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Short communication

Combinatory effects of PBDEs and 17β-estradiol on MCF-7 cell proliferation and apoptosis

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Abstract:
In the present work, we analyzed whether polybrominated diphenyl ethers (PBDEs) (47, 99, 100 and 209) interfere with the effect of 17β-estradiol on the proliferation and apoptosis of the MCF-7 cell line. MCF-7 cells were cultured in DMEM without phenol red supplemented with 5% charcoal-treated fetal bovine serum for 3 days with 10 nM 17β-estradiol; with 0.1 μM, 0.5 μM or 1 μM of the tested PBDE congeners; or with both 17β-estradiol and a congener. Cell proliferation was determined by measuring BrdU incorporation, and cell apoptosis was measured by caspase-9 activity.
No PBDE congener had an effect on basal cell proliferation, but they all significantly decreased basal caspase-9 activity. An additive anti-apoptotic activity and ability to induce cell proliferation was observed in the presence of 17β-estradiol.

Key words:
PBDEs, 17β-estradiol, MCF-7 cells, proliferation, apoptosis

Introduction

The low potency of many estrogenic chemicals, known as xenoestrogens, has led to the suggestion that the risks arising from exposure to individual chemicals are negligible. However, there is an increasing amount of data showing that xenoestrogens can interfere with endogenous estrogens. In a study of mixtures of 17β-estradiol and weak xenoestrogens (bisphenol A or *o,p´*-DDT), Rajapakse et al. [12] found that both 17β-estradiol and the xenoestrogens contributed in equal measure to the observed effects when combined in concentrations that produce similar responses. Wang and Kurzer [17] showed that the interaction of phytoestrogens with 17β-estradiol was dose dependent. Low concentrations of genistein and coumestrol significantly enhanced 17β-estradiol-induced DNA synthesis in MCF-7 cells, while high concentrations caused inhibition. In another report, the dose dependent combined effect of the phytoestrogens (genistein, daidzein and coumestrol) and 17β-estradiol on cell proliferation and apoptosis induction in human MCF-7 breast cancer cells was studied [14]. In the presence of phytoestrogens and low concentrations of 17β-estradiol, no additive or antagonistic effects on proliferation were observed; however, the observed increase in cell number was explained by apoptosis inhibition. Hewitt et al. [6] showed that lindane increased the ratio of an anti-apoptotic factor (Bcl-2) to a pro-apoptotic factor (Bax), as did 17β-estradiol; however, a mixture of 17β-estradiol and lindane induced a marked reduction in the ratio of Bcl-2-positive cells to Bax-positive cells due to an apparent increase in the number of Bax-positive

cells, while the percentage of Bcl-2-positive cells remained unchanged. Rajapakse et al. [13] showed that weak xenoestrogens can significantly modulate the effects of 17β-estradiol, even when each compound was present below the concentration at which no effect was observed. The results of our previously published data showed that, among all PCB congeners tested, PCB138 and PCB153 had the highest stimulatory effects on basal cell proliferation and the greatest inhibitory effect on basal caspase-9 activity. In addition, the proliferative and anti-apoptotic effects of PCB138 and PCB153 were still observed in the presence of 17β-estradiol, while the effects of PCB118 and PCB180 were reversed in the presence of 17β-estradiol [11].

The structure of non-planar polybrominated diphenyl ethers (PBDEs) is very similar to that of environmental contaminants, such as non-planar polychlorinated biphenyls (PCBs) [8]. Chemicals with similar structures often have similar effects on an organism. Because a large number of health effects have been associated with non-planar PCB exposure, there is concern that non-planar PBDE exposure may result in similar health effects. Although few studies have investigated whether people exposed to PBDEs are at higher risk for breast cancer, researchers have started to investigate whether PBDEs are estrogen mimics.

In the present study, we compared the effects of several non-planar brominated diphenyl ethers – BDEs (47, 99, 100 and 209) on MCF-7 cell proliferation and apoptosis. In addition, we investigated the contribution of these xenoestrogens to the estrogenic effect of endogenous 17β-estradiol in the breast cancer cell line MCF-7.

Materials and Methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM) without phenol red, fetal bovine serum (FBS, heat inactivated), penicillin, streptomycin and charcoal-dextran were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The compound 17β-estradiol (E2) was obtained from Steraloids, Inc. (Newport, RI, USA). The compounds 2,2',4,4'-tetrabromodiphenyl ether (BDE47), 2,2'4,4',5-pentabromodiphenyl ether (BDE99), 2,2',4,4',6-pentabromo- diphenyl ether (BDE100) and decabromodiphenyl ether (BDE209) were obtained from LGC Promochem (Wesel, Germany).

