

Apigenin inhibits antiestrogen-resistant breast cancer cell growth through estrogen receptor- α -dependent and estrogen receptor- α -independent mechanisms

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Abstract

Breast cancer resistance to the antiestrogens tamoxifen (OHT) and fulvestrant is accompanied by alterations in both estrogen-dependent and estrogen-independent signaling pathways. Consequently, effective inhibition of both pathways may be necessary to block proliferation of antiestrogen-resistant breast cancer cells. In this study, we examined the effects of apigenin, a dietary plant flavonoid with potential anticancer properties, on estrogen-responsive, antiestrogen-sensitive MCF7 breast cancer cells and two MCF7 sublines with acquired resistance to either OHT or fulvestrant. We found that apigenin can function as both an estrogen and an antiestrogen in a dose-dependent manner. At low concentrations (1 $\mu\text{mol/L}$), apigenin stimulated MCF7 cell growth but had no effect on the antiestrogen-resistant MCF7 sublines. In contrast, at high concentrations (> 10 $\mu\text{mol/L}$), the drug inhibited growth of MCF7 cells and the antiestrogen-resistant sublines, and the combination of apigenin with either OHT or fulvestrant showed synergistic, growth-inhibitory effects on both antiestrogen-sensitive and antiestrogen-resistant breast cancer cells. To further elucidate the molecular mechanism of apigenin as either an estrogen or an antiestrogen, effects of the drug on estrogen receptor- α (ER α); transactivation activity, mobility, stability, and ER α -coactivator interac-

tions were investigated. Low-dose apigenin enhanced receptor transcriptional activity by promoting interaction between ER α and its coactivator amplified in breast cancer-1. However, higher doses (> 10 $\mu\text{mol/L}$) of apigenin inhibited ER α mobility (as determined by fluorescence recovery after photobleaching assays), down-regulated ER α and amplified in breast cancer-1 expression levels, and inhibited multiple protein kinases, including p38, protein kinase A, mitogen-activated protein kinase, and AKT. Collectively, these results show that apigenin can function as both an antiestrogen and a protein kinase inhibitor with activity against breast cancer cells with acquired resistance to OHT or fulvestrant. We conclude that apigenin, through its ability to target both ER α -dependent and ER α -independent pathways, holds promise as a new therapeutic agent against antiestrogen-resistant breast cancer. [Mol Cancer Ther 2008;7(7):2096–108]

Introduction

The majority of human breast tumors are estrogen receptor- α (ER α) positive and thus depend primarily on estrogens for growth (1). Currently, the first-line agent for treatment of ER α + breast cancer in both premenopausal and postmenopausal women is the antiestrogen tamoxifen (OHT). The second-line drug given to postmenopausal women with receptor-positive, OHT-resistant tumors is fulvestrant (Faslodex; ICI 182,780), a selective ER down-regulator (2). These two antiestrogens are extremely important breast cancer therapeutics; however, not all ER α + breast cancers respond to OHT and fulvestrant, and for those women who do respond, initial positive responses can be of short duration (3), with most tumors eventually developing complete resistance to both of these agents (4). Clearly, more effective drugs are needed to enhance the efficacy of OHT and fulvestrant in antiestrogen-insensitive and/or antiestrogen-resistant breast tumors.

The development of antiestrogen-resistant breast cancer is associated with a myriad of cellular, molecular, and biochemical alterations (5). Multiple changes in ER α signaling and protein kinase pathways, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, and cyclic AMP protein kinase A (PKA; refs. 6, 7), have all been reported. The receptor coactivator amplified in breast cancer-1 (AIB1), which is often overexpressed in breast cancer, functions as an oncogene by transmitting kinase-mediating growth factor signaling to the ER α (8). Previously, we established and characterized both OHT-resistant (MCF7-T) and fulvestrant-resistant (MCF7-F) breast cancer cell lines (9). We further showed significant changes in ER α , AIB1, protein kinases, and growth factor pathways in the antiestrogen-resistant

Received 11/30/07; revised 3/10/08; accepted 3/18/08.

Grant support: NIH/National Cancer Institute grants CA 085289 and CA 113001, U.S. Army Medical Research Acquisition Activity awards DAMD 17-02-1-0418 and DAMD 17-02-1-0419, American Cancer Society Research and Alaska Run for Woman grant TBE-104125, and Walther Cancer Institute.

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doi:10.1158/1535-7163.MCT-07-2350

sublines compared with the parental MCF7 cell line (9). As ER α and protein kinases play critical roles in breast cancer cell proliferation and antiestrogen resistance, targeting both pathways simultaneously could likely inhibit both genomic and nongenomic effects of estrogen and also serve as a potential strategy for the treatment or prevention of antiestrogen-resistant breast cancer.

Apigenin [chemical name 5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one], a known phytoestrogenic compound (10–12), is a naturally occurring, nontoxic, nonmutagenic, plant flavonoid commonly present in various fruits and vegetables (13). Apigenin has shown both anti-inflammatory and anticarcinogenic effects in various animal tumor model systems, including breast, colon, skin, thyroid, leukemia, and prostate (14–16). Epidemiologic studies suggest that flavonoids play an important role in reducing the risk of breast cancer (17, 18). Although the effects of apigenin and other flavonoids appear to be mediated through ER α binding-dependent and binding-independent pathways (19), the precise mechanism of apigenin on breast cancer cell growth inhibition is not clear. Interestingly, apigenin displays dose-dependent alternate effects, enhancing 17 β -estradiol (E₂)-induced DNA synthesis at low concentrations while inhibiting DNA synthesis at high concentrations (12). Furthermore, by competing with ATP, apigenin has been shown to be an inhibitor of protein kinases (20). Although the antiproliferative activity of apigenin is widely accepted, it has also been shown to stimulate breast cancer cell growth (21), further showing its complexity.

In the current study, we examined the effect of apigenin on antiestrogen-sensitive and antiestrogen-resistant breast cancer cells. We investigated whether apigenin can enhance the growth-inhibitory efficiency of both OHT and fulvestrant in hormone-sensitive and antiestrogen-resistant breast cancer cells. We showed that low concentrations of apigenin stimulated proliferation of hormone-sensitive MCF7 cells, high doses of apigenin inhibited both hormone-sensitive and drug-resistant breast cancer cell growth, and the synergistic action with OHT and fulvestrant in antiestrogen-resistant breast cancer cells. We further showed that high doses of apigenin blocked ER α mobility and transcriptional activity, induced degradation of ER α and its coactivator AIB1, and inhibited the activities of multiple protein kinases involved in antiestrogen resistance, including MAPK, PKA, p38, and AKT. This is the first study to show that apigenin can function as both an antiestrogen and a protein kinase inhibitor and has activity against breast cancer cells with acquired resistance to OHT and fulvestrant.

Materials and Methods

Materials

The following antibodies and reagents were used in this study: anti-PKAB β and anti-ER α (Santa Cruz Biotechnology); monoclonal anti-human ER α and mouse anti-glyceraldehyde phosphate dehydrogenase (Chemicon International); mono-

clonal anti-AIB1 antibody (BD Biosciences); protease inhibitor mixture set III (Calbiochem-Novabiochem); LipofectAMINE Plus reagent and cell culture medium (Invitrogen); FuGene (Roche Molecular Biochemicals); apigenin, E₂, 4-hydroxytamoxifen, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and MG132 (Sigma); ICI 182,780 (Tocris Cookson); passive lysis buffer and Luciferase Assay System (Promega), Galaco-Star luminescent assay kit (PE Applied Biosystems); IRDye 700DX-conjugated affinity-purified anti-mouse IgG (H&L) and IRDye 800-conjugated affinity-purified anti-rabbit IgG (H&L; Rockland Immunochemicals).

Plasmid Construction

pBD-Gal4-ER α AF2 was constructed by cloning ER α AF2 into pBD-Gal4 vector (Stratagene; ref. 22). Creation of the ERE2pS2-Luc reporter plasmid was described previously (22). Wild-type ER α pSG5-ER α (HEGO) was kindly provided by Dr. Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire) and GFP-ER α was provided by Dr. Michael Mancini (Baylor College of Medicine; ref. 23). Constitutively active AKT was kindly provided by Dr. Harikrishna Nakshatri (Indiana University School of Medicine; ref. 24). The plasmids pFA-CHOP, pFA2-CREB, pFA2-Elk1, and pFR-Luc for PathDetect Trans-Reporting Systems were purchased from Stratagene. pcDNA3-AIB1 was kindly provided by Dr. Myles Brown (Dana-Farber Cancer Institute, Harvard Medical School). The plasmid pAD-Gal4-rAIB1 was described previously by cloning rat coactivator AIB1 (rAIB1) into pAD-Gal4 vector (22).

