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ORIGINAL ARTICLE

Inhibition of NF-κB-mediated transcription and induction of apoptosis in human breast cancer cells by epoxypseudoisoeugenol-2-methyl butyrate

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Abstract
Purpose Breast cancer is one of the most prevalent woman cancers. Genomic instability, accumulative mutations, and subsequent changes in intracellular signaling cascades play key roles in the development of human breast cancers. Activation of nuclear factor-κB (NF-κB) has been implicated in oncogenesis of breast cancers and is known to be associated with resistance to anticancer agents and apoptosis. Blocking NF-κB signaling may represent a therapeutic strategy in breast cancer therapy. The objective of this study is to investigate the in vitro effects of epoxypseudoisoeugenol-2-methyl butyrate (EPB), a phenylpropranoid isolated from *Pimpinella corymbosa*, on the activation of NF-κB, cell growth, cell cycle progression and apoptosis in MCF-7 (estrogen-dependent) and BT-549 (estrogen-independent) breast cancer cells.

Methods Transcriptional activity of NF-κB was measured by cell based reporter gene assay. Cell proliferation was determined by MTT assay. Cell cycle analysis was carried out by flow cytometry and apoptosis was observed by DAPI staining assy.

Results EPB inhibited the NF-κB-mediated transcription activity induced by tumor necrosis factor-α (TNF-α) and phorbol myristate acetate (PMA) in MCF-7 cells. EPB also inhibited constitutive NF-κB transcriptional activity in BT-549 cells. EPB inhibited the proliferation of both MCF-7 and BT-549 cells in a concentration- and time-dependent manner. EPB induced cell cycle arrest in G_1/G_0 phase and apoptosis in both MCF-7 and BT 549 cells.

Conclusions These in vitro results indicated that EPB has a potential for use against both hormone-dependent and hormone-independent breast cancers and its effects seem to be mediated by inhibiting the NF-κB activity.

Keywords Phenylpropranoid · NF-κB · Cyclin D_1 · Breast cancer · Cell cycle · Apoptosis

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Introduction

Breast cancer is a leading cause of morbidity and mortality of women in developed as well as in developing countries [1, 2]. Genomic instability, accumulative *mutation*, and subsequent changes in intracellular signaling cascades play key roles in the development of human breast cancers [3, 4]. Evidences suggest that members of nuclear factor kappa B (NF-κB) family genes are involved in tumor growth, differentiation, metastasis and reduced apoptosis [5–7]. Breast cancer cells contain high levels of NF-κB DNA-binding activity, which is essential for the survival of these cells in culture [8–10]. Of note, chemically induced

breast cancers in rats and many primary human breast cancers have high levels of NF-κB. High levels of activated NF-κB are also associated with the progression of breast cancer cells from estrogen-dependent phenotype to estrogen-independent phenotype [11, 12]. Inhibition of NF-κB may have a therapeutic potential for control of breast cancer progression and NF-κB may be a molecular target for breast cancer chemotherapy. The current therapeutic approach with antihormones, targeted at hormone receptors, is not always effective. All estrogen receptor negative (ER-) and also a fraction of ER positive (ER+) tumors do not respond to antihormone treatment [13, 14]. Thus, alternative treatment protocols aimed at different targets for these classes of anti-hormone non-responsive breast cancers need to be explored.

Pimpinella species have been traditionally used to treat various diseases related to inflammation and cardiovascular dysfunction for many years without having any adverse effects [15, 16]. To evaluate medicinal properties of plants from these species, a series of phenylpropanoids have been isolated from Turkish *Pimpinella* species and their structures have been determined by spectral methods [17–20]. Our preliminary study showed that some of these compounds inhibited activation of NF-κB in human chondrosarcoma SW 1353 cells. Of them, epoxypseudoisoeugenol-2-methyl butyrate (EPB, Fig. 1) isolated from *P. corymbosa*, showed the strongest inhibitory activity towards NF-κB [20]. In the present study, we evaluated the effects of EPB on the proliferation of estrogen-dependent, poorly invasive breast cancer cells (MCF-7) and estrogen-independent, invasive breast cancer cells (BT-549). Its effects on PMA and TNF-α stimulated transcriptional activity of NF-κB in MCF-7 cells, and on the constitutive activity of NF-κB in BT 549 cells were determined. Cell cycle progression was also monitored as a result of EPB treatment in both cell lines.

