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BDE-99 congener induces cell death by apoptosis of human hepatoblastoma cell line – HepG2

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ABSTRACT

Polybrominated Diphenyl Ethers (PBDEs) are an important class of flame retardants with a wide range of toxic effects on biotic and abiotic systems. The toxic mechanisms of PBDEs are still not completely understood because there are several different congeners with different chemical and biological characteristics. BDE-99 is one of these, widely found in the environment and biological samples, showing evidence of neurotoxic and endocrine disruption activities, but with little information about its action mechanism described in the current literature. This work investigated the effects of BDE-99 on the HepG2 cell line in order to clarify its toxic mechanism, using concentrations of 0.5–25 μM (24 and 48 h). Our results showed that BDE-99 could cause cell death in the higher concentrations, its activity being related to a decrease in mitochondrial membrane potential and an accumulation of ROS. It was also shown that BDE-99 induced the exposure of phosphatidylserine, caspases 3 and 9 activation and DNA fragmentation in HepG2 cells, without causing the release of LDH. Thus it was shown that BDE-99 could cause HepG2 cell death by apoptosis, suggesting its toxicity to the human liver.

1. Introduction

Scientific and technological development brings benefits and advantages to our modern lifestyle. Innovation is currently a necessity due to the great demand for new consumer products, but this also brings serious consequences to the current and future generations due to factors such as air, soil and water pollution as related to the release of several chemicals potentially harmful to the environment and human health.

Amongst these compounds are the brominated flame retardants (BFRs) that represent a class of contaminants widely used in consumer products due to their high efficiency in inhibiting or minimizing the effects caused by fires, and their low cost; representing 25% of the world market of flame retardants (Hardy, 1999). However it has been shown that they persist in the environment and show high bioaccumulation potential, being classified as persistent organic pollutants (POPs).

Polybrominated Diphenyl Ethers (PBDEs) are a class of BFRs used as additives in plastics, textiles, electronic circuits and equipments, building materials and many other consumer goods. They are added during the manufacture of various products in daily use, but no effective chemical bonds occurred during the process which would cause their release into the environment during manipulation or improper disposal (Mcdonald, 2002).

The bioaccumulation potential of PBDEs and their persistence in the environment are due to their lipophilicity, and high levels of these compounds have been detected in samples of animal fats, blood, placenta and breast milk. (Covaci et al., 2009; Hites, 2004; Li et al., 2008; Ma et al., 2012; Shen et al., 2010; Letcher et al., 2010; Toms et al., 2007). The main contamination routes for humans are house dust and contaminated foods (Branchi et al., 2003; Talsness, 2008).

Amongst the effects described as caused by exposure to PBDEs, there is evidence of a neurotoxic potential (Branchi et al., 2003; Madia et al., 2004; Verner et al., 2011) and changes in the endocrine system, by acting on hormone receptors such as estrogen and progesterone, and decreasing the levels of the thyroid hormones (Costa and Giordano, 2007; Costa et al., 2008; Madia et al., 2004; McDonald, 2002; Zhang et al., 2008). They have also being related to the development of liver toxicity and thyroid cancer (Albina et al., 2010; Hu et al., 2007; Zhang et al., 2008), but the mechanisms underlying these effects are still not completely understood.

2,2′,4,4′ Tetrabromodiphenyl ether (BDE-47) and 2,2′,4,4′,5 pentabromodiphenyl ether (BDE-99) are the most commonly found congeners in environmental samples and biological systems, and show high levels of toxicity. *In vitro* investigations have shown

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that some PBDE congeners, such as BDE-47 and BDE-209, present cytotoxic potential in several cell lines such as HepG2 (Madia et al., 2004; Jing et al., 2010; Weihong et al., 2008; Hu et al., 2007, 2009; Yan et al., 2011), however little is known about the initial events that trigger these effects. In addition, there are no data about the effects of BDE-99 on HepG2 cells, a fact that makes it difficult to compare the different congeners. Therefore an investigation of the toxic effects of congeners with different amounts of bromine substituents is required, in order to better understand the mechanism of action of this class of compounds.

Reports have demonstrated that BDE-99 is found mainly in the liver of humans or animals, and is related to the development of hepatoxicity (Albina et al., 2010) which can be due to the original compound or to the metabolites that can be more toxic than the original congener (Gandhi et al., 2011). So, hepatic cell models are important experimental tools to investigate their action mechanism.

HepG2 cells are derived from human hepatoblastoma and are widely used in several *in vitro* assays (Knasmuller et al., 1998). Due to the need for more data about the toxicity of the PBDEs and particularly about the consequences of exposure to BDE-99, this work proposed to investigate its effects on HepG2 cells.

2. Materials and methods

2.1. Cell culture

HepG2 cells (American Type Culture Collection, n° HB8065) were cultured in "Minimum Essential Medium" MEM supplemented with 10% fetal calf serum in an atmosphere containing 5% CO_2 at 37 °C until the cells reached a confluence suitable for starting testing. After this procedure, adequate amounts of cells were plated and incubated for 24 h to ensure good adhesion before initiating the experiments.

