Cytotoxicity and gene expression profiling of two hydroxylated polybrominated diphenyl ethers in human H295R adrenocortical carcinoma cells

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Abbreviations: AHR, aryl hydrocarbon receptor; ERAD, ER-associated degradation; ER, endoplasmic reticulum; PBDE, Polybrominated diphenyl ether; PCB, polychlorinated biphenyl; TCDD, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin; UPR, unfolded protein response.

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Abstract

Polybrominated diphenyl ethers (PBDEs) are commonly used as flame retardants in a variety of commercial and household products. They have been detected in the environment and accumulate in mammalian tissues and fluids. PBDE toxicity is thought to be associated with endocrine disruption developmental neurotoxicity and changes in fetal development. Although humans are exposed to PBDEs, our knowledge of the effects of PBDE metabolites on human cells with respect to health risk is insufficient. Two hydroxylated PBDEs (OH-PBDEs), 2-OH-BDE47 and 2-OH-BDE85, were investigated for their effects on cell viability/proliferation, DNA damage, cell cycle distribution and gene expression profiling in H295R adrenocortical carcinoma cells. We show that the two agents are cytotoxic in a dose-dependent manner only at micromolar concentrations, with 2-OH-BDE85 being more toxic than 2-OH-BDE47. However, no DNA damage was observed for either chemical, suggesting that the biological effects of OH-PBDEs occur primarily via non-genotoxic routes.

Furthermore, no evidence of aryl hydrocarbon receptor (AHR)-mediated, dioxin-like toxicity was observed. Instead, we report that a micromolar concentration of OH-PBDEs induces transcriptional changes associated with endoplasmic reticulum stress and the unfolded protein response. We discuss whether OH-PBDE bioaccumulation could result in impairment of the adrenocortical secretory function.

Keywords: OH-PBDE; H295R adrenocortical carcinoma cells; toxicity; endoplasmic reticulum stress; unfolded protein response.

1. Introduction

Polybrominated biphenyl ethers (PBDEs) are synthetic compounds used as flame retardants in a variety of construction materials, textiles, and polymers for electronic equipment. PBDEs are hydrophobic, stable and persist in the environment (Hites, 2004). PBDEs and their metabolites, hydroxylated PBDEs (OH-PBDEs), have been found in blood, adipose tissues and liver of fish, birds, and mammals (Teuten et al., 2005; Wolkers et al., 2004), as well as in human blood, serum, adipose tissue, breast milk, placental tissue and the brain (Siddiqi et al., 2003; Athanasiadou et al., 2008; Domingo et al. 2008). Importantly, concentrations of PBDEs in breast milk (Hooper and McDonald, 2000; Kodavanti and Derr-Yellin, 2002; Hites, 2004) or blood (Schechter et al., 2005) increased exponentially in the last decades. Although in some cases human exposure has decreased in recent years (e.g. Lind et al. 2003; Domingo et al. 2008; Polder et al. 2008), PBDEs still present a potential risk to both the environment and to public health.

PBDEs have a similar chemical structure to other environmental, persistent, organic pollutants such as polychlorinated biphenyls (PCBs) and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). Exposure to PCBs is associated with adverse effects on human health. PCBs are complete carcinogens in rodents and display genotoxic activity *in vitro* and *in vivo* (Safe, 1984; Silberhorn et al., 1990). PBDEs and their metabolites are thought to be toxic to humans, but little is known about their mechanisms of toxicology. PBDEs cause developmental neurotoxicity in rodents (Viberg et al., 2003), which could be related to the high content of PBDEs in the brain (Naert et al., 2007) and in breast milk, from where it is transferred to nursing infants who are in the early stages of brain development (Coburn et al., 2007). There is increasing concern about the potential endocrine-disrupting properties of brominated flame retardants and their derivatives, which could affect steroidogenic enzymes responsible for the biosynthesis of various steroid hormones, and eventually cause reproductive problems, cancer and other pathological conditions related to growth and development. In this respect, some

PBDEs were determined to have endocrine disrupting effects *in vitro* (Meerts et al., 2001) and *in vivo* (Bonefeld-Jorgensen et al., 2006; Hardell et al., 2006). OH-PBDE congeners have structural similarities with thyroid hormones and bind human thyroid hormone receptors and serum thyroid hormone binding proteins (Siddiqi et al., 2003). OH-PBDEs also affect the activity of two key enzymes in the production of estrogens and androgens, aromatase (CYP19) and CYP17, in H295R adrenocortical carcinoma cells at concentrations in the low micromolar range (between 0.5 and 10 μ M) (Canton et al. 2005, 2006; He et al. 2008a). Moreover, certain OH-PBDEs are able to bind to cytosolic aryl hydrocarbon receptors (AHR), becoming AHR agonists or antagonists (Chen and Bunce, 2003).