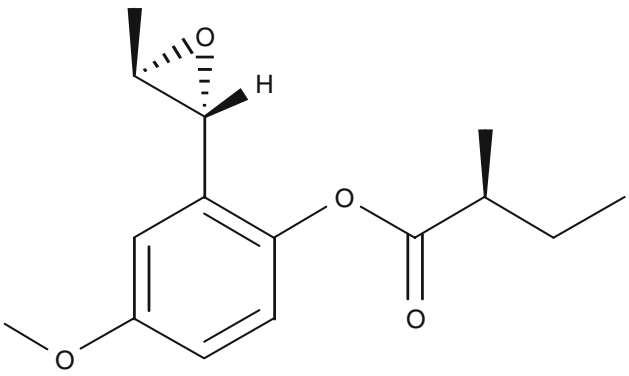

Fig. 1 The chemical structure of Epoxypseudoisoeugenol-2-methyl butyrate (EPB)

Materials and methods

Chemicals and reagents

EPB was isolated from *Pimpinella corymbosa* as described previously [19]. RPMI 1640 medium was from Gibco and fetal bovine serum (FBS) was from Atlanta Biologicals, USA. The human recombinant TNF-α, phorbol myristate acetate (PMA), 4′-6′-diamidino-2-phenylidol (DAPI), 3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolim bromide (MTT), propidium iodide (PI) and ribonuclease-type1A were from Sigma Chemical Co (ST. Louis, MO, USA). Isoton II, carrier fluid used in flow cytometry was from Becton Dickinson.

Cell culture

Human breast cancer MCF-7 (hormone-dependent) and BT-549 cells (hormone-independent) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown as monolayer in RPMI 1640 medium, supplemented with 10% FBS, and 100 U/mL penicillin G sodium and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% CO_2 and 95% humidity.

Plasmids

The nuclear factor-κB (NF-κB) reporter construct contained two copies of the element from the immunoglobulin K promoter (pBIIXLUC) and was a gift from Dr. Riccardo Dalla-Favera [21]. The Sp-1 reporter plasmid (pGL3-promoter) was obtained from Promega.

Cell based reporter gene assay

The reporter gene assay was carried out as described previously [22]. Conditions were optimized for transfection of NF-κB into the MCF-7 or BT-549 cells by electroporation. A voltage of 140 or 150 V and a single 70 ms pulse was found to be optimal for transfection of NF-κB into the MCF-7 or BT-549 cells, respectively. For the assay, 5×10^6 cells were washed once in an antibiotic and FBS-free medium (RPMI1640) and then resuspended in 500 μL of antibiotic-free medium containing 2.5% FBS. The *pBIIXLUC reporter plasmid* was added to the cell suspension at a concentration of 50 μg/mL and incubated for 5 min at room temperature. The cells were then electroporated in 500 μL medium at the optimal vo1tage (140 V for MCF-7 cells and 150 V for BT-549 cell) and one 70 ms pulse. After 10 min, cells (5×10^4 cells/mL) were seeded to the wells of 96-well plates (200 μL/well) and incubated at 37°C. After 24 h, the cells were exposed to different concentrations of EPB for 0.5 h followed by the treatment with 70 ng/mL of PMA or 50 ng/mL of TNF-α for an additional

8 h. Media were aspirated and the transfected cells were lysed by adding 40 µL of a 1:1 mixture of lucLite reagent (Promega) and PBS containing 1 mM calcium and magnesium. Light output was detected on a TopCount in a single-photon counting mode (Packard Instrument Company, Meriden, CT).

Cell growth inhibitory assay

MCF-7 and BT-549 cells in logarithmic growth were seeded at a density of 2,500 cells/well in a 96-well plate and incubated at 37°C for 24 h. Test compound was diluted in serum-free medium supplemented with antibiotics and added to the wells to achieve the desired concentrations in a 200 µL final volume. After 48 h, cell viability was determined using MTT assay [23]. 50 µL of MTT (1 mg/mL) was added to each well and the plates were incubated at 37°C under 5% CO_2 for 4 h. The blue formazan crystals formed in each well were dissolved by adding 150 µL of DMSO. The absorbance was measured at 540 nm using EL 312E microplate reader (Bio-Tek Instruments). The cell growth inhibitory activity of compounds was expressed as IC_{50} values (the concentration that caused inhibition of growth by 50% compared to control).

