

Mitochondrial damage and apoptosis: Key features in BDE-153-induced hepatotoxicity

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A B S T R A C T

Brominated flame retardants are used in consumer goods to increase product resistance to fire and/or high temperatures. Polybrominated diphenyl ethers (PBDEs) are the most commonly employed class of brominated flame retardants because they are inexpensive and can effectively prevent flame from spreading. PBDEs are persistent, can bioaccumulate, are transported over long distances, and display toxicity. However, their toxic mechanisms of action have not been well established. Because mitochondria are recognized as the main energy-producing cell organelle and play a vital role in cellular function maintenance, here we apply mitochondria as an experimental model to evaluate the toxic effects of the PBDE congener BDE-153 (Hexa-BDE) at concentrations ranging from 0.1 to 25 μ M. We also assess BDE-153 cytotoxicity to HepG2 cells in order to elucidate its mechanisms of toxicity. Exposure to BDE-153 affects isolated mitochondria: this congener can interact with the mitochondrial membrane, to dissipate the membrane potential and to induce significant ATP depletion. Furthermore, BDE-153 can diminish MTT reduction and cell proliferation and can interfere in cell cycle, as evaluated in cell cultures. These cytotoxic effects are related to mitochondrial dysfunction due to mitochondrial membrane potential dissipation and reactive oxygen species accumulation. These effects result in apoptotic cell death, as demonstrated by phosphatidylserine maintenance on the cell membrane external surface, nuclear condensation and fragmentation, and presence of pro-apoptotic factors such as cytochrome *c* and Apoptosis-inducing Factor (AIF) plus caspase 3 activation in the cytosol. Together, our results show PBDEs can induce cytotoxicity, reinforcing the idea that these compounds pose a risk to the exposed population.

1. Introduction

Modern life has been constantly affecting the human health. Environmental pollution has increased wildly in the industrialized world, which is reflected in the development of some diseases and in the increased incidence of other diseases. Considering the modern lifestyle and the variety of currently available technologies, an environment free of chemical compounds is utopia. Human exposure to compounds regarded as contaminants has become a matter of great

concern and can occur in various ways, such as inhalation, ingestion of water or contaminated food, and dermal contact [11]; [37].

Polybrominated diphenyl ethers (PBDEs) represent the class of brominated flame retardants. PBDEs are considered as emerging contaminants and are known to be toxic. Flame retardants can enhance the safety of numerous products and meet flammability requirements, so they have been used in several industrial and consumer products to prevent fire-related injury and property damage. In general, these compounds are employed as additive in plastics, foam, textiles,

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furniture, and building materials [4]; [30]. Many studies have shown PBDEs bioaccumulate and persist in the environment and can undergo long-range transport in the atmosphere [12], rendering them a subset of persistent organic pollutants (POPs) of recognized interest in recent years [3]. Environmental pollution caused by POPs is a well-established risk factor for cell death [53].

Cytotoxicity is the manifestation of adverse effects on the structure and/or function of one or more cell components. Toxicity due to exposure to chemicals results from non-specific changes in cellular functions, which induce a specific effect on certain organs [16]. Toxic compounds may induce cytotoxicity through different mechanisms. A number of emerging contaminants have been reported to disrupt eukaryotic cell organelles, including the mitochondrion. Mitochondria are best known for their critical role in energy production by oxidative phosphorylation (OXPHOS). Contaminants can affect OXPHOS, disrupting the mitochondrial bioenergetics or structure [33].

Functionally, mitochondria are versatile and essential organelles best noted for their participation in ATP production, but they are also important for other metabolic pathways, like intracellular calcium regulation, innate immunity, thermogenesis, and fever responses, not to mention their involvement in cell death [32,33]; [9]. Mitochondrial function depends on mitochondrion organization, maintenance, and dynamics [45]; [50]. Therefore, chemicals that damage or alter these mitochondrial features can have serious health consequences [32] [7]; [43].

Mitochondria vary among tissues [5]; [49]. Additionally, their morphology will depend on cell type, developmental stage, and environmental threats [32]; [21]. Furthermore, mitochondria undergo remodeling through fusion and fission, and this balance controls their structure and number [6]. Thus, their morphology can range from fragmented to networked, and this morphological dynamics is critical to the maintenance of their correct functions and can be affected by stressors such as contaminants [18]; [32].

Research into mitochondrial pathways of chemical interactions and toxicity is accelerating. Sporadic reports of mitochondrial toxicity of various chemicals have been published along decades. In this context, this work aims to elucidate what happens at the mitochondrial and cellular levels after cell exposure to BDE-153. We evaluate the toxic effects induced by the hexa-brominated pollutant BDE-153 by means of *in vitro* tests. For this purpose, we exposed mitochondria isolated from rat liver and HepG2 cells cultured as monolayer to BDE-153 at concentrations ranging from 0.1 to 25 μ M, to find that this flame retardant is toxic to mitochondria and triggers cell death.

2. Material and methods

2.1. Chemicals

The BDE-153 congener (CAS Number 68631-49-2) was purchased from AccuStandard (New Haven, USA). Sulforhodamine B (SRB); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); dimethyl sulfoxide (DMSO); propidium iodide (PI); tert-butyl-hydroperoxide solution (TBHP); Triton X-100; bis-benzimide H 33342 trihydrochloride (Hoechst 33342); rotenone; carbonylcyanide-3-chlorophenylhydrazone (CCCP); succinate; glutamate; malate; adenosine 5'-diphosphate sodium salt (ADP); safranin-O; *o*-phthalaldehyde (OPT); N-ethylmaleimide (NEM); 1-anilino-8-naphthalene sulfonate salt (ANS); 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH); 1,6-diphenyl-1,3,5-hexatriene (DPH); ethyleneglycol bis-(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA); 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB); and Ruthenium Red were acquired from Sigma-Aldrich (USA). 2',7'-Dichlorodihydrofluorescein diacetate (H2DCFDA) and Calcium Green 5N were purchased from Molecular Probes (OR, USA). Tetramethylrhodamine methyl ester (TMRM); fetal bovine serum (FBS, GIBCO); 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; acetyl ester (CM-H₂DCFDA); and "Minimum

Essential Medium" (MEM, GIBCO) were purchased from Invitrogen (USA). Annexin V-FITC was obtained from Proteimax (Brazil). Cisplatin solution was purchased from Cirúgica Mafra (Brazil). All the other reagents were of the highest commercially available degree. The amounts of DMSO required to solubilize BDE-153 did not affect the assays (0.1% DMSO for all experiments). All stock solutions were prepared with glass-distilled deionized water.

2.2. Mitochondrial assays

2.2.1. Animals

Male Wistar rats weighing 180–200 g were used. The Committee for Experimental Animal Care and Use of the University of Sao Paulo, Brazil, approved all the experimental procedures (Protocol number 11.1.90.53.3). The animals were kept under a 12-h light/dark cycle at an ambient temperature of $24 \pm 2^\circ\text{C}$, with free access to food and water. After sacrifice, rat livers (10–15 g) were immediately removed, sliced, and maintained at 4°C .

2.2.2. Isolation of mitochondria

Rat liver mitochondria were isolated by standard differential centrifugation [35]. Livers (10–15 g) were extracted and sliced. The slices were placed in 50 mL of medium containing sucrose 250 mM, EGTA 1 mM, and HEPES-KOH 10 mM, pH 7.2, and homogenized three times for 15 s in a Potter-Elvehjem homogenizer at 1-min intervals. The homogenates were centrifuged at $580 \times g$ for 5 min, and the resulting supernatant was centrifuged at $10,300 \times g$ for 10 min. The pellets were suspended in 10 mL of medium containing sucrose 250 mM, EGTA 0.3 mM, and HEPES-KOH 10 mM, pH 7.2, which was followed by centrifugation at $3400 \times g$ for 15 min. The final mitochondrial pellet was suspended in 1 mL of medium containing sucrose 250 mM and HEPES-KOH 10 mM, pH 7.2, and used within 3 h. All procedures were conducted at 4°C , and the mitochondrial protein content was determined by the biuret reaction.

2.2.3. Evaluation of interactions with the mitochondrial membrane

Insertion of DPH, ANS, and TMA-DPH into membranes elicits fluorescence (F) whose static quenching is described by the Stern-Volmer equation: $F_0/F = 1 + K_{SV} [Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, and K_{SV} is the Stern-Volmer constant. To evaluate the BDE-153 interactions with the mitochondrial membrane, the mitochondria (1 mg of protein) were incubated with DPH 0.5 μ M and ANS 75 μ M in the standard incubation medium at 30°C for 30 min, which was followed by addition of BDE-153 to a final volume of 2 mL and kinetics monitoring for 10 min. Fluorescence was measured with an F-4500 spectrofluorometer (Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 362/432 nm, 360/430 nm, and 380/485 nm for TMA-DPH, DPH, and ANS, respectively.

