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RESEARCH PAPER

Differences in the rate of oestrogen-induced apoptosis in breast cancer by oestradiol and the triphenylethylene bisphenol

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BACKGROUND AND PURPOSE

Triphenylethylene (TPE)-like compounds were the first agents to be used in the treatment of metastatic breast cancer in postmenopausal women. Although structurally related to the anti-oestrogen, 4-hydroxytamoxifen, TPEs possess oestrogenic properties in fully oestrogenized breast cancer cells but do not induce apoptosis with short-term treatment in long-term oestrogen-deprived breast cancer cells. This study determined the differential effects of bisphenol, a TPE, on growth and apoptosis based on the modulation of the shape of the ligand–oestrogen receptor complex.

EXPERIMENTAL APPROACH

Apoptotic flow cytometric studies were used to evaluate apoptosis over time. Proliferation of the breast cancer cells was assessed using DNA quantification and cell cycle analysis. Real-time PCR was performed to quantify mRNA levels of apoptotic genes. Regulation of cell cycle and apoptotic genes was determined using PCR-based arrays.

KEY RESULTS

Bisphenol induced an up-regulation of cell cycle genes similar to those induced by 17β oestradiol (E_2). Unlike the changes induced by E_2 that occur after 24 h, the apoptosis evoked by bisphenol occurred after 4 days, with quantifiable apoptotic changes noted at 6 days. A prolonged up-regulation of endoplasmic reticulum stress and inflammatory stress response genes was observed with subsequent activation of apoptosis-related genes in the second week of treatment with bisphenol.

CONCLUSIONS AND IMPLICATIONS

The bisphenol: ERα complex induces delayed biological effects on the growth and apoptosis of breast cancer cells. Both the shape of the complex and the duration of treatment control the initiation of apoptosis.

Abbreviations

4OHT, 4-hydroxytamoxifen; E_2, 17β oestradiol; ER, oestrogen receptor (also known as NR3B); ERS, endoplasmic reticulum stress; IS, inflammatory stress; RT-PCR, real-time PCR; TPE, triphenylethylene

Introduction

Apoptosis triggered by physiological oestrogen levels in antihormone-resistant oestrogen receptor (ER)-positive breast cancer (Wolf and Jordan, 1993; Yao *et al.*, 2000; Song *et al.*, 2001; Lewis *et al.*, 2005a) is a well-documented laboratory phenomenon, which has clinical significance. Low-dose oestradiol produces 30% clinical benefit for the treatment of

aromatase-resistant breast cancer (Ellis *et al.*, 2009), and treatment with conjugated equine oestrogen mediates a decrease in breast cancer incidence and mortality (Anderson *et al.*, 2012) in oestrogen-deprived cancer in women in their 60s who are 15 years postmenopausal. It is proposed that the key to triggering oestrogen-induced apoptosis is the selection of vulnerable ER-positive tumour cell populations that evolve and eventually dominate the tumour during long oestrogen deprivation or antihormone therapy (Jordan, 2004; 2008; Jordan *et al.*, 2011; Obiorah and Jordan, 2013). However, unlike the immediate and catastrophic initiation of apoptosis by paclitaxel, there is a delayed commitment of the cell for 24 h with 17β oestradiol (E_2) but the process is completed by 96 h (Obiorah *et al.*, 2014b). Oestrogen-induced apoptosis is heralded by endoplasmic reticulum stress (ERS) and an unfolded protein response (Ariazi *et al.*, 2011; Fan *et al.*, 2013).

Oestrogen can be classified into planar (class I) and angular (class II) ligands that create different shapes when complexed with the ERα (also known as NR3B1, see Alexander *et al.*, 2013; Jordan *et al.*, 2001). A class I oestrogen such as E_2 causes cell replication and apoptosis because the ligand is sealed by helix 12 in the ligand-binding domain of the ERα complex. By contrast, a class II angular oestrogen such as bisphenol (Supporting Information Fig. S1) causes cell replication but cannot evoke oestrogen-induced apoptosis in a short-term 7 day *in vitro* assay (Maximov *et al.*, 2010; Sengupta *et al.*, 2013). In fact, bisphenol blocks oestradiol-induced apoptosis in a manner similar to 4-hydroxytamoxifen (4OHT) (Sengupta *et al.*, 2013). It appears that bisphenol can adopt the conformation of the 4OHT–ERα complex (Bourgoin-Voillard *et al.*, 2010). However, these laboratory data are paradoxical as they do not conform to the known antitumour effects of class I and II oestrogens in the successful treatment of metastatic breast cancer in postmenopausal women (Haddow *et al.*, 1944).

High-dose oestrogen therapy was the first 'chemical therapy' to be used successfully to treat any cancer. Haddow *et al.* (1944) demonstrated, after preliminary laboratory studies, that high doses of two structurally different oestrogens diethylstilboestrol (class I) and triphenyl chloroethylene (class II) were both effective in producing a 30% response rate in postmenopausal women with metastatic breast cancer. Haddow (1970) also noted that responses were more likely in breast cancer if the patient was more than 5 years postmenopause. Today, it is recognized that oestrogen deprivation caused by menopause creates a selection pressure for breast tumour cells that results in the outgrowth of cellular populations more likely to die in the presence of oestrogens than grow (Obiorah and Jordan, 2013).

We have addressed the paradox that an angular class II oestrogen, bisphenol, can act as an inhibitor of oestrogen-induced apoptosis by adopting an 'anti-oestrogenic conformation' for the bisphenol: ERα complex (Bourgoin-Voillard *et al.*, 2010; Sengupta *et al.*, 2013), but related triphenylethylenes (TPEs) are effective antitumour agents in patients (Haddow *et al.*, 1944). We found that the trigger for oestrogen-induced apoptosis is dependent not only on the shape of the oestrogen–ERα complex but also on the duration of oestrogen exposure.

Methods

Cell growth assay

The cell growth was monitored by measuring the total DNA content per well in 24-well plates. Fifteen thousand cells were plated per well, and treatment with either the indicated concentrations of the compounds or the vehicle control (0.1% ethanol) was started after 24 h in triplicate. Media containing the specific treatments were changed every 48 h. The cells were harvested and total DNA was assessed using a fluorescent DNA quantification kit (Cat #170-2480; Bio-Rad, Hercules, CA, USA) and was performed as previously described (Lewis *et al.*, 2005a). All timings for determining select genes by real-time PCR (RT-PCR), real-time profiler assays or apoptosis assays were based on results from cell growth assays.

