The nuclear factor κB inhibitor parthenolide restores ICI 182,780 (Faslodex; fulvestrant)–induced apoptosis in antiestrogen-resistant breast cancer cells

Rebecca B. Riggins, Alan Zwart, Ruchi Nehra, and Robert Clarke

Lombardi Comprehensive Cancer Center and Department of Oncology, Georgetown University School of Medicine, Washington, District Columbia

Abstract

The molecular mechanisms underlying the acquisition of resistance to the antiestrogen Faslodex are poorly understood, although enhanced expression and activity of nuclear factor κB (NFκB) have been implicated as a critical element of this phenotype. The purpose of this study was to elucidate the mechanism by which NFκB up-regulation contributes to Faslodex resistance and to determine whether pharmacologic inhibition of NFκB by the small molecule parthenolide could restore Faslodex-mediated suppression of cell growth. Basal expression of multiple NFκB-related molecules in MCF7-derived LCC1 (antiestrogen-sensitive) and LCC9 (antiestrogen-resistant) breast cancer cells was determined, and cells were treated with Faslodex or parthenolide. The effect of these drugs either singly or in combination was assessed by cell proliferation, estrogen receptor (ER)–dependent transcriptional activation, cell cycle analysis, and apoptosis assays. Expression of the p65 NFκB subunit and the upstream NFκB regulator IκB kinase γ/NFκB essential modulator were increased in the resistant MCF7/LCC9 cells ($P = 0.001$ and 0.04, respectively). Whereas MCF7/LCC9 cells were unresponsive to Faslodex alone, parthenolide effectively inhibited MCF7/LCC9 cell proliferation and the combination of Faslodex and parthenolide resulted in a 4-fold synergistic reduction in cell growth ($P = 0.03$). This corresponded to a restoration of Faslodex-induced apoptosis ($P = 0.001$), with no observable changes in ER-dependent transcription or cell cycle phase distribution. Because parthenolide has shown safety in Phase I clinical trials, these findings have direct clinical relevance and provide support for the design of clinical studies combining antiestrogens and parthenolide in ER-positive breast cancer. [Mol Cancer Ther 2005; 4(1):33–41]

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Requests for reprints: Robert Clarke, Lombardi Comprehensive Cancer Center, Georgetown University School of Medicine, Room W405A Research Building, 3970 Reservoir Road NW, Washington, DC 20057. Phone: 202-687-3755; Fax: 202-687-7505.
E-mail: clarker@georgetown.edu

Introduction

Antiestrogens inhibit the function of the estrogen receptor (ER), a nuclear transcription factor that directs the expression of genes that contribute to proliferation and cell growth (reviewed in refs. 1, 2). The most frequently prescribed is the nonsteroidal antiestrogen tamoxifen, which has been shown to be highly effective in both the treatment of ER-positive breast tumors and in reducing breast cancer incidence in women at high risk for the disease. However, most ER-positive tumors become estrogen independent and develop resistance to antiestrogen therapy, whereas the remainder (~30%) exhibit *de novo* or intrinsic resistance. Once resistance has developed, treatment with most nonsteroidal antiestrogens is usually unsuccessful.

In contrast, the steroidal antiestrogen Faslodex (ICI 182,780; ICI) induces significant clinical responses in patients whose tumors have acquired tamoxifen resistance (3). The effectiveness of Faslodex in patients with tamoxifen-resistant disease is similar to that of the aromatase inhibitor anastrozole, and several clinical trials have shown that Faslodex may be a viable alternative to nonsteroidal antiestrogens and aromatase inhibitors as a first-line endocrine treatment (4). Faslodex stimulates degradation of the ER and prevents receptor dimerization, inhibiting estrogen-dependent gene transcription (5, 6). As a pure antagonist of the ER, Faslodex is not associated with the increased risk for endometrial cancer that is seen with tamoxifen (7).

The antiestrogen resistance phenotype is complex, involving many changes at the cellular and molecular levels. Antiestrogens are cytostatic, inducing a G_0-G_1 block in breast cancer cells in culture (1, 8). However, these drugs are also capable of actively inducing programmed cell death or apoptosis, which is consistent with the ability of antiestrogens to increase overall survival (9). One way in which breast cancer cells may become antiestrogen resistant is through changes in gene networks that control cell proliferation and apoptosis (10). To test this hypothesis,

we developed several variant cell lines from the estrogen-dependent and antiestrogen-sensitive MCF-7 breast cancer cells (11, 12). MCF7/LCC1 cells are estrogen independent but remain responsive to antiestrogens; MCF7/LCC9 cells are derivatives of MCF7/LCC1 that have acquired resistance to Faslodex. Similar to what has been observed in breast cancer patients, MCF7/LCC9 cells are cross-resistant to the nonsteroidal antiestrogen tamoxifen (2).

Several genes were found to be altered in the resistant MCF7/LCC9 cells, when their transcriptomes were compared with that of their antiestrogen-sensitive MCF7/LCC1 parental cells by serial analysis of gene expression and microarray analysis (10). For example, we implicated loss of the putative tumor suppressor interferon regulatory factor-1 (IRFI) in acquired resistance and have recently shown IRFI to be a key mediator of the proapoptotic effects of Faslodex in MCF-7 cells (13).

