking water of China: comparison to the parent compounds, Environ. Sci. Technol. 47 (2013) 10841–10850.
[13] U.J. Kim, J.-E. Oh, Tetrabromobisphenol A and hexabromocyclododecane flame retardants in infant-mother paired serum samples, and their relationships with thyroid hormones and environmental factors, Environ. Pollut. 184 (2014) 193–200.
[14] K.A. Szychowski, A.K. Wójtowicz, TBBPA causes neurotoxic and the apoptotic responses in cultured mouse hippocampal neurons in vitro, Pharmacol. Rep. 68 (2016) 20–26.
[15] K. Halldin, C. Berg, A. Bergman, I. Brandt, B. Brunström, Distribution of bisphenol A and tetrabromobisphenol A in quail eggs, embryos and laying birds and studies on reproduction variables in adults following in ovo exposure, Arch. Toxicol. 75 (2001) 597–603.
[16] J. Gu, X. Chen, Y. Wang, L. Wang, K. Szlavecz, Y. Ma, R. Ji, Bioaccumulation, physiological distribution, and biotransformation of tetrabromobisphenol a (TBBPA) in the geophagous earthworm Metaphire guillelmi – hint for detoxification strategy, J. Hazard Mater. 388 (2020), 122027.
[17] G.A. Knudsen, J.M. Sanders, A.M. Sadik, L.S. Birnbaum, Disposition and kinetics of tetrabromobisphenol A in female Wistar han rats, Toxicol Rep 1 (2014) 214–223.
[18] L. Lyu, X. Jin, Z. Li, S. Liu, Y. Li, R. Su, H. Su, TBBPA regulates calcium-mediated lysosomal exocytosis and thereby promotes invasion and migration in hepatocellular carcinoma, Ecotoxicol. Environ. Saf. 192 (2020), 110255.
[19] Y. Yu, Y. Hou, Y. Dang, X. Zhu, Z. Li, H. Chen, M. Xiang, Z. Li, G. Hu, Exposure of adult zebrafish (Danio rerio) to Tetrabromobisphenol A causes neurotoxicity in larval offspring, an adverse transgenerational effect, J. Hazard Mater. 414 (2021), 125408.
[20] J.S. Siracusa, L. Yin, E. Measel, S. Liang, X. Yu, Effects of bisphenol A and its analogs on reproductive health: a mini review, Reprod. Toxicol. 79 (2018) 96–123.
[21] C. Ji, H. Wu, L. Wei, J. Zhao, iTRAQ-based quantitative proteomic analyses on the gender-specific responses in mussel Mytilus galloprovincialis to tetrabromobisphenol A, Aquat. Toxicol. 157 (2014) 30–40.
[22] S. Kitamura, N. Jinno, S. Ohta, H. Kuroki, N. Fujimoto, Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A, Biochem. Biophys. Res. Commun. 293 (2002) 554–559.
[23] A. Riu, M. Grimaldi, A. le Maire, G. Bey, K. Phillips, A. Boulahtouf, E. Perdu, D. Zalko, W. Bourguet, P. Balaguer, Peroxisome proliferator-activated receptor γ is a target for alogenated analogues of bisphenol A, Environ. Health Perspect. 119 (2011) 1227–1232.
[24] A. Riu, A. le Maire, M. Grimaldi, M. Audebert, A. Hillenweck, W. Bourguet, P. Balaguer, D. Zalko, Characterization of novel ligands of ERα, ERβ, and PPARγ: the case of halogenated bisphenol A and their conjugated metabolites, Toxicol. Sci. 122 (2011) 372–382.
[25] Z. Arbeli, Z. Ronen, M.C. Díaz-Báez, Reductive dehalogenation of tetrabromobisphenol-A by sediment from a contaminated ephemeral streambed and an enrichment culture, Chemosphere 64 (2006) 1472–1478.
[26] L. Iasur-Kruh, Z. Ronen, Z. Arbeli, A. Nejidat, Characterization of an enrichment culture debrominating tetrabromobisphenol A and optimization of its activity under anaerobic conditions, J. Appl. Microbiol. 109 (2010) 707–715.
[27] R. Allanou, K. Aschberger, S.J. Munn, 2,2',6,6'-tetrabromo-4,4'-isopropylidenediphenol (tetrabromobisphenol-A or TBBP-A), in: 4th Priority List. Part II, Human Health. European Union Risk Assessment Report, 2007. https://echa.europa.eu/documents/10162/32b000fe-b4fe-4828-b3d3-93c24c1cdd51.
[28] E. Akiyama, H. Kakutani, T. Nakao, Y. Motomura, Y. Takano, R. Sorakubo, A. Mizuno, O. Aozasa, K. Tachibana, T. Doi, S. Ohta, Facilitation of adipocyte differentiation of 3T3-L1 cells by debrominated tetrabromobisphenol A compounds detected in Japanese breast milk, Environ. Res. 140 (2015) 157–164.
[29] H. Takemura, J. Ma, K. Sayama, Y. Terao, B.T. Zhu, K. Shimoi, In vitro and in vivo estrogenic activity of chlorinated derivatives of bisphenol A, Toxicology 207 (2005) 215–221.
[30] Y. Mutou, Y. Ibuki, Y. Terao, S. Kojima, R. Goto, Change of estrogenic activity and release of chloride ion in chlorinated bisphenol a after exposure to ultraviolet B, Biol. Pharm. Bull. 29 (2006) 2116–2119.