Although human tissues contain measurable levels of PBDEs, little is known about the toxicological effects of PBDE metabolites in humans (Darnerud et al., 2005). Adrenal glands, which have massive blood flow and high lipid content, represent a major accumulation site for dioxins, PCBs and PBDEs in the body. Recent work has shown that PBDEs and their derivatives affect adrenal steroidogenesis in H295R adrenocortical carcinoma cells (Canton et al. 2005, 2006; He et al. 2008a), but information on their toxicities and mechanisms of action is limited. In this work, we evaluated the effects of two environmental PBDE metabolites (2-OH-BDE47 and 2-OH-BDE85) on DNA damage, cell proliferation, cell cycle distribution and gene expression profiling in H295R adrenocortical carcinoma cells. BDE47 is one of the most widespread and persistent PBDE congeners in the environment (Law et al. 2003). In vertebrates, it undergoes phase I metabolism leading to the formation of OH-BDE47 (Qiu et al. 2007). BDE85, on the other hand, has a similar structure to TCDD and affinity for the AHR (Chen et al. 2001).

2. Materials and Methods

2.1 Chemicals and Reagents

2-OH-BDE47 and 2-OH-BDE85 were synthesized and provided by Dr. Michael H.W. Lam, Department of Biology and Chemistry, City University of Hong Kong, Hong Kong. All other chemicals, tissue culture media and reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

2.2 Cell culture and Chemicals Exposure

The H295R adrenocortical carcinoma cell line was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with Ham's nutrient mixture F-12 with 1 % Insulin Transferrin Selenium (ITS) Premix and 2.5 % Nu-Serum at 37 °C in a 5 % CO₂ atmosphere. Cells were exposed to chemicals dissolved in 0.1 %

dimethyl sulfoxide (DMSO) (final concentration) for different incubation periods. Control cells were incubated with the vehicle (DMSO) only.

2.3 Single Cell Gel Electrophoresis (Comet assay)

DNA damage was measured with the alkaline comet assay, which is a straightforward and sensitive method for measuring a variety of DNA lesions in individual cells (Singh et al., 1988), and expressed as the percentage of tail DNA, as described (Duarte et al., 2007).

2.4 Cell proliferation assay

Cell proliferation was studied by using the CyQUANT NF cell proliferation assay (Invitrogen, California, USA), which measures cellular DNA content via fluorescent dye binding, according to the manufacturers' instructions. Briefly, 10,000 cells in 100 μ L were seeded in a 96-well plate and left to attach for 24 h. Cells were then treated with different concentrations of chemicals in 100 μ L of medium for 24 h. The agent-containing medium was removed and 100 μ L of dye binding solution directly added and incubated for 60 min. Fluorescence was recorded using a FLUOstar OPTIMA fluorescence microplate reader (Offenburg, Germany) with excitation at 480 nm and emission detection at 520 nm.

2.5 Cell Cycle Analysis

Cells were plated in 6-well plates at 100,000 cells per well. After 24 h, cells were treated with test agents or vehicle (control) for up to 72 h. Cells were harvested at each incubation period of interest, fixed in 70% ethanol and subsequently resuspended in PBS containing 0.1 mg/ml RNase A and 5 μ g/ml PI, prior to analysis. Cellular DNA content was analysed using a Becton Dickinson FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA), plotting 10,000 events per sample. Cell cycle distribution data analysis was subsequently performed using ModFit LT software (Becton Dickinson, Cowley, UK).

2.6 Statistical Analysis

For the above Comet, cell proliferation and cell cycle assays, statistical analysis was performed with SPSS14 (SPSS Inc., Chicago, Illinois, USA). Statistically significant differences from control were calculated by a one-way ANOVA followed by the post hoc Tukey's test.

2.7 Microarray Analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, UK) and 5 µg were reverse transcribed using the One-cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, California, USA). Biotin-labeled cRNA was synthesised by *in vitro* transcription with the Affymetrix IVT labeling kit. Fragmented, biotin-labeled cRNAs (10 µg) were hybridised to GeneChip Affymetrix Human U133 Plus 2.0 arrays and these were washed and stained according to Affymetrix EukGE-WS2v5 Gene Chip protocol. Probe arrays were scanned at 570 nm using an Affymetrix GeneChip scanner and the fluorescence intensity of the scanned image registered in CEL intensity files. The data were deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE8588. DNA-Chip Analyser (dChip) software (Li and Wong, 2001) was used to normalise the fluorescence intensity over multiple arrays, to calculate model-based expression values from the differences between perfect match and mismatch probes in the cell intensity files, and to filter a set of 2-OH-BDE47- and 2-OH-BDE85-responsive genes for which the expression levels were significantly altered in three independent experiments (paired t-test, $P < 0.05$), with an average fold change ≥ 3 or ≤ -3 when comparing with the respective control. In addition, the software was employed to determine the probability of having a particular number of genes of a certain Gene Ontology (GO) biological process in the generated list of filtered genes, using hypergeometric distribution; GO enrichment was assumed at $P < 0.001$.

2.8 Real Time RT-PCR

Real-time reverse transcription-PCR (RT-PCR) was performed as described (Duarte and Jones, 2007), with the following modifications. The reaction mix consisted of Brilliant SYBRGreen QPCR Master Mix (Stratagene), 2.5 mM MgCl₂, 150 nM each primer and 1 µL of cDNA. Primer sequences are shown in Table 1. The amplification protocol consisted of a denaturation step at 95 °C for 10 min and 40 cycles of 95 °C for 30 sec, 57 °C for 1 min and 72 °C for 1 min.