Cell Culture and Proliferation Assays

The OHT- and fulvestrant-resistant sublines MCF7-T and MCF7-F have been described previously (9). MCF7 human breast cancer cells were purchased from the American Type Culture Collection. To assess the effects of apigenin, E₂, ICI 182,780, or 4-hydroxytamoxifen on cell proliferation, cells (2,000 per well) were plated in 96-well dishes in hormone-free medium for 3 days before drug exposure. Cell numbers were determined by MTT (25) assay at the indicated times after drug treatment. Following drug treatment, cells were exposed to MTT for 4 h and solubilized in DMSO, and the MTT metabolite formazan was quantitated at 600 nm using a Bio-Tek ELX-800 microplate absorbance reader.

Western Blot and Quantitation

Whole-cell lysates were prepared in 1 \times SDS sample buffer by sonication, and the supernatant protein concentration was then determined using a Bio-Rad protein assay kit. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were then incubated with primary antibody followed by incubation with infrared dye IR700-labeled goat anti-mouse IgG or IR800-labeled goat anti-rabbit IgG (LI-COR) secondary antibodies and quantified with LI-COR imaging system and Odyssey software. All Western blots were done at least two times.

Coimmunoprecipitation

Lysates from MCF7 cells were prepared in lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP-40, 0.5% Triton X-100, 1 mmol/L Na₃VO₄,

protease inhibitor] and incubated with protein G-agarose for 30 min at 4°C. After centrifugation at 12,000 × *g* for 15 s, the precleared supernatants were incubated with 5 μL anti-ERα antibody or IgG at 4°C for 3 h followed by incubation with 30 μL protein G-agarose beads for 30 min. The beads were then pelleted by brief centrifugation, washed three times with TBS, and finally resuspended in 30 μL SDS-PAGE loading buffer for SDS-PAGE and Western blotting.

Transient Transfection Assay and Reporter Enzyme Assays

MDA-MB-231 or HeLa cells were cultured in hormone-free medium for 3 days and then transfected with equal amounts of total plasmid DNA (adjusted by the corresponding empty vectors) using LipofectAMINE Plus reagent or FuGene according to the manufacturer's guidelines. Unless stated otherwise, 24 h after transfection, cells were treated with the specified drug. At the end of the experiment, cell lysates were prepared for reporter enzyme assays using passive lysis buffer according to the manufacturer's instructions. Luciferase and β-galactosidase activities were determined using the Luciferase Assay System (Promega) and Galaco-Star assay kit (Applied Biosystems), respectively. Luciferase activity was corrected for transfection efficiency by expression as ratio of luciferase to β-galactosidase activities.

Quantitative Real-time PCR

Cells were cultured in basal medium for 3 days and treated with apigenin (10 μmol/L). Total RNA was prepared by a RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA (2 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) according to the instructions of the manufacturer. The resulting cDNA (equivalent to 40 ng total RNA) was used in quantitative real-time PCR using FastStart DNA MasterPLUS SYBR Green I (Roche Applied Science) and LightCycler according to the instructions of the manufacturer.

Yeast Two-Hybrid Reporter Assays

The yeast two-hybrid reporter assay was done as described previously (26). Briefly, yeast cells (strain PJ69-4A; a gift from Dr. Philip James, University of Wisconsin-Madison) were transformed with pAD-Gal4-rAIB1 and pBD-Gal4-ERαAF2. To measure the strength of the interaction of AIB1 with ERα in the presence of apigenin, β-galactosidase expression levels in liquid yeast cultures from three independent transformants were determined using Galaco-Star luminescent assay kit (Applied Biosystems).

Yeast Estrogen Reporter Assay

The yeast estrogen-responsive reporter assay was done as described previously (22). Briefly, yeast cells (RS188N) were transformed with vectors for ERα, ERE-β-gal reporter gene and either empty pAD-Gal4 vector or pAD-Gal4-rAIB1. Cells were grown overnight in selection medium (lacking uracil, tryptophan, and/or leucine) and then divided into treatment groups. Test compounds were dissolved in DMSO. The β-galactosidase activity in yeast

was assayed 18 h later using a Galaco-Star luminescence assay kit (Applied Biosystems).

Live-Cell Microscopy and Fluorescence Recovery after Photobleaching

Live-cell fluorescence microscopy and fluorescence recovery after photobleaching (FRAP) were done on MCF7 cells transfected with GFP-ERα. Cells were grown on 12-mm coverslips in six-well plates and transfected with GFP-ERα plasmid using FuGene (27) and subsequently maintained in MEM with 5% dextran-coated charcoal-stripped fetal bovine serum at 37°C. Transfected cells were then treated for 1 h with apigenin (10 μmol/L), E₂ (10 nmol/L), 4-hydroxytamoxifen (100 nmol/L), or ICI 182,780 (100 nmol/L), and FRAP analysis was carried out on a Spinning disk confocal microscope (Yokogawa CSU10/Nikon TE 2000) using Metamorph7.1 software (Molecular Devices) and the MOSAIC Digital Diaphragm System (Photonic Instruments). A single z-section was imaged before and at various time intervals after the 2-s bleach. Images were taken every 5 s for a 5-min period.

Statistical Analyses

P values were determined by ANOVA statistical test and Student's *t* test.

Results

Dose-Dependent Effects of Apigenin on Growth of Breast Cancer MCF7 Cells

The common flavonoid apigenin has been well established to have antiproliferative activity against numerous cancer types, including breast cancer (28, 29), and several mechanisms have been proposed for its antineoplastic effects (15, 30). However, apigenin was also paradoxically shown to stimulate proliferation of breast cancer cells (21), and consequently, we sought to further investigate the divergent effects of this drug. To examine the effects of apigenin on the growth of breast cancer cells, ERα+ MCF7 breast cancer cells were treated with various concentrations of apigenin. At low (0.1 and 1 μmol/L) concentrations, apigenin stimulated MCF7 cell growth (Fig. 1A); in contrast, apigenin inhibited cell growth at a higher (10 μmol/L) concentration (Fig. 1A), revealing a dose-dependent activity of apigenin on cell growth. To test the effect of apigenin on E₂-induced proliferation, MCF7 cells were treated with a combination of E₂ and various doses of apigenin. Treatment with 10 μmol/L apigenin blocked E₂-induced cell proliferation, whereas 1 μmol/L apigenin had no effect (Fig. 1B). To examine whether the growth-stimulatory effect of apigenin was mediated by ERα, MCF7 cells were treated with 1 μmol/L apigenin in the presence of increasing doses of ICI 182,780 (fulvestrant) or OHT. Apigenin-induced cell growth was inhibited by both ICI 182,780 and OHT (Fig. 1C and D), showing that the growth-stimulatory effect of apigenin was mediated by ERα. It was also notable that only 1 to 10 nmol/L antiestrogen was required to inhibit 1 μmol/L apigenin, suggesting that ERα has a weak binding affinity for this flavonoid.

Growth Inhibition of Antiestrogen-Resistant Breast Cancer Cells by Apigenin

We next investigated whether apigenin can inhibit proliferation of antiestrogen-resistant breast cancer cells. MCF7 and its OHT- and fulvestrant-resistant derivatives (MCF7-T and MCF7-F, respectively) were grown in hormone-free medium for 3 days and then treated with

various doses of apigenin. Low concentrations of apigenin again stimulated MCF7 cell growth but had no effect on growth of either MCF7-T or MCF7-F (Fig. 1E). High doses of apigenin inhibited proliferation of all three cell lines but to a differing extent. Apigenin at 20 $\mu\text{mol/L}$ inhibited the growth of MCF7 cells and MCF7-T cells by 80% but only by 40% for MCF7-F cells (Fig. 1E). Because the level of ER α in

