Apoptosis induction on human hepatoma cells Hep G2 of decabrominated diphenyl ether (PBDE-209)

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Abstract

Polybrominated diphenyl ethers (PBDEs) are an important class of halogenated organic brominated flame retardants. Because of their presence in abiotic and biotic environments widely and their structural similarity to polychlorinated biphenyls (PCBs), concern has been raised on their possible adverse health effects to humans. This study was designed to determine the anti-proliferative, apoptotic properties of decabrominated diphenyl ether (PBDE-209), using a human hepatoma Hep G2 line as a model system. Hep G2 cells were cultured in the presence of PBDE-209 at various concentrations (1.0–100.0 \mu mol/L) for 72 h and the percentage of cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The results showed that PBDE-209 inhibited the cells viability in time and concentration-dependent characteristics at concentrations (10.0–100.0 \mu mol/L). We found that anti-proliferative effect of PBDE-209 was associated with apoptosis on Hep G2 cells by determinations of morphological changes, cell cycle and apoptosis. Mechanism study showed that PBDE-209 could increase the generation of intracellular reactive oxygen species (ROS) concentration-dependently. Antioxidant N-acetylcycteine partially inhibited the increase of ROS. The mechanism for its hepatoma-inhibitory effects was the induction of cellular apoptosis through ROS generation. In addition, activity of lactate dehydrogenase (LDH) release increased when the cells incubated with PBDE-209 at various concentrations and times. These results suggested that PBDE-209 had the toxicity activity of anti-proliferation and induction of apoptosis in tumor cells in vitro.

Keywords: PBDE-209; Hep G2 cells; Cell viability; Apoptosis; LDH release; Reactive oxygen species

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are one class of halogenated organic brominated flame retardants (BFRs), and have been used industrially in large volumes for flame protection purposes in various commercial products such as electronic equipment and textiles. The commercial PBDE products predominantly consist of so-called penta-, octa- and decabromodiphenyl ether products. Today, decabromodiphenyl ether (DecaBDE) is the largest product on the market and makes up over 80% of the total production of PBDEs, whereas pentabromodiphenyl ether (PentaBDE) and octabromodiphenyl ether (OctaBDE) products constitute about

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12% and 6%, respectively, of the total PBDEs production (De Wit, 2002). Because of their physical, chemical and bio-accumulative characteristics, such as environmental persistence and high lipophilicity, they have become widely distributed in the environment, where they are found to persist for a long time (De Wit, 2002; Watanabe and Sakai, 2003; Alaeae et al., 2003; Alcock et al., 2003). PBDEs have recently been found in indoor and outdoor air (Wilford et al., 2004) and dust samples (Stapleton et al., 2005). Furthermore, time trend studies from several regions have indicated increasing concentrations in the biotic environment, such as gull eggs, wildlife and fish (Norstrom et al., 2000; Law et al., 2003; Sellstrom et al., 2003; Kirkegaard et al., 2004), and in human milk, body tissue, and serum samples (Meironyte et al., 1999; Petreas et al., 2003; Sjödin et al., 2003; Hites, 2004; Noren and Meironyte, 2000; Meneses et al., 1999; Strandman et al., 2000; Ryan and Party, 2000; She et al., 2002; Mazdai et al., 2003). Generally, in most cases of abiotic environments, such as sediment, sewage sludge and air, the dominant congeners are PBDE-209 and PBDE-47. And the most prevalent PBDE congeners in humans and biotic samples are PBDE-47 and PBDE-99. So the widespread uses of PBDEs and increased contamination in the environment have led to the rising concern about the possible adverse health effects to humans.