3. Results and Discussion

3.1 Cytotoxicity of OH-PBDEs

The effects of the two OH-PBDEs on H295R cell proliferation were evaluated after 24-h exposure by comparison with untreated, control cells (Figure 1). At concentrations below 10 µM, both OH-PBDEs inhibited H295R cell proliferation, with only 2-OH-BDE85 causing a slight reduction in cell number when compared to the number of cells at 0 h. From 10 µM onwards both compounds induced cell death in a concentration-dependent manner, with 2-

OH-BDE85 clearly being the more cytotoxic of the two.

3.2 Cell cycle effects of OH-PBDEs

Flow cytometric analysis of cellular DNA content was subsequently performed to determine whether the cytotoxic effects of the two OH-PBDEs were accompanied by cell cycle arrest at specific checkpoints. Cell cycle distribution changes were monitored in H295R cells following exposure to 10 μ M 2-OH-BDE47 or 2-OH-BDE85 over 24 to 72 h. In the controls, the percentage of cells in the G₀/G₁, S and G₂/M phases of the cell cycle was 60-70 %, 20 % and 10-20 %, respectively. Notably, both compounds (10 μ M) caused a significant decrease in the percentage of G₂/M cells and concomitant increase in G₀/G₁ cells at 24 h of exposure (Figure 2); however, there was no significant difference noted between the exposed and control cells at 48 and 72 h.

3.3 Genotoxicity of OH-PBDEs

Barber et al. (2006) reported increased chromosomal damage in the micronucleus test in MCF7 cells treated with low-dose PBDEs. Recent reports showed that incubation of human neuroblastoma cells with the parental compound BDE-47 induces genotoxicity and apoptotic cell death in a dose-dependent manner (He et al. 2008b), through the formation of reactive oxygen species (Gao et al. 2008). To investigate whether the anti-proliferative effects of OH-PBDEs were produced via a genotoxic route, we employed the alkaline comet assay. We exposed H295R cells to different concentrations of 2-OH-BDE47 or 2-OH-BDE85 for 12 h. In the assay, DNA lesions including single strand breaks, double strand breaks and alkali labile sites are detected as an increase in the percentage of DNA in the 'tail' of the comets. However, we found no prominent tail formation in the treated samples, which was no different from the control (Figure 3A). Even at a cytotoxic OH-PBDE concentration (20 μ M) there was no significant formation of DNA damage compared to control ($P > 0.05$; ANOVA). We could not exclude, however, the possibility that cells repaired any DNA damage formed during the incubation period. Therefore, in a subsequent experiment we compared the DNA damage accumulation in the presence or absence of two DNA repair inhibitors, hydroxyurea and cytosine arabinoside, which inhibit DNA synthesis during excision repair (Martin et al., 1999). Figure 3B shows the accumulation of DNA damage when H295R cells were incubated with 30 μ M hydrogen peroxide (positive control) or 10 μ M 2-OH-BDE85 over a 24 h time period. When cells were incubated with 10 μ M 2-OH-BDE85 in the presence of the DNA repair inhibitors the slight increase in tail DNA observed at 24 h was not greater than that observed in control cells. Similar results were obtained when cells were incubated with 10 μ M 2-OH-BDE47 (Supplementary figure 1). Importantly, this modified version of the assay also allows the detection of DNA strand breaks and alkali labile sites generated by the

excision repair of most DNA lesions. Hence our data suggest that the toxicity of OH-PBDEs in H295R cells cannot be explained by a genotoxic mechanism.

3.4 Gene expression profiling

To investigate the toxicological action of the two OH-PBDEs, we incubated H295R cells with 10 μ M 2-OH-BDE47 or 2-OH-BDE85 for 24 h, a treatment that severely inhibited cell proliferation (Figure 1), and examined changes in global mRNA profiles using Affymetrix high-density oligonucleotide arrays. Experiments were performed on three separate occasions and, in each experiment, control cells were incubated with vehicle (DMSO) for the same period of time. The HG-U133 Plus 2.0 arrays contain probes for over 47,000 transcripts and variants. A total of 430 annotated genes and 152 genes or expressed sequence tags with unknown function had their expression significantly ($P < 0.05$) altered by at least 3-fold after exposure to either 2-OH-BDE47 or 2-OH-BDE85 (Supplementary data table 1). Despite the higher number of significant changes induced by 2-OH-BDE85 (312 annotated and 104 unknown genes vs. 208 annotated and 67 unknown genes for 2-OH-BDE47), it was apparent from the two-way hierarchical clustering analysis of the filtered genes and samples according to their patterns of gene expression that both compounds induced similar expression profiles in H295R cells (Figure 4). The gene expression changes induced by both compounds could be classified into five significantly enriched Gene Ontology biological processes: response to unfolded protein, apoptosis, cell cycle, transcription and organic acid metabolism (Table 2).

Given the analogous chemical structures, it has been anticipated that PBDEs could have similar toxicological properties to TCDD, whose toxic effects are mostly due to alterations in gene expression mediated by the transcription factor AHR. In this study, whilst we observed the induction of 3 dioxin-responsive genes (AHR, CYP1B1 and TIPARP), we did not observe changes in the expression of many well established AHR-responsive genes like CYP1A1, CYP1A2, NAD(P)H dehydrogenase, quinone 1, UGT1A1/6, aldehyde dehydrogenase family 3, member A1 or glutathione S-transferase Ya (Fletcher et al., 2005). Our results with 2-OH-BDE47 and 2-OH-BDE85 agree with previous observations that both parental compounds (BDE47 and BDE85) have a negligible effect on AHR binding to the dioxin response element and on CYP1A1 and CYP1B1 expression or activity (Chen and Bunce, 2003; Peters et al., 2004; Sanders et al., 2005).