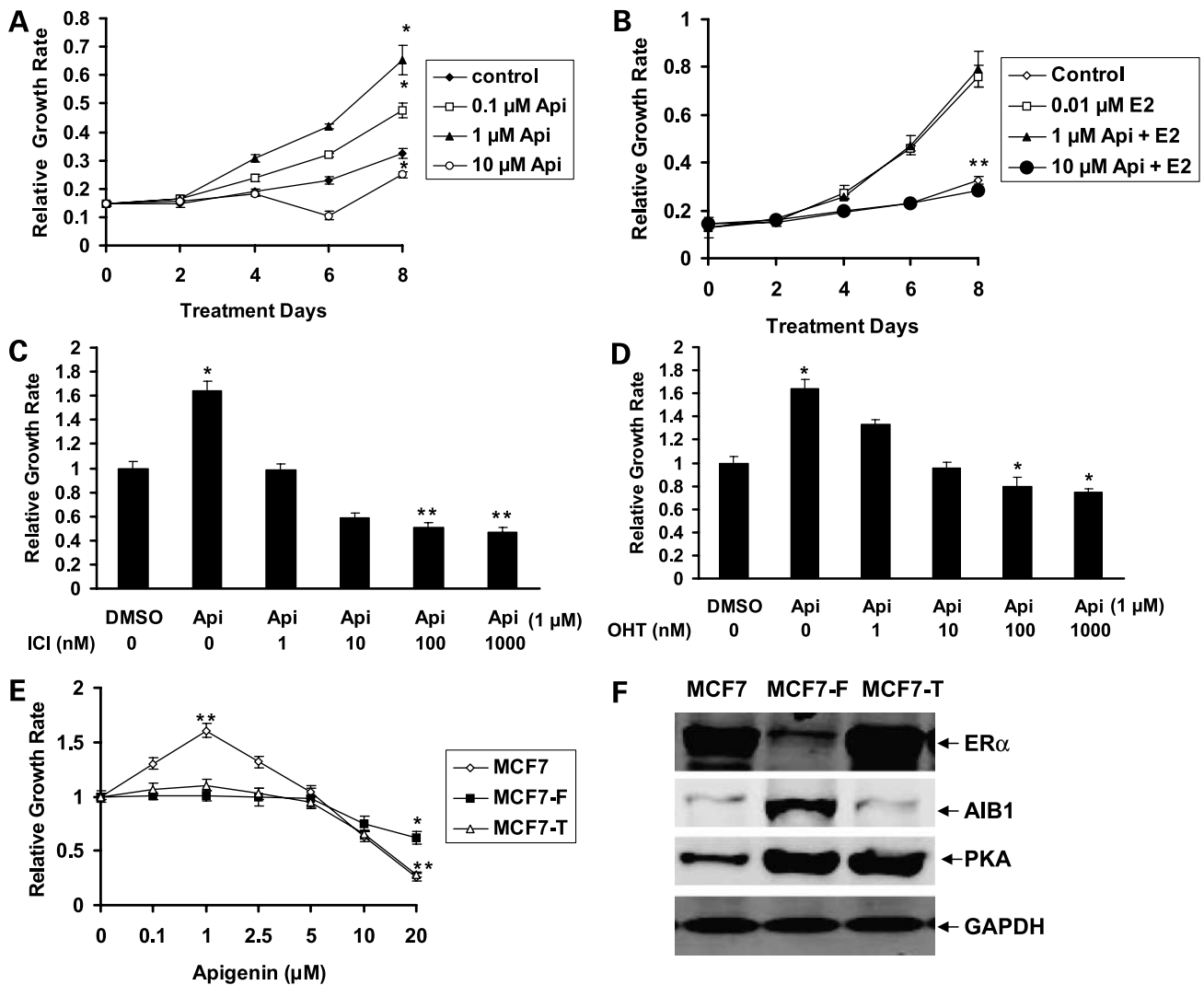


Figure 1. **A**, dose-dependent effects of apigenin on breast cancer MCF7 cell growth. To determine growth rates in the presence of apigenin (Api), cells were plated in 96-well dishes (2,000 per well) in basal medium for the indicated times and cell numbers were determined by MTT assay. Relative cell growth rates (drug versus vehicle) are shown in the presence of the indicated doses of apigenin compared with DMSO-treated controls. *, $P < 0.05$, Student's t test. **B**, dose-dependent effects of apigenin on E₂-induced breast cancer cell growth. MCF7 cells were treated with E₂ or combinations of E₂ with the indicated concentrations of apigenin and cell numbers were determined by MTT assay after treatment. Points, mean ($n = 6$); bars, SE. **, $P < 0.01$, 10 $\mu\text{mol/L}$ apigenin + E₂ compared with 10 nmol/L E₂ (control), Student's t test. **C** and **D**, fulvestrant and OHT inhibit apigenin-induced breast cancer cell growth. MCF7 cells were treated with combinations of apigenin and various concentrations of fulvestrant (ICI 182,780) or OHT. Cell numbers were determined by MTT assay after treatment. Points, mean ($n = 6$); bars, SE. *, $P < 0.05$; **, $P < 0.01$, compared with DMSO-treated controls, Student's t test. **E**, dose-dependent effects of apigenin on MCF7, MCF7-F, and MCF7-T cells and differential inhibitory effects of apigenin on growth of drug-sensitive and drug-resistant breast cancer cells. To determine growth rates in the presence of apigenin, MCF7, MCF7-T, and MCF7-F cells were plated in 96-well dishes (2,000 per well) in basal medium for the indicated times and treated with various doses of apigenin for 7 d. Cell numbers were then determined by MTT assay and relative cell growth rates (drug versus vehicle) were then determined. Points, mean ($n = 6$); bars, SE. *, $P < 0.05$; **, $P < 0.01$, compared with DMSO-treated controls, Student's t test. **F**, differential expression of ER α , AIB1, and PKA in drug-resistant breast cancer cell lines. ER α , AIB1, and PKA protein levels in MCF7, MCF7-F, and MCF7-T cells were determined by immunoblotting using specific antibodies. Glycerinaldehyde phosphate dehydrogenase was used as a loading control. Representative results of two independent experiments, each done in duplicate.

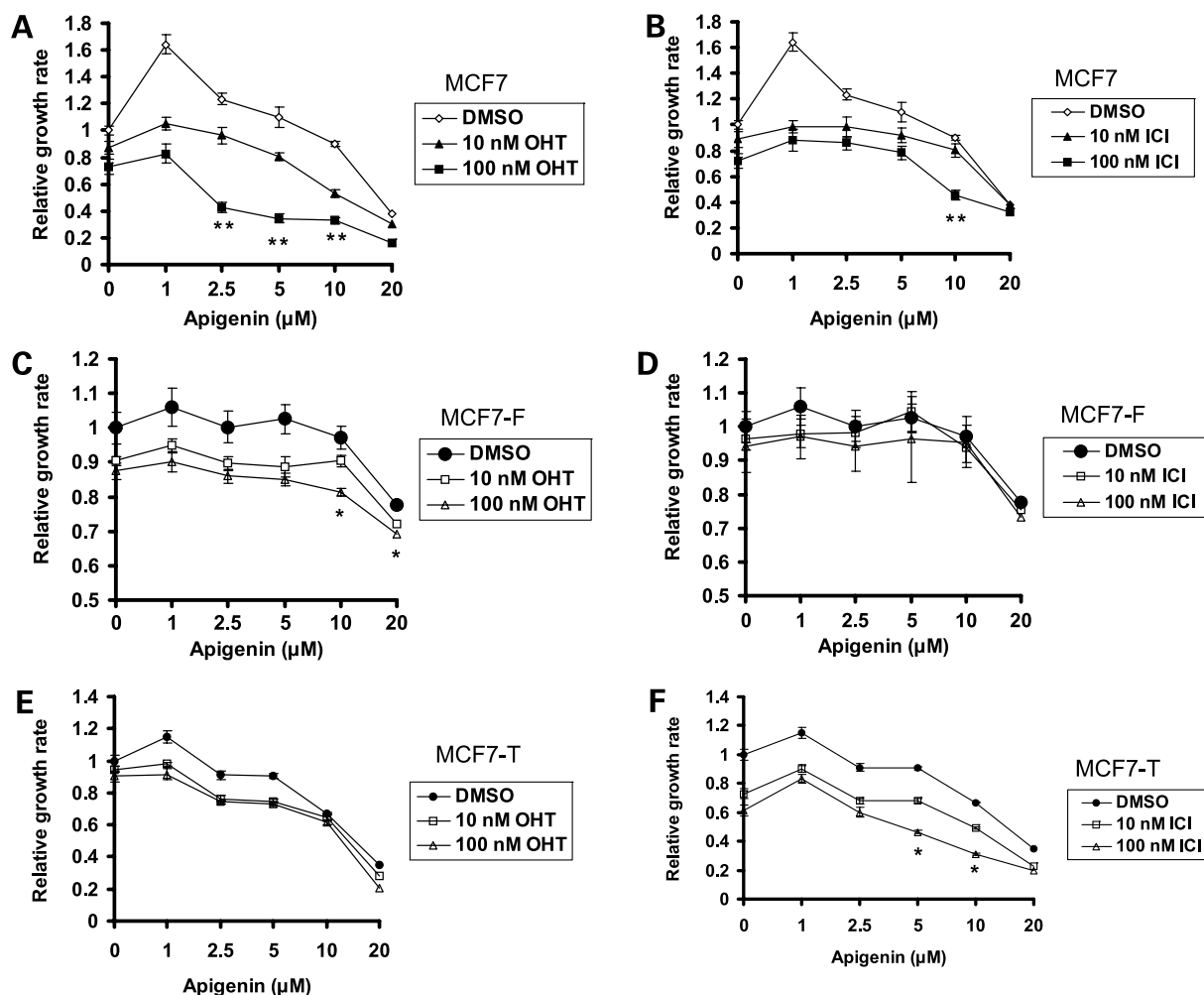


Figure 2. Synergistic effects of apigenin and OHT or fulvestrant (ICI 182,780) on MCF7 (A and B), MCF7-F (C and D), and MCF7-T (E and F) cells. Growth curves for 0, 1, 2.5, 5, 10, and 20 $\mu\text{mol/L}$ apigenin in combination with varying dosages of OHT (A, D, and E) and ICI 182,780 (B, D, and F). Growth was determined by MTT assay. Points, mean of at least six replicates; bars, SE. *, $P < 0.05$; **, $P < 0.01$, compared with DMSO-treated controls by ANOVA.