RNA isolation and RT-PCR

Total RNA was isolated using RNAeasy kit (Qiagen, Valencia, CA, USA) and was converted to first strand using a kit from Applied Biosystems (Foster City, CA, USA). Quantitative RT-PCR assays were performed with the SYBR Green PCR Master Mixes (Applied Biosystems) and a 7900 HT fast RT-PCR system (Applied Biosystems). RT-PCR was performed as previously described (Sengupta *et al.*, 2010). The sequences for all primers are as follows: BCL2L11 (BCL2-like 11) (Bim) forward: TCGGACTGAGAAACGCAAG; reverse: CTCGGTCACTCAG AACTTAC. TNF forward: ACTTTGGAGTGATCGGCC; reverse: GCTTGAGGGTTTGCTACAAC. FAS (Fas cell surface death receptor) forward AAGCTCTTTCACTTCGGAGG; reverse GGGCATTAACACTTTTGGACG. FADD (Fas-associated via death domain) forward CCTGGTACAAGAGGTTCAGC; reverse CTGTGTAGATGCCTGTGGTC. Caspase 4 forward CCATAGAACGACTGTCCATGAC; reverse GCTGTACTAAT GAAGGTGCTCC. LTA (lymphotoxin alpha) forward TCTTCTTTGGAGCCTTCGC; reverse AGACTTGAGCTGTT GGAATGG. The change in expression of transcripts was determined as described previously and the ribosomal protein 36B4 mRNA was used as the internal control (Sengupta *et al.*, 2010).

Real-time profiler assay

RT-PCR profiler assay kits for apoptosis and cell cycle was used from a commercial vendor that uses 384-well plates (Cat# PAHS-3012E; Qiagen; SABiosciences Corp., Fredrick, MD, USA) to profile the expression of 370 apoptosis-related human genes and 4 × 96-well plates to profile the expression of 84 cell cycle-related genes (Cat# PAHS-020; Qiagen; SABiosciences Corp.). All the procedures were followed as previously described (Sengupta *et al.*, 2013). Briefly, cells were treated with indicated compounds (in triplicate) for the indicated time points. To identify cell cycle or apoptosis-related genes, total RNA was isolated using the method mentioned earlier. Two micrograms of total RNA was reverse transcribed and RT-PCR was performed using ABI 7900 HT sequence detection system (Applied Biosystems, Foster City, CA, USA). We created a gene signature throughout the indicated time points after comparing them with vehicle treatment. This gene signature was generated by comparing the expression level of all the genes with vehicle treatment and selecting genes that were at least 2.5-fold overexpressed or underexpressed as compared with vehicle-treated cells at a statistical

significance *P*-value of 0.05. The fold change was calculated by ΔΔCt method (Qiagen; SABiosciences Corp.).

Apoptosis assay

Apoptosis was verified in MCF7:5C cells in response to bisphenol based on loss of plasma membrane integrity. This verification was determined by flow cytometric analysis of cells stained with either FITC-labelled annexin V or DNA-binding dye, YO-PRO-1. Viable cells excluded these dyes, whereas apoptotic cells allowed moderate staining. In brief, MCF7:5C cells were treated with 0.1% ethanol vehicle (control) or bisphenol (1 μM) for 6 days and then harvested in cold PBS (Invitrogen) and resuspended in 1X binding buffer and stained simultaneously with FITC-labelled annexin V and propidium iodide (PI) (Life Technologies, (Grand Island, NY, USA). The experiment was repeated and the cells were stained with YO-PRO-1 and PI (Pharmingen, San Diego, CA, USA). Cells were analysed using a FACS flow cytometer (Becton Dickinson, San Jose, CA, USA). The % of apoptotic cells was calculated as the addition of the right upper and lower quadrants.

Cell cycles analysis

MCF7:5C cells were cultured in dishes and were treated with vehicle control (0.1% ethanol), E_2 (1 nM) and bisphenol (1 μM) for 24, 48, 72 and 96 h respectively. Cells were harvested and gradually fixed with 75% EtOH on ice. After staining with PI, cells were analysed using a FACS flow cytometer (Becton Dickinson), and the data were analysed with ModFit software package (Verity Software House, Topsham, ME, USA).

Statistical analysis

All data are expressed as the mean of at least three determinations, unless otherwise stated. The differences between the treatment groups and the control group were determined by two-sample *t*-test or one-factor or two-way ANOVA using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Cell culture and reagents

Cell culture media were purchased from Invitrogen, Inc. (Grand Island, NY, USA), and fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT, USA). Compounds E_2, 4OHT and ICI 182,780 were obtained from Sigma (St. Louis, MO, USA), and bisphenol (Supporting Information Fig. S1) was obtained as previously described (Maximov *et al.*, 2010). Caspase 4 inhibitor with the peptide sequence z-LEVD-fmk (z,benzyloxycarbonyl; LEVD-Leu-Glu(OMe)-Val-Asp(OMe),fmk, fluoromethyl ketone) was from Biovision (Milpitas, CA, USA). Drug/molecular target nomenclature used conforms to the required guide to receptors and channels (Alexander *et al.*, 2013). MCF7:5C were derived from MCF7 cells obtained from the Drs Dean Edwards and Bill McGuire, San Antonio, Texas, as reported previously (Lewis *et al.*, 2005b). MCF7 cells were maintained in RPMI media supplemented with 10% FCS, 6 ng·mL^{-1} bovine insulin and penicillin and streptomycin. They have been maintained for more than 20 years. Before the experiments were started, MCF-7 cells were cultured and maintained in phenol-red free RPMI media containing 10% charcoal dextran-treated FCS, 6 ng·mL^{-1} bovine insulin and penicillin and streptomycin. MCF7:5C cells were maintained in phenol-red free RPMI media containing 10% charcoal dextran-treated FCS, 6 ng·mL^{-1} bovine insulin and penicillin and streptomycin. The cells were treated with indicated compounds (with media changes every 48 h) for the specified time and were subsequently harvested for tissue culture experiments.

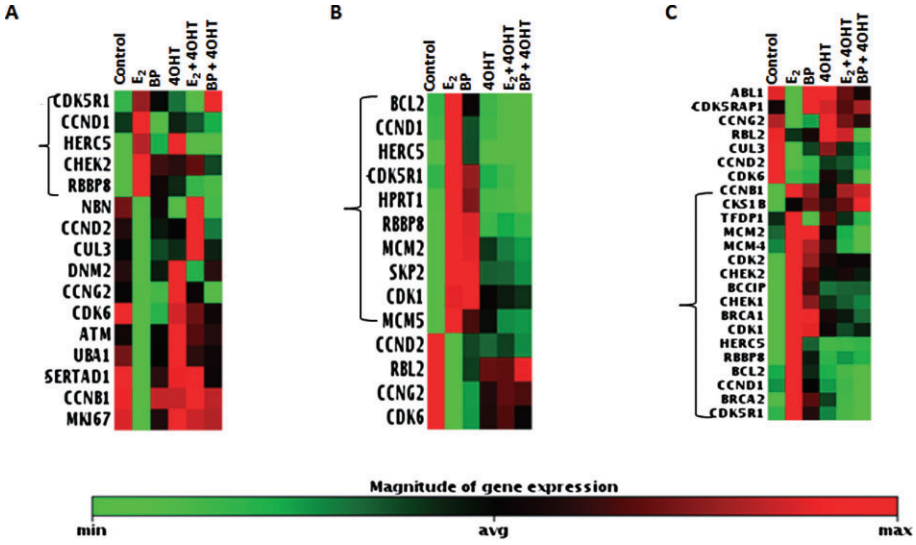

Figure 1

Heat map of the time course pattern of E_2 and bisphenol (BP)-regulated expression of cell cycle genes. MCF7 breast cancer cells were treated with either control, E_2 (1 nM), bisphenol (1 μM) or 4OHT (1 μM) over a period of 24 h, and 4OHT was used to block the effects of E_2 and bisphenol. Genes that are at least 2.5-fold overexpressed (red) or underexpressed (green) as compared with control at *P*-value of 0.05 at (A) 6, (B) 12 and (C) 24 h are presented. Cell cycle genes induced by E_2 and bisphenol are indicated in black.