Cell cycle analysis by flow cytometry

Cell cycle analysis was conducted as described previously [24]. Briefly, MCF-7 and BT-549 cells (1×10^5/mL) were seeded in 25-cm^2 tissue culture flasks (8 mL/flask) 24 h prior to experiment. After 6, 12, 24 and 48 h incubation with 3 µM of EPB at 37°C, approximately 2×10^5 cells were collected by centrifugation at 1,000 rpm for 5 min. Cell pellets were fixed in 1 mL ice cold ethanol (70%) and stored at −20°C. Cells were washed twice with PBS before cell cycle assay. DNA staining was performed by addition of 200 µL propidium iodide (50 µg/mL in PBS) and 50 µL RNase A (final concentration 40 µg/mL) to each sample. Cellular DNA content was measured by a Facscan flow cytometer. Approximately 10^4 cells were analysed for each DNA content histogram. The samples were excited at 380–410 nm and the resulting fluorescence was measured at wavelengths >550 nm. Analysis of the percentage of cells in G_1/G_0, S and G_2/M phases of the cell cycle was made by the Cellquest computer program (Becton Dickinson, CA, USA).

Detection of apoptosis (DAPI staining)

DAPI staining assay was used to determine whether the mechanism of growth inhibition was related to induction of apoptosis. MCF-7 and BT-549 cells grown on chambered coverslips were treated with either vehicle or 3 µM of EPB for 48 h and then fixed with 3.7% formaldehyde for 10 min and methanol for 20 min. Fixed cells were stained with 4 µg/mL of DAPI for 15 min. The nuclear morphology of cells was observed under a fluorescence microscope.

Results

Inhibition of inducible NF-κB activity by EPB in MCF-7 cells

Effect of EPB on the transcriptional activity of NF-κB in human breast cancer MCF-7 cells was determined by transient transfection assay using luciferase reporter plasmid. MCF-7 cells were transfected with NF-κB reporter construct pBIIXLUC and treated with PMA (70 ng/mL) or TNF-α (50 ng/ml) for 8 h. As shown in Fig. 2, both PMA and TNF-α induced NF-κB-dependent transcription. EPB inhibited both PMA-induced and TNF-α-induced transcriptional activity of NF-κB in a concentration-dependent fashion. The IC_{50} values were 0.4 ± 0.1 µM for PMA and 0.6 ± 0.1 µM for TNF-α, respectively.

Inhibition of constitutive NF-κB activity by EPB in BT-549 cells

BT-549 cells were transfected with NF-κB reporter construct pBIIXLUC, and treated with PMA (70 ng/mL) or TNF-α (50 ng/ml). As shown in Fig. 3, neither TNF-α nor

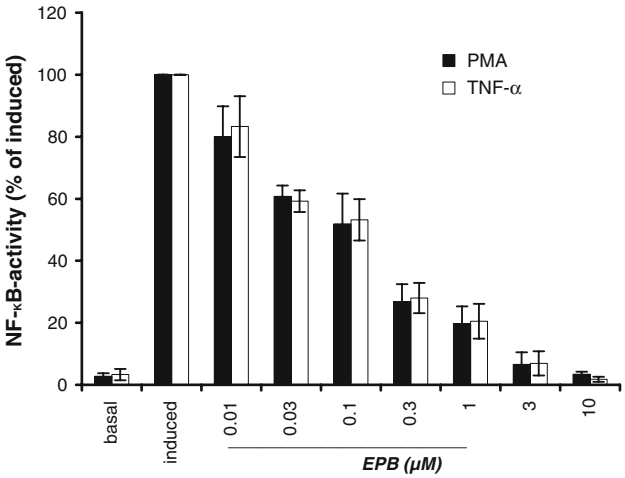

Fig. 2 Inhibition of PMA- and TNF-alpha-induced transcription of NF-κB by EPB in MCF-7 cells. Cells were transfected with reporter construct pBIIXLUC (50 µg/mL). Twenty-four hours after transfection, cells were treated with *DMSO* or EPB (0–10 µM) for 0.5 h followed by 8 h incubation with *TNF-α* (50 ng/mL) or PMA (70 ng/mL). Luciferase activity was measured as described in "Materials and methods". The results are expressed as percentage of NF-κB activity. Each bar represents the mean ± SD of three experiments. TNF-α and PMA stimulated the NF-κB activity in MCF-7 cells which was inhibited by EPB. Note: Basal = 0.25% DMSO control; Induced = 0.25% DMSO + inducers (PMA or TNF-α)