On the other hand, we show that OH-PBDE-mediated cytotoxicity in H295R cells is accompanied by the induction of genes involved in endoplasmic reticulum (ER) stress response, including those that mediate correct protein folding, cell cycle arrest and cell death/apoptosis. Adrenocortical cells express several pathways of secretory protein

biosynthesis and the ER is the cellular compartment where these proteins are synthesised and correctly folded, with the assistance of molecular chaperones and folding enzymes. Unfolded/mis-folded proteins are retained in the ER, translocated back to the cytoplasm by the ER-associated degradation (ERAD) machinery, and degraded by the ubiquitin-proteasome system. The excessive accumulation of unfolded proteins into the ER, however, causes ER stress and is associated with several conformational diseases. To alleviate ER stress, mammalian cells activate the unfolded protein response (UPR), which inhibits translation to prevent further accumulation of unfolded proteins, and induces the transcription of ER chaperone and ERAD component genes. In some cases, apoptotic cell death is induced to safely dispose of injured cells (Pahl, 1999; Yoshida, 2007).

OH-PBDEs induced key regulators of ER stress, such as a well-known ER chaperone that belongs to the heat shock protein 70 family, HSPA5 (also known as BiP or GRP78), and an ER transmembrane protein that senses the accumulation of unfolded proteins, EIF2AK3/PERK. PERK induces ATF4-mediated dramatic increases in the expression of the pro-apoptotic transcription factor DDIT3/CHOP and of proteins involved in amino-acid metabolism such as asparagine synthetase and amino acid transporters (Ron and Walter, 2007; Yoshida, 2007). In the current study, we observed the modulation of metabolic genes such as glutamate decarboxylase 1, methylenetetrahydrofolate dehydrogenase, phosphoenolpyruvate carboxykinase 2 and six aminoacyl tRNA synthetases, all of which were previously shown to be regulated during ER stress in human cells (Murray et al., 2004). In addition to CHOP (14-fold), both OH-PBDEs dramatically increased the expression of a number of stress-responsive, pro-apoptotic and cell cycle arrest genes like protein phosphatase GADD34 (6- to 10-fold), GADD45a (about 9-fold) and TRIB3 (10-fold), which are also induced during ER stress via the PERK pathway (Ohoka et al., 2005). The induction of these and other genes involved in apoptosis/cell death (e.g. BCL10, PDCD5) was generally more significant for 2-OH-BDE85, which may explain the higher cytotoxicity of this compound (Figure 1).

2-OH-BDE85 also modulated a greater number of transcription factors and regulators. The highest induction corresponded to a stress response gene, ATF3 (12- to 18-fold). Also highly up-regulated by both compounds was XBP1 (about 7-fold), a transcription factor that is essential to the function of cells that produce large amounts of secretory proteins and another important mediator of the ER stress response. In agreement with our results, XBP1 is transcriptionally activated during ER stress and it induces the expression of several ER chaperones and ERAD component genes (Ron and Walter, 2007; Yoshida, 2007). This may explain the up-regulation of two members of the heat shock protein family that act as molecular chaperones to restore correct protein folding during ER stress (DNAJB9, DNAJC3)

and a member of the ERAD machinery (HERPUD1) in our study. We have highlighted known ER stress-responsive genes in Table 2 (in bold).

3.5 Validation of gene expression changes

Microarray data were validated by real-time quantitative RT-PCR. We chose nine genes that were up-regulated in the microarray experiment because of their involvement in ER stress response (ATF3, DDIT3, GADD45A, HSPA5, TRIB3) or in response to dioxin (CIP1B1, TIPARP). In addition, we analysed two genes that were down-regulated in the microarray experiment, which are known to play important roles in the normal function of adrenocortical carcinoma cells (ADRA2A, SREBF1). We monitored the expression of these genes in H295R cells incubated with a range of concentrations of 2-OH-BDE47 or 2-OH-BDE85 (0.01, 0.1, 1 and 10 μ M). At 10 μ M, there was a good agreement between gene regulation in the Affymetrix GeneChip analysis and in the real-time RT-PCR (Figure 5). Fold changes of expression derived from both techniques were generally similar, with the exception of DDIT, ATF3 and TRIB3, for which the RT-PCR fold change was much higher than that in the microarray experiments. This is likely to be due to the higher sensitivity of the RT-PCR. No expression changes were detected, however, when H295R cells were incubated with lower concentrations of 2-OH-BDE47 or 2-OH-BDE85 (0.01-1 μ M) (supplementary data, Figure 2). Further studies are thus warranted not only to identify the exact mechanism by which OH-PBDEs induce ER stress, but also to determine whether the UPR can be elicited *in vivo* as a consequence of OH-PBDE bioaccumulation. Estimates based on the accumulation of certain OH-PBDEs, like OH-BDE47, in human serum samples (ranging between 0.5 and 6 ng g⁻¹ lipid weight; Athanasiadou et al. 2008) indicate that the serum concentration is within the picomolar range, which is several orders of magnitude lower than the medium concentration of OH-BDEs that elicited toxicity to H295R cells in this study. However, more information is required on the concentration of these compounds in human blood before the possible *in vivo* relevance of this toxic effect can be accurately determined.