Results

Differential expression of cell cycle genes induced by bisphenol and E_2

We have previously shown that bisphenol, a TPE, can induce the growth of MCF7 breast cancer cells as effectively as E_2 (Maximov *et al.*, 2010; Sengupta *et al.*, 2013). To identify cell cycle genes associated with bisphenol-induced cell growth, MCF7 cells were treated with 1 μM bisphenol for 6, 12 and 24 h and compared with 1 nM E_2 and 1 μM 4OHT as positive and negative regulators of cell replication respectively. The anti-oestrogen, 4OHT, was used to block the stimulatory effects of bisphenol and E_2. We used RT-PCR array kits that contain 4 × 96-well plates to profile the expression of 84 genes key to cell cycle regulation. At 6 h, E_2 induces several genes such as *cyclin D1* (*CCND1*), *CDK5R1*, *HERC5*, *CHEK2* and *RBBP8* (Figure 1A). bisphenol and 4OHT only induced HERC5. Interestingly, CCND1 was down-regulated by bisphenol at this time point. There was increased expression of cell cycle-related genes by E_2 at 12 h (Figure 1B), which further increased by almost twofold at 24 h (Figure 1C). Similarly, bisphenol induced 60 and 50% of the cell cycle-related genes that were up-regulated by E_2 at 12 and 24 h respectively. The rest of the cell cycle-related genes induced by bisphenol showed an obvious trend of overexpression when compared with the control. Similarly, all cell cycle genes down-regulated by bisphenol were equally decreased by E_2 treatment. The list of genes induced by E_2 and bisphenol are presented in Supporting Information Table S1. Furthermore, E_2 and bisphenol decrease retinoblastoma protein mRNA levels in a time-dependent manner (Supporting Information Fig. S2). Unlike the oestrogens, 4OHT did not activate the cell cycle-related genes but rather blocked the effects of E_2 and bisphenol. These results demonstrate that bisphenol induces similar cell cycle-related genes as E_2, although not as effectively.

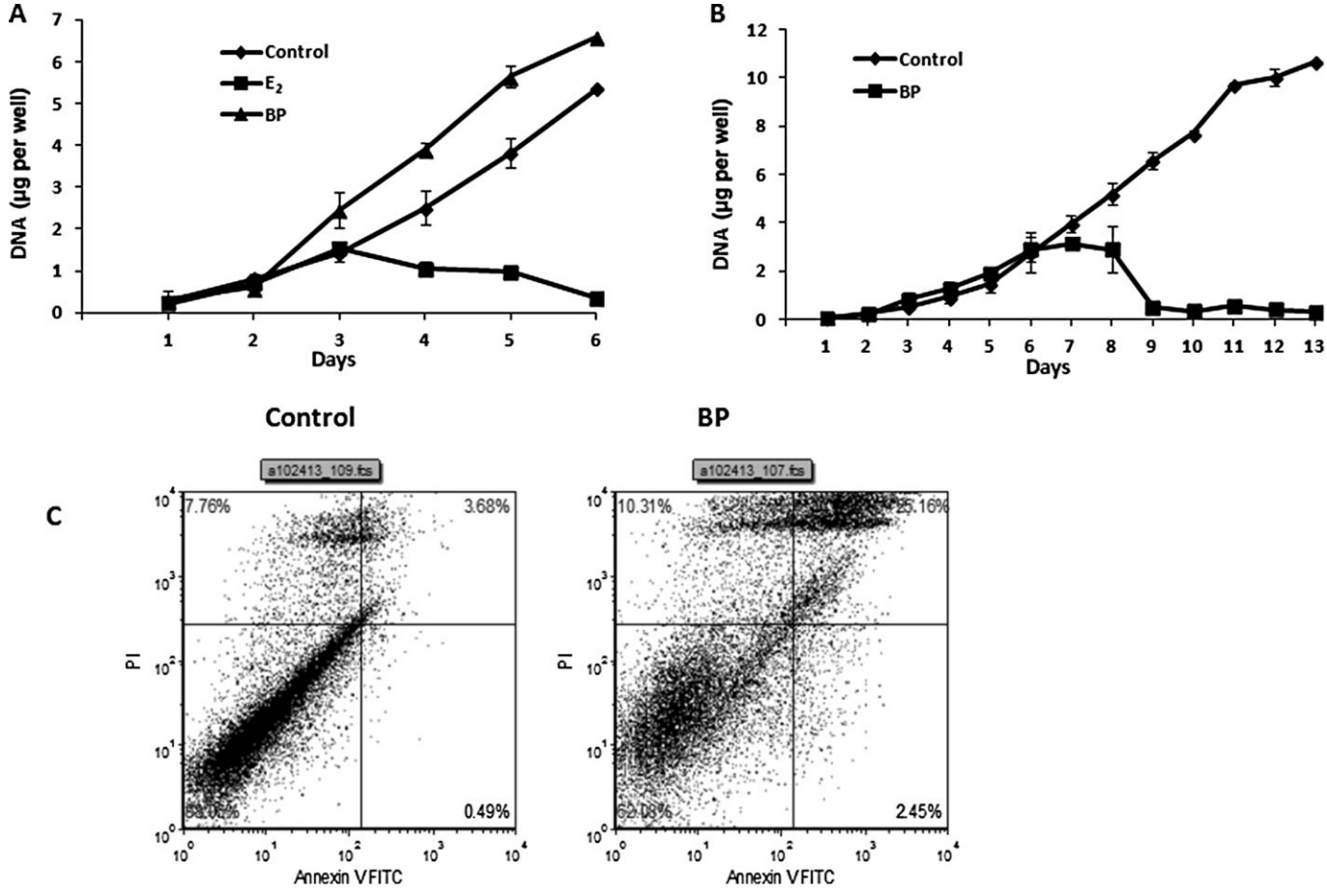

Figure 2

Effect of bisphenol on the growth and apoptosis of MCF7:5C breast cancer cells. (A) Cells were seeded in triplicate and treated with either control, E_2 (1 nM) or bisphenol (BP, 1 μM), and the cells were harvested daily for 6 days. (B) Treatment with bisphenol versus control was extended for 13 days, and the DNA content of the remaining cells in each well was quantified. The data represent the mean of three independent experiments. (C) MCF7:5C cells were treated with control or bisphenol (1 μM) for 6 days and then stained with annexin V–FITC and propidium iodide and analysed by flow cytometry. Viable cells (left lower quadrant) are annexin V–FITC– and PI–; early apoptotic cells (right lower quadrant) are annexin V–FITC+ and PI–; dead cells (left upper quadrant) are PI+ and late apoptotic cells (right upper quadrant) are annexin V–FITC+ and PI+. An increased, late apoptotic effect is observed in the right upper quadrant.