MCF7-F is very low compared with MCF7 and MCF7-T (Fig. 1F), this observation suggests that ER α levels may determine cell sensitivity to apigenin.

Previously, it was suggested that the antiestrogenic activity of flavonoid phytochemicals may be mediated by ER α -independent pathways (19). The above results, including the ability of apigenin to inhibit the growth of MCF7-F cells at high doses, suggests that apigenin may act by both ER α -dependent and ER α -independent mechanisms. Our previous results (9), including gene expression analysis showing overexpression of PKA, MAPK, PI3K, and coactivator AIB1 in MCF7-T and MCF7-F compared with MCF7 (Supplementary Table S1),⁶ suggested that these

antiestrogen-resistant sublines were not completely dependent on ER α for growth. To validate those microarray results at the protein level, we did Western blot analysis for AIB1 and the PKA β catalytic subunit. We observed overexpression of AIB1 in MCF7-F cells, and both MCF7-F and MCF7-T cells displayed up-regulation of the PKA β catalytic subunit (Fig. 1F), consistent with our previous observation (9).

Effects of Apigenin Combined with OHT or ICI 182,780 on Breast Cancer Cell Growth

We next investigated whether apigenin can enhance the inhibition of breast cancer cell growth by the antiestrogens OHT or ICI 182,780 in a synergistic manner. We first evaluated the effects of various combinations of apigenin (1.0, 2.5, 5, 10, and 20 $\mu\text{mol/L}$) with OHT or ICI 182,780 on the growth of MCF7 cells. Again, apigenin treatment alone showed dose-dependent effects on MCF7 cell growth (Fig. 2A and B). Treatment of MCF7 cells with apigenin

⁶ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

(2.5-10 $\mu\text{mol/L}$) and OHT (100 nmol/L) resulted in greater ($P < 0.01$) growth inhibition than apigenin or OHT alone (Fig. 2A). The combination of apigenin (10 $\mu\text{mol/L}$) plus ICI 182,780 (100 nmol/L) also showed synergistic growth inhibition of MCF7 cells ($P < 0.01$; Fig. 2B).

We next examined the effects of combinations of apigenin with OHT or ICI 182,780 on the growth of MCF7-F cells. We have reported previously that this subline was refractory to the inhibitory effects of both fulvestrant and OHT (9). As shown in Fig. 2C, MCF7-F cell growth was inhibited by 20 $\mu\text{mol/L}$ apigenin, and treatment of MCF7-F cells with

both apigenin and OHT showed greater ($P < 0.05$) growth inhibition than either apigenin or OHT alone (Fig. 2C). However, treatment with ICI 182,780 showed no effect on apigenin-mediated growth inhibition (Fig. 2D).

Finally, we tested the effects of combinations of apigenin with OHT or ICI 182,780 on the growth of OHT-resistant MCF7-T cells, which express high levels of ER α . At doses of 10 to 20 $\mu\text{mol/L}$, apigenin alone inhibited growth of MCF7-T cells (Fig. 2E), and the presence of OHT showed no effect on apigenin-mediated growth inhibition of this subline (Fig. 2E). The combination of apigenin (5-10 $\mu\text{mol/L}$) and

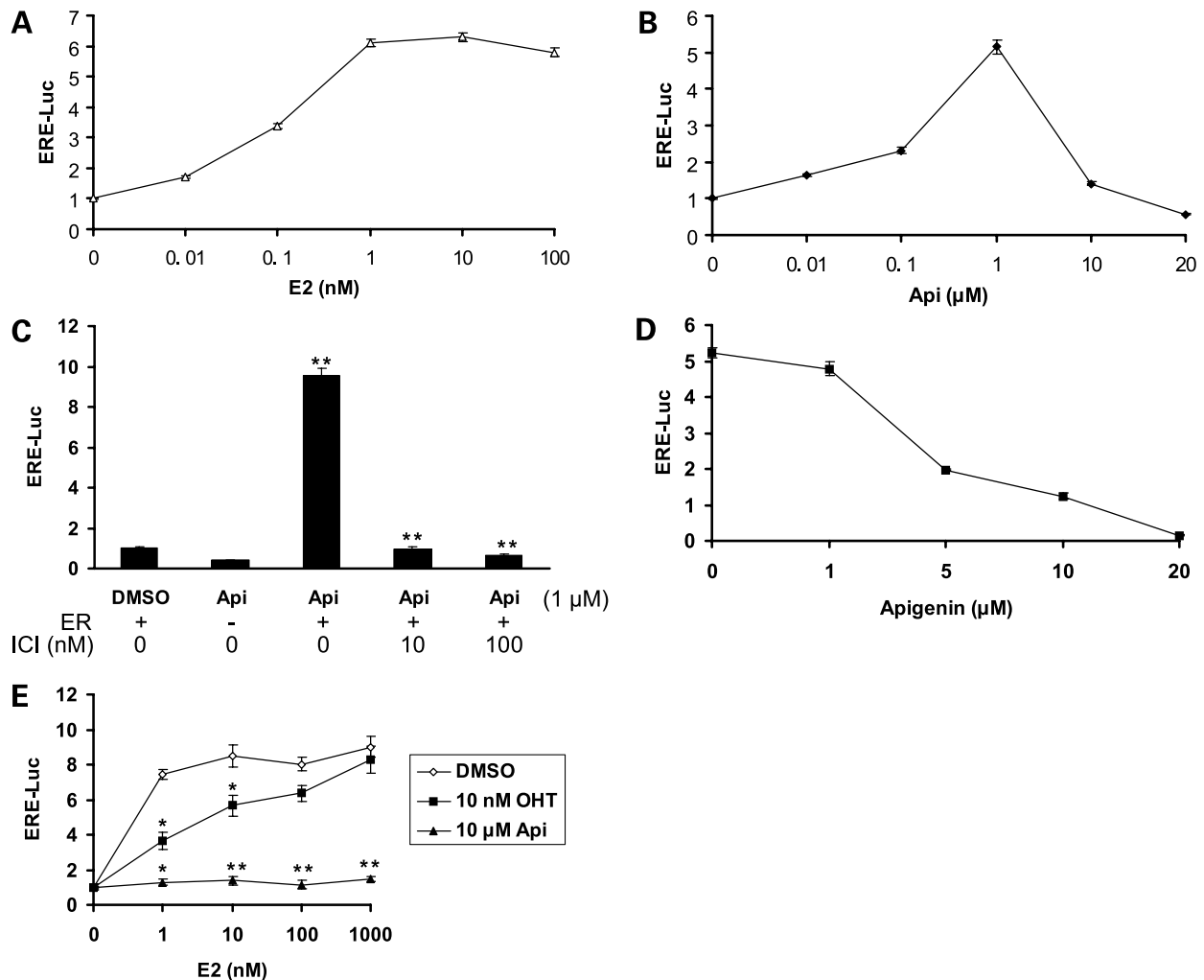


Figure 3. A and B, effects of apigenin on ER α transcriptional activity is dose dependent. ER α transcriptional activity was determined by measuring luciferase activity in MDA-MB-231 cells transfected with ERE2pS2-Luc, pSG5-ER α , and pCMV- β -gal. Cells were treated with the indicated doses of E₂ alone (A) or the indicated doses of apigenin alone (B) for 24 h. To correct for transfection efficiency, luciferase and β -galactosidase activities were expressed as a ratio. Mean \pm SD ($n = 4$). C, apigenin-induced ERE2pS2-Luc expression is ER α dependent and is blocked by the pure antiestrogen fulvestrant. MDA-MB-231 cells were transfected with or without ER α and then treated with 1 $\mu\text{mol/L}$ apigenin alone or with a combination of apigenin and fulvestrant (ICI 182,780) for 24 h. To correct for transfection efficiency, luciferase and β -galactosidase activities were expressed as a ratio. Mean \pm SD ($n = 4$). D, antiestrogenic effect of apigenin is dose dependent. MDA-MB-231 cells transfected with ER α were treated with 0, 1, 5, 10, and 20 $\mu\text{mol/L}$ apigenin in the presence of 10 nmol/L E₂. To correct for transfection efficiency, luciferase and β -galactosidase activities were expressed as a ratio. Mean \pm SD ($n = 4$). E, antiestrogenic activity of apigenin is not attributed to competition with estrogen for ER α binding. Transfected MDA-MB-231 cells were treated with the indicated doses of E₂ alone, 10⁻⁸ mol/L OHT in combination with indicated doses of E₂, or 10⁻⁵ mol/L apigenin in combination with the indicated doses of E₂. To correct for transfection efficiency, luciferase and β -galactosidase activities were expressed as a ratio. Mean \pm SD ($n = 4$). *, $P < 0.05$; **, $P < 0.01$, compared with DMSO-treated controls using the Student's t test.