Although the toxicology of PBDEs is still under investigation, toxicity studies indicate that the liver, thyroid gland and possibly also developing reproductive organs are particular targets of PBDEs toxicity (Darnerud et al., 2001; Kuriyama et al., 2005). Evidence is emerging that PBDEs may be developmental neurotoxins, as behavioural, neurochemical and hormonal deficits have been found following perinatal exposure (Erikkson et al., 2001; Viberg et al., 2002; Zhou et al., 2002; Branchi et al., 2002, 2003). Carcinogenicity studies on PBDE-209, revealing some effects at very high doses, have resulted in an IARC (International Agency for Research on Cancer) classification stating limited evidence for carcinogenicity of PBDE in experimental animals (IARC, 1990). PBDEs are capable to induce cell death of cerebellar granule cells in culture (Reistad et al., 2006). Midia et al. reports PBDE-99 can induce apoptosis in astrocytoma cells assessed by the TUNEL method and by Hoechst 33258 staining, via a p53 dependent mechanism (Midia et al., 2004). Shin et al. reports polychlorinated biphenyls (PCBs) induce apoptotic cell death of human promyelocytic leukemia line (HL-60) with the involvement of PKC activity (Shin et al., 2002).

Human hepatoma cells Hep G2 (American Type Culture Collection, ATCC, Rockville, MD, USA) is a human hepatoblastoma cell line with a wide variety of signal responses to different kinds of drugs (Knowles et al., 1980). Based on increased contamination of PBDEs in the environment, its possible adverse health effects to humans and its structural similarity to PCBs, in this paper, Hep G2 cells are selected as a model system and apoptosis induction and mechanism of a purified single congener PBDE-209 of PBDEs isomers on the cells are investigated.

2. Materials and methods

2.1. Materials

Hep G2 cells were purchased from the Cell Storage Center of Wuhan University (China). DMEM culture medium, heat-inactivated fetal calf serum, 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, propidium iodide (PI) and acridine orange (AO) were purchased from Gibco (USA). 2, 7-Dichlorofluorescin-diacetate (DCFH-DA) and N-acetylcycteine (NAC) were from Sigma (USA). Dimethyl sulfoxide (DMSO) was from Amresco (99.9% purity) (USA). Olympus IX-70 fluorescent inversion microscope was made in Japan. Confocal laser-scanning microscope was from Bio-Rad Company (USA). FACSort flow cytometer and data analysis/analysis software CELLQuest were from Becton Dickinson Company (USA). When not otherwise stated, all chemicals used were of highest available purity obtained from Merck (Germany) or Sigma (USA) or of analytical pure grade from China.

The pure congener PBDE-209 (2,2′,3,3′,4,4′,5,5′,6,6′-decabrominated diphenyl ether) was synthesised (>99.5% purity) and supplied by Dr. J. Heidrich at the labor Dr. Ehrenstorfer-Schafers, Augsburg, Germany. 48.0 mg PBDE-209 was dissolved in 5 mL DMSO to give a stock solution of 10.0 mmol/L, then the solution was placed in an ultrasonic machine for 30 min and was also placed in the ultrasonic machine for 15 min before used. The final culture concentration of DMSO in all experiments was 1.0% (v/v) or less.

2.2. Cell culture and viability assay

Hep G2 Cells were cultured in DMEM medium supplemented with 5% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in an incubator at 37°C and 5% CO2. The cells with medium changed every other day were subcultured at 1:2 every 3 days, and were then plated at an appropriate density according to each experimental scale. Cells were used for experiments within eight passages to ensure cell line stability. Stock of cells were routinely frozen and stored in liquid N2. To study the effect of PBDE-209 on hepatomas, Hep G2 cells were seeded in 24-well culturing plates at a density of 5 × 104 cells/well in 1 mL of culture medium. The cells were divided into three groups as followings: (1) The blank control group; (2) DMSO control group; (3) PBDE-209 group supplemented at final PBDE-209 concentrations of 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 μmol/L, respectively. Each
sample had at least three replicates. Cells were cultured for 24, 48, or 72 h separately before thiazol blue was added to assay the viability by MTT method. Absorbance at 570 nm was read and cell viability (%) was calculated as \( \frac{A_{570 \text{ of drug-treated sample}}}{A_{570 \text{ of control}}} \times 100 \) (Shen et al., 1995).