Effect of bisphenol on apoptosis in MCF7:5C cells

The planar type 1 oestrogen, E_2, induced apoptosis in long-term oestrogen-deprived MCF7 (MCF7:5C) cells. In contrast, the angular oestrogen bisphenol did not initially induce apoptosis in MCF7:5C cells and blocked E_2-induced apoptosis in a similar manner to 4OHT (Sengupta *et al.*, 2013). To evaluate the long-term effects of bisphenol, we treated MCF7:5C cells with 1 μM bisphenol, 1 nM E_2 and 0.1% ethanol vehicle (control). Growth of the cells was inhibited by E_2 after 3 days of treatment and the effect became maximal by 6 days of treatment (Figure 2A). In contrast, bisphenol increased the growth of the cells up to 6 days of treatment (Figure 2A) but caused 100% inhibition of growth by 9 days of treatment (Figure 2B). The inhibition of growth observed with bisphenol was further investigated for apoptosis using flow cytometry. Following 6 days of treatment, bisphenol caused a sevenfold increase in the % of cells (4.15 vs. 27.61%) undergoing apoptosis compared with the control (Figure 2C) using annexin V staining. A similar effect was observed using a DNA-binding stain, YO-PRO-1 (Supporting Information Fig. S3).

Determination of the point of commitment for bisphenol-induced apoptosis

Next, we investigated the delayed response to bisphenol; MCF7:5C cells were treated with bisphenol (1 μM) and 4OHT (1 μM) was used to block the antiproliferative and apoptotic effects of bisphenol at daily intervals over a range of 9 days. Cells were harvested after 13 days of treatment and total DNA was quantified using a fluorescent DNA quantification kit. Apoptosis induced by bisphenol was blocked by daily additions of 4OHT for up to 3 days and afterwards the cells became committed to apoptosis mediated by bisphenol (Figure 3). 4OHT alone caused a small decrease in DNA similar to that observed at days 1, 2 and 3. After day 3, an irreversible decline occurred with bisphenol that was not rescued. The day 4 value was about 50% of the control or 4OHT alone values. Cells could not be rescued from bisphenol-induced apoptosis by 4OHT after 4 days of treatment, suggesting that the cell commitment trigger for apoptosis had occurred. The experiment was repeated with the oestrogen antagonist ICI 182,780 and similar results were obtained (Supporting Information Fig. S4). It is important to emphasize that in each of the two 'rescue' experiments anti-oestrogens 4OHT or ICI 182,780 were added at specific days after bisphenol and cellular DNA measured at 13 days. MCF7:5C cells were both committed to apoptosis after day 3 with either anti-oestrogen (Figure 3; Supporting Information Fig. S4).

Apoptosis-related genes induced by bisphenol

To determine the early events preceding bisphenol-induced apoptosis, the induction of apoptosis-related genes was investigated in MCF7:5C cells treated with bisphenol (1 μM), 0.1% ethanol vehicle (control), 1 μM 4OHT, and bisphenol in combination with 4OHT (in triplicate) for 3, 4 and 5 days. We used 384-well RT-PCR profiler plates to monitor the expression of 370 apoptosis-related human genes (see Methods section). Comparative analysis showed that significant evidence of apoptotic gene induction did not occur until after 3 days of treatment. At 4 days (Figure 4A), bisphenol induced an up-regulation of ERS-related genes; DDIT3 and inflammatory stress (IS) response genes such as *CEBPB*, *IFI6*, *IFI16* and *DAPK1*. At 5 days of treatment (Figure 4B), the up-regulation

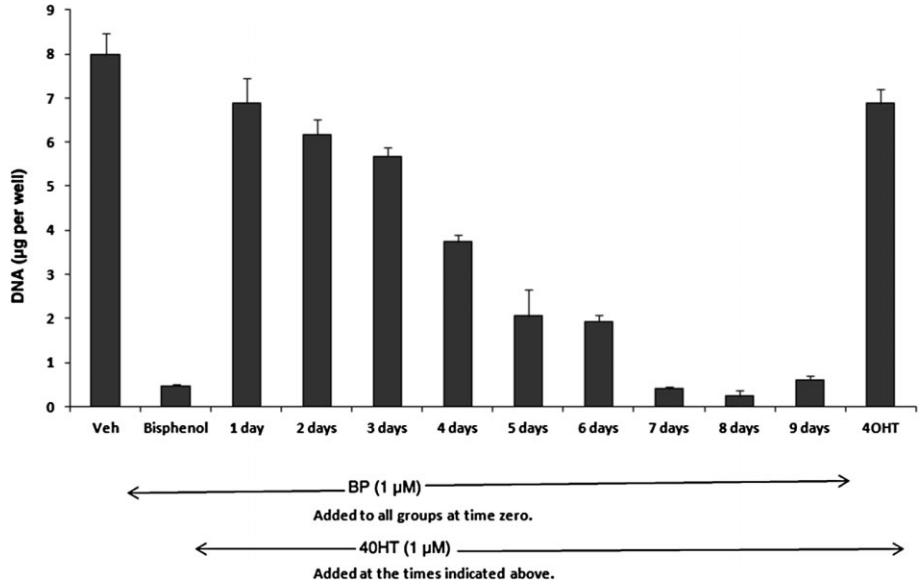

Figure 3

Determination of the trigger point for bisphenol-induced apoptosis. MCF7:5C cells were treated with bisphenol (BP, 1 μM) alone and 1 μM 4OHT was added and used to block and reverse bisphenol's action daily over a period of 9 days. The cells were harvested after 14 days of treatment. The DNA content of the remaining cells was quantified using a fluorescent DNA quantification kit. The point of trigger for apoptosis induced by bisphenol was determined as the time when the apoptotic effects of bisphenol could not be blocked by 4OHT.