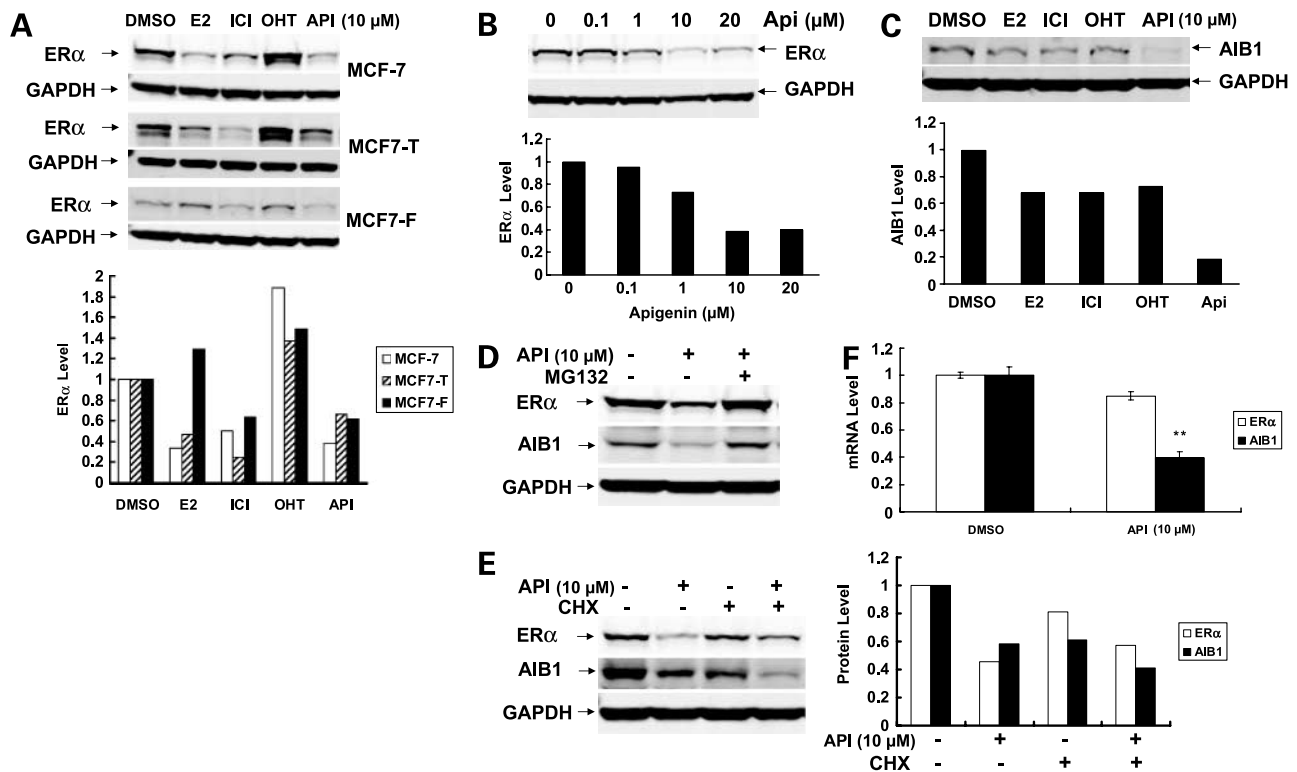


Figure 4. **A**, apigenin can induce ER α degradation in breast cancer cell lines. Breast cancer MCF7, MCF7-T, and MCF7-F cells were treated with 10 nmol/L E₂, 100 nmol/L fulvestrant (ICI 182,780), 100 nmol/L OHT, or 10 μ mol/L apigenin for 24 h. Whole-cell lysates were then prepared, subjected to SDS-PAGE, and blotted. ER α levels were measured and analyzed using Western blotting and a LI-COR imaging system (described in Materials and Methods). *Top*, Western blot image; *bottom*, quantitative analysis of Western blot of ER α protein from *top*, normalized to glyceraldehyde phosphate dehydrogenase using LI-COR Odyssey software. **B**, apigenin-induced ER α degradation is dose dependent. MCF7 cells were treated with the indicated doses of apigenin for 24 h and ER α levels were analyzed using Western blotting and LI-COR imaging system (*top*, Western blot image; *bottom*, quantitative analysis of Western blot normalized to glyceraldehyde phosphate dehydrogenase). **C**, apigenin can induce coactivator AIB1 degradation. Breast cancer MCF7 cells were treated with 10 nmol/L E₂, 100 nmol/L ICI 182,780, 100 nmol/L OHT, or 10 μ mol/L apigenin for 24 h. Whole-cell lysates were prepared, subjected to SDS-PAGE, and blotted. AIB1 levels were measured and analyzed using Western blotting and LI-COR imaging (*top*, Western blot image; *bottom*, quantitative analysis of Western blot of AIB1 protein from *top*, normalized to glyceraldehyde phosphate dehydrogenase). For all experiments, representative results of two independent experiments, with each done in duplicate, are shown. **D**, apigenin induces ER α and AIB1 degradation in breast cancer cell lines through the proteasome. Breast cancer MCF7 cells were treated with 10 μ mol/L apigenin and MG132 for 8 h as indicated. ER α levels were analyzed by Western blotting and a LI-COR imaging system. **E**, *de novo* protein synthesis is not required for apigenin-induced ER α and AIB1 degradation. MCF7 cells were treated with apigenin and CHX for 8 h as indicated. ER α and AIB1 levels were analyzed using Western blotting and LI-COR imaging system. **F**, AIB1 mRNA is down-regulated by apigenin. MCF7 cells were treated by 10 μ mol/L apigenin for 8 h. ER α mRNA and AIB1 mRNA were quantified by quantitative reverse transcription-PCR. **, $P < 0.01$, compared with DMSO-treated controls, Student's t test.

ICI 182,780 (100 nmol/L) resulted in greater ($P < 0.05$) growth inhibition of MCF7-T compared with apigenin or ICI 182,780 alone (Fig. 2F). These results show that apigenin alone can inhibit cell proliferation of MCF7-F and MCF7-T cells and is also capable of acting synergistically with both OHT and ICI 182,780 in these drug-resistant cells.

Dose-Dependent Effect of Apigenin on ER α Transcription Activity

Based on the observation that a low concentration of apigenin stimulated proliferation of hormone-sensitive MCF7 cells, we hypothesized that apigenin may activate E₂-responsive genes. To test this hypothesis, we investigated the effect of apigenin on ER α -mediated gene transcription using estrogen-responsive reporter assays. For these experiments, we used MDA-MB-231 cells transfected with wild-type ER α (pSG5-ER α) and estrogen-responsive reporter gene (ERE2pS2-Luc) and then treated with various

concentrations of either E₂ or apigenin. As expected, a dose-dependent increase in reporter gene activity was observed after E₂ treatment (Fig. 3A). Consistent with the dose-dependent effect of apigenin on the growth of MCF7 cells (Fig. 1A and B), a biphasic effect of apigenin on ER α activity was observed. Low concentrations (0.1-1 μ mol/L) of apigenin increased ERE2pS2-Luc expression (Fig. 3B), whereas high concentrations (10-20 μ mol/L) decreased expression.

To further investigate whether apigenin-induced ERE2pS2-Luc is ER α dependent, MDA-MB-231 cells were transfected with or without receptor and then treated with apigenin. Apigenin did not induce luciferase activity in the absence of ER α (Fig. 3C, lane 2), whereas apigenin-induced luciferase activity in the presence of ER α was inhibited by ICI 182,780 (Fig. 3C), showing that low-dose apigenin activity is ER α dependent. To examine whether apigenin

can eliminate E₂-induced ER α activity, MDA-MB-231 cells transfected with pSG5-ER α and ERE2pS2-Luc were treated with E₂ either alone or with various doses of apigenin. As shown in Fig. 3D, apigenin also inhibited E₂-induced luciferase activity in a dose-dependent manner.

To understand whether the antagonistic activity of apigenin is dependent on binding to ER α , MDA-MB-231 cells transfected with pSG5-ER α and ERE2pS2-Luc were treated with various concentrations of E₂ alone or in combination with apigenin or OHT. Although the antagonistic effect of OHT was reduced by increasing E₂ concentration, the antagonistic activity of apigenin was independent of E₂ concentration (Fig. 3E). Therefore, unlike OHT, whose antagonistic activity is dependent on competing with estrogen for ER α binding, the antiestrogenic effect

of apigenin does not appear to be due to competition with estrogen for ER α binding.

