Inhibition of estradiol-induced mammary proliferation by dibenzoylmethane through the $E_2$–ER–ERE-dependent pathway

Chuan-Chuan Lin, Yun-Luen Tsai¹, Mou-Tuan Huang², Yao-Ping Lu², Chi-Tang Ho³, Shun-Fu Tseng¹ and Shu-Chun Teng¹,4*

Department of Food Science, China Institute of Technology, Taipei 115, Taiwan, ¹Department of Microbiology, College of Medicine, National Taiwan University, Taipei 100, Taiwan, ²Laboratory for Cancer Research, School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854-8020, USA, ³Department of Food Science and Center for Advanced Food Technology, Rutgers University, New Brunswick, NJ 08901-8520, USA and 4Institute of Internal Medicine, National Taiwan University Hospital, Taipei 100, Taiwan

¹To whom correspondence should be addressed at: Department of Microbiology, National Taiwan University College of Medicine, No. 1, Sec. 1, Jen-Ai Road, Taipei 100, Taiwan.
Tel: +886 2 2312 3456 ext. 8282; Fax: +886 2 23915293; Email: scteng@ha.mc.ntu.edu.tw

The phytochemical dibenzoylmethane (DBM) has been shown to inhibit 7,12-dimethylbenz[a]anthracene induced mammary tumorigenesis in Sencar mice. However, the molecular basis of this activity is still elusive. In the present study, we demonstrated that DBM inhibits estradiol ($E_2$)-induced incorporation of bromodeoxyuridine into mammary DNA in immature female Sencar mice by 52%, when 10 $\mu$mol of DBM was intraperitoneally injected into mice prior to the injection of $E_2$. Examination of the influence of DBM on the expressions of $E_2$–ERE-dependent oncogenes in MCF-7 cells indicated that DBM inhibits the $E_2$-induced cell growth as well as the expressions of four oncogenes, telomerase, c-myc, Ha-ras and bcl-2. Further mechanistic study using chromatin immunoprecipitation assay demonstrated that DBM acts as a pure antagonist by attenuating the binding of estrogen receptor to the estrogen response elements in the regulatory regions of c-myc, hTERT and bcl-2 genes in vivo. Taken together, our results strongly suggest that DBM plays an inhibitory role in $E_2$-induced proliferations, which establishes DBM as a model molecule for studying the antiestrogenic drugs.

Introduction

Breast cancer is the second leading cause in cancer-related deaths among American women (1). Most breast cancers (70%) are estrogen receptor (ER) positive (2). Estrogen is a steroidal hormone essential in physiological regulation as well as a potential culprit for the occurrence of breast cancer in hormone replacement therapy (3). Two recent clinical trials indicated that taking estrogen alone or in combination with progesterone for the treatments of post-menopausal symptoms would incur the increased rate of breast and ovarian cancers (4,5). The main action of estrogen is mediated by binding to a ligand-dependent ER, thus inducing its conformational change and subsequently interacting with estrogen response elements (EREs) together with multiprotein complexes of transcription factors and co-activators to activate transcription (6). Although several alternative pathways have been reported, the $E_2$–ERE pathway is believed to play the crucial role in regulating the oncogenic expression, which results in the mitogenic effect of estradiol ($E_2$) (7–10). Therefore, there is a great deal of interest in developing drugs to block the undesired activity of estrogens.

Selective ER modulators (SERMs), a group of pharmacologically active ER ligands, have been developed and designed to mimic estrogen’s beneficial actions and prevent unwanted effects in specific tissues (11,12). The clinical application of tamoxifen and raloxifen for breast cancer prevention and therapy has brought intensive research on searching for novel tissue-SERMs, which act as estrogen agonists in the mammary as well as endometrial tissues and display estrogenic activity in other tissues in order to relieve post-menopausal symptoms (11,12). Several reports indicated that the molecular determinant for the selectivity of tamoxifen relies on its recruitment of co-activator and/or co-repressor in a tissue-specific manner (13). Recent studies showed that tamoxifen resistance correlates with high levels of SRC-3 and HER2/neu in patients (14). These findings encourage researchers to investigate new agents that can destroy the ER complex without the side effect of endocrine resistance. Fulvestrant, for example, is a pure antiestrogenic drug and has been reported for effectively treating the tamoxifen-resistant breast cancers (15).