2.2.4. Mitochondrial respiration

Mitochondrial respiration was monitored by polarography on an oxygraph (Hansatech, Norfolk, England) equipped with a Clark-type oxygen electrode. The mitochondria (1 mg of protein) were incubated in 1 mL of standard medium containing sucrose 125 mM, KCl 65 mM, HEPES-KOH 10 mM, EGTA 0.5 mM, and K_2HPO_4 10 mM, pH 7.4, at 30°C . The oxidizable substrates for complexes I and II were glutamate and malate 5 mM or potassium succinate 5 mM (+ rotenone 2.5 μ M), respectively.

2.2.5. Mitochondrial membrane potential

The Mitochondrial Membrane Potential ($\Delta\Psi$) was monitored on an F-4500 spectrofluorometer (Hitachi, Tokyo, Japan) by using 1 mg of protein, safranin-o 10 μ M as probe, and the 495/586 nm excitation/emission wavelength pair [1]; [17].

2.2.6. Mitochondrial swelling

Mitochondrial swelling was estimated from the decrease in apparent absorbance of 0.4 mg of protein at 540 nm. A spectrophotometer Model DU-70 (Beckman, Coulter Inc., Fullerton, CA, U.S.A.) was employed to monitor changes in absorbance for 10 min (incubation time with BDE-153).

2.2.7. Calcium efflux

Mitochondrial Ca^{2+} efflux was spectrofluorimetrically monitored by using Calcium Green 5 N 150 nM (Molecular Probes, OR, USA) as probe, the 506/531 nm excitation/emission wavelength pair, and 1 mg of protein [17].

2.2.8. ATP level determination

Mitochondrial ATP was determined by means of the firefly luciferin–luciferase assay system. The mitochondrial suspension (1 mg of protein in 1 mL) was centrifuged at $9000 \times g$ and 4°C for 5 min, and the pellet was treated with 1 mL of ice-cold HClO_4 1 M. After centrifugation at $14,000 \times g$ and 4°C for 5 min, aliquots (100 μL) of the supernatant were neutralized with 70 μL of KOH 2 M suspended in Tris–HCl 100 mM, pH 7.8 (final volume of 1 mL) and centrifuged again. Bioluminescence was measured in the supernatant by using a Sigma–Aldrich assay kit (according to the manufacturer's instructions) and an AutoLumat LB953 Luminescence photometer (Perkin Elmer Life Sciences, Wildbad, Germany).

2.3. HepG2 cell monolayer culture

2.3.1. Cell culture

HepG2 cells (American Type Culture Collection, n° HB8065) were cultured in Dubelcos Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum, at 37°C , in atmosphere containing 5% CO_2 , until the cells reached confluence that was suitable (80%) to begin the tests. Then, adequate amounts of the cells were plated for 24 h to ensure good adhesion before the start of the experiments.

2.3.2. Cell metabolic activity and total cell content assays

Cell metabolic activity was assessed by the MTT assay, and total cell content was assessed by sulforhodamine B (SRB) assay as indicators of BDE 153-induced effects on cell proliferation and cytotoxicity.

MTT assay was assessed according to [13]. HepG2 cells were cultured at a density of 5×10^4 cells per well in the same conditions described above. Subsequently, the cells were incubated with MTT 0.5% (5 mg/mL) in atmosphere containing 5% CO_2 at 37°C for 3 h. After this period, the medium from the wells was discarded, and the resulting formazan crystals were solubilized with DMSO and glycine buffer 0.2 M, pH 10.2. The final absorbance was measured in a microplate reader (Varian Cary 50MPR, Varian, USA) at 540 and 570 nm for proliferation and viability, respectively. The results are given as % values in treated cells as compared to non-treated (control) cells, taken as 100%.

To evaluate how BDE-153 affects cell number, the influence of BDE-153 concentrations ranging from 0.1 to 25 μM was determined by the SRB colorimetric assay described by Ref. [44]. Briefly, HepG2 cells were cultured at a density of 5×10^4 cells per well and exposed to BDE-153 for 24 or 48 h. The medium was discarded, and the cells were washed once with phosphate buffer saline (PBS) and twice with distilled water to remove salts. Next, the cells were dried at room temperature and subsequently fixed with 1% methanol in acetic acid solution (1%) for 2 h. Fixed cells were stained with SRB 0.5% in acetic acid 1% and washed with acetic acid 1% to remove excess probe. The SRB that attached to the cell membrane was extracted with 1 mL of Tris 10 mM, pH 10.

2.3.3. Mitochondrial membrane potential in HepG2 cells

Mitochondrial depolarization was examined on the basis of cell retention of the fluorescent dye TMRM as described by Ref. [20]. The cells (1×10^5 cells per well) were incubated in the absence (control) or presence of BDE-153 at final concentrations ranging from 0.1 to 25 μM . The cell suspensions were washed with PBS, trypsinized, and incubated with TMRM 6.6 μM at 37°C for 30 min. Subsequently, the samples were lysed with Triton X-100 0.1% (v/v), and the TMRM that was captured and retained by the mitochondria was measured at 485 and 590 nm of excitation and emission, respectively, with a F-4500 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan). Carbonylcyanide-3-chlorophenylhydrazone (CCCP) was used as positive control and the results are expressed as % fluorescence in relation to the control taking the control as 100%.

2.3.4. Reactive oxygen and nitrogen species (RONS) accumulation

Intracellular $\text{H}_2\text{DCF-DA}$ oxidation to 2,7-dichlorofluorescein (DCF) by ROS was assessed as indicator of intracellular ROS accumulation [8]. The cells (1×10^5 cells per well) were incubated in the same conditions described above. Then, the cells were incubated with CM- $\text{H}_2\text{DCF-DA}$ 2 mM at 37°C for 1 h. Subsequently, CM- $\text{H}_2\text{DCF-DA}$ fluorescence was measured with an F-4500 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) at 503 and 528 nm of excitation and emission, respectively. The results are presented as the difference from the control group. TBHP solution (100 μM) was used to induce oxidative stress.

2.3.5. Phosphatidylserine exposure on the outer cell membrane (Annexin V assay) and nuclear fragmentation

Phosphatidylserine exposure was evaluated by using Annexin-V combined with a fluorescent agent to identify an apoptotic process [52]. HepG2 cells (1×10^5) were cultured and treated in the absence (control) and presence of BDE-153 (1–25 μM). Then, the cells were incubated with FITC-Annexin-V 0.25 $\mu\text{g/mL}$ for 15 min, and PI 0.5 $\mu\text{g/mL}$ was added immediately before the analysis. The cells were analyzed with a BD-FACSCANTO™ flow cytometer (BD Bioscience, CA, USA) and BD-FACSDIVA software (BD Bioscience, CA, USA).

Nuclear fragmentation was also investigated by using the fluorescent dye Hoechst 33342. Briefly, HepG2 cells were seeded at a density of 1×10^4 cells on glass coverslips and treated with BDE-153 at final concentrations ranging from 0.1 to 25 μM for 24 or 48 h. Each sample was assayed in at least three replicates. The cells on the coverslips were fixed with methanol at -20°C for 2 h, which was followed by staining with Hoechst 33342 5 $\mu\text{g/mL}$ at 37°C for 30 min. The nuclear fragments were observed by fluorescence microscopy with a Leica DM 5000B microscope (Germany); 300 cells were quantified in each slide.

2.3.6. Western Blotting analysis of pro-apoptotic proteins

HepG2 cells were seeded on culture dishes, as described above, and treated with BDE-153. After exposure to BDE-153 for 24 or 48 h, the cells were collected and washed with cold PBS and homogenized in RIPA lysis buffer (Tris/HCl 50 mM, pH: 7.4; NaCl 150 mM; EDTA 1 mM; triton 1%; deoxycholate 1%; sodium dodecyl sulfate (SDS) 0.1%; and SIGMAFast protease inhibitor tablet cocktail (Reference S8820 with dilution 1:1000)), and they were subsequently sonicated for 10 s and centrifuged at 13,000 g and 4°C , for 10 min. Protein concentration was determined by the bicinchoninic acid assay; bovine serum albumin was the standard. Aliquots with equivalent amounts of the cell lysates were fractionated in sodium dodecyl sulfate-polyacrylamide gel 8–15%, transferred to polyvinylidene difluoride membranes, and incubated with primary antibody (dilution 1:500) and an adequate secondary antibody, anti-tubulin or anti-actin antibody (dilution 1:5000) conjugated with biotin (Life technologies), chosen as pro-apoptotic markers. The membranes were incubated with the Qdot detection system (Life technologies). The tubulin content was used as loading control for the total protein content. There were no differences between groups.