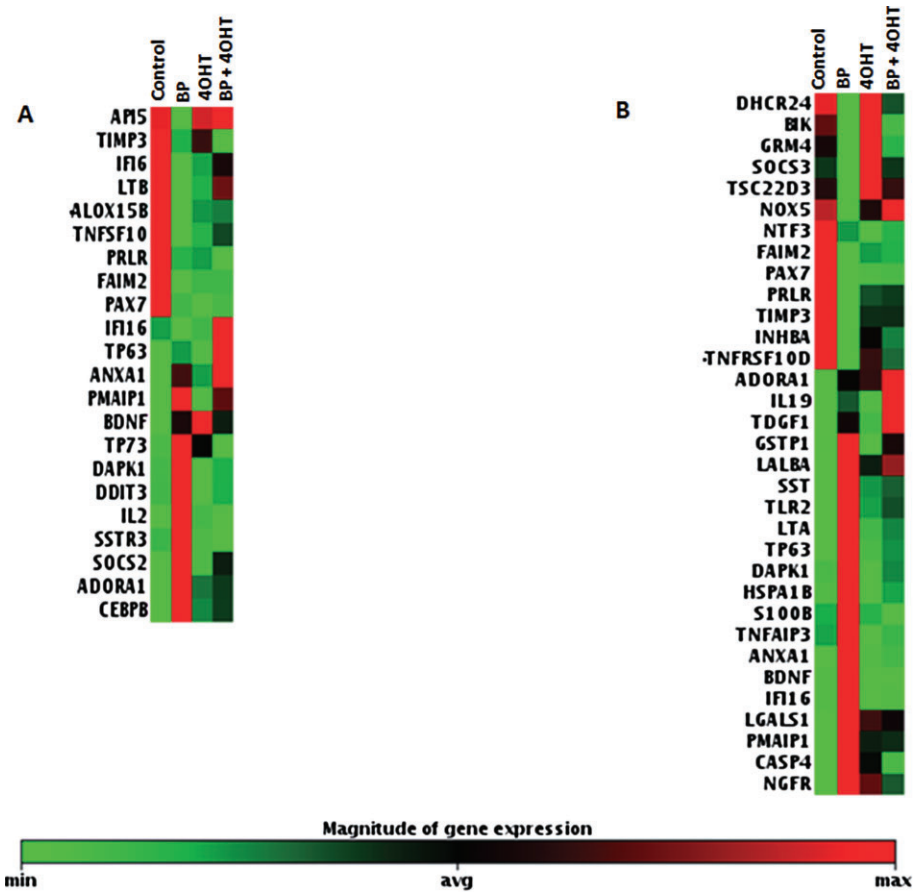

Figure 4

Determination of apoptotic genes differentially expressed by bisphenol (BP) treatment in MCF7:5C cells. MCF7:5C cells were treated with vehicle (control), 1 μM bisphenol and 1 μM 4OHT in the presence or absence of bisphenol over a period of 5 days. Gene expression values were obtained and analysed in comparison with the controls, and heat maps were generated after (A) 96 and (B) 120 h of treatment and the expressed genes listed. The selected genes were at least 2.5-fold overexpressed (red) or underexpressed (green) as compared with control at a *P*-value of 0.05.

of ERS and IS-associated genes, including LTA and caspase 4, an inflammatory caspase, continued to increase. Levels of LTA and caspase 4 mRNA were elucidated using RT-PCR (Supporting Information Fig. S5). The apoptosis-related genes detected using the PCR arrays are listed in Supporting Information Tables S2 and S3. Bim/BCL2L11 is important for E_2-induced apoptosis. Its activation by E_2 occurs by 36 h of treatment (Obiorah *et al.*, 2014b), and E_2 subsequently induces the TNF family of pro-apoptosis-related genes. The induction of these genes by bisphenol was investigated by extending the duration of treatment for 7, 8 and 9 days; mRNA levels of BCL2L11 and TNF were quantified by RT-PCR. Up-regulation of Bim/BCL2L11 (Figure 5A), TNF (Figure 5B), FAS (Figure 5C) and FADD (Figure 5D) was observed by 8 days of treatment with continued increase of all genes at 9 days of treatment with bisphenol. These data indicate that there is a prolonged induction of ERS and IS-associated genes by 4 days of treatment with subsequent up-regulation of mitochondrial and TNF-related apoptosis genes.

Differential effect of bisphenol on cell cycle

Because the bisphenol-induced apoptosis was not apparent until the second week of treatment, we evaluated the effect of bisphenol on the regulation of the cell cycle. MCF7:5C cells were treated with either vehicle control (0.1% ethanol), 1 nM E_2 or 1 μM bisphenol for 24, 48 and 96 h and a cell cycle analysis was performed using flow cytometry (Figure 6). As suspected, bisphenol and E_2 cause a consistent increase in the S phase when compared with the control. Although the trigger for apoptosis occurred for E_2 and bisphenol at 36 (Obiorah *et al.*, 2014b) and 96 h (Figure 3 and Supporting Information Fig. S4), respectively, no checkpoint blockade was noted after treatment with either compound, which contrasts dramatically with early cell cycle arrest at G2/M with paclitaxel (Obiorah *et al.*, 2014b).

Functional importance of caspase 4 in bisphenol-induced apoptosis

Caspase 4, an inflammatory caspase, was up-regulated in the MCF7:5C cells by 5 days of treatment with bisphenol. To determine the role of caspase 4 in bisphenol-induced apoptosis, cells were treated with control solution or bisphenol (1 μM) and the effects of caspase 4 were blocked by caspase 4 inhibitor, z-LEVD-fmk (10 μM). Growth inhibited by bisphenol was reversed by z-LEVD-fmk (Figure 7A). Proliferation was determined after 12 days of exposure to bisphenol and