[31] S. Takayanagi, T. Tokunaga, X. Liu, H. Okada, A. Matsushima, Y. Shimohigashi, Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERRγ) with high constitutive activity, Toxicol. Lett. 167 (2006) 95–105.

[32] N.J. Cabaton, C. Canlet, P.R. Wadia, M. Tremblay-Franco, R. Gautier, J. Molina, C. Sonnenschein, J.-P. Cravedi, B.S. Rubin, A.M. Soto, D. Zalko, Effects of low doses of bisphenol A on the metabolome of perinatally exposed CD-1 mice, Environ. Health Perspect. 121 (2013) 586–593.
[33] M. Yeo, K. Berglund, M. Hanna, J.U. Guo, J. Kittur, M.D. Torres, J. Abramowitz, J. Busciglio, Y. Gao, L. Birnbaumer, W.B. Liedtke, Bisphenol A delays the perinatal chloride shift in cortical neurons by epigenetic effects on the Kcc2 promoter, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 4315–4320.
[34] K. Suyama, S. Kaneko, H. Kesamaru, X. Liu, A. Matsushima, Y. Kakuta, T. Okubo, K. Kasatani, T. Nose, Evaluation of the influence of halogenation on the binding of bisphenol A to the estrogen-related receptor γ, Chem. Res. Toxicol. 33 (2020) 889–902.
[35] M.C. Cuellar, T.W. Zijlmans, A.J.J. Straathof, J.J. Heijnen, L.A.M. van der Wielen, Model-based evaluation of cell retention by crossflow ultrafiltration during fed-batch fermentations with *Escherichia coli*, Biochem. Eng. J. 44 (2009) 280–288.
[36] Y. Ibuki, Y. Tani, T. Toyooka, UVB-exposed chlorinated bisphenol A generates phosphorylated histone H2AX in human skin cells, Chem. Res. Toxicol. 21 (2008) 1770–1776.
[37] S. Lee, K. Suk, I.K. Kim, I.S. Jang, J.W. Park, V.J. Johnson, T.K. Kwon, B.J. Choi, S.H. Kim, Signaling pathways of bisphenol A-induced apoptosis in hippocampal neuronal cells: role of calcium-induced reactive oxygen species, mitogen-activated protein kinases, and nuclear factor-κB, J. Neurosci. Res. 86 (2008) 2932–2942.
[38] A. Bouskine, M. Nebout, F. Brücker-Davis, M. Benahmed, P. Fenichel, Low doses of bisphenol A promote human seminoma cell proliferation by activating PKA and PKG via a membrane G-protein-coupled estrogen receptor, Environ. Health Perspect. 117 (2009) 1053–1058.
[39] E. Bosch-Panadero, S. Mas, E. Civantos, P. Abaigar, V. Camarero, A. Ruiz-Priego, A. Ortiz, J. Egido, E. González-Parra, Bisphenol A is an exogenous toxin that promotes mitochondrial injury and death in tubular cells, Environ. Toxicol. 33 (2018) 325–332.
[40] T. Debenest, F. Gagné, A.-N. Petit, C. André, M. Kohli, C. Blaise, Ecotoxicity of a brominated flame retardant (tetrabromobisphenol A) and its derivatives to aquatic organisms, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152 (2010) 407–412.
[41] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Adv. Drug Deliv. Rev. 46 (2001) 3–26.
[42] N.J. Yang, M.J. Hinner, Getting across the cell membrane: an overview for small molecules, peptides, and proteins, Methods Mol. Biol. 1266 (2015) 29–53.
[43] J.H. Kang, F. Kondo, Y. Katayama, Human exposure to bisphenol A, Toxicology 226 (2006) 79–89.
[44] S. Kacew, A. Wallace Hayes, Absence of neurotoxicity and lack of neurobehavioral consequences due to exposure to tetrabromobisphenol A (TBBPA) exposure in humans, animals and zebrafish, Arch. Toxicol. 94 (2020) 59–66.
[45] S. Liang, S. Liang, N. Yin, B. Hu, F. Faiola, Toxicogenomic analyses of the effects of BDE-47/209, TBBPA/S and TCBPA on early neural development with a human embryonic stem cell in vitro differentiation system, Toxicol. Appl. Pharmacol. 379 (2019), 114685.
[46] P.J. Hughes, H. McLellan, D.A. Lowes, S.Z. Kahn, J.G. Bilmen, S.C. Tovey, R.E. Godfrey, R.H. Michell, C.J. Kirk, F. Michelangeli, Estrogenic alkylphenols induce cell death by inhibiting testis endoplasmic reticulum Ca^{2+} pumps, Biochem. Biophys. Res. Commun. 277 (2000) 568–574.
[47] T. Oka, N. Adati, T. Shinkai, K. Sakuma, T. Nishimura, K. Kurose, Bisphenol A induces apoptosis in central neural cells during early development of Xenopus laevis, Biochem. Biophys. Res. Commun. 312 (2003) 877–882.
[48] A. Mansur, M. Adir, C. Racowsky, C.M. Combelles, N. Landa, R. Machtinger, Susceptibility of human cumulus cells to bisphenol A in vitro, Reprod. Toxicol. 74 (2017) 189–194.