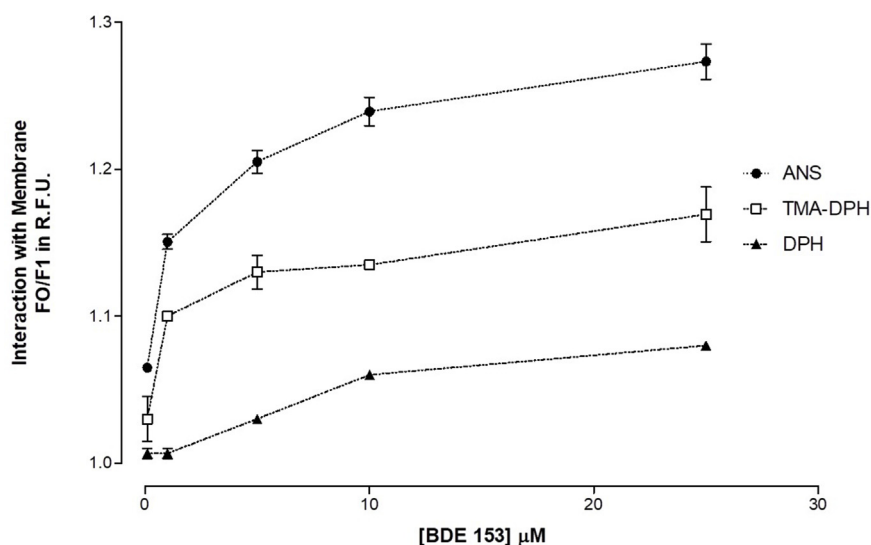


Fig. 1. Interaction of BDE-153 with the inner mitochondrial membrane as measured with the aid of the probes ANS, TMA-DPH, and DPH as described in materials and methods. The relative fluorescence units (R.F.U.) were measured before and after BDE-153 addition and are presented as the F₀/F ratio, where F₀ and F are the fluorescence intensities in the absence and presence of the compound. The data are presented as the means \pm SEM of a series of three experiments.

Table 1

Respiratory parameters.

Parameters of oxidative phosphorylation				
[BDE-153] μ M	RCR with G + M	RCR with Suc	ADP/O with G + M	ADP/O with Suc
0	5.26 \pm 0.26	4 \pm 0.21	2.83 \pm 0.29	1.6 \pm 0.65
0.1	3.66 \pm 0.26	5.1 \pm 0.21	2.83 \pm 0.28	1.9 \pm 0.73
1	3.86 \pm 0.28	5.6 \pm 0.21	3.32 \pm 0.28	1.90 \pm 0.73
5	4.36 \pm 0.26	4.5 \pm 0.21	2.83 \pm 0.29	1.9 \pm 0.74
10	4.96 \pm 0.26	5.6 \pm 0.21	2.73 \pm 0.28	1.5 \pm 0.50
25	5.06 \pm 0.26	5.6 \pm 0.21	2.83 \pm 0.28	1.9 \pm 0.73

2.4. Statistical analysis

Data were evaluated by analysis of variance (ANOVA) followed by Dunnett's test to compare the several treated groups to their control by means of the program GraphPrism, version 5.1 for Windows. Results with $p < 0.05$ were considered statistically significant.

3. Results

The results indicated that mitochondria could be a target for BDE-153 congener-induced toxicity. BDE-153 interacted with the inner mitochondrial membrane (Fig. 1). The probes DPH and ANS have affinities for mitochondrial membrane. While DPH interacts with the hydrophobic portion of the mitochondrial membrane, ANS interacts with

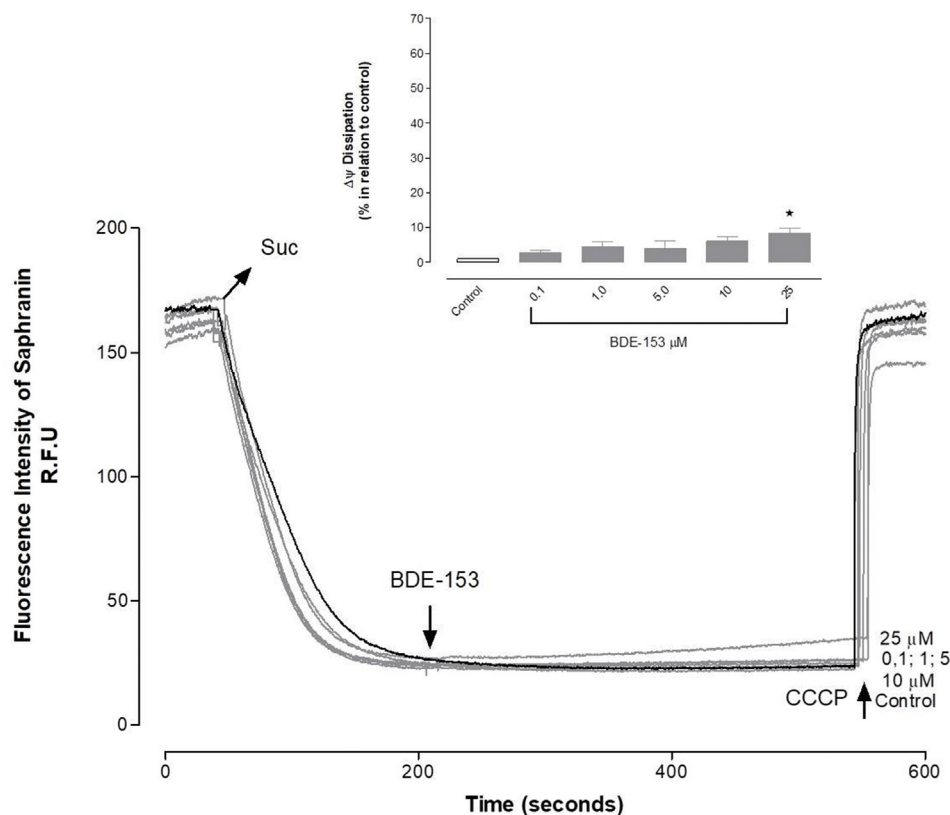


Fig. 2. The effect of BDE-153 concentration-response on the mitochondrial membrane potential (MMP) dissipation in mitochondria isolated from rat liver (1.0 mg protein. mL⁻¹) was evaluated with the aid of the Saphranin-o probe. Incubation is described in materials and methods. The data represent the mean \pm SEM of three determinations with different mitochondrial preparations, relative to the control in the absence of the compound. *Statistically significant results ($p < 0.05$) as compared to the control.

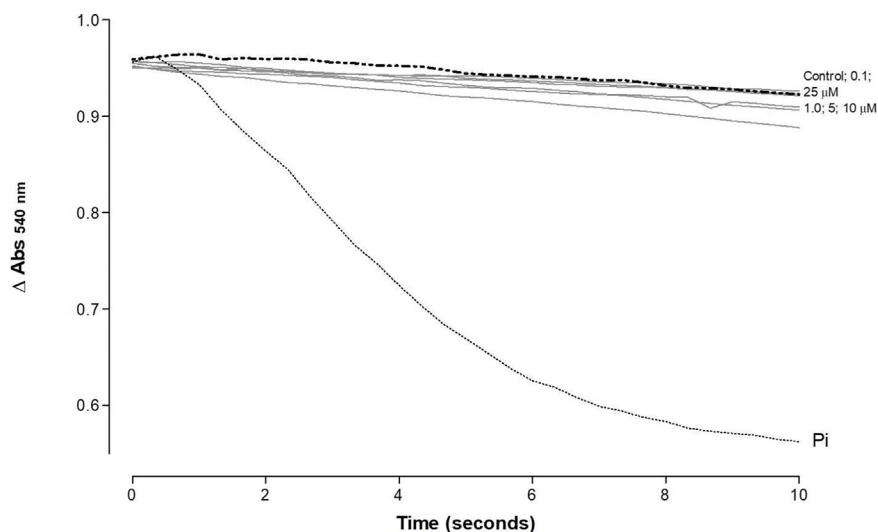


Fig. 3. Effect of BDE-153 on mitochondrial swelling in mitochondria isolated from rat liver (0.4 mg protein. mL⁻¹) incubated as described in Materials and Methods. Maximum swelling was observed with inorganic phosphate (Pi) 1 μM. The data represent the mean ± SEM of three determinations with different mitochondrial preparations, relative to the control in the absence of the compound.