Cell culture

MCF-7 human breast cancer cells (ATCC, Manassas, VA, USA) were routinely cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 IU/ml of penicillin and 100 μg of streptomycin. Twenty-four hours before the experiments, the medium was removed and replaced with DMEM without phenol red supplemented with 5% dextran-coated, charcoal-treated FBS (5% DC-FBS) to exclude estrogenic effects caused by the medium. Then, cells were plated in the same medium and allowed to attach overnight.

Cell proliferation analysis

DNA synthesis in proliferating cells was determined by measuring BrdU incorporation with the commercial Cell Proliferation ELISA System (Roche Molecular Biochemicals, Mannheim, Germany). The cells were seeded in 96-well culture plates at a density of 2.5 $\times 10^4$ cells/well and then incubated in DMEM supplemented with 5% DC-FBS as a control medium or with three different doses (0.1 μM, 0.5 μM or 1 μM) of PBDE congeners alone or in combination with 10 nM 17β-estradiol for 72 h. After 72 h, the BrdU incorporation assay was performed according to the manufacturer's instructions. Absorbance values were measured at 450 nm using an ELISA reader (ELx808 BIO-TEK Instruments, USA). Culture medium alone was used as a control for nonspecific binding. To estimate the predicted effect of PBDE congeners in co-treatment with 17β-estradiol on cell proliferation, the independent effects of the individual congeners and the effect of 17β-estradiol were added together.

Caspase-9 activity analysis

Caspase assays were performed using the Caspase-9/Mch6/Apaf-3 Colorimetric Assay Kit (BioSource International, Inc. USA). MCF-7 cells were seeded in 48-well culture plates at a density of 13 $\times 10^4$ cell/well and then incubated in DMEM supplemented with 5% DC-FBS as a control medium or with three different doses (0.1 μM, 0.5 μM or 1 μM) of PBDE congeners alone or in combination with 10 nM of 17β-estradiol for 24 h. After 24 h, the cells were pretreated for 3 h

in the presence of staurosporine. The media were then removed, and the cells were lysed with the buffer provided in the kit. The assay was performed according to the manufacturer's instructions. Absorbance values were measured at 405 nm using an ELISA reader (ELx808 BIO-TEK Instruments, USA). Appropriate controls were included as described in the manufacturer's instructions. To estimate the predicted effect of PBDE congeners in co-treatment with 17β-estradiol on caspase-9 activity, the sum of the independent effects of individual congeners and the effect of 17 -estradiol was evaluated.

Statistical analysis

Each treatment was repeated two times (n = 2), and each repetition was run in quadruplicate. The average of four values was used for statistical calculations. Statistical analyses were performed using GraphPad Prism 5. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple range test.

Results

Effect of PBDEs (47, 99, 100 and 209) on MCF-7 cell proliferation

17β-Estradiol alone, at a concentration of 10 nM, significantly increased MCF-7 cell proliferation (25% above control values, $p < 0.05$). An inhibitory effect on cell proliferation was noted only under the influence of the highest doses of BDE99 and BDE100. BDE47 and BDE209, at all doses used, had no effect on cell proliferation (Fig. 1). An increase in cell proliferation was noted after combined treatment with PBDEs and 17β-estradiol (Fig. 1). The predicted effect on cell proliferation was lower than that observed after combined treatment with PBDEs and 17β-estradiol (Tab. 1).

Effect of PBDEs (47, 99, 100 and 209) on MCF-7 caspase-9 activity

All PBDE congeners, at all doses used, decreased caspase-9 activity in a significant manner, (from 31% to 67% *vs.* the control value of 100%) (Fig. 2). Decreases in caspase-9 activity of 2- to 2.5-fold were noted in cells exposed to combined treatments of 17β-estradiol and all doses of all PBDE congeners. The predicted effect on caspase-9 activity was comparable to that observed for the combined treatments (Tab. 1).

Discussion

Our results showed a lack of effect on cell proliferation for all PBDE congeners tested, with the exception of an inhibitory effect observed with the highest doses of BDE99 and BDE100. These results are not in agreement with those of Song et al. [15], who observed an inhibitory effect of the hydroxylated metabolites of PBDE (2OH-BDE47 and 2OH-BDE85) on H295R adrenocortical carcinoma cell proliferation at concentrations of 2–10 μM. Hu et al. [7] observed a dose- and time-dependent inhibitory effect of BDE209 on HepG2 hepatoma cell proliferation, in parallel with an increase in apoptosis. The differences between our observations and those of Hu et al. [7] can be explained by the different cell types used (breast cancer and hepatoma) and, most importantly, the different doses employed. In our study, we used doses from 0.1–1.0 μM, while Hu et al. [7] used doses from 1–100 μM. Hu et al. [7] observed that, at a BDE209 concentration of 1 μM, there were no observed differences in cell viability. Barber et al. [1] showed that low doses (1 pM–1 nM) of BDE47, BDE99 and BDE209 did not influence MCF-7 cell proliferation, confirming our observations. The opposite effect was described by Mercado-Feliciano et al. [10], who showed that the PBDE congener mixture DE-71 (containing BDE47, 99, 100, 153 and 154) caused an increase in MCF-7 cell proliferation. The differences could be due to differences in incubation time. After 10 days of incubation, which was 3 times longer than in our study, CYP enzymes, expressed in MCF-7 cells [2, 16, 18], can metabolize PBDE congeners to yield more active hydroxylated forms.

