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Induction and Inhibition of Cytochrome P450 1A1, 1B1, and Ethoxyresorufin-O-deethylation activity by Polybrominated Diphenyl Ethers (PBDE) in MCF7 cells

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Introduction

Flame-retardants are added to materials to increase their resistance to ignition and thus make them more fire-safe. Polybrominated diphenylethers (PBDEs) are an important group of flame-retardants. Three different PBDE products, PentaBDE, OctaBDE and DecaBDE are in commercial use. They are used as additive flame-retardants in plastic materials, paints, and textile fabrics. Some PBDEs have been found to be lipophilic, and may consequently bioaccumulate. Recently, levels of some PBDEs, mainly related to the commercial PentaBDE mixture, have been increasing in fish, wildlife, and in human tissue [4].

The structural similarity of certain PBDE congeners to other polyhalogenated aromatic hydrocarbons such as PCBs and PCDFs has raised concerns that these compounds might act as agonists for the aryl hydrocarbon (Ah) receptor, though this aspect of their toxicology is still unclear. If some of these PBDEs were to act as Ah receptor agonists, they would warrant inclusion in the toxic equivalence factor (TEF) concept [5].

The Ah receptor is a steroid hormone-like receptor that is present in almost every vertebrate. The receptor binds effectively dioxin-like compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); its endogenous ligand is unknown. Planar dioxin-like compounds bind with higher affinity than non-planar molecules. The unbound Ah receptor is present in the cytosolic compartment of the cell as a multiprotein complex. Following ligand binding, the ligand-receptor complex migrates to the nucleus of the cell and binds to a nuclear protein Arnt (Ah receptor nuclear translocator). This newly formed complex has a high affinity for certain binding regions on the DNA. After binding to these specific dioxin responsive elements (DRE) an increase in the transcription of various genes occurs, including that of the *CYP1A1* and *1B1* gene [6,7].

CYP1A1 and 1B1 are cytochrome P450 (CYP) enzymes that are involved in phase 1 biotransformation of xenobiotics and endogenous compounds such as estrogens. The CYP enzymes detoxify xenobiotics or bioactivate xenobiotics to reactive intermediates. Although CYP1A1 is expressed in all mammals, there are differences in expression levels among species [8].

To study the possible dioxin-like effects of environmentally most relevant PBDEs (BDE47, 77, 99, 100, 153, 154, 183, 209), the Ah receptor-mediated induction of the CYP enzymes 1A1 and 1B1 were studied in human breast carcinoma (MCF7) cells. CYP 1A1 is the major enzyme that catalyses the deethylation of 7-ethoxyresorufin to resorufin. This ethoxyresorufin-O-deethylation (EROD) activity was used as a marker for CYP1A1 and 1B1 activity [9].

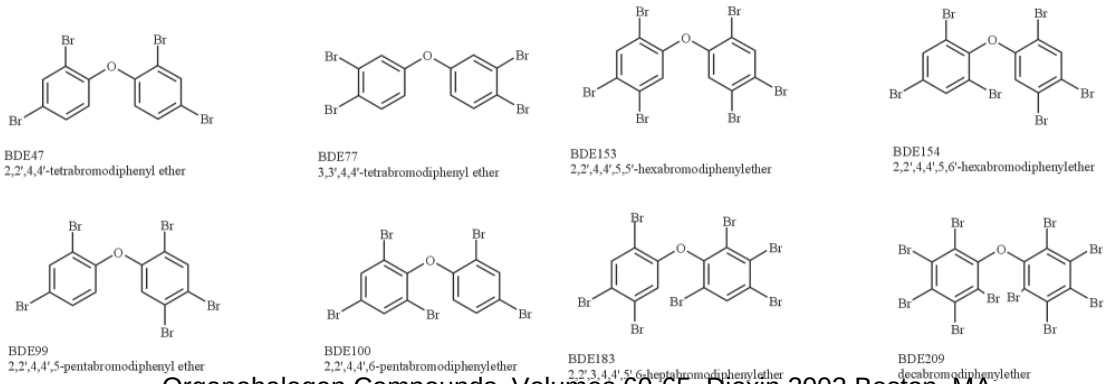

Figure 1. Structure of several environmentally occurring PBDEs

Methods and Materials

Cell culture: The MCF7 cell line was ordered at American Type Culture Collection (ATCC). The cells were cultured in DMEM-MOD supplemented with 5% foetal calf serum, 100U/ml penicillin, 100U/ml streptomycin (all Gibco), and 10 ng/ml insulin (Sigma Chemical Company).
We optimised the well size for the EROD assay in the MCF7 cell line by testing positive controls, TCDD (0.001-2.5 nanoM) and PCB 126 (0.01-1 microM) in different well plates (Greiner). The optimal plate size for the EROD assay proved to be a 24-well plate, $1*10^5$ cells per well (using Trypan Blue in a counting chamber with light microscope).