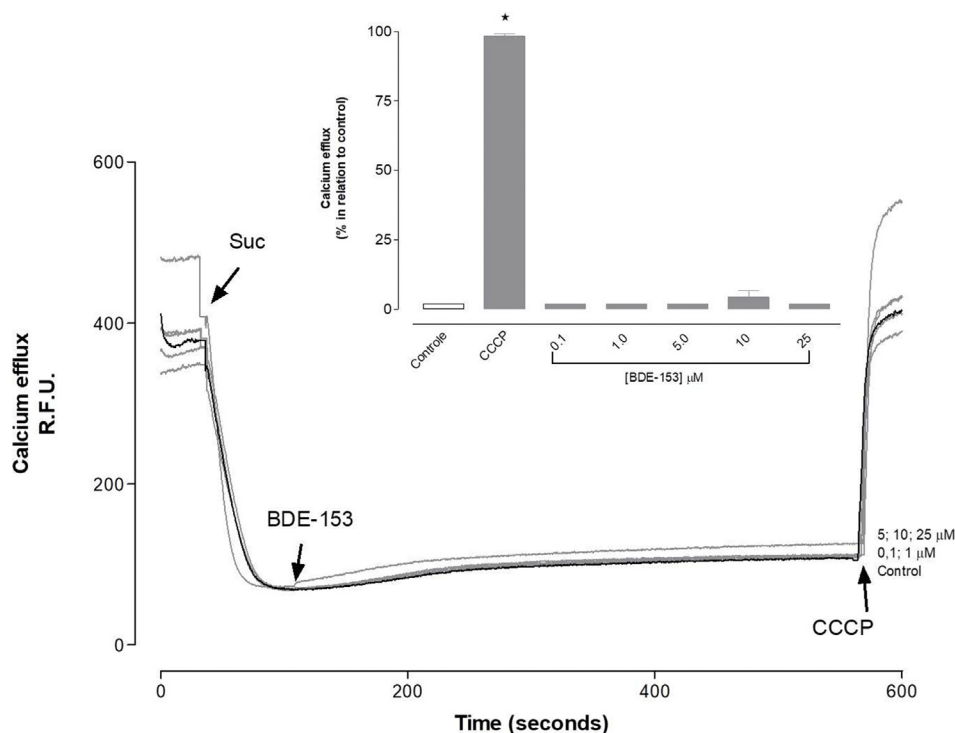


Fig. 4. The BDE-153 concentration-response effect on calcium efflux from mitochondria isolated from rat liver (1.0 mg protein. mL⁻¹) was measured with Calcium Green 5 N probe and incubated as described in Materials and Methods. The points represent the mean ± SEM of three determinations with different mitochondrial preparations as compared to the control. *Statistically significant results ($p < 0.05$) as compared to the control. Total calcium release was induced with CCCP 1 μM.

the hydrophilic portion, and TMA-DPH remains between both portions. When the probes interact with mitochondria, they emit fluorescence. Thus, BDE-153 was able to penetrate into the mitochondria and to interact with the hydrophilic portion of the internal mitochondrial membrane, given that it interfered with the ANS fluorescence more pronouncedly. However, this insertion in the hydrophilic portion did not affect the mitochondrial OXPHOS capacity (Table 1).

Respiratory control ratio (RCR) and ADP/O obtained by using glutamate and malate for complex I and succinate for complex II in mitochondria isolated from rat liver, under the influence of BDE-153. Data represent the mean ± SEM of three determinations with different mitochondrial preparations. *Means different from the control (no addition of BDE-153) in accordance with Dunett's test ($p < 0.05$). RCR = Respiratory control ratio; G + M = Glutamate 5 mM + Malate 5 mM; Suc = Succinate 5 mM.

On the other hand, the time during which BDE-153 and mitochondria interacted was sufficient to initiate dissipation of the mitochondrial

membrane potential ($\Delta\psi$). Fig. 2 shows the highest BDE-153 concentration tested here (25 μM) affected $\Delta\psi$. Evaluation of mitochondrial swelling was then used to assess whether BDE-153 alters mitochondrial permeability. Fig. 3 reveals the compound did not decrease the mitochondrial suspension apparent absorbance at 540 nm.

Because we did not detect mitochondrial swelling, we expected there would be no calcium efflux (see Fig. 4). Many emerging contaminants can influence the cell energy balance by increasing ATP consumption and/or reducing ATP production. To investigate whether BDE-153 mitochondrial insertion affects ATP production, we determined the mitochondrial ATP content. The data in Fig. 5 demonstrate that incubation with BDE-153 significantly decreased the mitochondrial ATP levels at all the tested BDE-153 concentrations.

To verify the BDE-153 effects on mitochondria, we directly exposed these organelles to the compound for short periods (10–30 min). To verify the BDE-153 effects on a cellular model and to verify the consequences of this kind of exposure, we exposed liver cells to the

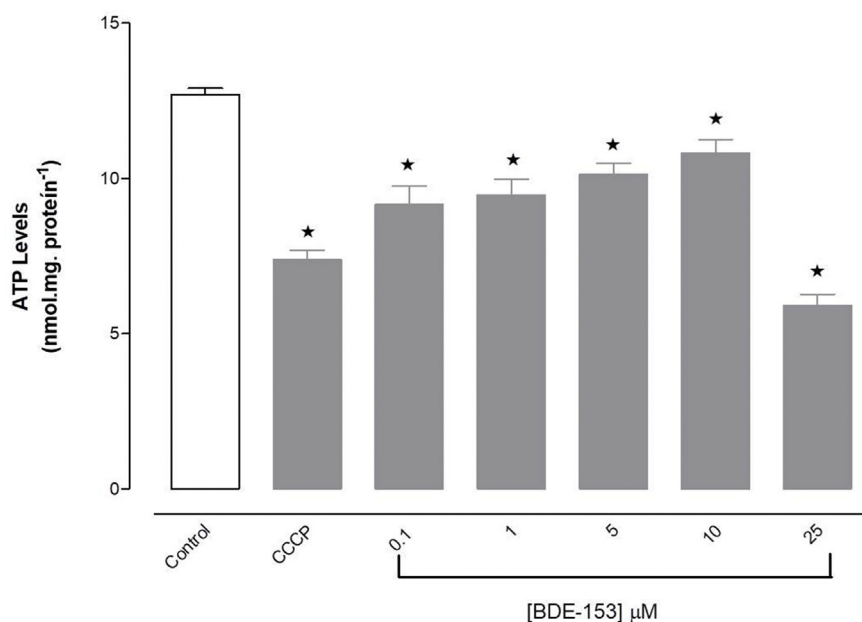


Fig. 5. The BDE-153 concentration-response effect on ATP levels in mitochondria isolated from rat liver (1.0 mg protein. mL⁻¹) was evaluated by using the luminescence obtained from the luciferin/luciferase reaction, incubated as described in Materials and Methods. The data represent the mean \pm SEM of three determinations with different mitochondrial preparations, relative to the control in the absence of the compound. *Statistically significant results ($p < 0.05$) as compared to the control. The fall in ATP levels was induced by CCCP 1 μ M.