Recently, Van Boxtel et al. (2008) performed Affymetrix microarray analysis in embryonic fibroblasts from Zebrafish exposed to another BDE47 metabolite, 6-OH-BDE47. The main result was a mild altered expression of two functional groups of genes involved in proton transport and carbohydrate metabolism, which agreed with their proposed mechanism of toxicity for 6-OH-BDE47 in Zebrafish involving the uncoupling of oxidative phosphorylation. However, it is difficult to compare their results with the current ones, since the authors used a different model organism, a different PBDE metabolite and a lower concentration (1 μ M) of the agent.

3.6 Summary

In summary, the two OH-PBDE congeners tested in the current study were not toxic to adrenocortical cells at $< 1 \mu\text{M}$. However, both OH-PBDEs were able to inhibit the proliferation of H295R adrenocortical carcinoma cells in a dose-dependent manner in the micromolar range. This was accompanied by a significant cell cycle arrest at G_0/G_1 . Despite their cytotoxicity, we did not observe the formation of DNA lesions or any evidence of excision repair activity. Instead, our results suggest that ER stress is implicated in the OH-PBDE-mediated adrenocortical toxicity. In particular, OH-PBDEs ($10 \mu\text{M}$) activate the molecular signature of one of the arms of UPR, namely the PERK pathway. We therefore propose that the bioaccumulation of OH-PBDEs may cause disruption of the adrenocortical secretory pathways via ER stress. Whilst ER stress explains the cytotoxicity of these compounds *in vitro*, more work is required to establish whether this could result in impairment of the adrenocortical gland *in vivo*.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Figure 1. H295R adrenocortical carcinoma cell proliferation. Cells were incubated with different concentrations of 2-OH-BDE47 or 2-OH-BDE85 for 24 h. Viable cell numbers were determined as described in Materials and Methods and expressed as a percentage of control. Data are expressed as mean $\pm$ SD of three independent experiments. The dashed line represents cell number at 0 h treatment.

Figure 2. H295R adrenocortical carcinoma cell cycle distribution. Cells were incubated with the two OH-BDEs at 10 μ M for 24, 48 or 72 h. Control cells were incubated with vehicle alone. The percentage of cells in different phases of the cell cycle is expressed as the mean $\pm$ SD of three independent experiments. * $p < 0.05$.

Figure 3. DNA damage formation in H295R adrenocortical carcinoma cells. A, Cells were incubated with increasing concentrations of 2-OH-BDE47 or 2-OH-BDE85 for 12 h and DNA damage was measured with the alkaline comet assay. Control cells were only given the vehicle. The percentage of DNA in the tail is expressed as the mean $\pm$ SEM of 100 cells measured (2 x 50 cells) in a single experiment, representative of 3 independent experiments. B, Cells were incubated with either 30 μ M hydrogen peroxide (P), 10 μ M 2-OH-BDE85 or vehicle (C) in the presence (+) or absence of the DNA repair inhibitors hydroxyurea (10 mM) and cytosine arabinoside (1.8 mM). DNA damage was measured with the alkaline comet assay. The percentage of tail DNA is expressed as the mean $\pm$ SD of three independent experiments.

Figure 4. Hierarchical clustering diagram of OH-BDE-induced expression changes. H295R cells were incubated with 10 μ M 2-OH-BDE47, 2-OH-BDE85 or DMSO (control) for 24 h, all in triplicate. In the heatmap, rows represent individual genes and columns display the gene expression levels in each independent sample. Red colour represents an expression level above the mean value of expression of a gene across all samples, black represents the mean and green colour represents an expression level below the mean.

Figure 5. Validation of gene expression changes by RT-PCR. Seven genes that were up-regulated (A) and two genes that were down-regulated (B) by OH-BDEs in the microarray experiments were analysed by RT-PCR. H295R cells were incubated with 10 μ M 2-OH-BDE47 or 2-OH-BDE85 for 24 h. Control cells were only given the vehicle. Gene expression levels were normalised against the endogenous control gene, hypoxanthine phosphoribosyltransferase 1 (HPRT1). The data are shown as the mean $\pm$ SD of three

independent experiments.

Table 1. RT-PCR primer sequences

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Length (bp)
ATF3	ATGGGCAGTATGATGGCAGGTCC	TGAGTGGAGAGTCCTAACCCCTTTGGA	101
DDIT3	AGAGCCCTCACTCTCCAGATTCCA	TCTGTTTCCGTTTCCTGGTTCTCC	90
HSPA5	AAGCCACCAAGATGCTGACATTGA	GCCTGCACTTCCATAGAGTTTGCTG	105
TIPARP	AATGGAGGCTGTGAGCAATGTGAG	CTTTGGACAGGGAATACGCATGG	95
TRIB3	GCTGCCAACAGTGGATTGAGTTTG	GGCTTCTGCCTTTCTCCCTTCTG	103
CYP1B1	ACAATAACGCAACCAAGTGTGCT	GAAACCCTGCTTCATTTCCATGTC	83
ADRA2A	TTTCTGCCTCACATCAGCCCTGT	GGGAGAGAGGGAACACTGGAAAGAG	105
SREBF1	CTATGTGGCGGCTGCATTGAGAGT	GCACTGCTCAGGAAGAAGCGTGTC	81
GADD45 α	AGGATTACAGAACTGATGCCAAGGG	TCCATCTGCAAAGTCATCTATCTCCG	85
HPRT1	GCAGACTTTGCTTTCCTTGGTCAG	GTCTGGCTTATATCCAACACTTGGTG	103