Cytotoxicity: Cell viability, as an indicator for cytotoxicity, was determined by measuring the capacity of the cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Chemical Company) to formazan [10]. This occurs in the presence of the mitochondrial enzymes succinate dehydrogenase. Damage to this enzyme due to cytotoxicity will result in less formazan production. The cells in each well of a 24-well plate were exposed to MTT (0.5 ml, 1 mg/ml) dissolved in serum-free medium for 30 min at 37°C. After the MTT solution was removed, the cells were washed with phosphate-buffered saline (PBS). The formazan was extracted from the cells by adding 1 ml of isopropanol and incubating for 10 min. The isopropanol was transferred to a cuvette and the formazan concentration was determined spectrophotometrically at an absorbance wavelength of 560 nm.

EROD assay: Ethoxyresorufin-O-deethylation (EROD) activity was used as a marker for CYP1A1 and 1B1 activities, using a modification of the method described by Burke and Mayer (1974) [11]. The cells were exposed for 72h to several concentrations (0.01-25 microM) of PBDEs (Åke Bergman, Sweden) and both controls TCDD (0.001-10 nanoM) and PCB126 (0.001-1 microM), and negative control (DMSO 0.1%). Then, medium was removed and the cells were washed twice with warm PBS. The cells were then exposed to 5 mM $MgCl_2$, 5 microM 7-ethoxyresorufin, and 10 microM dicumarol (all Sigma Chemical Company) in 0.5 ml serum-free medium. The conversion of ethoxyresorufin to resorufin was followed for 10 min using an excitation wavelength of 530 nm and an emission wavelength of 590 nm at 37°C (Fluostar, BMG). The EROD activity was corrected for the amount of protein/ well [12], which allows for a better comparison among assays.

Real time RT-PCR: RNA isolation (RNA Instapure, Eurogentec) was performed after 72 hours exposure of the cells to the positive controls TCDD and PCB126, and several PBDEs with and without co-exposure to TCDD in 24 well plates. The amount of RNA was measured photospectrometrically and checked for DNA impurities on a 3% agarose gel. Primers for CYP1A1 and CYP1B1 were found using Genebank and Primer Express (ABI Prism). Beta-actin was used as an endogenous control.

For quantitative comparison of CYP1A1 mRNA levels, we used real-time PCR technology with an ABI Prism 7700 Sequence Detector. The amount of template cDNA obtained from reverse transcription was expressed by the threshold cycle (C_T) determined by the amplification curve (exponential phase) and by the threshold level of PCR product detection. A comparison of CYP1A1 mRNA levels among the different treatments was made by the comparative C_T method using separate tubes (Applied Biosystems User Bulletin). The larger the C_T value, the lesser the amount of transcript present. The assay was performed using One-step RT-PCR Mastermix (Applied Biosystems).

Statistical analysis: Each concentration was tested in triplicate, all experiments were carried out in duplicate. Statistical differences among treatments were determined by a two-tailed Student t test, with a level of probability of 95% ($p<0.05$). The data are expressed as mean ± standard deviations.

Results and Discussion

Cytotoxicity: There was no inhibition of MTT reduction after exposure of MCF7 cells to the positive controls TCDD and PCB126, or the PBDEs (BDE47, 77, 99, 100, 153, 154, 183, 209). This indicates that the compounds were not cytotoxic at the concentrations tested.

EROD activity: There was a concentration-dependent increase in EROD activity measured after a 72h exposure to the positive controls TCDD and PCB126. The PBDEs (BDE47, 77, 99, 100, 153, 154, 183) did not show any induction of EROD activity (see figure 2). This indicates that BDE47, 77, 99, 100, 153, 154, and 183 were not agonists of the Ah receptor.

However, when the cells were exposed to various concentrations of BDE153 in combination with TCDD for 72h, there was a concentration dependent antagonistic effect on EROD activity (see figure 3). The positive control TCDD caused maximal EROD induction at 1 nM (100% efficacy). In the presence of BDE153 at concentrations of 1 and 10 microM, the efficacy of TCDD to induce EROD activity was reduced in MCF7 cells to 75% and 42% of the maximum. All PBDEs tested (BDE47, 77, 99, 100, 153, 154, 183) proved to have concentration-dependent antagonistic properties in the MCF7

cell line, though not always statistically significant. A similar antagonistic effect was observed for PCB153 [11]. BDE209 could not be tested in the concentration range where other PBDEs showed antagonistic effects, due to its insolubility.

To further assess these possible antagonistic activities, EROD activity was measured in MCF7 cells in which PBDEs were added directly to TCDD induced cells (72h). Direct inhibition of EROD activity was observed which was similar to the effects seen in fig 3.