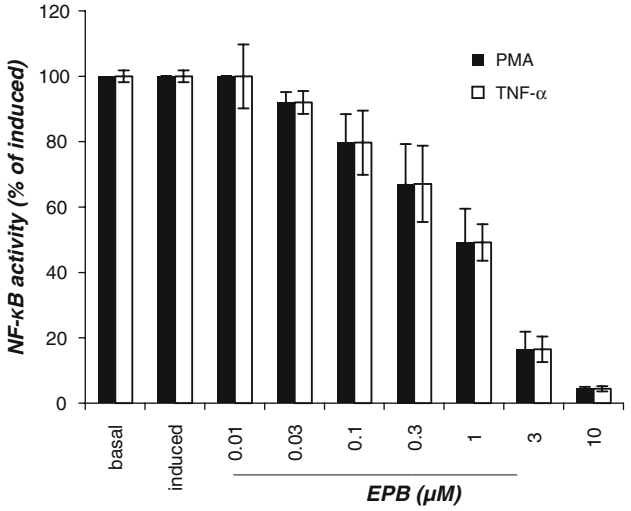

Fig. 3 Inhibition of transcription of constitutive NF-κB activity by EPB in BT-549 cells. Cells were transfected with reporter construct pBIIXLUC (50 μg/mL). Twenty-four hours after transfection, the cells were treated with DMSO or EPB (0 – 10 μM) for 0.5 h followed by 8 h incubation with TNF-α (50 ng/mL) and PMA (70 ng/mL). The luciferase activity was measured as described in "Materials and methods". The results are expressed as percentage of NF-κB activity. Each *bar* represents the mean ± SD of three experiments. Neither PMA nor TNF-α stimulated the NF-κB activity in BT 549 cells. The constitutive NF-κB activity was inhibited by EPB. Note: Basal = 0.25% DMSO control; Induced = 0.25% DMSO + inducers (PMA or TNF-α)

PMA stimulated NF-κB transcription in these estrogen-independent breast cancer cells, which indicated that NF-κB is constitutively activated in these cells. EPB dose-dependently inhibited the steady-state transcriptional activity of NF-κB. The IC_{50} values were 0.5 ± 0.2 μM for PMA and 0.7 ± 0.2 μM for TNF-α.

A luciferase construct with binding sites for Sp-1 was used as a control because this transcription factor is relatively unresponsive to inflammatory mediators. Hence, measurement of Sp-1-mediated luciferase expression is useful for detecting agents that nonspecifically inhibit luciferase expression because of cytotoxicity or inhibition of luciferase enzyme activity or light output. As shown in Fig. 4a and b, neither PMA nor TNF-α induced Sp-1 activity in MCF-7 and BT-549 cells and EPB did not inhibit transcriptional activity of Sp-1.

Inhibition of proliferation of human breast cancer cells

In order to evaluate effects of EPB on the growth of non-invasive, estrogen-dependent (MCF-7) and highly invasive, estrogen-independent (BT-549) breast cancer cells, cells were treated with EPB (0–10 μM) for 48 h. As shown in Fig. 5, EPB suppressed the growth of MCF-7 and BT- 549 cells. The inhibitory effects were dose-dependent, and the IC_{50} values were 0.7 ± 0.1 μM in MCF-7 cells and 0.9 ± 0.2 μM in BT-549 cells, respectively.