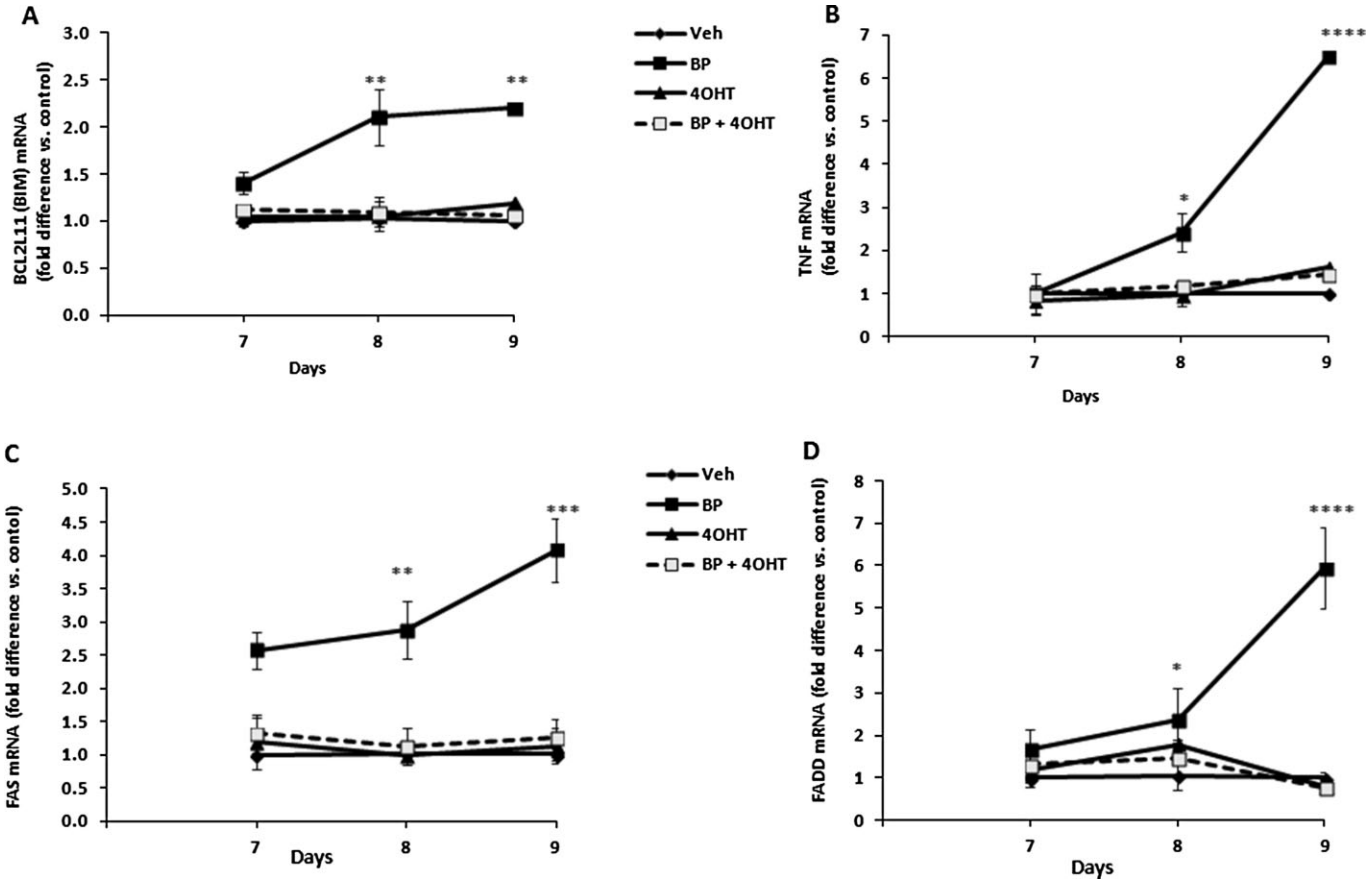

Figure 5

Induction of apoptotic genes by bisphenol (BP). Bisphenol induces an up-regulation of apoptotic genes after 7 days of treatment. MCF7:5C cells were treated with vehicle (Veh), bisphenol (1 μM), 4OHT, 1 μM or combination treatment of bisphenol and 4OHT for 7–9 days. Total RNA was isolated and reverse transcribed, and (A) BIM and (B) TNF, (C) FAS and (D) FADD mRNA levels was determined using RT-PCR. PCR data values are presented as fold difference versus vehicle-treated cells ± SEM; $*P < 0.05$, $**P < 0.005$, $***P < 0.0001$, $****P < 0.0005$.

quantified by determining DNA mass per well. Apoptosis induced after 6 days of exposure to bisphenol was completely reversed by z-LEVD-fmk (Figure 7B). Thus, the blockade of bisphenol-induced apoptosis by the caspase 4 inhibitor z-LEVD-fmk indicates that caspase 4 plays an important role in the induction of apoptosis evoked by bisphenol.

Discussion and conclusions

The aim of our study was to elucidate the growth and induction of apoptosis mediated by bisphenol in fully oestrogenized and long-term oestrogen-deprived breast cancer cells. The ERα in breast cancer cells can either initiate replication or trigger apoptosis based on the context of cell selection in oestrogen replete or deprived environments (Lewis-Wambi and Jordan, 2009). Originally, oestrogens including E_2 and TPE derivatives were discovered using a bioassay of the induction of vaginal cornification in ovarectomized mice. Replication and cornification of vaginal cells in the mouse was the early appropriate method for establishing the structure–function relationships of an oestrogenic TPE molecule (Robson and Schonberg, 1937). Initial structure–function studies *in vitro* established an ERα-mediated mechanism for E_2 stimulate prolactin (an oestrogen-responsive gene) synthesis in rat pituitary cells (Lieberman *et al.*, 1983). However, bisphenol and other TPE derivatives were found to act as partial agonists with anti-oestrogenic properties at the prolactin gene *in vitro* (Jordan and Lieberman, 1984; Jordan *et al.*, 1984). Structure–function relationship studies to modulate prolactin synthesis by extending the length of the 'anti-oestrogenic side chain' created an anti-oestrogen that blocked oestrogen-stimulated prolactin synthesis (Jordan *et al.*, 1984; 1986). These are the basic early facts of the pharmacological function of the oestrogen–ERα complex that now allows us to interpret our current findings on the modulation of apoptosis.

Our results show that bisphenol induces cell cycle-regulated genes that are similar to those activated by E_2 in MCF7 cells. This correlates with the ability of bisphenol to induce replication of MCF7 cells in a comparable manner to that of E_2 (Maximov *et al.*, 2010). In contrast, 4OHT, which possesses a bulky alkylaminoethoxy side chain (Shiau *et al.*, 1998), failed to induce cell cycle-regulated genes in a time-dependent manner but rather blocked E_2- and bisphenol-mediated activation of cell cycle genes; therefore, confirming