Overexpressions of oncogenes induced by $E_2$ have been suggested to lead to the mammary tumorigenesis in animal and proliferation of human cancerous cells (7–10). Specifically, the synergistic effects of oncogenic expressions, e.g. c-myc, ras, bcl-2 and telomerase were observed in both transgenic mice and cultured cells, resulting in abrupt cellular proliferation and tumor formation (16–18). Consistently, EREs, which are required for gene expressions, have been identified in either promoter positions or coding sequences in several oncogenes (7–10).

Dibenzoylmethane (DBM), a $\beta$-diketone structural analog of curcumin, has been reported to exhibit antitumorigenic and chemopreventive activities for the past few years (19–25). Both DBM and its derivatives have been used as sun-screening agents (26). In biological aspects, DBM would modulate the Phase I/Phase II enzymatic systems, induce apoptosis in various cancer cells and, as a metal-chelator, exert beneficial effects for the ischemic diseases (19,21,23,27). In both rat and mouse models, DBM has been reported to have inhibitory effects toward carcinogen-induced mammary tumorigenesis (20,24,25).

Abbreviations: BrdU, bromodeoxyuridine; ChIP, chromatin immunoprecipitation; DBM, dibenzoylmethane; DMBA, 7,12-dimethylbenz[a]anthracene; $E_2$, estradiol; ER, estrogen receptor; EREs, estrogen response elements; i.p., intraperitoneal.

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Our previous study indicated that dietary DBM inhibits the incidence of 7,12-dimethylbenz[a]anthracene (DMBA)-induced mouse mammary tumorigenesis and decreases the number of breast tumors per mouse as well as the formation of DMBA–DNA adducts in mammary glands (20). In vitro competitive ER binding studies demonstrated a direct binding between DBM and ER in vitro (28). Further in vivo proliferation studies implicated the potential role of DBM as an antiestrogenic agent (28). The underlying mechanisms might be complex and have not been well characterized, especially its function at the molecular level. Therefore, we decided to investigate the specific role of DBM on E2-induced cell proliferation in both the mouse model and one ER-positive human breast cancer cell line, MCF7. In the current study, the expressions of four oncogenes, hTERT, c-myc, Ha-ras and bcl-2, with their ERs having been identified in ER-positive MCF-7 cells, were examined. Furthermore, chromatin immunoprecipitation (ChIP), a powerful technique for detecting the dynamic binding of transcription factors to DNA responsive elements, was employed to examine the influence of DBM on the ER binding to EREs within the promoter regions in target genes (29). The results demonstrated that DBM strongly inhibits the E2-induced proliferation in both the human breast cancer cell line MCF7 as well as in a mouse model, reduces the expression of bcl-2, c-myc, Ha-ras and hTERT, and acts as an antiestrogenic agent by attenuating the ER–ERE binding within the regulatory regions of these oncogenes. Taken together, our data suggest that DBM serves as a valuable model molecule for chemopreventive and antiestrogenic drugs in tamoxifen-resistant cases.

Materials and methods

Materials

DBM (98–99% purity) was purchased from Aldrich (Milwaukee, WI). Bromodeoxyuridine (BrDU) staining kit was purchased from Oncogene Science (Cambridge, MA). E2, tamoxifen and other chemicals were obtained from Sigma Chemical (St Louis, MO).

Cell culture and proliferation assay

MCF-7 cells were grown and passed routinely as monolayer cultures in 10-cm cell-culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum, MEM non-essential amino acids, penicillin and streptomycin in the presence of 5% of CO2 at 37°C. MCF-7 cells were grown and passaged routinely as monolayer cultures in 10-cm cell-culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum, MEM non-essential amino acids, penicillin and streptomycin in the presence of 5% of CO2 at 37°C.

Soft agar colony assay

MCF-7 cells were seeded at cell densities of 10⁴ and 2 × 10⁴ in agar media containing 10 mM E2 and 1 μM DBM, alone or together. Agar medium was prepared according to the standard assay conditions except that 15% fetal calf serum was used (30).