Apigenin Reduces Steady-State Protein Levels of ER α and AIB1

We and others have shown previously that agonist/antagonist binding can lead to changes in ER α stability (27, 31). To examine whether apigenin can affect ER α degradation, we used three cell lines with different levels of receptor: MCF7-T cells, having a higher level of ER α than MCF7 cells, and MCF7-F cells, having very low ER α levels (Fig. 1F). To test receptor stability in the presence or absence of apigenin, MCF7, MCF7-T, and MCF7-F were treated with 10 μ mol/L apigenin, 10 nmol/L E₂, 100 nmol/L ICI 182,780, or 100 nmol/L OHT for 24 h, and Western blot analysis for ER α was then done. Steady-state levels of

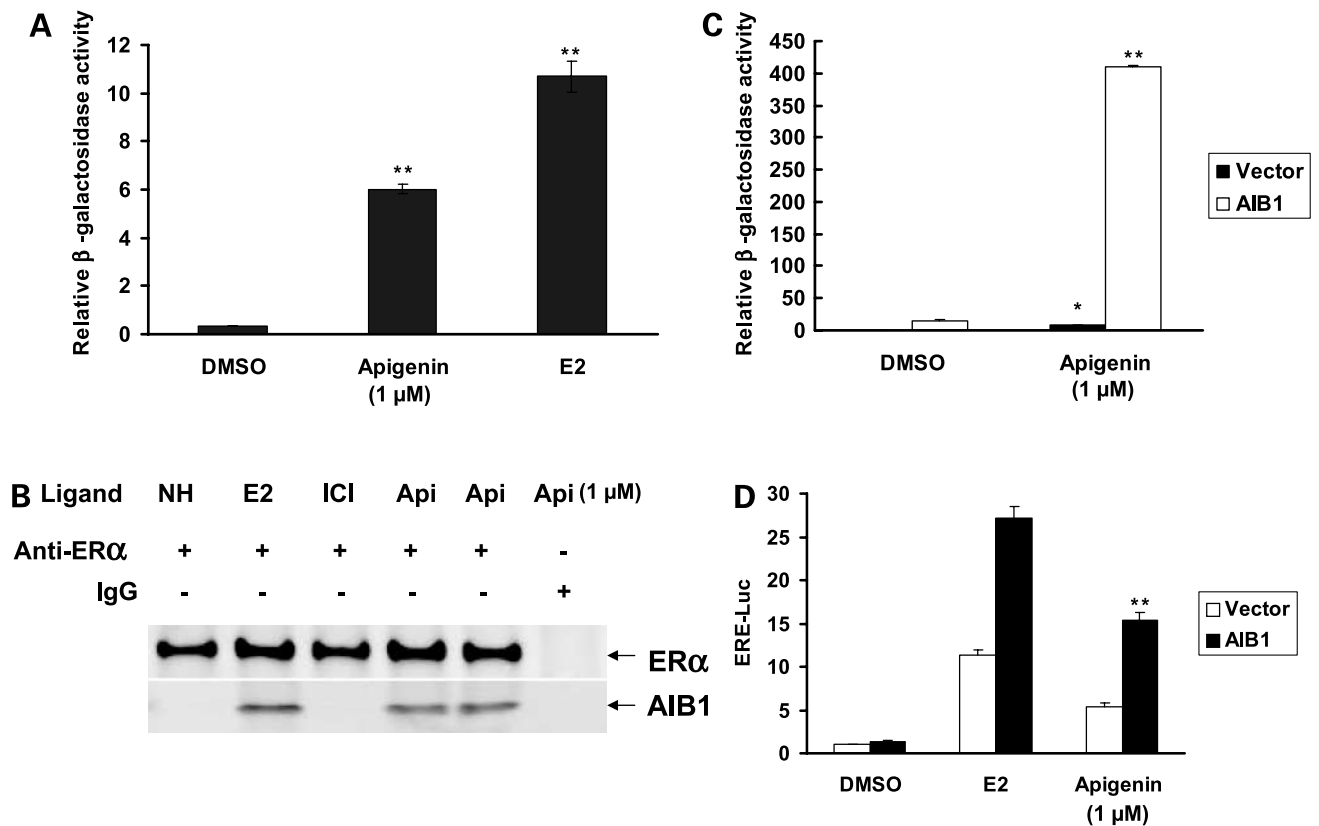


Figure 5. **A**, apigenin can induce an ER α -AIB1 interaction. Protein interactions were examined by yeast two-hybrid system. The yeast strain PJ69-4A was transformed with pAD-Gal4-rAIB1 and pBD-Gal4-ER α AF2 and grown in liquid yeast culture with the appropriate ligand. The strength of the interaction of AIB1 with the receptor was determined by measuring β -galactosidase activity from three independent transformants. β -Galactosidase activity represents the level of interaction between AIB1 and ER α in the absence or presence of apigenin (10⁻⁶ mol/L) or E₂ (10⁻⁹ mol/L). **, $P < 0.01$, compared with DMSO-treated controls, Student's t test. **B**, apigenin-induced ER α interaction with AIB1 in MCF7 cells. Coimmunoprecipitation was used to examine the ER α -AIB1 interaction in MCF7 cells. ER α was precipitated from cell lysates using an anti-ER α antibody in the presence or absence of ligand (1 μ mol/L E₂, ICI 182,780, and apigenin; NH, no hormone). The presence of AIB1 in the pull-down complex was examined by immunoblotting using an AIB1-specific antibody. To assess the amount of precipitated ER α in the complex, the same membrane was then re probed with an ER α antibody. Normal rabbit IgG was used as a negative control. Representative results of two independent experiments, each done in duplicate, are shown. **C**, AIB1 can enhance apigenin-induced ER α transcription activity in the yeast system. ER α transcription activity was examined in a yeast estrogen reporter assay. Yeast cells (RS188N) were transformed with expression vectors for ER α and an estrogen-responsive reporter construct (ERE- β -gal) in the absence or presence of the expression vector for AIB1. Cells were treated with or without apigenin. Mean \pm SD ($n = 4$). *, $P < 0.05$; **, $P < 0.01$, compared with DMSO-treated controls, Student's t test. **D**, AIB1 can enhance apigenin-induced ER α transcription activity in HeLa cells. ER α transcriptional activity was determined by measuring luciferase activity in HeLa cells transfected with or without pcDNA3-AIB1, ERE2pS2-Luc, pSG5-ER α , and pCMV- β -Gal. Cells were treated with 10 nmol/L E₂ or 1 μ mol/L apigenin for 24 h. Luciferase activity was corrected for transfection efficiency by expressing it as ratio of luciferase to β -galactosidase activities. Mean \pm SD ($n = 4$). **, $P < 0.01$, AIB1 compared with the vector control, Student's t test.