Table 2. Significant functional gene cluster modulation by 2-OH-BDE in H295R adrenocortical carcinoma cells

Probe set ID	Gene ID ¹	Gene title	Fold change	
			2-OH-BDE47 ²	2-OH-BDE85 ²
Response to unfolded protein				
226665_at	130872	activator of heat shock 90kDa protein ATPase homolog 2 (AHSA2)	3.4	
202842_s_at	4189	DNAJ (Hsp40) homolog, subfamily B, member 9 (DNAJB9)	4.3	6.1
208499_s_at	5611	DNAJ (Hsp40) homolog, subfamily C, member 3 (DNAJC3)		3.5
218696_at	9451	eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3/PERK)		3.5
241716_at	3329	heat shock 60kDa protein 1 (HSPD1)		8.4
202581_at	3304	heat shock 70kDa protein 1B (HSPA1B)	-4.1	-5.0
211538_s_at	3306	heat shock 70kDa protein 2 (HSPA2)		-4.3
230031_at	3309	heat shock 70kDa protein 5 (HSPA5/GRP78)	4.5	5.1
1569380_a_at	9709	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (HERPUD1)		6.4
Apoptosis				
210538_s_at	330	baculoviral IAP repeat-containing 3 (BIRC3)		26.1
1557257_at	8915	B-cell CLL/lymphoma 10 (BCL10)		5.3
207829_s_at	662	BCL2/adenovirus E1B 19kDa interacting protein 1 (BNIP1)		3.0
209406_at	9532	BCL2-associated athanogene 2 (BAG2)		6.8
224414_s_at	84674	caspase recruitment domain family, member 6 (CARD6)		-6.5
203989_x_at	2149	coagulation factor II (thrombin) receptor (F2R)		-3.4
209383_at	1649	DNA-damage-inducible transcript 3 (DDIT3/CHOP)	13.6	13.7
202887_s_at	54541	DNA-damage-inducible transcript 4 (DDIT4)	3.4	
212374_at	10116	fem-1 homolog b (C. elegans) (FEM1B)		3.1
210655_s_at	2309	forkhead box O3A (FOXO3)		3.2
206865_at	8739	Harakiri, BCL2 interacting protein (HRK)	5.8	
231733_at	59082	ICEBERG caspase-1 inhibitor (ICEBERG)	3.6	5.4
226352_at	133746	Junction-mediating and regulatory protein (JMY)	3.2	4.1
209193_at	5292	pim-1 oncogene (PIM1)		4.8
227751_at	9141	Programmed cell death 5 (PDCD5)	3.8	5.8
202014_at	23645	protein phosphatase 1, regulatory (inhibitor) subunit 15A (PPP1R15A/GADD34)	6.0	9.7
212884_x_at	10452	Translocase of outer mitochondrial membrane 40 homolog (TOMM40)		3.7

1555788_a_at	57761	tribbles homolog 3 (<i>Drosophila</i>) (TRIB3)	10.2	10.4
Cell cycle				
1555785_a_at	54464	5'-3' exoribonuclease 1 (XRN1)		3.4
227896_at	56647	BRCA2 and CDKN1A interacting protein (BCCIP)	-4.3	
1556006_s_at	1452	Casein kinase 1, alpha 1 (CSNK1A1)		3.3
214683_s_at	1195	CDC-like kinase 1 (CLK1)		6.7
217010_s_at	995	cell division cycle 25C (CDC25C)		-3.8
217988_at	57820	cyclin B1 interacting protein 1 (CCNB1IP1)		3.2
204826_at	899	cyclin F (CCNF)	-5.3	-5.8
220046_s_at	57018	cyclin L1 (CCNL1)		4.0
206967_at	904	cyclin T1 (CCNT1)		3.4
210021_s_at	10309	cyclin U (CCNU/UDG2)		3.7
236313_at	1030	cyclin-dependent kinase inhibitor 2B (CDKN2B/p15)		-3.1
204015_s_at	1846	dual specificity phosphatase 4 (DUSP4)		10.3
217370_x_at	2521	fusion (involved in t(12;16) in malignant liposarcoma) (FUS)	3.7	4.3
203725_at	1647	growth arrest and DNA-damage-inducible, alpha (GADD45A)	8.9	8.2
1560402_at	60674	growth arrest-specific 5 (GAS5)	3.9	3.6
202934_at	3099	hexokinase 2 (HK2)	4.4	4.5
207361_at	26959	HMG-box transcription factor 1 (HBP1)		7.2
227013_at	26524	LATS, large tumor suppressor, homolog 2 (<i>Drosophila</i>) (LATS2)		3.2
203036_s_at	9788	metastasis suppressor 1 (MTSS1)		3.4
221527_s_at	56288	par-3 partitioning defective 3 homolog (<i>C. elegans</i>) (PARD3)		3.6
202883_s_at	5519	protein phosphatase 2, regulatory subunit A (PR 65), beta (PPP2R1B)		-3.2
200730_s_at	7803	protein tyrosine phosphatase type IVA, member 1 (PTP4A1)		3.3
204346_s_at	11186	Ras association (RalGDS/AF-6) domain family 1 (RASSF1)		4.1
237626_at	9821	RB1-inducible coiled-coil 1 (RB1CC1)		4.5
223195_s_at	83667	sestrin 2 (SESN2)	5.5	6.4
220961_s_at	9238	transforming growth factor beta regulator 4 (TBRG4)		-3.8
225610_at	115426	ubiquitin-like, containing PHD and RING finger domains, 2 (UHRF2)		3.0
Transcription				
202672_s_at	467	activating transcription factor 3 (ATF3)	11.8	18.0
238476_at	153222	adult retina protein (LOC153222)	3.9	6.1
202820_at	196	aryl hydrocarbon receptor (AHR)		3.7
201170_s_at	8553	basic helix-loop-helix domain containing, class B, 2 (BHLHB2)	3.6	8.8
221530_s_at	79365	basic helix-loop-helix domain containing, class B, 3 (BHLHB3)		3.2
235965_at	168620	Basic helix-loop-helix domain containing, class B, 8 (BHLHB8)	25.1	