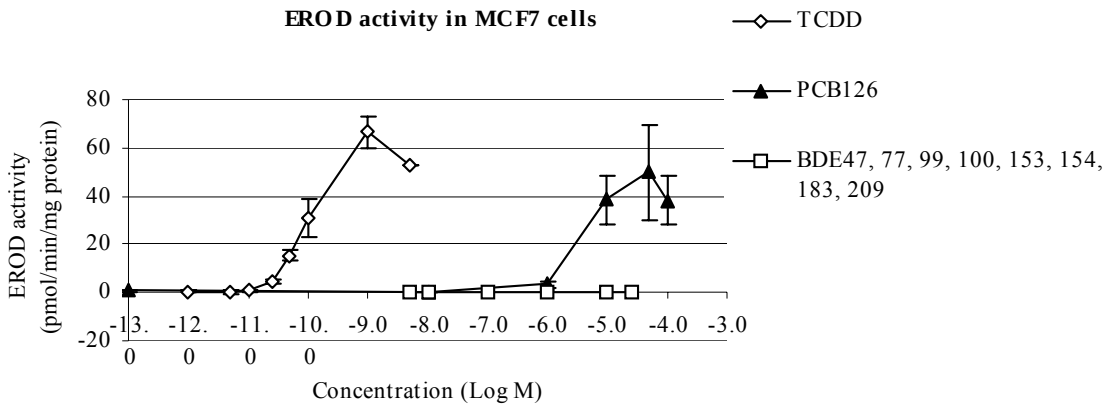

Figure 2. EROD activity in MCF7 cells; exposure to PBDEs, positive and negative controls (72h)

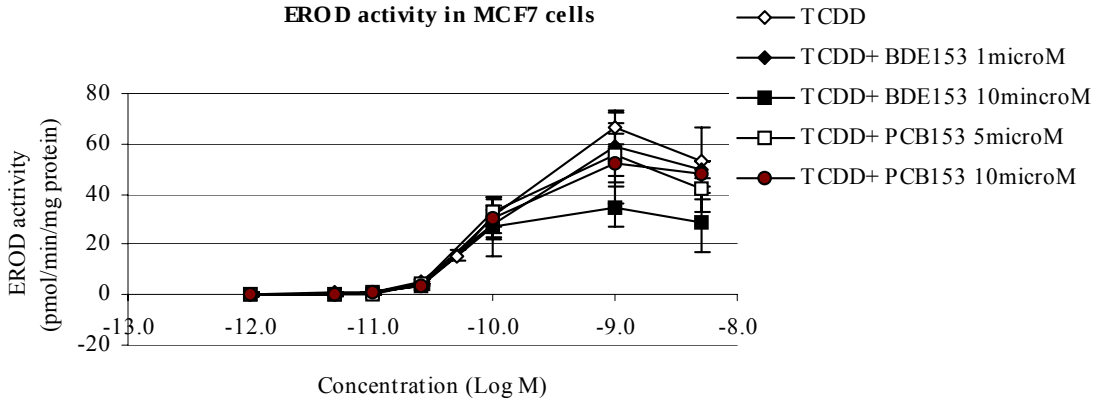

Figure 3. EROD activity in MCF7 cells after exposure of the cells to mixtures of TCDD and individual PBDEs (72h)

Real time RT-PCR: Real time RT-PCR was performed to assess whether the antagonistic effects of the PBDEs were due to catalytic inhibition of CYP1A1 or whether there was direct interaction between the PBDEs and the Ah receptor.

The C_T values were dependent on the input amount of RNA; increased input amount of RNA, resulted in lower the C_T values for CYP1A1, 1B1, and beta-actin. When the MCF7 cells were exposed to higher concentrations of the positive control TCDD, the C_T values for CYP1A1 and 1B1 decreased concentration dependently. However, when the RNA of MCF7 cells exposed to various PBDEs was amplified, there was no statistically significant difference between the RNA from PBDE-exposed cells C_T values and the blanks (vehicle alone, solvent control DMSO 0.1%). There was also no statistically significant difference in the mRNA levels of CYP1A1 and 1B1 from cells exposed to TCDD or cells exposed to mixtures of both TCDD and individual PBDEs. The expression of the endogenous control beta-actin was not affected by any of the treatments.

This concludes that the PBDEs did not induce CYP1A1 or CYP1B1, and thus appeared to have no agonistic effect on the Ah receptor. Moreover, the observed antagonism of EROD activity after co-exposure to mixtures of TCDD (1 nanoM) and PBDEs (1-10 microM) could not be explained by reduced mRNA levels and thus appeared to be due to catalytic inhibition and not by interaction of these compounds at AhR level.

One of the TEF system criteria is that if there is no Ah receptor binding, compounds do not need to be assigned TEF values [5]. The lack of CYP1A1 and 1B1 induction by these PBDEs supports the idea that these compounds do not need to be included in the TEF concept for dioxin-like compounds. After co-exposure of the MCF7 cells to mixtures of individual PBDEs and TCDD, an inhibitory effect on CYP1A1 activity was observed. This inhibition was probably not caused by antagonism of the AhR receptor, but may be due to catalytic inhibition of CYP1A1 and 1B1 by these PBDEs. The lack of AhR antagonism was supported by mRNA analysis. The inhibition of CYP1A1 and 1B1 activity by these PBDEs could compromise the use of this enzyme activity as a biomarker for exposure to dioxin-like compounds. At present this inhibitory effect on CYP1A1 and 1B1 activity is also under investigation in other in vitro and ex vivo cell systems in our laboratory.

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