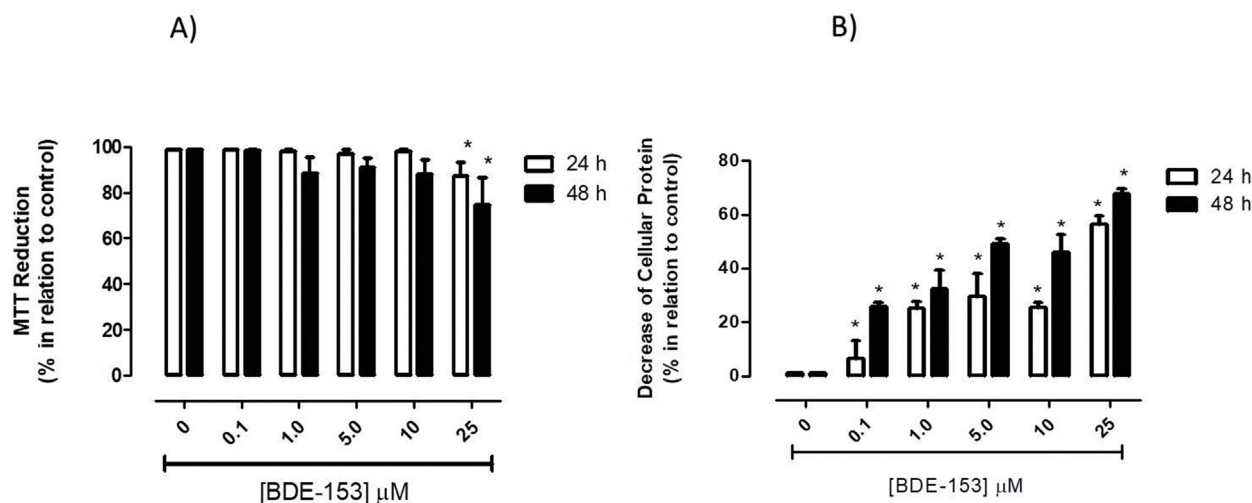


Fig. 6. (A) BDE-153 (0.1–25 μ M) effects on the HepG2 MTT reduction after 24 or 48 h of incubation. Cell viability was assessed by the MTT assay, as described in Materials and Methods. The data are presented as the means \pm SEM of a series of three experiments. * Significantly different ($p < 0.05$) from the negative control (without BDE-153). (B) BDE-153 (0.1–25 μ M) effects on HepG2 cell proliferation after 24 or 48 h of incubation. Cell proliferation was assessed by using sulforhodamine B (SRB), as described in Materials and Methods.

compound for 24 or 48 h. The liver operates important functions in the body, especially xenobiotics biotransformation and metabolism, so this organ is the main target of xenobiotics toxicity mechanisms. Our results show hepatotoxicity was closely linked to the observed mitochondrial damage.

After 24 or 48 h of exposure to BDE-153 25 μ M, the ability of HepG2 cells to reduce the MTT dye diminished, which pointed to the lower cell metabolic viability (Fig. 6A). Furthermore, the amount of cellular protein decreased significantly at all the assayed BDE-153 concentrations and exposure times (Fig. 6B), probably due to the BDE-153 cytotoxic and cytostatic effect. It is possible to assess the cytostatic effect by stopping the cell cycle.

As observed previously, mitochondrial membrane potential dissipation also occurred in the cellular model (Fig. 7A). Exposure to BDE-153 10 or 25 μ M dissipated the membrane potential significantly. In the micrographs shown in Fig. 7B, the fluorescence intensity of the cationic probe TMRM, which labels mitochondria with viable membrane potential, decreased. Bearing in mind that mitochondria are the main site

of reactive oxygen species formation and that the presence of xenobiotics alters their structure and function to increase reactive species formation, we investigated reactive oxygen and nitrogen species formation and accumulation by using the CM-H₂DCF-DA probe in HepG2 cells exposed to BDE-153. The results (Fig. 8) demonstrated that reactive species significantly accumulated after HepG2 cell exposure to BDE-153 in a time- and concentration-dependent manner as compared to the control.

We monitored the effects of mitochondrial damage and cytotoxicity by examining phosphatidylserine exposure in the outer membrane, which characterizes apoptotic cell death (Fig. 9). There was no PI staining, so no necrotic cell death took place. To confirm that there was no necrosis after exposure to BDE-153, we assessed release of the cytosolic protein lactate dehydrogenase (LDH), which did not occur as judged from the absence of cell membrane rupture (data not shown). We also examined nuclear condensation and fragmentation, which are morphological characteristics of apoptosis. The data presented in Fig. 10 confirmed apoptosis happened after exposure to BDE-153 at

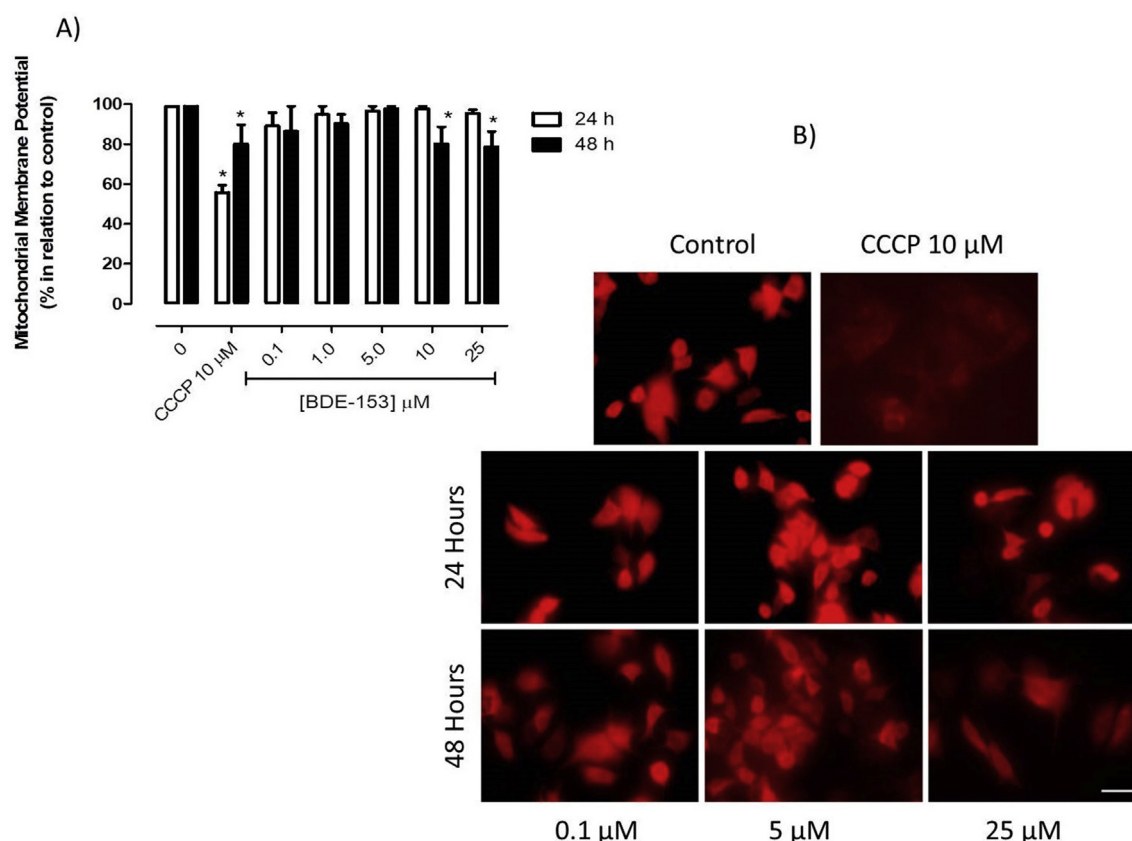


Fig. 7. BDE-153 (0.1–25 μM) effects on the mitochondrial membrane potential (MMP) of HepG2 cells after 24 or 48 h of incubation A) The MMP was assessed by using TMRM (tetramethylrhodamine, methyl ester) as described in Materials and Methods. B) Fluorescence microscopy at 400 \times magnification showing TMRM probe the accumulation in HepG2 cells with intact mitochondria after exposure to BDE-153 for 24 or 48 h. The data are presented as the mean \pm SEM of a series of three experiments. * Significantly different ($p < 0.05$) from the negative control (without BDE-209). CCCP 10 μM was used as the positive control.

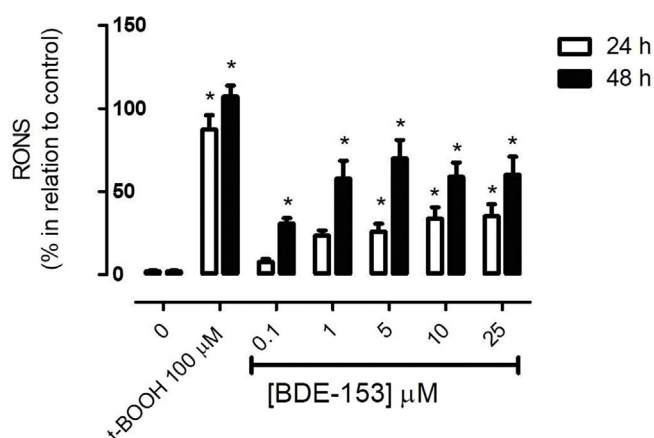


Fig. 8. BDE-153 (0.1–25 μM) effects on ROS induction in HepG2 cells after 24 or 48 h of incubation. ROS accumulation was assessed with the fluorescence probe 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) as described in Materials and Methods. The data are presented as the means \pm SEM of a series of three experiments. * Significantly different ($p < 0.05$) from the negative control (without BDE-153). The positive control was tert-butyl hydroperoxide (TBHP) 100 μM .

concentrations as low as 1 μM .