1558560_s_at	8548	basic leucine zipper nuclear factor 1 (JEM-1)		3.1
228289_at	29117	Bromodomain containing 7 (BRD7)	-3.6	-4.6
209357_at	10370	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (CITED2)		3.1
203973_s_at	1052	CCAAT/enhancer binding protein (C/EBP), delta (CEBPD)	4.4	4.1
204203_at	1054	CCAAT/enhancer binding protein (C/EBP), gamma (CEBPG)	4.5	6.7
202979_s_at	58487	CREB/ATF bZIP transcription factor (CREBZF)		3.2
219947_at	50856	C-type lectin domain family 4, member A (CLEC4A)	5.4	5.7
229069_at	84324	cytokine induced protein 29 kDa (CIP29)	3.6	3.2
210269_s_at	8227	DNA segment on chromosome X and Y (unique) 155 expressed sequence, isoform 1 (SFRS17A)		4.3
227404_s_at	1958	Early growth response 1 (EGR1)	3.8	6.6
222869_s_at	55520	elaC homolog 1 (E. coli) (ELAC1)		-3.1
230102_at	2119	Ets variant gene 5 (ETV5)		5.0
1569076_a_at	162962	FLJ16287 protein		4
206912_at	2304	forkhead box E1 (thyroid transcription factor 2) (FOXE1)		-3.8
225262_at	2355	FOS-like antigen 2 (FOSL2)		3.5
202949_s_at	2274	four and a half LIM domains 2 (FHL2)	3.4	3.4
204755_x_at	3131	hepatic leukemia factor (HLF)		5.3
213359_at	3184	Heterogeneous nuclear ribonucleoprotein D (HNRPD)	5.2	12
201209_at	3065	histone deacetylase 1 (HDAC1)		-3.1
223908_at	55869	histone deacetylase 8 (HDAC8)		-3.3
203543_s_at	687	Kruppel-like factor 9 (KL9)	4.7	8.1
203004_s_at	4209	MADS box transcription enhancer factor 2, polypeptide D (MEF2D)		3.9
228846_at	4084	MAX dimerization protein 1 (MXD1)		3.8
203238_s_at	4854	Notch homolog 3 (Drosophila) (NOTCH3)		-3.6
208003_s_at	10725	nuclear factor of activated T-cells 5 (NFAT5)		3.2
228388_at	4793	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (NFKBIB)		3.8
203574_at	4783	nuclear factor, interleukin 3 regulated	4.9	9.3
234471_s_at	57727	nuclear receptor coactivator 5 (NCOA5)		-3.5
202340_x_at	3164	nuclear receptor subfamily 4, group A, member 1 (NR4A1)		4.3
216248_s_at	4929	nuclear receptor subfamily 4, group A, member 2 (NR4A2)		5.8
227220_at	152518	nuclear transcription factor, X-box binding-like 1 (NFXL1)		3.0
202861_at	5187	period homolog 1 (Drosophila) (PER1)		4.8
203057_s_at	7799	PR domain containing 2, with ZNF domain (PRDM2)		-4.04
243624_at	9063	Protein inhibitor of activated STAT, 2 (PIAS2)	-3.4	-3.2
210191_s_at	10745	putative homeodomain transcription factor 1 (PHTF1)	-3.9	-3.1
210479_s_at	6095	RAR-related orphan receptor A (RORA)		4.2
232971_at	64783	RNA binding motif protein 15 (RBM15)		4.0