2.2. Reagents

Congener BDE-99 was purchased from AccuStandard (New Haven, USA). Sulforhodamine B (SRB), 3 (4,5 dimethylthiazol-2-il)-2,5 Diphenyltetrazolium Bromide (MTT), Dimethyl Sulfoxide (DMSO), Propidium Iodide (PI), tert-Butyl hydroperoxide solution (TBHP), Triton X-100 and bisBenzimide H 33342 trihydrochloride (Hoechst 33342) were purchased from Sigma–Aldrich (EUA). Tetramethylrhodamine Methyl Ester (TMRM), Fetal Bovine Serum (GIBCO), 5,6-Chloromethyl-2′,7′-Dichlorodihydrofluorescein Diacetate, Acetyl Ester (CM-H_2DCFDA) and "Minimum Essential Medium" MEM (GIBCO) were purchased from Invitrogen (USA). Annexin V-FITC was purchased from Proteimax (Brazil) and the Cisplatin Solution (Citoplax®) from Bergamo (Brazil). All other reagents were of the highest commercial degree. The amounts of Dimethyl Sulfoxide (DMSO) required to dissolve the BDE-99 had no effect on the assays. All stock solutions were prepared using glass-distilled deionized water.

2.3. Cell proliferation assay

In order to evaluate the effects of several concentrations of the BDE-99, cell proliferation was assessed using the SRB colorimetric assay according to Skehan et al. (1990).

Briefly, HepG2 cells were cultured to a density of 5×10^4 cells. The cultures were then exposed to BDE-99 at final concentrations ranging from 0.5 to 25 μM. Each sample had at least three replicates and was cultured for 24 and 48 h. The media were then discarded and the cells washed once with phosphate buffer saline (PBS) and twice with distilled water to remove the salts.

After this procedure, the cells were dried at room temperature and subsequently fixed in a 1% methanol in 1% acetic acid solution for 2 h. The fixed cells were stained with a 0.5% SRB in 1% acetic acid solution, and then washed with a 1% acetic acid solution to remove the excess probe. The SRB attached to the cell membranes was extracted using 1 ml of a 10 mM Tris solution, pH 10.0. The absorbance of the dye was then measured at a wavelength of 540 nm in a microplate reader (Varian Cary 50MPR, Varian, USA).

2.4. Cell viability assay (MTT assay)

Cell viability was assessed using a (4,5 dimethylthiazole-2-il)-2,5 diphenyltetrazolium bromide dye, according to Denizot and Lang (1986).

HepG2 cells were seeded with a density of 1×10^5 cells and exposed to BDE-99 at final concentrations ranging from 0.5 to 25 μM. At least three replicates were made for each sample and cultured for 24 and 48 h. The cells were subsequently incubated with a 0.5% MTT (5 mg/mL) solution in an atmosphere containing 5% CO_2 at 37 °C for 3 h. After this period, the medium in the wells was discarded and the formazan crystals formed dissolved in a DMSO solution in 0.2 M glycine buffer, pH 10.2. The final absorbance was evaluated at 570 nm wavelength in a microplate reader (Varian Cary 50MPR, Varian, USA). The results were shown as the percentage difference from the control group.

2.5. Mitochondrial membrane potential

Indications of cell damage can be evaluated by mitochondrial depolarization, since the collapse of the membrane potential compromises the cell energy and consequently damages cell integrity. Mitochondrial depolarization can be measured using the fluorescent dye TMRM, a cation compound permeable to cell membranes, which is rapidly sequestered by the mitochondria of intact cells, and produces a stoichiometric relationship between the fluorescence and the mitochondrial membrane potential (Imberti et al., 1993).

The HepG2 cells were cultured to a density of 1×10^5 cells and then exposed to BDE-99 at final concentrations ranging from 0.5 to 25 μM. Each sample was tested with at least three replicates. The cells were then washed with PBS, trypsinised and incubated with a 6.6 μM TMRM solution at 37 °C for 30 min. The samples were subsequently lysed with a 0.1% Triton X-100 solution (v/v) and the TMRM captured and retained by the mitochondria measured at the excitation and emission wavelengths of 485 and 590 nm, respectively, using a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The results are shown as the percentage of fluorescence in relation to the control group.

2.6. Accumulation of reactive oxygen species (ROS)

The accumulation of ROS can be evaluated using CM-H_2DCFDA, a reactive oxygen species indicator that becomes fluorescent in the presence of intracellular oxidation (Chernyak et al., 2006).

The HepG2 cells were cultured to a density of 1×10^5 cells. After incubation with BDE-99, the cells were further incubated with a 2 mM CM-H_2DCF-DA solution at 37 °C for 1 h. The fluorescence of the CM-H_2DCFDA was subsequently measured using a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) the excitation and emission wavelengths of at 503 and 528 nm, respectively. The results were shown as the difference from the control group. A tert-butyl hydroperoxide (100 μM) solution was used to induce oxidative stress.