The apoptotic cell death signaling pathway can be triggered in different ways. To elucidate the order of facts during BDE-153 hepatotoxicity, we evaluated some pro-apoptotic factors. Fig. 11A shows pro-caspase in protein caspase 3 was cleaved, which meant that there the effector caspase of the apoptotic pathway was activated after

exposure to BDE-153 at 5 or 25 μM . This finding corroborated our previous results and, together with cytochrome c release shown in Fig. 11B, indicated that the mitochondrial pathway induced apoptosis. Western Blotting helped us to assess Bax protein translocation to the mitochondrial membrane (Bax is an apoptosis-inducing cytosolic protein under mitochondrial stress conditions). In this context, Fig. 11C shows that BDE-153 at 5 or 25 μM promoted Bax protein translocation from the cytosol to the mitochondrial membrane. In addition, the AIF protein located in the mitochondrial matrix was detected and quantified in the cytosol, as demonstrated in Fig. 11D, which indicated pore opening in the mitochondrial membrane and justified cytochrome c release, which may have compensated Bax presence in the mitochondrial portion.

4. Discussion

Due to their high chemical stability, PBDEs have been widely used to reduce risks and damages caused by fire in countless consumer products. Even though chemical stability is an essential feature in flame retardants, it can also constitute a drawback that, allied with PBDE low vapor pressure and high hydrophobicity, render PBDEs persistent environmental contaminants [2]; [28]. Here, we have verified that one of the main representatives of this class of compounds, BDE-153, can induce hepatic damage. This congener has been reported to occur in environmental samples and biological fluids and to be toxic [14]; [46,47].

In recent years, our research group has attempted to elucidate the toxic effects of PBDEs. We have recently published that BDE-153 and other congeners can cause genomic instability, as revealed by comet assay results obtained after exposure to BDE-153 at 5, 10, or 25 μM for

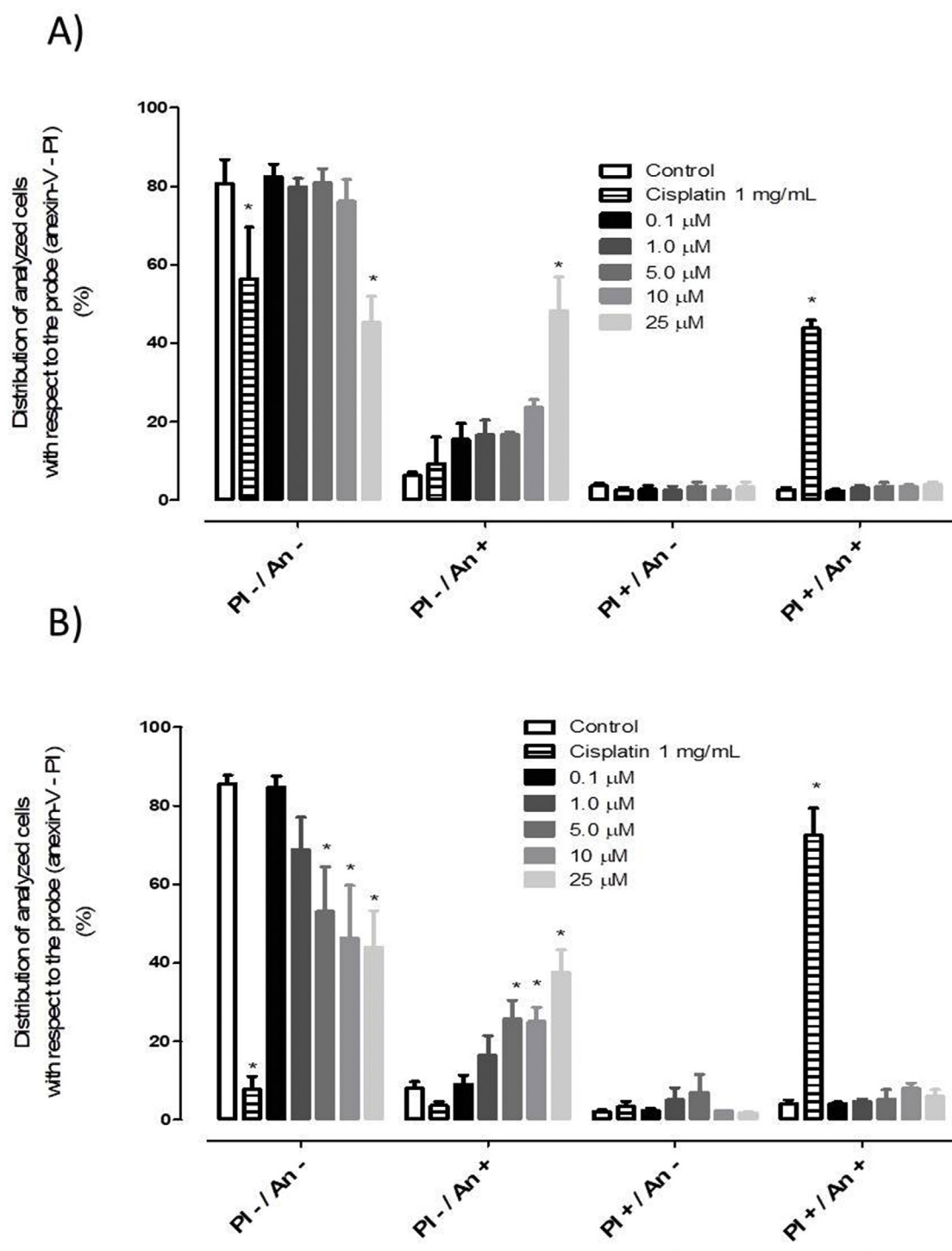


Fig. 9. (A) BDE-153 (0.1–25 µM) effects on phosphatidylserine (PS) exposure in HepG2 cells after 24 h of incubation. PS exposure was assessed by the Annexin-V (An.) / Propidium Iodide (PI) assay, as described in Materials and Methods. (B) BDE-153 (0.1–25 µM) effects on PS exposure of HepG2 cells after 48 h of incubation. PS exposure was assessed by the Annexin/PI assay, as described in Materials and Methods. Images are representative of three experiments with different cell cultures. Results are presented as the mean \pm SEM of a series of three experiments. * Significantly different ($p < 0.05$) from the negative control (without BDE-153). The positive control was cisplatin (cDDP) 1 mg/mL.

only 4 h [38]. In addition, we have shown the same compound can induce HepG2 cell autophagy [39,40] in an attempt to save cells from the damage caused by BDE-153. Autophagy is a pro-survival process that occurs under stressful conditions, such as xenobiotics impact [29]. In the present work, we can infer that the damage caused by BDE-153 to HepG2 cells goes beyond the capacity of the autophagic process to conduct repair. Actually, BDE-153 induces apoptotic cell death mainly

via the mitochondrial pathway.

The aforementioned mitochondrion-associated cellular processes, including OXPHOS, are highly sensitive to changes in the chemical and physical properties of the cellular milieu [22] because the mitochondrial function integrity is essential to cellular life [15]. Furthermore, damage to mitochondrial integrity may have even more negative effects such as decreased ATP production. Changes in the ANS fluorescence

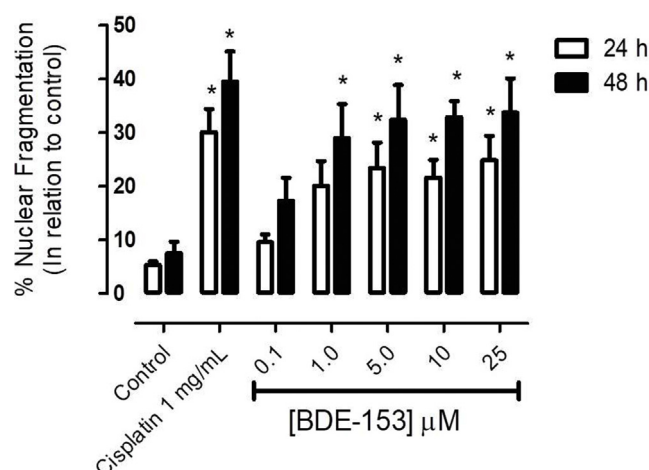


Fig. 10. BDE-153 (0.1–25 µM) effects on HepG2 cells after 24 or 48 h of incubation. Nuclear fragmentation was assessed by fluorescence microscopy; the fluorescent dye Hoechst 33342 was used as described in Materials and Methods. The data are presented as the mean \pm SEM of a series of three experiments. * Significantly different ($p < 0.05$) from the negative control (without BDE-153). The positive control was cisplatin (cDDP) 1 mg/mL.

demonstrate that BDE-153 can insert into the mitochondrial membrane due to its greater lipophilicity [2], which justifies decreased mitochondrial ATP production. Although we have not observed significant effects on the mitochondrial respiratory activity parameters, BDE-153 interaction with isolated mitochondria during a short time is enough to deplete ATP at all the assayed BDE-153 concentrations evaluated. ATP depletion leads to cell death by apoptosis or necrosis [19]; [26].