Co-treatment with 17β-estradiol additionally increased 17β-estradiol-stimulated cell proliferation. This result is not in agreement with those of Mercado-Feliciano et al. [10], who showed that when the PBDE mixture DE-71 was combined with 17β-estradiol, PBDEs reduced the increase in cell proliferation caused by 17 -estradiol alone, suggesting that the

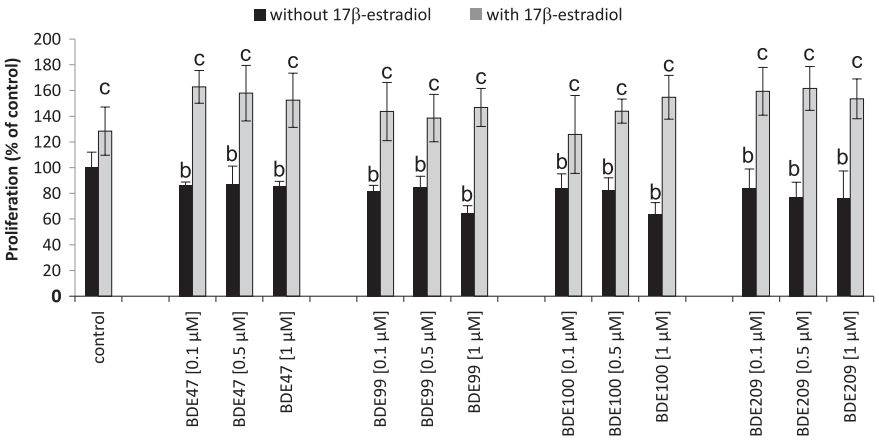

Fig. 1. The effect of PBDE congeners alone or in combination with 10 nM 17β-estradiol on MCF-7 cell proliferation. Each point represents the mean ± SEM of results from two independent experiments, each of which consisted of four replicates per treatment group. Statistically significant differences between points in each graph are indicated with different letters; the same letters indicate no significant difference, with a < b < c

Tab. 1. Effects of PBDE congeners alone or in combination with 10 nM 17β-estradiol (E2) on MCF-7 cell proliferation and caspase-9 activity versus control (set to 0%). Predicted effects were calculated based on the sum of the independent effects of individual congeners and 17β-estradiol

	Proliferation (% of control)			Caspase-9 activity (% of control)		
	PBDEs *vs.* control	PBDEs + E2 *vs.* control	predicted effect *vs.* control	PBDEs *vs.* control	PBDEs + E2 *vs.* control	predicted effect *vs.* control
BDE47 (0.1 μM)	–13.6 ± 2.4	+62.9 ± 12.8	+14.8 ± 2.4	–41.40 ± 4.1	–68.20 ± 3.0	–68.30 ± 4.0
BDE47 (0.5 μM)	–12.8 ± 14.0	+57.9 ± 21.6	+15.6 ± 14.0	–31.50 ± 14.4	–61.50 ± 5.4	–58.40 ± 14.4
BDE47 (1 μM)	–14.8 ± 4.1	+52.4 ± 21.0	+13.6 ± 4.1	–48.70 ± 5.5	–66.90 ± 4.2	–75.60 ± 5.5
BDE99 (0.1 μM)	–18.2 ± 4.4	+43.7 ± 22.6	+10.2 ± 4.4	–42.60 ± 6.1	–57.10 ± 6.0	–69.50 ± 6.1
BDE99 (0.5 μM)	–15.1 ± 8.3	+38.6 ± 18.4	+13.3 ± 8.3	–42.20 ± 19.5	–64.50 ± 6.6	–69.10 ± 19.4
BDE99 (1 μM)	–35.7 ± 6.1	+46.8 ± 14.8	–7.3 ± 6.1	–48.10 ± 10.9	–64.80 ± 6.4	–75.00 ± 10.9
BDE100 (0.1 μM)	–16.2 ± 11.4	+25.8 ± 30.2	+12.2 ± 11.4	–67.30 ± 5.7	–64.90 ± 4.7	–94.20 ± 5.7
BDE100 (0.5 μM)	–17.9 ± 10.0	+44.0 ± 9.3	+10.5 ± 10.0	–50.80 ± 9.8	–68.10 ± 5.8	–77.70 ± 9.8
BDE100 (1 μM)	–36.3 ± 9.0	+54.7 ± 17.0	–7.9 ± 9.0	–45.40 ± 12.2	–65.40 ± 7.7	–72.30 ± 12.2
BDE209 (0.1 μM)	–15.8 ± 14.8	+59.4 ± 18.6	+12.6 ± 14.8	–54.10 ± 5.5	–67.60 ± 3.7	–81.00 ± 5.5
BDE209 (0.5 μM)	–22.8 ± 11.5	+61.7 ± 17.0	+5.6 ± 11.5	–48.50 ± 10.4	–53.60 ± 8.7	–75.40 ± 10.4
BDE209 (1 μM)	–23.9 ± 21.4	+53.5 ± 15.4	+4.5 ± 21.4	–41.80 ± 20.9	–70.60 ± 10.9	–68.70 ± 20.9