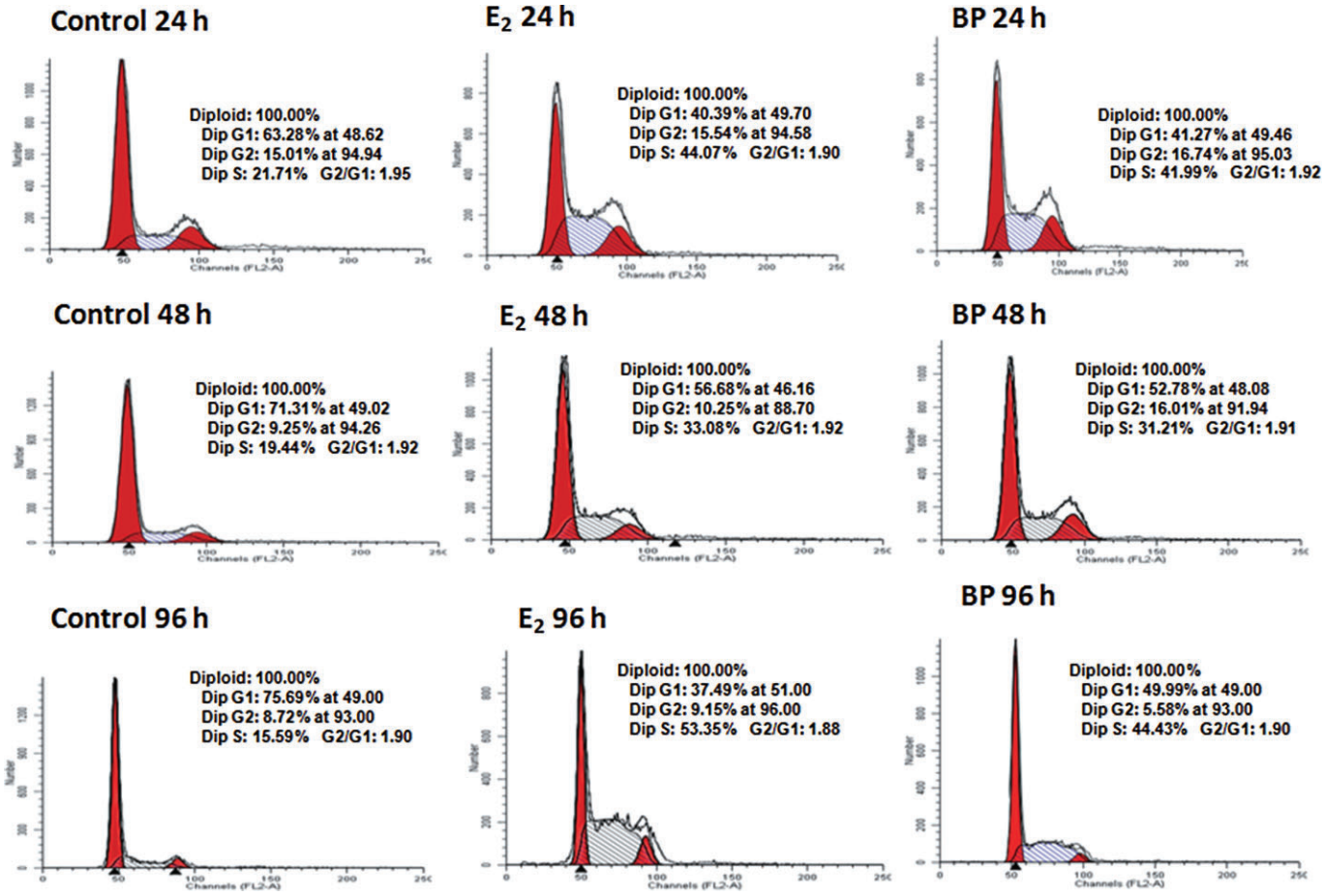

Figure 6

Diverse effects of bisphenol (BP) and E_2 on cell cycle progression. Distribution of the cells through the cell cycle phases was analysed by flow cytometry in cells treated with E_2 (1 nM), bisphenol (1 μM) or vehicle (control) for 24, 48 and 96 h. The percentage of cells in each fraction was calculated using the ModFit software. The *y*-axis represents the number of cells and FL2-A represents the intensity of propidium iodide.

its role as an anti-oestrogen. Although bisphenol possesses a bulky phenyl substituent, it does not have an akylaminoethoxy side chain. Molecular modelling studies suggest that the phenyl component of TPEs prevent the complete sealing of the ligand-binding domain of the ERα by helix 12 (Maximov *et al.*, 2010; Sengupta *et al.*, 2013). The reduced number of gene changes noted with bisphenol treatment compared with E_2 (Figure 1) may be caused by differences in the structure of the ligand–ERα complex, thus resulting in a reduction in the full oestrogenic potential of bisphenol-induced replication. Additionally, bisphenol unlike E_2 does not readily induce apoptosis in long-term oestrogen-deprived MCF7 cells but rather appears to possess early anti-oestrogenic properties (Sengupta *et al.*, 2013). Using cell proliferation assays, we demonstrated that bisphenol induces growth of MCF7:5C cells in the first week of treatment. In contrast, growth inhibition occurred after the third day of treatment with E_2. Inhibition of growth in oestrogen-deprived MCF7:5C cells with bisphenol was seen after 8 days of treatment. Similarly, apoptotic effects of bisphenol were observed following 6 days of bisphenol treatment using flow cytometry studies (Figure 2; Supporting Information Fig. S3). Previous studies have shown that MCF7:5C cells are resistant to the actions of 4OHT, which has the ability to reverse and block E_2-mediated apoptosis (Maximov *et al.*, 2011). 4OHT or ICI 182,780 blocked and rescued the cells from bisphenol-induced apoptosis, which suggests that the trigger for apoptosis occurs after 4 days of treatment with bisphenol.

There was no evidence of cell cycle arrest with either E_2 or bisphenol (Figure 6) before apoptosis. This contrasts dramatically with our previous findings for E_2 and the rapid G2 blockade triggered by paclitaxel before apoptosis; no checkpoint blockade was noted after treatment with either compound and contrasts dramatically with early cell cycle arrest at G2/M with paclitaxel (Obiorah *et al.*, 2014b).

The apoptosis-related genes clearly demonstrate that the majority of genes that are up-regulated by bisphenol at 4 days of treatment are ERS and IS response genes. DDIT3 also known as CHOP or GADD153 is a key ERS protein associated with cell death (Oyadomari and Mori, 2003; Kim *et al.*, 2006), whereas CEBPB, which is known to induce pro-inflammatory cytokines such as IL-6 (Akira *et al.*, 1990), is activated by ERS and is important for nuclear transport of DDIT3 (Ron and Habener, 1992; Styner *et al.*, 2012). The induction of similar

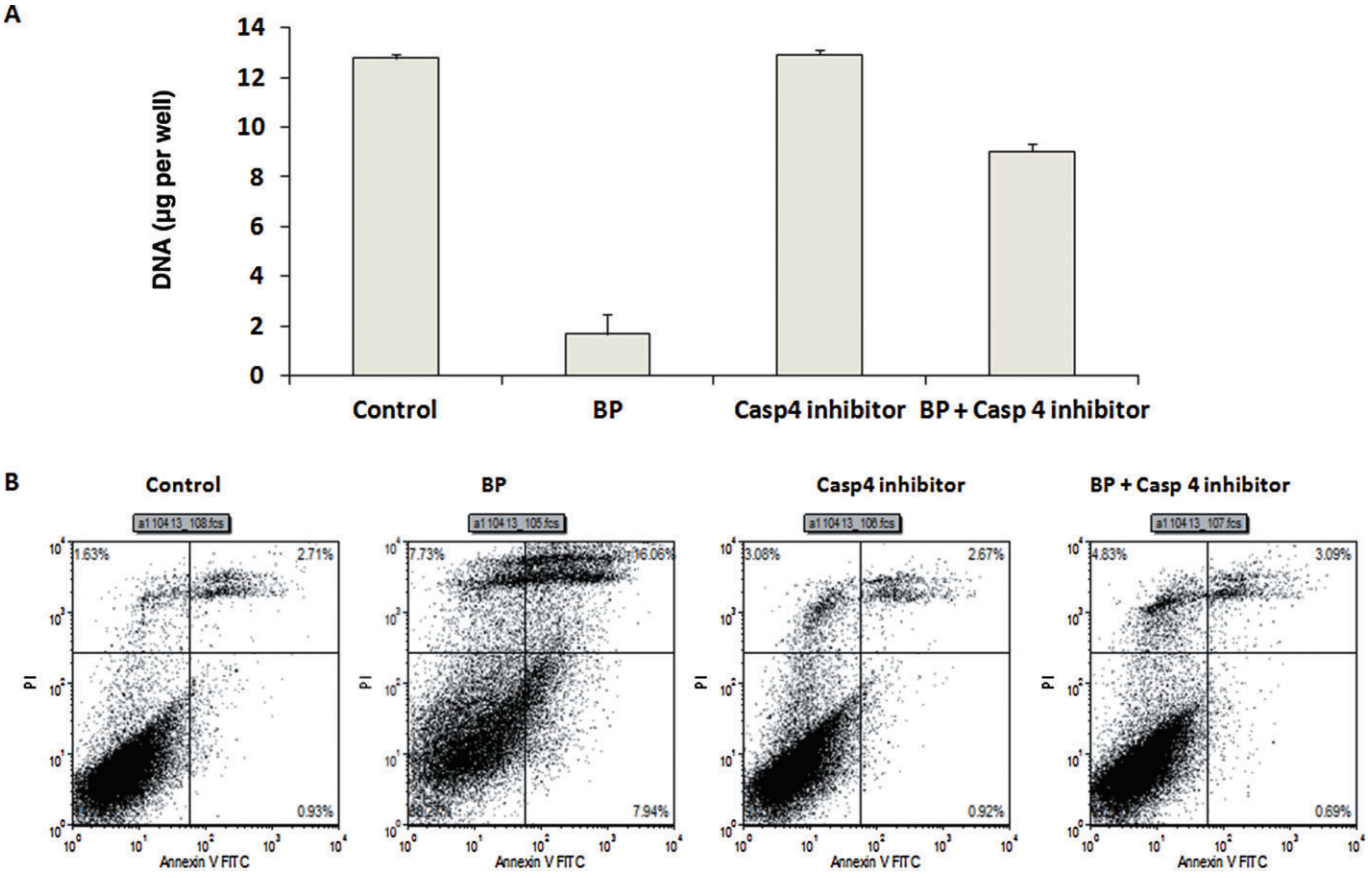

Figure 7

Caspase 4 is important for bisphenol (BP)-induced apoptosis. MCF7:5C cells were treated with control (0.1% ethanol) or bisphenol (1 µM) or caspase 4 (casp4) inhibitor with or without bisphenol for either (A) 12 days and assessed for cell proliferation or (B) for 6 days and evaluated for apoptosis. Apoptosis and inhibition of growth of cells were blocked by caspase 4 inhibitor z-LEVD-fmk (10 µM).