Quantitative RT–PCR analysis

Cells were harvested by trypsin solution and collected by centrifugation. Total cellular RNAs were extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) as described by the manufacturer’s protocol and cDNA was then synthesized according to the standard protocol. The expressions of hTERT, c-myc, bcl-2, Ha-ras and GADPH genes were determined quantitatively by real-time PCR using the SYBR Green PCR Master Mix in an ABI PRISM 7700 Sequence Detector. Briefly, hTERT, c-myc, bcl-2, Ha-ras and GADPH cDNAs were amplified using the primer pairs: 5′-GGACAAACGCTTGGCAGG-G3′ and 5′-TTGGTTTCCCGTGGTG-3′ for hTERT; 5′-CTCTCCAACGCCCCACTGTGTC-3′ and 5′-CTTTGGCAGCAGATTCTCTCC-3′ for c-myc; 5′-AGGCTGAGATTCCAGCGA-AGTACA-3′ and 5′-AGGACAGCAGCTCACAAGCT-3′ for bcl-2; 5′-ACGGTTGCGTGAGATCC-3′ and 5′-CGTCAAGGACACACATCTTG-3′ for Ha-ras; and 5′-GGAGTTGAAAGTGCTGGATC-3′ and 5′-GAAGATGGTGATGGGATTCTC-3′ for GADPH. Relative gene expressions were normalized to the expression of GADPH.

ChIP assay

Cells were grown to 95% confluence in phenol red-free DMEM supplemented with 10% charcoal-dextran-stripped fetal bovine serum for at least 3 days. Following the addition of hormone, cells were washed with PBS and cross-linked with 1% formaldehyde at room temperature for 10 min and the reaction was stopped by adding glycerine to a final concentration of 0.125 M. Cells were washed subsequently with 1 ml of ice-cold PBS, and then resuspended in 5 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1, 1× protease inhibitor), pelleted by centrifugation and suspended in 2 ml of IP buffer (100 mM Tris, pH 8.6, 0.5% SDS, 1.7% Triton X-100, 5 mM EDTA). The cell suspension was sonicated with a 0.25 in. diameter probe for 15 s twice using an MNE-Soniprep1500 sonicator (setting 18) and then was collected by centrifugation for 10 min. For each immunoprecipitation, 1 ml of lysate was precleared by adding 50 μl of blocked Protein A beads (50% Protein A-Sepharose, Amersham Biosciences; 0.5 mg/ml BSA, 0.2 mg/ml salmon sperm DNA) at 4°C for 1 h. The supernatants were incubated at 4°C for 1 h with specific antibodies. Immunocomplexes were recovered by adding 50 μl of blocked Protein A beads and incubated overnight at 4°C. After immunoprecipitation, beads were washed sequentially with 1 ml of mixed micelle buffer (20 mM Tris, pH 8.1, 150 mM NaCl, 5 mM EDTA, 5% w/v sucrose, 1% Triton X-100, 0.2% SDS), buffer 500 (50 mM HEPES, pH 7.5, 0.1% w/v deoxycholic acid, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA), and LiCl detergent wash buffer (10 mM Tris, pH 8, 0.5% deoxycholic acid, 0.5% Nonidet P-40, 250 mM LiCl, 1 mM EDTA). Beads were then washed two times with TE buffer (10 mM Tris, 1 mM EDTA) and extracted two times with 1% SDS and 0.1 M NaHCO3. Elutes were pooled and incubated at 65°C for 6 h to remove the formaldehyde cross-linking. After digestion with protease K, the DNA was phenol/chloroform extracted and ethanol precipitated. DNA fragments were resuspended in 50 μl of water and 5 μl of DNA extract was subjected to RT–PCR analysis with 21–28 cycles of amplification. The primers used were 5′-GATCCCTCTCCTGTAATCTC-3′ and 5′-CTCGCTGGAAAT- TACTACACG-3′ for c-myc; 5′-CAACCTCCTGCTCCTGGGT-3′ and 5′- GGTAAATGAGCCAGGTCGAT-3′ for hTERT; and 5′-CCCATC- CACCGCCTACCC-3′ and 5′-AAGAAGGCCAATCCTCCC-3′ for bcl-2. Input was 2% of total input lysates.

Animals

Female Sencar mice (26–28 days old) were purchased from the Charles River Breeding Laboratories (Kingston, NY). The animals were kept in our animal facility for at least 1 week before use. Purina Laboratory Chow 5001 diet was given and water ad libitum. The mice were kept at a 12 h light/12 h dark cycle during this adaptative period.