2.3. Assessment of cell injury

Hep G2 injury was quantitated by measuring the lactate dehydrogenase (LDH) released from lysed cells into the bathing medium, utilizing a commercial available kit (Papadopoulos et al., 1997; Koh and Choi, 1987). Total LDH release corresponding to complete Hep G2 death was determined at the end of each experiment following freezing at \(-70^\circ C\) and rapid thawing. LDH release (%) = \( \frac{\text{LDH activity in media}}{\text{LDH activity in media} + \text{LDH activity in total death cells}} \times 100\% \).

2.4. Morphological observation

Hep G2 cells were seeded in 24-well culturing plates at a density of \( 5 \times 10^4 \) cells/well in 1 mL of culture medium. The cells were divided into three groups as followings: (1) The blank control group; (2) DMSO control group; (3) PBDE-209 group supplemented at final PBDE-209 concentrations of 10.0, 25.0, 50.0 and 100.0 \( \mu \text{mol/L} \), respectively. Each sample had at least three replicates. Cells were cultured for 48 h. Then the mediums were discarded with a pipet and cells were washed twice with phosphate buffer saline (PBS). One milliliter of 5 \( \mu \text{g/mL} \) AO was added for 15 min at room temperature. The AO solutions were discarded before cellular morphology was observed under inverted fluorescence microscopy and recorded by the image system at 400 x magnification.

2.5. Detection of apoptosis

Hep G2 cells were seeded into 6-well culturing plates at a density of \( 1 \times 10^5 \) cells/well. The cells were treated with PBDE-209 at concentrations mentioned above. After culturing, the cells were digested with trypsin and washed with PBS. Then they were fixed with ice-cold 70% ethanol overnight, washed with PBS, and treated with 200 \( \mu \text{L} \) of 50 mg/mL RNase for 30 min at 37\(^\circ \)C. Eight hundred microliters of PI staining solution containing 0.1% Triton X-100 and 100 mg/mL PI was added and left for 1 h before DNA quantification by FACSort flow cytometry. The recorded data were analyzed by a software CELLQuest (Gorczyca et al., 1993; Jing et al., 1994; Hu et al., 2005).

2.6. Assay of reactive oxygen species

The generation of ROS in vivo was detected by using DCFH-DA (Guo et al., 1998; Cathcart et al., 1983). DCFH-DA was cleaved by nonspecific esterases inside cells to form DCFH, a non-fluorescent compound, and it was oxidized to the fluorescent compound DCF by intracellular ROS. After the treatment of PBDE-209, cell cultures were loaded with 10 \( \mu \text{mol/L} \) DCFH-DA in Hank’s balanced saline solution (HBSS) for 20 min at room temperature and observed on an inverted microscope under bright-field optics and scanned once with laser (488 nm excitation and 510 nm emission). The fluorescent images of cells were acquired by using a confocal laser-scanning microscope. The intensity of laser was held constantly to allow quantitative comparisons of relative fluorescence intensity of cells from treated groups. Values of cellular fluorescence were obtained by using software Winview 32.

2.7. Data analysis

Data are expressed as mean ± standard deviation (S.D.) of at least three independent experiments performed in triplicate. One-way analysis of variance (ANOVA), followed by Dunnet’s significant difference test was used to determine statistically significant (*) \( P < 0.05 \); ** \( P < 0.01 \) differences from untreated controls. Two-way ANOVA, followed by Holm–Sidak method was used to determine statistically significance of difference for multiple comparisons between treatments (§ \( P < 0.05 \); level of significance only).

3. Results

3.1. Inhibition of cell growth by PBDE-209

Effects of PBDE-209 on the growth of Hep G2 cells were investigated by MTT method. Hep G2 cells were not affected when exposed to low concentrations of
PBDE-209 (1.0–5.0 μmol/L), and the cells exposed to high concentrations of PBDE-209 (10.0–100.0 μmol/L) revealed that cell proliferation was inhibited in the time and concentration-dependent manner for up to 72 h (Fig. 1).