The potential effects of exposure to mitotoxins that may have long-term consequences to all organisms are particularly important [23]; [48]. Regardless of mechanism, mitochondrial impairment likely has a significant impact on cell survival, causing cell death. In this context, our results show that BDE-153 is cytotoxic, as already demonstrated for other congeners, such as BDE-154 and BDE-47 [46,47]. BDE-71 and BDE-47 can cause oxidative stress and cell death in human neutrophils [42].

In addition to mitochondrial ATP depletion, liver damage can originate from time- and concentration-dependent RONS accumulation upon exposure to BDE-153. An important consequence of RONS accumulation is apoptotic and necrotic cell death. Mitochondria-generated RONS induce release of cytochrome *c* and other pro-apoptotic proteins that elicit cellular apoptotic response and caspase activation [34]. Formation of reactive compounds can cause lipid peroxidation, protein oxidation, and DNA damage inside and outside mitochondria [41]. In fact, we have observed RONS accumulation and cytochrome *c* in the

cytosol after exposure to BDE-153, which characterizes intrinsic apoptosis.

Cell death due to mitochondrion-mediated apoptosis may occur as a consequence of mitochondrial membrane potential dissipation and increased RONS accumulation which, in association, impair OXPHOS and ATP production [24]. BDE-153 at 25 and 10 µM can dissipate the membrane potential of isolated mitochondria and HepG2 cell mitochondria, respectively. This difference in effect intensity may be due to the exposure time, which was 10 min for isolated mitochondria and 48 h for mitochondria in HepG2 cells.

Bax protein translocation to the mitochondrial membrane, caused by the extrinsic pathway, is another mechanism underlying the release of pro-apoptotic factors. Bax protein in the membrane contributes to pore opening in the outer mitochondrial membrane, facilitating the release of apoptotic proteins [10]; [27], and our results demonstrate BAX is present in the mitochondrial portion.

In parallel, AIF release points to us another pathway of BDE-153 toxicity mechanism. AIF is normally located in the mitochondrion intermembrane space, but apoptotic stimulation causes its release into the cell cytoplasm and triggers cell death by independent apoptosis and caspase activation after exposure to BDE-153 for 24 or 48 h.

Although our previous results have shown autophagic induction [39,40], persistent mitochondrial damage may have led these cells to death by apoptosis elicited by different stimuli, to culminate in phosphatidylserine exposure and nuclear condensation and fragmentation. Anthropogenic chemicals and other stressors can impact macromolecular mitochondrial structures via molecular interference or mechanical damage [22]. Our results corroborate with this notion and demonstrate that mitochondria function as important biosensors of environmental contaminants. In this context, given that BDE-153 accumulates in the environment and organisms, this congener remains a cause of concern for the general public.

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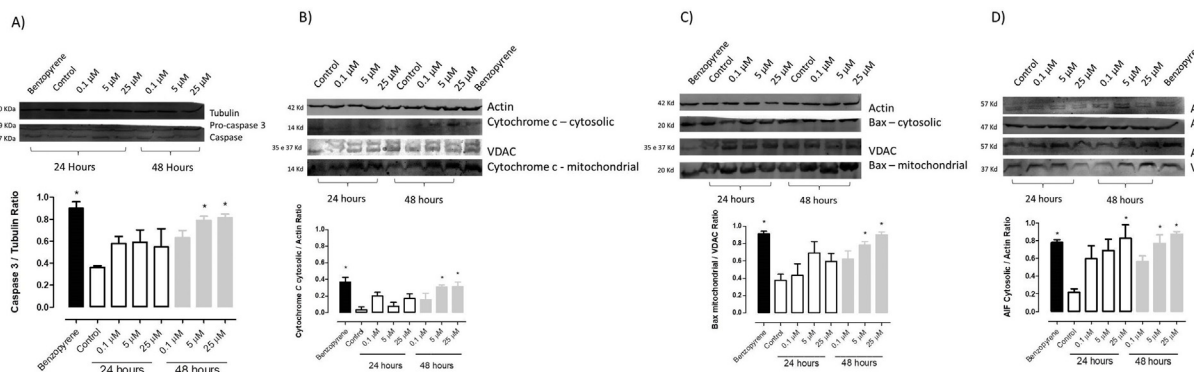


Fig. 11. Effects of BDE-153 (0.5–25 µM) after 24 and 48 h of incubation of HepG2 cells with the target compound. The caspase-3 (A), cytochrome *c* (B), Bax (C) and AIF (D) contents were assessed by western blotting as described in Materials and Methods. Data are presented as the means \pm SEM of a series of three experiments. * Significantly different ($p < 0.05$) from the negative control (without BDE-153).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.cbi.2018.06.021>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.cbi.2018.06.021>.