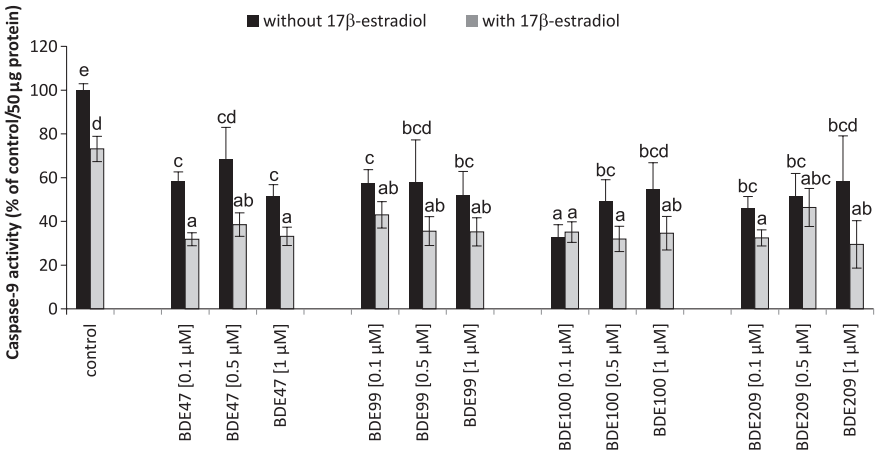

Fig. 2. The effect of PBDE congeners alone or in combination with 10 nM 17β-estradiol on caspase-9 activity. Each point represents the mean ± SEM of results from two independent experiments, each of which consisted of four replicates per treatment group. Statistically significant differences between points in each graph are indicated with different letters; the same letters indicating no significant difference, with a < b < c

PBDEs had an antagonistic effect on 17β-estradiol--induced cell proliferation. This reduction was dose dependent, and the highest dose caused the largest decrease in cell proliferation. The discrepancy between our results and those of Mercado-Feliciano et al. [10] could be explained by the fact that, instead of using single congeners, they used a mixture; in addition, they used higher doses than those used in our experiment.

All PBDE congeners used in this study decreased caspase-9 activity in a statistically significant manner. The predicted effect on caspase-9 activity (calculated based on the sum of the independent effects of individual congeners and 17β-estradiol) was smaller than that observed with combined treatment of single congeners and 17β-estradiol, suggesting an additive effect. To our knowledge, no studies have shown that PBDE congeners possess anti-apoptotic activity in any cell type. Some studies have shown that PBDEs cause apoptosis in different cells [5, 7, 9], but in all of these studies, the concentrations used were higher than 10 μM. The concentrations used in this study are equal to the concentrations of PBDEs isolated from liver oil waste products from burbot in Mjosa, Norway, where PBDEs were major contaminants [3]. Barber et al. [1] observed that PBDE concentrations in human blood range from 1 pM–1 nM, while the concentrations used by other studies that resulted in an increase in apoptosis were in the range of 10–100 μM; these concentrations are described as cytotoxic [15].

Co-treatment with 17β-estradiol did not change 17β-estradiol-stimulated cell proliferation but caused an additional decrease in 17β-estradiol-induced apoptosis. The decrease in apoptosis can be explained by the inhibition of 17β-estradiol sulfation. As described previously, brominated flame retardants (e.g., some PBDE congeners) can inhibit sulfotransferase activity [4] and can disrupt the effect of 17β-estradiol on cells.

Taken together, our data show for the first time that (1) none of PBDE congeners used alone have an effect on basal cell proliferation, but co-treatment with 17β-estradiol causes an additional increase in cell proliferation, and (2) all PBDE congeners cause a significant decrease in basal caspase-9 activity and, moreover, show an additive effect with 17β-estradiol with regard to caspase-9 activity.

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