Incorporation of BrDU into mammary gland DNA in female immature Sencar mice

Female Sencar mice were given a single intraperitoneal (i.p.) injection of DBM, tamoxifen or vehicle control in 0.1 ml corn oil. The mice were injected subcutaneously with E2 (0.3 μg in 0.1 ml of 10% ethanol in saline), 3 h before they were killed.

Incorporation of BrDU into DNA was performed as described previously (3). BrDU, a thymidine analog that is incorporated into proliferating cells during the S-phase, is detected by digestion of the DNA by a biotinylated mononclonal anti-BrDU antibody and visualized using streptavidin peroxidase and 3,3′-diaminobenzidine, which stains BrDU-containing nuclei a dark brown using a staining kit from Oncogene Science (Cambridge, MA). Briefly, all the animals were injected intraperitoneally with BrDU (50 mg/kg body wt) and killed 1 h later. Mammary glands and uterus were removed and stored in a −80°C freezer for the BrDU labeling index assay.

Statistical methods

All values are expressed as means ± standard error of the mean. Differences between groups were tested using Student’s t-test and Fisher’s exact test. P values <0.05 were considered significant.
Results

**DBM inhibited the growth of E2-induced ER-positive MCF-7 breast cancer cells**

In previous studies, we observed that the structural resemblance between DBM and E2 might result in the antagonistic action of DBM toward binding to the ER in mouse mammary glands. To examine this possibility, we first tested the effect of DBM on E2-stimulated growth of ER-positive MCF-7 breast cancer cells in both liquid- and solid-based media. As shown in Figure 1A, after 6 days of incubation with 10 nM E2 or 1 μM DBM, alone or in combination, the proliferation of MCF-7 induced by E2 was significantly inhibited by DBM, although no growth effect was observed on the treatment of DBM alone. In soft agar assays, the number of colonies was remarkably increased by ~3-fold at two different cell concentrations (10⁴ and 2 × 10⁴) of MCF-7 cells seeded in an agar media containing 10 nM of E2. Figure 1B illustrated that 1 μM of DBM treatments exhibited inhibitory effects on the colony formation abilities stimulated by E2 at both seeding conditions. These results demonstrated that DBM inhibits the growth of E2-induced ER-positive MCF-7 cells.

**DBM inhibited the expressions of the E2–ER–ERE-dependent oncogenes**

To examine how DBM affects E2-dependent cell proliferation, the expressions of four oncogenes, bcl-2, c-myc, Ha-ras and hTERT, with their EREs having been identified in ER-positive MCF-7 cells, were examined by quantitative RT–PCR, a technique for quantitative analysis of gene expression with high specificity. In a time-course study, MCF-7 cells were treated with 100 nM of E2 for several time intervals and the results indicated that E2-induced c-myc, Ha-ras and bcl-2 reached their maximum expression levels after induction for 2 h, whereas hTERT required 8 h to aggrandize its climax (data not shown). The expression levels of hTERT, c-myc, Ha-ras and bcl-2 in E2-treated cells were increased by 4.6-, 4.1-, 2.4- and 5.4-fold, respectively, compared with the E2-untreated control (Figure 2A). Treatment of 10 μM of DBM together with 100 nM E2 reduced the expression of these four oncogenes to the basal levels, for c-myc, where a lesser extent of attenuation was observed. The inhibitory pattern of DBM was also compared with that of tamoxifen, a SERM that antagonizes the estrogenic action of E2 in MCF-7 cells. Both DBM and tamoxifen exhibited similar patterns of inhibition as shown in Figure 2. Tamoxifen decreased the expressions of hTERT, c-myc and bcl-2. However, tamoxifen did not show significant reduction of expression in Ha-ras. Expression of hTERT, Ha-ras and bcl-2 were examined using a range of DBM concentrations (1, 5, 10 and 20 μM) (Figure 3). We observed concentration-dependent inhibitory pattern of DBM. Treatment of DBM together with 100 nM E2 strongly reduced the expression of these three oncogenes to the basal levels at ~5 μM of DBM. These results suggested that DBM inhibits the expression of E2-regulated oncogenes, which might attribute to its inhibitory effect on E2-stimulated cellular proliferation.