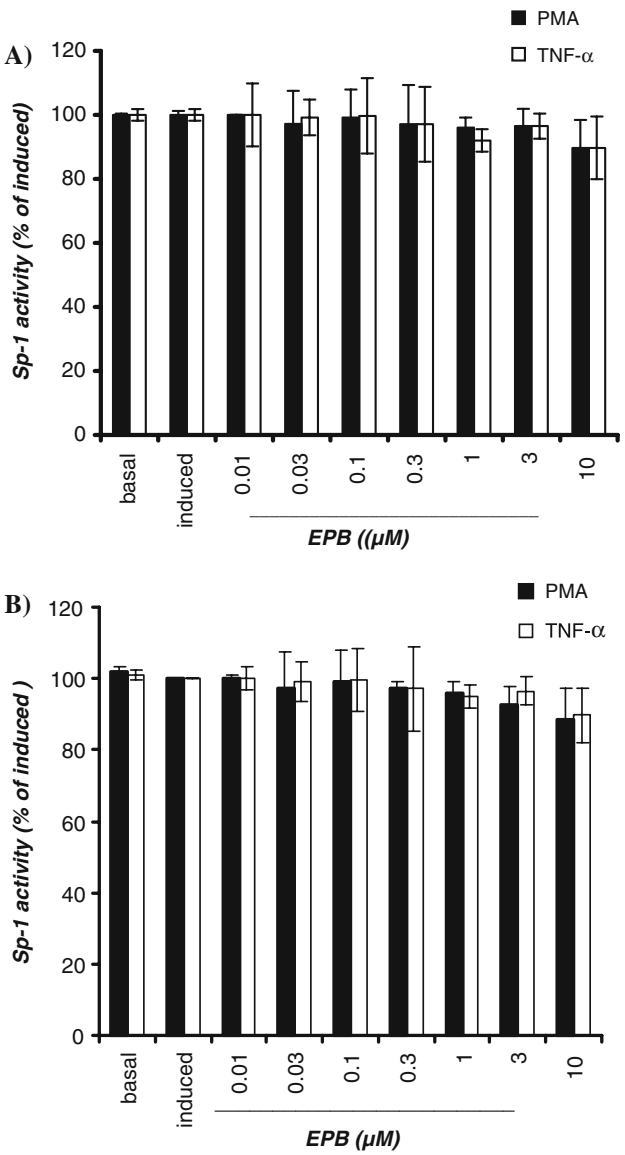

Fig. 4 Effects of EPB on transcription of Sp-1 in MCF-7 (**a**) and BT 549 (**b**) cells. MCF-7 cells and BT-549 cells were transfected with reporter construct Sp-1 plasmid (50 μg/mL). Twenty-four hours after transfection, the cells were treated with DMSO or EPB (0–10 μM) for 0.5 h followed by 8 h incubation with *TNF-α* (50 ng/mL) and *PMA* (70 ng/mL). The luciferase activity was measured as described in "Materials and methods". The results are expressed as percentage of Sp-1 activity. Each bars represents the mean ± SD of three experiments. Neither PMA nor TNF-α stimulated Sp-1 activity in MCF-7 or BT 549 cells and EPB did not affect the activity of Sp-1. Note: Basal = 0.25% DMSO control; Induced = 0.25% DMSO + inducers (PMA or TNF-α)

Effects of EPB on cell cycle

The ability of EPB to inhibit cell cycle progression was determined by flow cytometry. As shown in Fig. 6, treatment of MCF-7 cells with EPB (3 μM) for 6 h resulted in the accumulation of cells in G_1/G_0 phase, with a

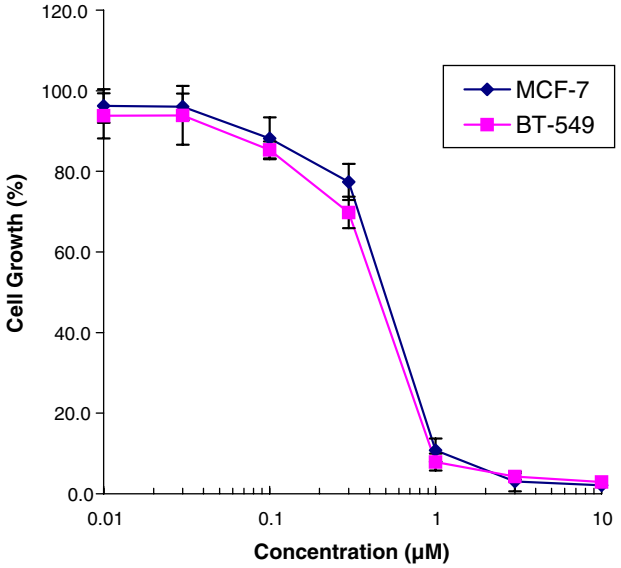

Fig. 5 Dose-response curves of EPB inhibiting the growth of MCF-7 and BT-549 cells. Each *point* is mean of three experiments performed in duplicate and the *bars* represent standard deviation