206554_x_at	6419	SET domain and mariner transposase fusion gene (SETMAR)		-3.3
202308_at	6720	sterol regulatory element binding transcription factor 1 (SREBF1)		-6.5
207678_s_at	11063	SRY (sex determining region Y)-box 30 (SOX30)	3.9	3.9
215048_at	140883	suppressor of hairy wing homolog 2 (Drosophila) (SUHW2)		3.4
1558208_at	23435	TAR DNA binding protein (TARDBP)		6.2
242243_at	7110	TATA element modulatory factor 1 (TMF1)		4.1
223393_s_at	57616	teashirt family zinc finger 3 (TSHZ3)		-3.1
236978_at	9969	Thyroid hormone receptor associated protein 1 (THRAP1)		-3.3
232097_at	9878	TOX high mobility group box family member 4 (TOX4)		3.3
242538_at	7027	Transcription factor Dp-1 (TFDP1)		-3.7
36711_at	23764	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (MAFF)	5.9	15.9
238496_at	54904	Wolf-Hirschhorn syndrome candidate 1-like 1 (WHSC1L1)		4.0
200670_at	7494	X-box binding protein 1 (XBP1)	6.3	6.7
222863_at	65986	zinc finger and BTB domain containing 10 (ZBTB10)		5.4
226284_at	57621	zinc finger and BTB domain containing 2 (ZBTB2)		4.7
1554045_at	7572	zinc finger protein 24 (ZNF24)		8.0
219595_at	7574	zinc finger protein 26 (ZNF26)		5.3
228711_at	7587	zinc finger protein 37A (ZNF37A)		-3.0
236429_at	55769	Zinc finger protein 83 (ZNF83)		-4.6
1569241_a_at	81931	Zinc finger protein 93 (ZNF93)	3.6	3.9
216960_s_at	7692	zinc finger protein 133 (ZNF133)		3.4
215887_at	11179	zinc finger protein 277 pseudogene (ZNF277P)		4.2
225539_at	49854	zinc finger protein 295 (ZNF295)		4.0
229136_s_at	55900	Zinc finger protein 302 (ZNF302)		4.2
1554985_at	252884	zinc finger protein 396 (ZNF396)	6.8	3.9
219848_s_at	9668	zinc finger protein 432 (ZNF432)	3.1	3.2
233175_at	10794	zinc finger protein 460 (ZNF460)		3.9
217593_at	65982	zinc finger protein 447 (ZNF447)		3.5
229901_at	118738	zinc finger protein 488 (ZNF488)	-3.8	-5.3
228099_at	162972	zinc finger protein 550 (ZNF550)		3.4
211721_s_at	90233	zinc finger protein 551 (ZNF551)		-3.5
224518_s_at	84527	zinc finger protein 559 (ZNF559)		-3.3
242028_at	163050	Zinc finger protein 564 (ZNF564)		-3.3
1570038_at	152687	Zinc finger protein 595 (ZNF595)		5.2
239482_x_at	7562	zinc finger protein 708 (ZNF708)		-5.5
238907_at	284323	zinc finger protein 780A (ZNF780A)		-5.6

Organic acid metabolism

201000_at	16	alanyl-tRNA synthetase (AAR)	3.6	
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203946_s_at	384	arginase, type II (ARG2)	4.6	3.9
205047_s_at	440	asparagine synthetase (ASNS)	9.7	4.8
243283_at	54677	Carnitine O-octanoyltransferase (CROT)	-3.5	
202402_s_at	833	cysteinyl-tRNA synthetase (CARS)	4.2	
231790_at	29958	dimethylglycine dehydrogenase (DMGDH)	30.4	
213712_at	54898	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2 (ELOVL2)	-3.0	
234513_at	83401	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3 (ELOVL3)	-4.4	
205278_at	2571	glutamate decarboxylase 1 (GAD1)	4.4	
202721_s_at	2673	glutamine-fructose-6-phosphate transaminase 1 (GFPT1)	3.9	4.0
1560013_at	23042	pyridoxal-dependent decarboxylase domain containing 1 (PDXDC1)		6.2
242775_at	22949	Leukotriene B4 12-hydroxydehydrogenase (LTB4DH)	-3.9	
213672_at	4141	Methionine-tRNA synthetase (MARS)	5.4	11.7
225520_at	25902	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like (MTHFD1L)	3.2	3.6
206340_at	9971	nuclear receptor subfamily 1, group H, member 4 (NR1H4)	3.3	
202847_at	5106	phosphoenolpyruvate carboxykinase 2 (PCK2)	5.1	
223062_s_at	29968	phosphoserine aminotransferase 1 (PSAT1)	3.0	
214474_at	5565	protein kinase, AMP-activated, beta 2 non-catalytic subunit (PRKAB2)	3.5	
214437_s_at	6472	serine hydroxymethyltransferase 2 (SHMT2)	3.2	
200802_at	6301	seryl-tRNA synthetase (SARS)	3.0	
225779_at	10999	solute carrier family 27 (fatty acid transporter), member 4 (SLC27A4)	-4.2	-5.4
200628_s_at	7453	tryptophanyl-tRNA synthetase (WARS)	4.2	
212048_s_at	8565	tyrosyl-tRNA synthetase (YARS)	3.6	

1, Gene ID according with Entrez Gene; 2, Cells were incubated with test compounds at 10 μ M for 24 h as described in Materials and Methods. Significant ($P < 0.05$) changes in mRNA expression levels in treatment group over control (fold changes) are listed. ER stress-responsive genes are highlighted in bold.

Figure1
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A

B

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A

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