**DBM attenuated the binding of ER to the EREs of c-myc, hTERT and bcl-2**

To further investigate the underlying mechanism of DBM as an antiestrogenic agent, ChIP was employed to examine the DBM on the ER binding to EREs within the regulatory regions. DBM inhibited the growth of MCF-7 breast cancer cells. The number of viable cells was determined by the trypan blue exclusion. The numbers of colonies were counted after 10–14 days of incubation. The data are shown as the mean ± SD from three independent experiments. Asterisk indicates significantly different from E2-treated groups.

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Fig. 1. DBM inhibited the growth of MCF-7 cells. (A) MCF-7 cells were treated with 10 nM E2, 1 μM DBM or 10 nM E2 + 1 μM DBM during 6 days of incubation. The number of viable cells was determined by the trypan blue exclusion. (B) Soft agar assay of MCF-7 cells seeded at cell densities of 10⁴ and 2 × 10⁴ in an agar medium containing 10 nM E2 and 1 μM DBM, alone or together. The numbers of colonies were counted after 10–14 days of incubation. The data are shown as the mean ± SD from three independent experiments. Asterisk indicates significantly different from E2-treated groups.

Fig. 2. DBM and tamoxifen (Tam) affected the expressions of the E2–ER–ERE-dependent oncogenes in MCF-7 cells. MCF-7 cells were treated with EtOH, 100 nM E2, 100 nM E2 + 10 μM DBM and 10 μM DBM (A) or EtOH, 100 nM E2, 100 nM E2 + 1 μM Tam and 1 μM Tam (B) for 8 h to detect hTERT expression or 2 h to others. RNA was extracted and cDNA was then synthesized according to the standard protocol. The expression of hTERT, c-myc, Ha-ras and bcl-2 were measured by quantitative PCR analysis. Data are presented as the mean ± SD from triplicate determinations. Significant differences (P < 0.05) are indicated by asterisks. Asterisk indicates significantly different from vehicle-treated groups. Double asterisk indicates significantly different from E2-treated groups.

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of the target genes. Both hTERT and bcl-2 promoters harbor imperfect palindromic E2-responsive elements (at −2677 for hTERT and at +195 and +276 for bcl-2), which interact directly with ER. c-myc has been reported to contain a 116 bp GC-rich non-classical ERE (at +25 to +141). Our results showed that DBM alone or together with E2 attenuated ERα binding to the responsive elements in these three genes in comparison with the E2-treated control (Figure 4A). In real-time PCR analysis used to quantify the signals from ChIP, treatment of DBM exhibited at least 50% decrease in the binding efficiency of ER to the EREs (Figure 4B). In particular, a decrease of >50% in the binding efficiency was observed in the ERE of c-myc. Therefore, DBM significantly attenuates the binding of ER to the EREs.

DBM inhibited the E2-induced mammary gland proliferation in a mouse model

Finally, we examined the antiproliferative effect of DBM in the mammary glands of immature mice induced by E2. First, female Sencar mice were given a single injection (i.p.) of varying concentrations of DBM together with 0.3 µg of E2. Although E2 stimulated in a dose-dependent manner (Figure 5A), DBM inhibited E2-induced incorporation of BrdU into mammary DNA in immature female Sencar mice in a dose-dependent manner (Figure 5B). In another set of mouse model, female Sencar mice were injected intraperitoneally with 10 µmol of DBM, tamoxifen or vehicle control before injection of 0.3 µg of E2 once a day for 2 days. BrdU (50 mg/kg body wt) was injected intraperitoneally 2 h after the second administration of E2 and all mice were killed 1 h later. Figure 5C shows that DBM and tamoxifen reduced the BrdU incorporation in Sencar mice by 52 and 40%, respectively. These results demonstrated that DBM, similar to tamoxifen, inhibits the E2-induced mammary gland proliferation in a mouse model.