corresponding decrease in the number of cells in S and G_2/M phase. The number of cells in G_1/G_0 reached to a maximum at 12 h. After 24 h exposure, a significant increase occurred in the proportion of hypodiploid cells (peak prior to G_1 phase) while the proportion of cells at G_1/G_0 phase decreased relatively. By 48 h, most of the cells died and the cell number decreased in G_1, S, and G_2/M phase. The hypodiploid cells are likely dead cells with degraded and/or apoptotic DNA (Fig. 6). The treatment of BT 549 with EPB (3 μM) for 6 h and 12 h also resulted in the accumulation of cells in G_1/G_0 phase, with a corresponding decrease in the number of cells in S and G_2/M phase.

Induction of apoptosis

The significant growth inhibitory activity of EPB led us to investigate whether part of this effect was a result of programmed cell death induction. The cell cycle assay indicated that the cells died at 48 h of drug treatment and hypodiploids (HP) rates (a peak before G_1) increased (Fig. 6). However, it was not clear if the cells died as a result of apoptosis or necrosis. Morphological examination after DAPI staining indicated that EPB significantly induced apoptosis, as shown by cell shrinkage and nuclear fragmentation, in both MCF-7 and BT-549 cells (Fig. 7).

Discussion

Numerous experimental and clinical studies have established that estrogen plays a major role in the initiation and progression of breast cancers [25, 26]. Estrogen receptor (ER) mediates the action of estrogen, and is required for estrogen-dependent growth of breast cancers. As the cancer progresses, tumor cells acquire growth autonomy, no longer require estrogen, and become resistant to antiestrogens such as tamoxifen. Tumor heterogeneity and clonal selection of ER-negative cells, down regulation of ER expression, mutation of ER, altered regulation of ER-response genes, and generations of ER variants that function as dominant positive and estrogen-independent transcription factor are believed to be responsible for antiestrogen-resistant growth of breast cancers. These antiestrogen-resistant tumors are generally invasive and metastatic and respond poorly to chemotherapy and traditional treatment. The activation of NF-κB contributes to the progression of breast cancer from estrogen-dependent to more aggressive estrogen-independent growth [12, 27]. Specific blockage of NF-κB signaling may inhibit breast cancer progression. Using MCF-7 (estrogen-dependent) and BT-549 (estrogen-independent) human breast cancer cells as models to represent hormone-dependent breast tumors and hormone-independent breast tumors, respectively, the effects of EPB on NF-κB-mediated transcription and cell proliferation were investigated. EPB inhibited not only NF-κB driven transcription activities induced by PMA and TNF-α in MCF-7 cells, but also suppressed constitutive NF-κB transcriptional activity in BT-549 cells. EPB did not suppress transcription mediated by Sp-1 under the conditions that it suppressed transcription mediated by NF-κB. Constitutive NF-κB activation has been found to be critical for the survival and proliferation of breast cancers [12]. Cell growth inhibition assay showed that EPB inhibited the growths of both MCF-7 and BT 549 cells to a similar extent. These results indicated that EPB might inhibit the growth of breast cancer cells and the progression of breast cancer from hormone-dependent phenotype to hormone-independent phenotype. EPB seems to act at a step in NF-κB activation pathway common to all NF-κB inducers. Meanwhile our study demonstrated that there is lower level of NF-κB in ER+ breast cancer (MCF-7) cells than in ER^- (BT-549) cells. Many stimuli such as PMA, TNF-α, cytokines such as IL-1 stimulate the growth of breast cancer cells via activation of NF-κB. EPB suppressed NF-κB driven transcription induced by PMA or TNF-α and the growth of MCF-7 cells, suggesting EPB could play an important role in cancer cells of low activity of NF-κB. EPB has a potential for using against both hormone-dependent and hormone-independent breast cancers.