Morphological changes induced by PBDE-209 were observed PBDE-209-concentration dependently at 10.0, 25.0, 50.0 and 100.0 μmol/L under fluorescence microscopy. After exposure to PBDE-209 for 48 h, Hep G2 cells shrank and retracted from their neighbors, accompanied with floating apoptotic cells in the culture medium (data not shown). By AO staining, cells with condensed chromatin or fragmented nuclei were clearly visualized, especially for those treated with 100.0 μmol/L PBDE-209 (Fig. 2). Meanwhile, the number of survival cells decreased significantly after exposure to 100.0 μmol/L PBDE-209 when it was compared with the control.
3.2. Assessment of cell injury

Hep G2 cells were cultured with a range of concentration of PBDE-209 (10.0–100.0 μmol/L) for up to 72 h, and release of LDH (an indicator of membrane integrity) was measured. Results indicated that increase of LDH release of Hep G2 cells took on both time and concentration characteristic (Fig. 3).

3.3. Cell apoptosis induced by PBDE-209

Apoptotic cells, i.e. sub-G1 cell population, could be separated from normal ones by their lower DNA contents. Since nuclear fragmentation was a hallmark of apoptosis, the intensity of nuclear DNA was detected in Hep G2 cells treated with PBDE-209 for 48 h. Flow cytometric analysis of DNA content showed that PBDE-209 induced cellular apoptosis effectively and dose-dependently (Fig. 4). The apoptotic rates induce by 10.0, 25.0, 50.0 and 100.0 μmol/L PBDE-209 were 18.27%, 23.26%, 32.12% and 53.52%, respectively. In contrast, the apoptotic rate for control cells and DMSO cells were 4.33% and 5.61%, respectively. Table 1 further showed that PBDE-209 resulted in the change of cell cycle. The fraction of cellular periods S was increased markedly while G0/G1 was decreased significantly after PBDE-209 were cultured with cells for 48 h. Effects of PBDE-209 on cell cycle changes were also dose-dependent in the PBDE-209 concentration range of 10.0–100.0 μmol/L.

3.4. ROS generation induced by PBDE-209

DCFH-DA was used to detect the generation of intracellular ROS induced by PBDE-209. A significant increase of ROS was observed 5, 10 and 15 h after the treatment of PBDE-209 (Fig. 5). ROS generation was also increased by PBDE-209 dose-dependently in a PBDE-209 range of 10.0–100.0 μmol/L (Fig. 5). To investigate whether the generation of ROS is a crucial step in PBDE-209-induced apoptosis, 5 mmol/L NAC, an antioxidant, was added to cell culture medium 10 min before the treatment with 10.0–100.0 μmol/L PBDE-209. We previously reported that NAC as an antioxidant could reduce ROS level of cells (Hu et al., 2005). As shown in Fig. 6A, PBDE-209 increased intracellular ROS level, while NAC reduced the ROS increase of PBDE-209 inducement. Accordingly, PBDE-209 remarkably reduced Hep G2 cells viability dose-dependently, whereas NAC recovered the cell viability (Fig. 6B). These results indicated that the viability decrease and cellular apoptosis induced by PBDE-209 were ROS dependent. NAC as an antioxidant could reverse the effect of PBDE-209 by partly inhibiting ROS generation inside cells.

4. Discussion

It is previous shown that PBDE-99 (1.0–100.0 μmol/L) can cause a concentration-dependent inhibition of MTT reduction, and induce apoptosis via a p53 dependent mechanism in human astrocytoma cells (Madia et al., 2004). On the contrary, it is not observed that commercial OctaBDE kill cells, cause an increase in intracellular calcium concentration, and cause an increase or decrease in membrane fluidity in mouse thymocytes (Sandal et al., 2004). It may be explained that the sensitivity of different cell lines exposed to different PBDEs isomers is different.