References

- [1] K.E.O. Akerman, M.K.F. Wikstrom, Safranina as a probe of the mitochondrial membrane potential, *FEBS Lett.* 68 (1976) 191–197.
- [2] D.L.R. Annuniação, F.A. Almeida, F.F. Sodr , Method development and validation for the determination of polybrominated diphenyl ether congeners in Brazilian aquatic sediments, *Microchem. J.* 133 (2017) 43–48.
- [3] F. Behnia, M.R. Peltier, G.R. Saade, R. Menon, Environmental pollutant polybrominated diphenyl ether, a flame retardant, induces primary amnion cell senescence, *Am. J. Reprod. Immunol.* 74 (2015) 398–406.
- [4] L.S. Birnbaum, D.F. Staskal, Brominated flame retardants: cause for concern? *Environ. Health Perspect.* 112 (2004) 9–17.
- [5] S.E. Calvo, V.K. Mootha, The mitochondrial proteome and human disease, *Annu. Rev. Genom. Hum. Genet.* 11 (2010) 25–44.
- [6] D.C. Chan, Fusion and fission: interlinked processes critical for mitochondrial health, *Annu. Rev. Genet.* 46 (2012) 265–287.
- [7] S.S.L. Chan, Inherited mitochondrial genomic instability and chemical exposures, *Toxicology* 391 (2017) 75–83.
- [8] B.V. Chernyak, D.S. Izumov, K.G. Ilyamzaev, A.A. Pashkovskaya, O.Y. Pletushkina, Y.N. Antonenko, D.V. Sakharov, K.W.A. Wirtz, V.P. Skulachev, Production of reactive oxygen species in mitochondria of HeLa cells under oxidative stress, *Biochim. Biophys. Acta* 1757 (2006) 525–534.
- [9] S.M. Cloonan, A.M. Choi, Mitochondria: sensors and mediators of innate immune receptor signaling, *Curr. Opin. Microbiol.* 16 (2013) 327–338.
- [10] M. Crompton, Bax, Bid and the permeabilization of the mitochondrial outer membrane in apoptosis, *Curr. Opin. Cell Biol.* 12 (2000) 414–419.
- [11] J.A. Daniels, *Advances in Environmental Research* vol. 28, Nova Science Publishers, New York, 2013, p. 274.
- [12] C.A. de Wit, An Overview of Brominated Flame Retardants in the Environment vol. 46, Chemosphere, Oxford, 2002, pp. 583–624.
- [13] F. Denizot, R. Lang, Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, *J. Immunol. Meth.* 89 (1986) 271–277.
- [14] D.J. Dorta, A.O. Souza, L.C. Pereira, M. Pazin, G.A.R. Oliveira, D.P. Oliveira, Polybrominated Diphenyl Ether (PBDE) Flame Retardants as Emergent Environmental Pollutants: an Overview of Their Environmental Contamination and Toxicological Properties. *Advances in Environmental Research*. 1ed vol. 28, Nova Science Publishers, Inc, Nova York, 2013, pp. 43–71.
- [15] M.R. Duchon, Mitochondria in health and disease: perspectives on a new mitochondrial biology, *Mol. Aspect. Med.* 4 (2004) 365–451.
- [16] B. Ekwall, Screening of toxic compounds in mammalian cell cultures, *Ann. N. Y. Acad. Sci.* 407 (1983) 64–77.
- [17] R.K. Emsa, R. Grunwald, J.J. Lemasters, Rhodamine 123 as a probe of transmembrane potential in isolated rat liver mitochondria: spectral and metabolic properties, *Biochim. Biophys. Acta* 850 (1986) 436–448.
- [18] D.R. Green, B. Van Houten, SnapShot: mitochondrial quality control, *Cell* 147 (2011) 950.
- [19] A. Halestrap, Biochemistry: a pore way to die, *Nature* 434 (2005) 578–579.
- [20] R. Imberti, A.L. Nieminen, B. Herman, J.J. Lemasters, Mitochondrial and glycolytic dysfunction in lethal injury to hepatocytes by t-butylhydroperoxide: protection by fructose, cyclosporin A and trifluoperazine, *J. Pharmacol. Exp. Therapeut.* 265 (1993) 392–400.
- [21] R.P. Jansen, K. de Boer, The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate, *Mol. Cell. Endocrinol.* 145 (1998) 81–88.
- [22] N. Jayasundara, Ecological significance of mitochondrial toxicants, *Toxicology* 391 (2017) 64–74.
- [23] J.S. Kozal, N.J. Jayasundara, R.T. Di Giulio, Transgenerational exposure to benzo(a)pyrene affects metabolic plasticity and thermal stress response capacity, *International Symposium on Pollutant Responses in Marine Organisms*. Matsuyama, Japan, 2017.
- [24] G. Kroemer, P. Petit, N. Zamzami, J.L. Vayssière, B. Mignotte, The biochemistry of programmed cell death, *Faseb. J.* 9 (1995) 1277–1287.
- [25] M. Leist, B. Single, A.F. Castoldi, S. Kuhnle, P. Nicotera, Intracellular adenosine Triphosphate (ATP) Concentration: a Switch in the decision between apoptosis and necrosis, *J. Exp. Clin. Med.* 185 (1997) 1481–1486.
- [26] L. Liu, Z. Zhang, D. Xing, Cell death via mitochondrial apoptotic pathway due to activation of Bax by lysosomal photodamage, *Free Radic. Biol. Med.* 51 (2011) 53–68.
- [27] D. Mackay, W.Y. Shiu, K.C. Ma, S.C. Lee, *Handbook of Physical-chemical Properties and Environmental Fate for Organic Chemicals*, second ed., CRC Press, Boca Raton, 2006.
- [28] M.C. Maiuri, E. Zalckvar, A. Kimchi, G. Kroemer, Self-eating and self-killing: crosstalk between autophagy and apoptosis, *Mol. Cell Biol.* 8 (2007) 741–752.
- [29] C.F. McDonald, A. Thomas, A perspective on the potential health risks of PBDEs, *Chemosphere* 46 (2002) 745–755.
- [30] J.N. Meyer, M.C.K. Leung, J.P. Rooney, A. Sendoel, M.O. Hengartner, G.E. Kisby, A.S. Bess, Mitochondria as a target of environmental toxicants, *Toxicol. Sci.* 134 (2013) 1–17.
- [31] P. Mishra, D.C. Chan, Mitochondrial dynamics and inheritance during cell division, development and disease, *Nat. Rev.* 15 (2014) 634–646.
- [32] M. Ott, V. Gogvadze, S. Orrenius, B. Zhivotovsky, Mitochondria, oxidative stress and cell death, *Apoptosis* 12 (5) (2007) 913–922.
- [33] P.L. Pedersen, J.W. Greenawalt, B. Reynafarje, J. Hulliher, G.L. Decker, J.W. Soper, E. Bustamante, Preparation and characterization of mitochondria and sub-mitochondrial particulates of rats liver - derived tissues, *Meth. Cell Biol.* 20 (1978) 411–481.
- [34] L.C. Pereira, A.O. de Souza, M.F. Franco-Bernardes, M. Pazin, M.J. Tasso, P.H. Pereira, D.J. Dorta, A perspective on the potential risks of emerging contaminants to human and environmental health, *Environ. Sci. Pollut. Res. Int.* 22 (2015) 13800–13823.
- [35] L.C. Pereira, A.O. de Souza, G. Meireles, M.M. Franco-Bernardes, M.J. Tasso, V. Bruno, D.J. Dorta, de oliveira D.P. Comparative Study of Genotoxicity Induced by Six Different PBDEs, *Basic Clin. Pharmacol. Toxicol.* 119 (2016) 396–404.
- [36] L.C. Pereira, A.O. Souza, M.J. Tasso, A.M.C. Oliveira, F.V. Duarte, C.M. Palmeira, D.J. Dorta, Exposure to decabromodiphenyl ether (BDE-209) produces mitochondrial dysfunction in rat liver and cell death, *J. Toxicol. Environ. Health* 80 (2017) 1129–1144.
- [37] L.C. Pereira, F.V. Duarte, A.T.I.F. Varela, A.P. Rolo, C.M.M. Palmeira, D.J. Dorta, Exposure to BDE-153 induces autophagy in HepG2 cells, *Toxicol. Vitro* 42 (2017) 61–68.
- [38] B.M. Polster, D.G. Nicholls, S.X. Ge, B.A. Roelofs, Use of potentiometric fluorophores in the measurement of mitochondrial reactive oxygen species, *Methods Enzymol.* 547 (2014) 225–250.
- [39] T. Reistad, E. Mariussen, F. Fonnum, A commercial mixture of the brominated flame retardant pentabrominated diphenyl ether (DE-71) induces respiratory burst in human neutrophil granulocytes in vitro, *Toxicol. Sci.* 87 (2005) 57–65.
- [40] D.A. Roubicek, N.C. Souza-Pinto, Mitochondria and mitochondrial DNA as relevant targets for environmental contaminants, *Toxicology* 391 (2017) 100–108.
- [41] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Keney, M.R. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, *J. Natl. Cancer Inst.* 82 (1990) 1107–1112.
- [42] R.A.J. Smith, R.C. Hartley, H.M. Cochem , M.P. Murphy, S.B. Vafai, V.K. Mootha, Mitochondrial disorders as windows into an ancient organelle, *Nature* 491 (2012) 374–383.
- [43] A.O. Souza, R.S. Ferrari, D. Annuniação, F.F. Sodr , D.J. Dorta, Polybrominated diphenyl ethers (PBDEs), emerging environmental pollutants, are detected on sediment samples of a water recharge point of Guarani Aquifer in Brazil, *Toxicol. Lett.* 259 (2016) S119–S120.
- [44] A.O. Souza, M.J. Tasso, A.M. Oliveira, L.C. Pereira, F.V. Duarte, D.P. Oliveira, C.M. Palmeira, D.J. Dorta, Evaluation of polybrominated diphenyl ether toxicity on HepG2 cells - hexabrominated congener (BDE-154) is less toxic than tetrabrominated congener (BDE-47), *Basic Clin. Pharmacol. Toxicol.* 119 (2016) 485–497.
- [45] P.A. Stapleton, C.E. Nichols, J. Yi, C.R. McBride, V.C. Minarchick, D.L. Shepherd, J.M. Hollander, T.R. Nurkiewicz, Microvascular and mitochondrial dysfunction in the female F1 generation after gestational TiO2 nanoparticle exposure, *Nanotoxicology* 9 (2015) 941–951.
- [46] S.B. Vafai, V.K. Mootha, Mitochondrial disorders as windows into an ancient organelle, *Nature* 15 (2012) 374–383.
- [47] D.C. Wallace, et al., Mitochondrial energetics and therapeutics, *Annu. Rev. Pathol.* 5 (2010) 297–348.
- [48] B. Zhivotovsky, A. Samali, A. Gahm, S. Orrenius, Caspases: their intracellular localization and translocation during apoptosis, *Cell Death Differ.* 6 (1999) 644–651.
- [49] J. Zhuang, H.Q. Yu, T.B. Henry, G.S. Saylor, Fate and toxic effects of environmental stressors: environmental control, *Ecotoxicology* 24 (2015) 2043–2048.