It has been shown that NF-κB is an important regulator of cell proliferation by its direct or indirect roles in cell cycle regulation through cyclin D_1, which is expressed early in cell cycle [27–29]. NF-κB activation also promotes

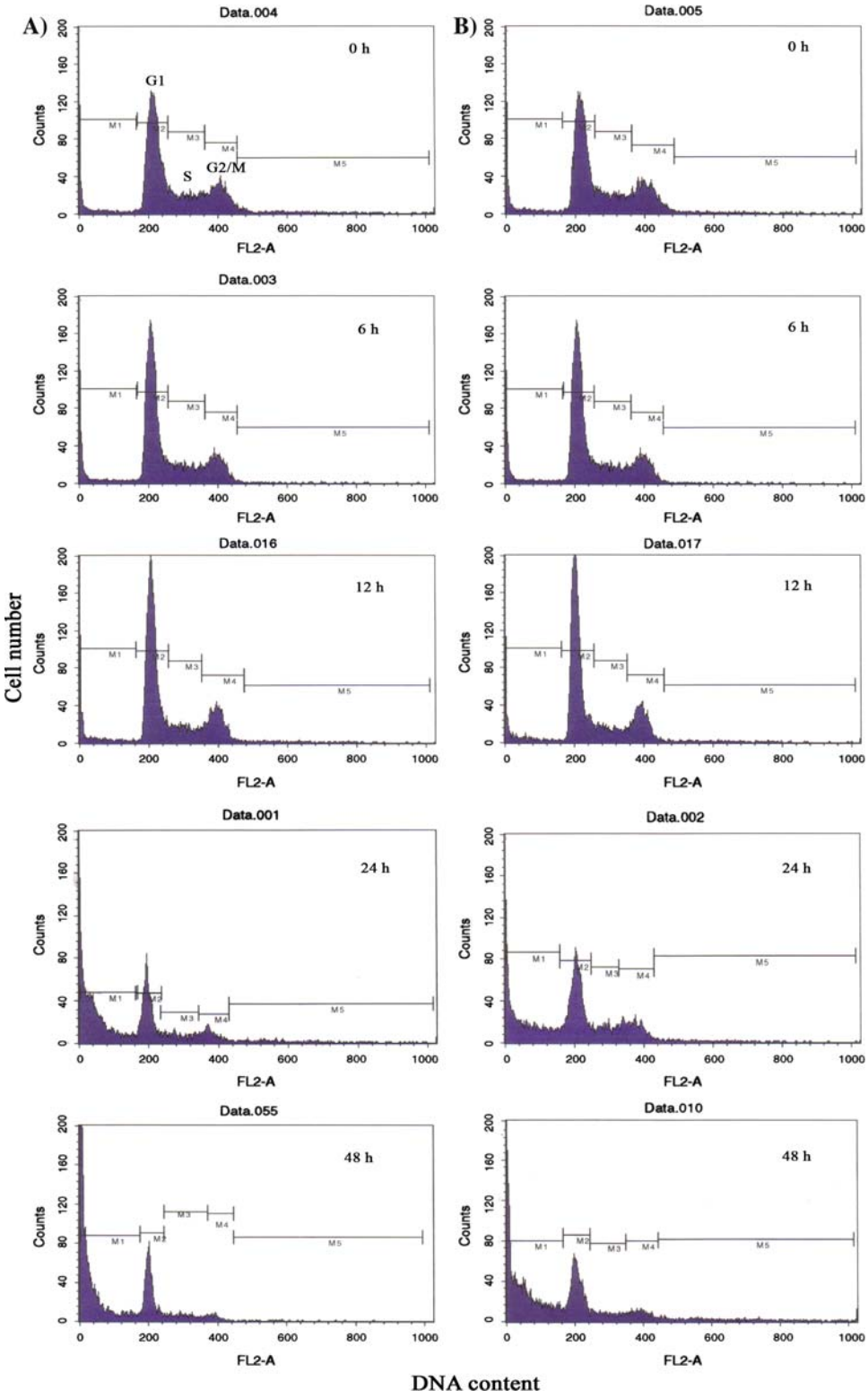

Fig. 6 Flow cytometry histograms of cell cycle distribution of MCF-7 (**a**) and BT-549 (**b**) cells treated with 3 μM EPB

the transition of the cell cycle from G_1/G_o to S phase by inducing the expression of cyclin D_1 [30–32]. Our observation that EPB blocks progression of cells in G_1/G_o phase while inhibiting NF-κB and the growth of MCF-7 and BT-549 cells, suggests that cell cycle arrest is related to inhibition of NF-κB-dependent transcription. EPB might inhibit cyclin D_1 expression through inactivation of NF-κB, finally blocking the transitions of cell cycle from the G_1/G_o to the S phase. Further studies on the effect of EPB on cyclin D_1 expression are warranted.