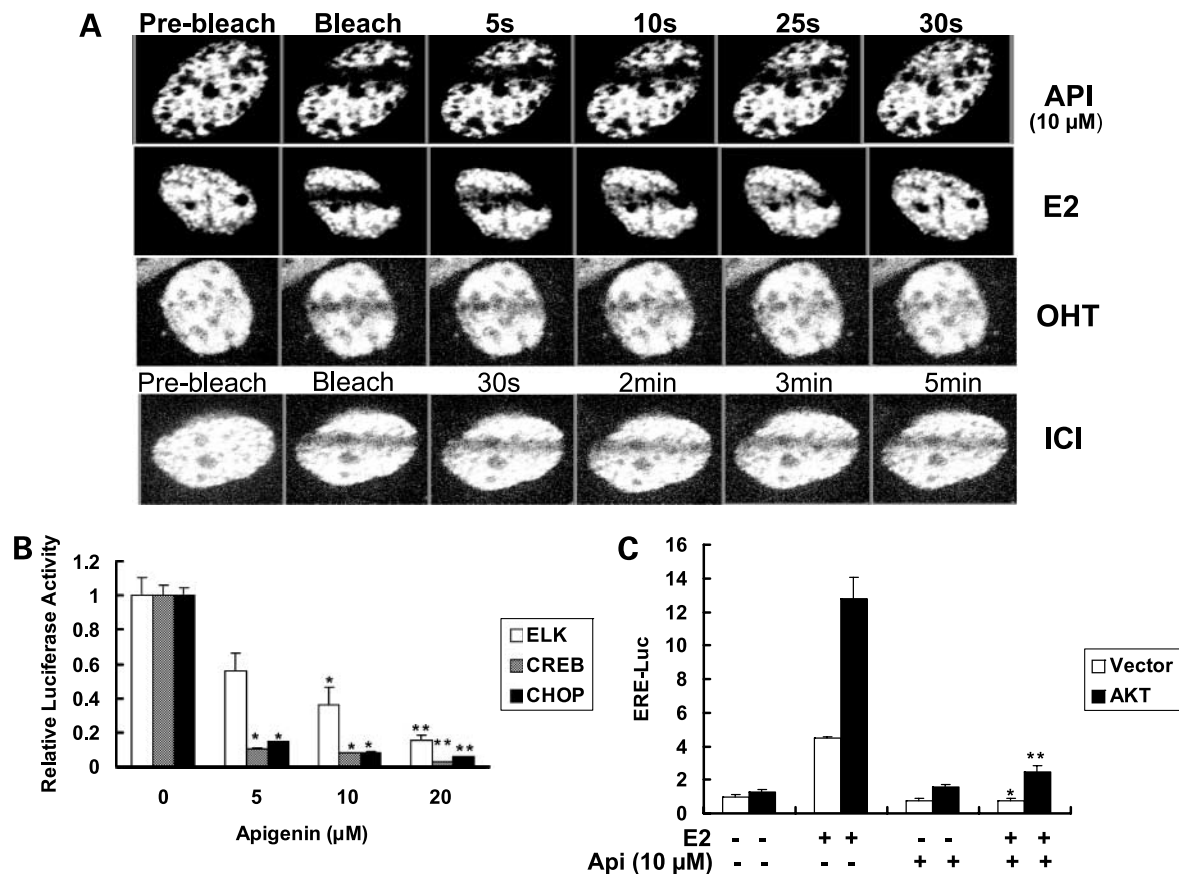


Figure 6. **A**, FRAP analysis of ER α mobility in the presence of apigenin. MCF7 cells transfected with GFP-ER α were subjected to FRAP analysis. Images show a single z-section and were obtained before bleaching at the indicated time points. After treatment with 10 μ mol/L apigenin for 1 h, a clear bleach zone was detected. Recovery began within 5 s and full recovery was seen within 30 s. In cells treated with E $_2$ and OHT (100 nmol/L for 1 h), GFP-ER α shows similar dynamics to those observed in apigenin-treated cells. After addition of the pure antagonist, ICI 182,780 (100 nmol/L for 30 min), no fluorescence recovery was observed, even after 5 min, indicating that ER α was immobilized. **B**, dose-dependent inhibition of p38, MAPK, and PKA pathways by apigenin. Effects of apigenin on transcription factors CHOP (p38 pathway), CREB (PKA pathway), and Elk1 (MAPK pathway) was monitored using a PathDetect Trans-Reporter system. MDA-MB-231 cells were cotransfected with pFA2-CHOP, pFA2-CREB, or pFA2-Elk1 and with pFR-Luc and pCMV- β -gal. After transfection, cells were treated with the indicated doses of apigenin for 20 h. To correct for transfection efficiency, luciferase and β -galactosidase activities were expressed as a ratio. **C**, inhibition of AKT activity by apigenin. AKT enhances estrogen-dependent ER α transcriptional activity, and apigenin (10 μ mol/L) can inhibit this effect. MDA-MB-231 cells were transfected with ERE2pS2-Luc and pcDNA3 vector or constitutively active AKT. Mean \pm SD ($n = 4$). *, $P < 0.05$; **, $P < 0.01$, compared with E $_2$ (10 nmol/L) with no apigenin controls, Student's t test.

ER α were decreased in all three cell lines after apigenin treatment (Fig. 4A); E $_2$ and ICI 182,780 also induced ER α degradation, whereas OHT stabilized ER α levels (Fig. 4A). A dose-response study of MCF7 cells revealed that apigenin markedly decreased steady-state ER α levels at 10 to 20 μ mol/L (Fig. 4B), doses that also inhibit both ER α transcriptional activity and cell growth (Figs. 1 and 3), further supporting our observations on the dose-dependent effects of apigenin on MCF7 cell growth and estrogen-responsive gene expression. Unlike E $_2$ and the antiestrogens, apigenin decreased the level of AIB1 in MCF7 cells (Fig. 4C), suggesting that apigenin can block estrogen signaling by down-regulating both ER α and AIB1.

To further examine the effect of apigenin on AIB1 and ER α protein degradation, MCF7 cells were treated with the proteasome inhibitor MG132 before apigenin treatment. As shown in Fig. 4D, treatment with MG132 blocked down-

regulation of ER α and AIB1 by apigenin. Treatment with the protein synthesis inhibitor CHX, however, had no effect on apigenin-induced down-regulation of AIB1 and ER α (Fig. 4E), suggesting that *de novo* protein synthesis is not required for apigenin-induced protein degradation. We then examined the effects of apigenin on ER α and AIB1 mRNA levels by real-time reverse transcription-PCR. Apigenin decreased ($P < 0.01$) the level of AIB1 mRNA but had no effect on ER α mRNA level (Fig. 4F). Taken together, these results showed that apigenin induces proteasomal degradation of both ER α and AIB1 as well as inhibits AIB1 transcriptional activity.

Apigenin Induces ER α -Coactivator Binding and AIB1 Enhances Estrogen Reporter Gene Response to Apigenin

It is now well known that estrogen promotes physical interactions between ER α and its coactivators, whereas

antiestrogens inhibit such interactions. As AIB1 is the dominant ER α coactivator in ER α + breast cancer (32), we examined the effect of apigenin on the interaction between AIB1 and ER α using a yeast two-hybrid assay to further elucidate the estrogenic and antiestrogenic characteristics of apigenin. To quantify the interaction of AIB1 and ER α , we measured β -galactosidase activity in liquid yeast culture. Yeast transformed with pBD-Gal4-ER α AF2 and pAD-Gal4-AIB1 displayed increased activity in the presence of apigenin (Fig. 5A), suggesting that apigenin can promote an ER α -AIB1 interaction. This interaction was further examined by coimmunoprecipitation, where even low doses of apigenin (1 μ mol/L) were sufficient to induce the receptor-coactivator interaction in MCF7 cells (Fig. 5B).

As AIB1 is overexpressed in breast cancer cells, and ER α enhances gene expression by forming a complex with the transcriptional machinery through a bridge formed by coactivator proteins (32), we examined whether AIB1 can enhance apigenin-induced ER α transcriptional activity. For this purpose, we used yeast cells transformed with ER α and ERE- β -gal, HeLa cells transfected with ER α and ERE2pS2-Luc, and also tested the effect of apigenin on ER α transcriptional activity in the presence or absence of AIB1. In both systems, the response of the estrogen reporter gene to apigenin was greatly enhanced by the addition of coactivator AIB1 (Fig. 5C and D), suggesting that apigenin, at lower concentrations, may enhance ER α transcriptional activity and stimulate breast cancer cell growth by recruiting AIB1 to ER α . High concentration (10 μ mol/L) of apigenin also induced AIB1 interaction with ER α in yeast cells transfected with ER α and ERE- β -gal (Supplementary Fig. S1),⁶ however, at this concentration, apigenin inhibited ER α transcription activity (Fig. 3) perhaps by down-regulating both ER α and AIB1 (Fig. 4) and inhibiting protein kinases activity (Fig. 6).

Effects of Apigenin on ER α Mobility

Depending on the nature of the ligand, ER α mobility and nuclear localization can be significantly altered (23), and we thus examined the effect of apigenin on ER α mobility. To characterize ER α mobility in the presence of apigenin and to compare the differences in ER α mobility induced by apigenin, E₂, OHT, and ICI 182,780, we treated GFP-ER α -transfected MCF7 cells with those agents followed by FRAP analysis. In cells treated with apigenin, E₂, or OHT, a distinct dark zone was detected immediately after 2-s bleaching, whereas fluorescence was fully recovered by 30 s after bleaching (Fig. 6A). In contrast, cells treated with ICI 182,780 showed very little recovery in a 5-min time frame (Fig. 6A, *bottom*). As it has been reported that ER α is extremely mobile in the absence of ligand (23), and FRAP analysis of DMSO-treated control cells resulted in immediate recovery of the bleach zone (data not shown), our results show that the mobility of ER α is similar in the presence of apigenin, E₂, and OHT. Although apigenin and ICI 182,780 can both down-regulate ER α protein levels, they have different effects on ER α mobility, suggesting that these compounds may reduce ER α protein levels by different mechanisms (27).

Effects of Apigenin on Kinase Activity

The antiproliferative effects of apigenin in prostate cancer appear to be mediated in part by inhibition of MAPK and PI3K/AKT (33), and genistein, an isoflavone, has been shown to inhibit breast cancer growth by inhibiting of p38, MAPK, and AKT kinase activity (34). As these kinases have been shown to become dramatically altered as breast cancers develop drug resistance (6, 9, 35), we next examined whether apigenin can inhibit the activity of p38, PKA, MAPK, and AKT. Here, PathDetect Trans-Reporting Systems and reporter genes for CHOP, CREB, and Elk1 were used to monitor the activity of p38, PKA, and MAPK, respectively. Each of the Elk1, CREB, and CHOP plasmids were cotransfected into MDA-MB-231 cells with a pFR-Luc reporter gene. As shown in Fig. 6B, apigenin inhibited CHOP (p38 kinase pathway), CREB (PKA pathway), and Elk1 (MAPK pathway) activities in a dose-dependent manner; these doses were consistent with a recent report showing kinase inhibition by apigenin in cancer cells (36). It has been reported previously that AKT protects breast cancer cells from antiestrogen-induced apoptosis (24), and increased AKT kinase activity was also reported in both OHT-resistant and ICI 182,780-resistant cell lines (7). To test the effect of apigenin on AKT activity, a construct expressing constitutively active AKT was cotransfected with ERE2pS2-Luc and ER α into MDA-MB-231 cells. As shown in Fig. 6C, AKT enhanced E₂-induced ERE-Luc activity, and this enhancement was inhibited by apigenin. Thus, by inhibiting the activity of p38, PKA, MAPK, and AKT, all of which have been associated with breast cancer drug resistance (24, 37), apigenin is capable of both blocking the growth of drug-resistant breast cancer and resensitization to OHT and ICI 182,780.