pro-apoptotic genes continued at 5 days of treatment; these included caspase 4, an inflammatory caspase that predominantly localizes to the endoplasmic reticulum and undergoes cleavage and induces effector caspases in response to ERS (Hitomi *et al.*, 2004; Bian *et al.*, 2009). Up-regulation of Bim, FAS, TNF and FADD mRNA was observed after 7 days of treatment with bisphenol. Microarray analysis has indicated ERS-mediated apoptosis as the top scoring pathway of apoptosis induced by E_2 in MCF7:5C cells (Ariazi *et al.*, 2011). Oestradiol induces ERS and IS response genes by 36 h of treatment, and apoptotic genes such as *Bim* and *TNF* are activated by 48 h of treatment (Obiorah *et al.*, 2014b). A similar trend was observed with bisphenol; however, there was a prolonged activation of ERS- and IS-related genes with subsequent induction of caspase 4 after 5 days of treatment and mitochondrial and extramitochondrial apoptotic genes after 7 days of treatment. After 48 h of treatment with bisphenol, there was no induction of apoptotic genes (Sengupta *et al.*, 2013) but we found there was an increase in growth (Figures 2A and 6), and the cells could be rescued from apoptosis with anti-oestrogens (Figure 3).

The initial resistance to trigger apoptosis may also result from the anti-oestrogenic conformation bisphenol creates with the ERα. Angular TPEs such as bisphenol have a reduced tendency to promote recruitment of co-activators containing the LxxLL motif (Bourgoin-Voillard *et al.*, 2010). We have previously shown that bisphenol recruits the ERα and SRC3 to the PS2 promoter ERE less efficiently when compared with planar oestrogens (Sengupta *et al.*, 2013; Obiorah *et al.*, 2014a), thus indicating that complete sealing of helix 12 of the LBD and interaction of co-activators with the TPE–ERα complex is necessary for the rapid activation of apoptosis observed with planar oestrogens (Maximov *et al.*, 2011). Depletion of SRC3 in the MCF7:5C cells and MCF7 cells leads to loss of E_2-induced apoptosis (Hu *et al.*, 2011) and growth (List *et al.*, 2001; Lahusen *et al.*, 2009) respectively.

Because caspase 4 is specifically activated by ERS (Hitomi *et al.*, 2004) and it was induced to increase twofold with E_2 within 24 h (Ariazi *et al.*, 2011) and twofold by bisphenol within 96 h, a specific caspase 4 inhibitor (Hitomi *et al.*, 2004) was used to block activation of caspase 4 in bisphenol-treated cells, and this resulted in a reversal of the inhibitory effects of bisphenol on growth and apoptosis (Figure 7). We previously reported that E_2-induced apoptosis can be blocked by a caspase 4 inhibitor (Ariazi *et al.*, 2011). Together, these results suggest that bisphenol activates IS and ERS-related genes, which interact with resultant induction of caspase 4 between 4 and 5 days of treatment and subsequent activation

of mitochondrial and extramitochodrial-related apoptotic genes in the second week of treatment. This delayed sequence for the effects of bisphenol contrasts with early activation by E_2 (Ariazi *et al.*, 2011; Obiorah *et al.*, 2014b).

In summary, we have used cell-based assays and gene profiling studies to demonstrate the biological response of the TPE, bisphenol, on both growth and apoptosis. TPEs were among the first chemical therapy used in the treatment of advanced breast cancer in postmenopausal women (Haddow *et al.*, 1944). These data support the apoptotic mechanism of TPEs in early clinical practice.

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Author contributions

I. E. O. and V. C. J. participated in research design. I. E. O. conducted the experiments. I. E. O. and V. C. J. performed data analysis and wrote or contributed to the writing of the manuscript.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Chemical structure of 17β bisphenol (BP).
Figure S2 Inhibition of (retinoblastoma protein) RB1 by E_2 and bisphenol (BP). MCF7 cells were treated with either Veh (0.1% ethanol), E_2 (1 nM) or bisphenol (1 μM) for 4, 8, 12 and 24 h. 4OHT (1 μM) and combination of 4OHT with either E_2 or bisphenol were used as negative controls. Retinoblastoma protein (RB) mRNA levels were determined using RT-PCR.
Figure S3 Apoptotic effect of bisphenol (BP) in MCF7:5C cells. MCF7:5C cells were treated with control (0.1% ethanol) or bisphenol (1 μM) for 5 days and then stained with nucleic acid dye YO-PRO-1 and propidium iodide and analysed by flow cytometry. Viable cells (left lower quadrant) are YO-PRO-1– and PI–; early apoptotic cells (right lower quadrant) are YO-PRO-1+ and PI–; dead cells (left upper quadrant) are PI+; and late apoptotic cells (right upper quadrant) are YO-PRO-1+ and PI+. Increased late apoptotic effect is observed in the right upper and lower quadrants.
Figure S4 Determination of the critical trigger of apoptosis. Cells were treated with bisphenol (BP, 1 μM) alone, and ICI 182,780 (1 μM) was added at the indicated time points and used to block and reverse bisphenol's action over a period of 9 days. The cells were harvested after 14 days of treatment. The DNA content of the remaining cells was quantified using a fluorescent DNA quantification kit.
Figure S5 mRNA levels of caspase 4 and LTA. Cells were treated with vehicle (0.1% ethanol), bisphenol (BP, 1 μM) and 4OHT (1 μM) in the presence or absence of bisphenol for 72, 96 and 120 h. Cells were harvested and caspase 4 and LTA mRNA levels were determined using RT-PCR.
Table S1 List of cell cycle-regulated genes induced by E_2 (1 nM), bisphenol (BP, 1 μM) and 4OHT (1 μM) combination treatments of 4OHT and E_2 and 4OHT and bisphenol in MCF7 cells after 24 h of treatment versus control.
Table S2 List of apoptosis-regulated genes induced by bisphenol (BP, 1 μM) and 4OHT (1 μM) in the presence or absence of bisphenol in MCF7: 5C cells after 96 h of treatment versus control.
Table S3 List of apoptosis-regulated genes induced by bisphenol (BP, 1 μM) and 4OHT (1 μM) in the presence or absence of bisphenol in MCF7: 5C cells after 120 h of treatment versus control.