For DecaBDE, one of three commercial polybrominated diphenyl ether (DecaBDE, PentaBDE and OctaBDE), its toxicology is reported in some literatures. Hardy previously summarizes the toxicology of DecaBDE in a review (Hardy, 2002). The product was not acutely toxic, was not irritating to the skin or eye, and did not induce skin sensitization. No evidence of genotoxic effects was detected in the Ames Salmonella, chromosome aberration, mouse lymphoma, or sister chromatid exchange tests. No cytogenic changes were observed in the bone marrow of rats (parents and
Fig. 4. Flow cytometry analysis of Hep G2 cells treated with PBDE-209. (A) Control cells, (B) DMSO control cells, (C)–(F) cells incubated with 10.0, 25.0, 50.0 and 100.0 μmol/L PBDE-209, respectively, for 48 h. M1 in figures represented a cell apoptotic peak.

offspring) undergoing a one-generation reproduction test. DecaBDE did not adversely affect development or reproduction in rats. DecaBDE’s no-adverse-effect-level (NOAEL) in repeated dose studies was ≥1000 mg/kg body weight. No, equivocal, or some evidence of carcinogenicity, dependent on genus and sex, was found in mice and rats at 2.5% and 5% of the diet administered for 2 years.

| PBDE-209 concentration (μmol/L) | Cell cycle | $G_0/G_1$ | $S$ | $G_2/M$
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<tr>
<td>0 (control)</td>
<td></td>
<td>86.03 ± 1.07</td>
<td>13.97 ± 1.54</td>
<td>0.00</td>
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<tr>
<td>DMSO</td>
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<td>80.21 ± 2.76</td>
<td>19.79 ± 1.21</td>
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<tr>
<td>10.0</td>
<td></td>
<td>75.46 ± 3.22*</td>
<td>24.54 ± 3.54*</td>
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<tr>
<td>25.0</td>
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<td>68.01 ± 2.29**</td>
<td>31.99 ± 3.12**</td>
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<tr>
<td>50.0</td>
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<td>60.91 ± 2.29**</td>
<td>39.09 ± 2.17**</td>
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<td>100.0</td>
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<td>53.47 ± 3.19**</td>
<td>46.53 ± 2.48**</td>
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Note: Significantly different from control, *$P<0.05$ and **$P<0.01$. Statistically significant difference between treatments, §$P<0.05$. 
However, recently, Tseng et al. reports that neonatal exposure to PBDE-209 reduces sperm epididymal sperm mitochondrial membrane potential (MMP), reduces amplitude of the lateral head displacement (ALH) and induces the generation of hydrogen peroxide (H$_2$O$_2$) in the sperm of sexually mature male mice (Tseng et al., 2006). The author deduces that the presence of the relationships between sperm ALH, MMP, and generation of H$_2$O$_2$ indicate toxic action possibly resulting from PBDE-209-induced oxidative stress. Viberg et al. reports that spontaneous behaviour, along with the cholinergic system during its developing stage, can be targets for PBDE-209 in the rat (Viberg et al., 2007). Neonatal oral exposure of male Sprague–Dawley rats, on postnatal day 3, to 6.7, and 20.1 mg PBDE-209/kg body weight, is shown to disrupt normal spontaneous behaviour at 2 months of age. Also, rats exposed to the high dose of PBDE-209 show a different response to adult nicotine treatment, compared to control rats. These results indicate that both lower and higher PBDE-209 can cause similar developmental neurotoxic effects in rats.

So far, no information exits on possible cellular and biochemical mechanism underlying effects of PBDE-209 since the compound widely exits environments, is one of the most prevalent PBDE congeners in environment compartments, and indicates its toxicities in animals.

In recent years, some reports reveal in most cases of abiotic environments, such as sediment, sewage sludge, water and air, PBDE-209 is one of the dominant congeners. For example, the PBDE-209 concentration is 12 µg/L in water (La Guardia et al., 2004), over 100 µg/kg in effluent particulates of water (La Guardia et al., 2004), 248 ± 81 µg/kg in sewage sludge (Christensen et al., 2003), and 0.5–132 µg/kg in sediments (Eljarrat et al., 2004; Sawal et al., 2004). In the paper, the range concentration of 1.0–100.0 µmol/L PBDE-209 was chose to be exposed to Hep G2 cells because the range concentration was also used in some literatures for toxicity activity studies of PBDEs and PCBs (Shin et al., 2002; Madia et al., 2004; Sandal et al., 2004), since the two kinds of compounds have similar structures, though the range concentration is above the reported concentrations of PBDE-209 in abiotic environments.
Apoptosis is a form of self-regulated cell death, which differs from necrosis (Kerr et al., 1972). Apoptosis is necessary for normal embryonic and tissue differentiation (Oppenheim, 1991; Barres et al., 1992). The apoptotic mode of cell death involves an active participation of the affected cell in its self-destruction via activation of a pre-programmed cascade of molecular events that culminate in DNA degradation, nuclear disintegration, and packaging of cell remnants into “apoptotic bodies,” which are then rapidly removed by macrophages. Cell death in tumors, whether spontaneous or treatment-induced, occurs predominantly via apoptosis rather than necrosis (Wyllie et al., 1980; Arend et al., 1990).