Discussion

Acquired resistance of breast cancer cells to the antiestrogens OHT and fulvestrant is accompanied by the dysregulation of ER α -dependent signaling molecules, such as coactivators, as well as altered receptor-independent growth pathways, such as protein kinases (6, 9). To effectively block proliferation of antiestrogen-resistant breast cancer cells, it may be necessary to identify a drug(s) that can target both of these important pathways. Apigenin, the most common flavonoid present in fruits and vegetables, has been shown to inhibit the growth of human tumor cell lines (16), including breast cancer cells (15, 18, 28, 38). However, the molecular mechanisms underlying that antiproliferative effect are not well understood. Recent evidence suggests that some plant flavones and isoflavones that prevent cancer can enhance the efficacy of cancer therapeutics by modifying the activity of cell survival pathways. The antiproliferative effects of apigenin in prostate cancer have been proposed to be mediated in part by inhibition of MAPK and PI3K/AKT (33, 38, 39). In this study, we showed a biphasic effect of apigenin on MCF7 breast cancer cell growth. At lower concentrations, apigenin stimulated cell growth by activating ER α -mediated

gene expression. However, at high concentrations, apigenin inhibited cell growth by reducing ER α and AIB1 protein levels and inhibiting multiple kinases. These observations are consistent with a recent report that antiestrogenic effects of flavonoids are mediated by both ER α -dependent and ER α -independent pathways (19). Although a recent study reported that ER β activation by apigenin suppresses prostate and breast cancer growth (40), whether the apigenin-elicited effects on ER β is dose dependent remains to be determined.

Because more than 60% of human breast tumors are ER α + and dependent on estrogens for growth (17), the first-line drug for these tumors is the selective ER modulator OHT, whereas fulvestrant is the second-line drug for ER α + tumors that are resistant to OHT. However, drug-sensitive breast cancer can eventually acquire resistance to both drugs, presenting a major challenge in disease management. Therefore, therapeutic agents that can be used to treat antiestrogen-resistant breast cancer and/or enhance sensitivity to OHT or fulvestrant would be of great value. Toward this objective, we recently generated breast cancer cell lines that recapitulate acquired resistance to OHT and fulvestrant, and we also reported dramatic changes in ER α and growth factor signaling pathways in those cell lines (9). The current study shows various differential effects of apigenin on the growth of MCF7, OHT-resistant MCF7-T, and fulvestrant-resistant MCF7-F cells. At lower concentrations, apigenin stimulated MCF7 cell growth but had no growth-stimulatory effects on drug-resistant MCF7-F and MCF7-T (Fig. 1E). At higher concentrations, apigenin inhibited the growth of all three breast cancer cell lines, with ER α + MCF7 and MCF7-T showing a greater response to high-dose apigenin than ER α - MCF7-F (Fig. 1E). In hormone-sensitive MCF7 cells, the combination of apigenin with antiestrogens resulted in greater growth inhibition than either drug alone. In addition, apigenin showed synergistic activity with antiestrogens in drug-resistant breast cancer cells (Fig. 2), indicating that apigenin has potential as a new therapeutic agent for antiestrogen-resistant breast cancer and further suggesting that apigenin can target both ER α -dependent and ER α -independent pathways. These hypotheses are further supported by a recent report showing that the isoflavone genistein can augment the inhibitory effect of OHT on various breast cancer cells (17).

We also observed that ER α mobility was similar in the presence of apigenin, E₂, and OHT but markedly different from receptor mobility in the presence of ICI 182,780 (Fig. 6A). This observation suggests that the mechanism of receptor down-regulation induced by apigenin is different from ICI 182,780, which induces ER α degradation by immobilizing the receptor to the nuclear matrix (27). Currently, the mechanism of ER α down-regulation by apigenin is unknown. However, a recent study reported that apigenin induces hypoxia-inducible factor-1 α degradation (41) by disrupting hypoxia-inducible factor-1 α association with chaperone complexes through competition with ATP (41). Furthermore, as heat shock protein 90 binding agents (geldanamycin and its derivative 17-allyl-

amino-geldanamycin) can inhibit breast cancer cell growth by ER α destabilization (42, 43), it is possible that apigenin induces ER α degradation by targeting heat shock and/or other chaperone proteins.

AIB1, a major ER α coactivator and oncogene in breast cancer (32, 44) that supports estrogen-independent growth in antiestrogen-resistant disease (45), is overexpressed in fulvestrant-resistant MCF7-F cells (see Fig. 1F; ref. 9). Apigenin, at low concentrations, promoted the interaction of ER α with AIB1 and also enhanced ER α transcriptional activity, resulting in overall growth stimulation. However, at high concentrations, apigenin down-regulated the level of AIB1 protein (Fig. 4C), suggesting that AIB1 is a potential target of apigenin. Degradation of AIB1 by RAD001, a derivative of the mTOR inhibitor rapamycin, has been reported to play a key role in breast tumor growth inhibition, and the combination of RAD001 with OHT was more effective than OHT alone (46), suggesting that degradation of AIB1 can contribute to inhibition of breast cancer cell proliferation. Although the mechanism of AIB1 down-regulation by apigenin is not known, the effect of the drug on heat shock protein and phosphorylation pathways (41, 47) may contribute to degradation of this coactivator.

In prostate cancer, apigenin was reported to induce cell cycle arrest by inhibiting both MAPK and PI3K/AKT (33), and the constitutive activation and increased activities of protein kinases p38, PKA, MAPK, and PI3K/AKT have also been associated with drug resistance in breast cancer (1, 6, 7, 24, 35, 48–50). Increased protein kinase activity not only enhances growth factor-dependent breast cancer survival but can also increase estrogen-stimulated breast cancer growth by modulating ER α activity (24). Thus, by inhibiting these protein kinases, apigenin has the potential to block both estrogen-dependent and estrogen-independent signaling pathways. The mechanism of protein kinase inhibition by apigenin is not clear, although some studies suggest that, by competing with ATP, apigenin may inhibit phosphorylation of kinases (51). This possibility is further supported by the structure of apigenin being similar to the drug PD98059, an inhibitor of MAPK phosphorylation (52). Additional studies suggest that apigenin can induce degradation of protein kinases by targeting heat shock proteins (18, 36). Apigenin has been reported to inhibit breast cancer cell growth by blocking proteasome activity (53). However, much higher concentrations of apigenin (50–100 μ mol/L) are required to inhibit proteasome activity compared with those sufficient to induce degradation of ER α and AIB1 (10–20 μ mol/L apigenin; Fig. 4).

In summary, this is the first study to show a biphasic effect of apigenin in hormone-sensitive breast cancer cells. At lower concentrations, apigenin activates ER α -mediated gene transcription by promoting AIB1 binding to ER α , resulting in growth stimulation of ER α + breast cancer cells. At higher concentrations, apigenin down-regulates protein levels of ER α and AIB1 and inhibits protein kinases p38, MAPK, PKA, and AKT/PI3K, leading to growth inhibition. An exciting aspect of this study is that apigenin has the potential to inhibit both ER α -dependent pathway and

protein kinase-mediated growth factor signaling pathways. As both pathways are commonly altered in anti-estrogen-resistant breast cancer, these broad effects of apigenin may be synergistic in combination with antiestrogens in growth inhibition of antiestrogen-resistant breast cancer cells. Apigenin thus fits into a recently proposed novel paradigm for the treatment of drug-resistant breast cancer by signal transduction inhibitors in combination with antiestrogen therapy (7, 46). Based on its ability to target both ER α -dependent and ER α -independent pathways, apigenin warrants further investigation as a therapeutic agent for both antiestrogen-sensitive and antiestrogen-resistant breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Pierre Chambon for providing pSG5-ER α (HEGO), Dr. Myles Brown for pcDNA3-AIB1, Dr. Harikrishna Nakshatri for constitutively active AKT, Dr. Michael A. Mancini for GFP-ER α , and Dr. Philip James for yeast strain PJ69-4A; Drs. Curtis Balch and Nicholas Berry and Cori Hartman-Frey for help with article preparation; and Jim Powers and Barry Stein (Biology Department, Indiana University) for technical support on confocal microscopy and FRAP analysis.

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