Discussion

Studies from our laboratory and others have shown that dietary DBM strongly inhibited DMBA-induced mammary carcinogenesis in mice and rats (24,25). Preliminary in vivo studies suggested that the decreased formation of DMBA–DNA adducts by DBM in mouse models could result from either its effect on the metabolism of DMBA and E2 through Phase I and/or Phase II enzymatic systems or on the proliferation rate of mammary gland via the hormonal mitogenic pathway (24,25). The former possibility has been confirmed and demonstrated previously (21–23,28). In vitro competitive ER binding studies have demonstrated a direct binding between
DBM and ER in vitro (28). The concentration–response curves of [3H]E2 versus DBM were compared (28). The results indicated that at 4 μM concentration E2 showed 50% of inhibition, whereas DBM exhibited weaker effect of ~30% inhibition.

Our current studies demonstrated for the first time that DBM lowers the proliferation rate of the mammary glands induced by E2 and inhibits the expressions of E2-regulated oncogenes in MCF-7 cells, via attenuation of the ER–ERE binding in vivo.

Tumor formation and maintenance require promoting pathways involving growth proliferation, telomerase activation and antiapoptosis (31). Overexpressions of oncogenes induced by E2 have been correlated to the mammary tumorigenesis in animals and proliferation of human cancerous cells (6). Several EREs present in either promoter positions or coding sequences for the transcription of oncogenes, have been identified, including those in hTERT, c-myc, bcl-2 and Ha-ras genes (7–10). The present study quantitatively demonstrates that DBM strongly reduced the expressions of the E2–ER–ERE-dependent oncogenes, hTERT, bcl-2 and Ha-ras to the basal levels. However, a weaker attenuation was observed in c-myc expression. Previous structure studies with respect to the variations among ligands revealed that the resulting conformational changes of ER may influence the abilities of co-activators and co-repressors to interact with the promoter in a cell-specific manner. Most of these studies used in vitro gel mobility assay or artificial reporter assay which is difficult to explain the in vivo effect in the context of cellular genes in their native chromatin forms. To address the direct role of DBM in cancer preventions, this is the first report using ChIP with antibody against ER to directly detect the effect of DBM on the E2–ER–ERE binding. Our results showed that DBM acts as an antiestrogenic agent by attenuating ~50% of ER binding to the EREs of c-myc, hTERT and bcl-2 (Figure 4B). However, the c-myc where the ER association with the ERE was reduced by DBM in ChIP assays showed less reduction of mRNA transcript (Figure 2A). Here, ER association with ERE does not relate to mRNA expression, implying other processes may dominate the regulation of c-myc expression in MCF-7 cells. Dubik and Shiu (10) have reported that the anti-ER complex may weakly transactivate the c-myc gene expression through ubiquitous and unknown interactions among ER, cellular cofactors and a specific ERE sequence in c-myc. Additionally, we observed that estradiol and/or DBM activated the expressions of c-myc in an ER-negative breast cancer cell line AU565 (data not shown), indicating that it exists in ER-independent pathways to coregulate the expression of c-myc.

In summary, a study on the anti-estrogenic effect of DBM against E2 was performed in both human breast cancer cell and mouse mammary gland. Our results indicated that in MCF-7 human breast cancer cells, DBM inhibits E2-induced proliferation through the reduction of expression of the E2–ER–ERE-dependent oncogenes. Further studies on its mechanism using ChIP demonstrated that DBM acted as a pure antagonist by attenuating the binding of ER to the EREs in the regulatory regions of c-myc, hTERT and bcl-2 in vivo. In an immature mouse model, DBM inhibited the E2-induced mammary gland proliferation. Recent studies showed that isoflavone genistein,
an estrogenic compound, has a biphasic effect (32). In our unpublished yeast-based studies (33), we found that DBM is not estrogenic, whereas genistein is. Taken together, our study demonstrated that DBM might act as a pure antagonist by reducing the ER binding to the EREs of the oncogenes in vivo. Recent works from both laboratory and clinical research indicated that the exhaustive use of tamoxifen resulted in drug-resistant cases by altering the SERM–ER inhibitory signal to a growth stimulatory one (11,14). High levels of SRC-3 and HER2/neu expression were observed in ER-positive breast cancer patients with tamoxifen resistance (14). Although estrogen purges may be prescribed cyclically in combination with tamoxifen for breast cancer patients in long-term treatments, any strategy to apply a single pure antagonist that entirely eliminates the oncogenic action of ER would be valuable (11). Therefore, our study provides evidence that DBM could be a valuable model molecule for chemopreventive and antiestrogenic drug in tamoxifen-resistant cases.

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