Data in the study from cell viability, morphological change, cell cycle and cell apoptosis showed that PBDE-209 (10.0–100.0 μmol/L) inhibited Hep G2 cells proliferation by apoptosis. A common feature of apoptosis is the activation of certain endonucleotidases leading to DNA fragmentation (Barry and Eastman, 1992; Wyllie et al., 1980; Arend et al., 1990). Aliquots of cells were removed from control and PBDE-209 treated Hep G2 cultures at appropriate times and fixed in suspension in 70% ethanol, at −20 °C, overnight. Following fixation, the cells were centrifuged and resuspended in PI staining solution for more than 1 h. This treatment extracts low MW DNA from apoptotic cells and has no effect on the DNA content of nonapoptotic cells (Gorczyca et al., 1992). At the same time, the cellular DNA was stained with PI, and the fluorescence of individual cells was measured quantification by FACSort flow cytometry. The recorded data were analyzed by a software CELLQuest (Gorczyca et al., 1993; Hu et al., 2005). Secondary DNA strand breaks (M1, cell apoptotic peak, Fig. 4), associated with apoptosis could be detected and were dose-dependently in cells treated with different concentrations of PBDE-209 though necrotic cells may happen since the cells came to exposure to high concentrations of PBDE-209.

In order to study the mechanism of cell apoptosis induced by PBDE-209, intracellular ROS was measured and the induction of ROS by PBDE-209 was observed at time and concentration-dependent. Since ROS mediated apoptotic mechanism is well used in many other anticancer reagents and chemopreventive agents against tumor cells (Gong et al., 1999; Jung et al., 2001). Lin et al. reports that PCBs induce concentration and time-dependent increase in cytotoxic response and ROS formation in both human T47D and MDA-MB-231 breast cancer cells (Lin and Lin, 2006). Since PBDEs are structural similarity to PCBs, it is also reasonable to deduce that PBDE-209 exerted its toxicity activity on Hep G2 cells through ROS generation mediated cell apoptosis.

LDH is a stable cytoplasmic enzyme present in cells and is released into the culture medium upon damage of the plasma membrane. Although LDH release in cell culture does not necessarily imply necrosis, plasma membrane damage is one feature of necrotic cells, which could serve among other parameters to determine necrosis. Since under the in vitro cell culture conditions the apoptotic cells cannot undergo rapid phagocytosis as in vivo in the intact tissue, it might also be that LDH release is a feature of late apoptotic cells (Grub et al., 2000). It is reported that the LDH release is increased after MAP (the loach, Misgurnus anguillicaudatus, polysaccharide) stimulation suggests MAP inducing HL-60 cells apoptosis (Zhang and Huang, 2005). Therefore, it is also possible to deduce that the LDH release in our experiments is increased after PBDE-209 stimulation suggests PBDE-209 inducing Hep G2 cells apoptosis, though PBDE-99 does not increase LDH release in other reports (Madia et al., 2004).

In conclusion, PBDE-209 inhibited the proliferation of Hep G2 cells by inducing apoptosis through ROS generation and at the same time LDH release was also be observed, and further research on the molecular mechanisms of PBDE-209 induced apoptosis in Hep G2 cells may be helpful for PBDE-209 toxicity activity in cellular and molecular level. In addition, it will be interestingly investigated whether other cell types, even primary cells are also susceptible to PBDE-209.

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References