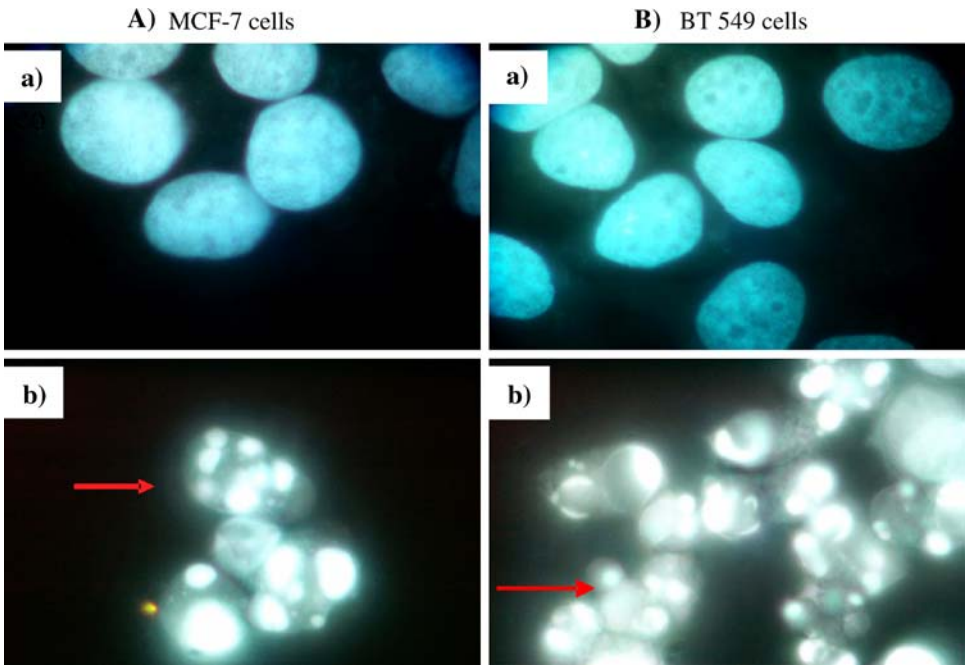

Fig. 7 Induction of apoptosis by EPB in human breast cancer MCF-7 (*A*) and BT-549 (*B*) cells. The cells were treated with vehicle (**a**) or with 3 μM EPB (**b**) for 48 h. Cells were harvested and washed with ice-cold PBS, followed by fixation in 3.7% formaldehyde and methanol. Fixed cells were incubated with 4 μg/ml of DAPI. Nuclear morphology was examined by using a fluorescent microscope. *Arrow* indicates apoptotic cells with condensed and fragmented nuclei

NF-κB has also been reported to play a pivotal role in the protection of cancer cells from apoptosis through activation of antiapoptotic genes [28, 33]. Inhibition of NF-κB activity may result in induction of apoptosis. Our cell cycle analysis results revealed that the sub-G_1 peak showing hypodiploid DNA increased after treatment with EPB for 24 h and 48 h, which is indicative of apoptotic cell death. DAPI staining further confirmed that EPB induced apoptosis in MCF-7 and BT-549 cells at the same concentration that inhibited NF-κB, suggesting that inhibition of NF-κB by EPB is associated with apoptosis.

In conclusion, EPB suppressed the proliferation of estrogen-dependent, poorly invasive breast cancer (MCF-7) and estrogen-independent, invasive breast cancer cells (BT-549). The compound also induced cell cycle arrest in G_1/G_0 phase and apoptosis. These activities are likely mediated by inhibition of NF-κB activation which could be correlated with anti-inflammatory activities reported for other phenylpropranoids [34]. Meanwhile, cell proliferation and apoptosis are complex processes which involve many pathways. NF-κB could be one of the important pathways. Therefore, inhibition of NF-κB could be one of the mechanisms involved in the inhibition of cell proliferation and induction of apoptosis by EPB. Possibility of involvement of other pathways could not be ruled out. Several preparations of *Pimpinella* species that are reported to contain EPB, have been used to treat various conditions in humans without any adverse effects [16], suggesting that it is not harmful to humans. However, further investigation on the exact mechanisms of the inhibitory effects of EPB on NF-κB and its in vivo therapeutic efficacy and toxicity are warranted.

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