Altered expression of the p65/RelA member of the nuclear factor κB (NFκB) transcription factor family, which can form functional heterodimers with IFN regulatory factor-1 (14), also was strongly implicated in acquired Faslodex resistance. mRNA levels of p65/RelA are up-regulated 2-fold in the MCF7/LCC9 cells, NFκB-dependent transcription are increased 10-fold, and MCF7/LCC9 cells exhibit a greater sensitivity to the growth inhibitory effects of parthenolide, a small molecule inhibitor of NFκB (10). These data strongly but indirectly implicate NFκB action in acquired antiestrogen resistance.

The NFκB family contains five members that form dimers and regulate the transcription of various genes including cytokines, cell adhesion molecules, the pro-proliferative proteins c-*myc* and cyclin D1, and several inhibitors of apoptosis (15). Inhibitors of the NFκB pathway show promise as anticancer and anti-inflammatory agents (16). Parthenolide, a sesquiterpene lactone that was first isolated from the feverfew herb (*Tanacetum parthenium*) native to Central America (17), is a relatively specific small molecule inhibitor of NFκB (18). Parthenolide and other members of the sesquiterpene lactone class have garnered recent attention as promising candidates for cancer treatment either as single agents or in combination with other cytotoxic drugs (19, 20). For example, parthenolide has anti-inflammatory, anticancer, and antiangiogenic properties and has successfully undergone phase I/II clinical trials (21, 22).

Constitutive NFκB activity is widely observed in many tumor types (23), including breast cancer where it is associated with resistance to apoptosis-inducing agents (24). In many tumor lines, autocrine secretion of cytokines and growth factors has recently been implicated in the constitutive activation of NFκB (25). Importantly, NFκB activity also increases in breast cancer cells as they acquire the ability to grow in the absence of estrogen (26, 27). These findings strongly implicate NFκB signaling in the control of breast cancer cell growth and response to antiestrogens.

In this study, we sought to clarify the mechanism by which NFκB up-regulation may affect resistance to Faslodex and determine whether pharmacologic inhibition of NFκB could restore sensitivity to the drug. We show here that in addition to p65/RelA, expression of the upstream regulator NFκB essential modulator/IκB kinase γ (NEMO/IKKγ) is also increased in the resistant cells. The NFκB inhibitor parthenolide efficiently inhibits cell growth and restores sensitivity to Faslodex by synergistically enhancing apoptosis. Our data indicate that inhibition of NFκB may be a successful approach in the treatment of ER-positive breast cancers that have acquired resistance to antiestrogen therapy. NFκB inhibition also may reduce the incidence or delay the onset of antiestrogen resistance. These data provide support for considering the design of clinical studies combining antiestrogens and parthenolide in ER+ breast cancer.

Materials and Methods

Cell Culture and Reagents

MCF-7–derived MCF7/LCC1 and MCF7/LCC9 cells (11, 12) were routinely cultured in phenol red-free improved minimal essential media (IMEM; Biofluids, Rockville, MD) supplemented with 5% charcoal-stripped calf serum (CCS; CCS-IMEM). Cells were maintained in a humidified atmosphere at 37°C and 95% air/5% CO_2. 17β-Estradiol (estradiol, E2) and parthenolide were purchased from Sigma (St. Louis, MO), and ICI 182,780 (ICI, Faslodex) was a kind gift of Dr. Alan Wakeling (AstraZeneca, Macclesfield, Cheshire, United Kingdom).

Cell Lysis, Immunoblotting, and Immunoprecipitation

Cells were grown in either 10-cm^2 dishes or T-75 cm^2 tissue culture flasks before lysis. To determine the effects of parthenolide and ICI 182,780 on protein expression, cells were treated with vehicle, 100 nmol/L ICI 182,780 (the IC_{50} for the control/parental LCC1 cells), or 600 nmol/L parthenolide singly or in combination in CCS-IMEM for 72 hours. Cells were then lysed in modified radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 1% Igepal CA-630, and 0.5% deoxycholate] supplemented with Complete Mini protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and 1 mmol/L sodium orthovanadate phosphatase inhibitor (Sigma). Lysates were clarified by centrifugation and total protein was quantitated using the bicinchoninic acid assay purchased from Pierce (Rockford, IL). Whole cell lysate (20 μg) was resolved by PAGE using NuPAGE 12% precast gels (Invitrogen, Carlsbad, CA). Proteins were then transferred to nitrocellulose membranes, which were probed with the following antibodies overnight at 4°C: p65 NFκB sc-109 (1:800; Santa Cruz Biotechnology, Santa Cruz, CA), p50 NFκB sc-8414 (1:200; Santa Cruz Biotechnology), p52 NFκB (1:200; Upstate Biotechnology, Charlottesville, VA), IKKγ/NEMO sc-8330 (1:200; Santa Cruz Biotechnology), IκBα sc-371 (1:200; Santa Cruz Biotechnology), phospho-Akt (Ser473; 1:1,000, Cell Signaling, Beverly, MA), or Akt (1:1,000, Cell Signaling). Membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ)

for 1 hour at room temperature before enhanced chemiluminescence (Amersham Biosciences) and exposure to film. To confirm equal loading of the gels, membranes were reprobed with antibodies for β-actin (1:5,000; Sigma).

For immunoprecipitations, 400 μg of cell lysate were incubated with 2.5 μL of p65 NFκB antibodies overnight at 4°C with rotation. The following day, 30 μL of Protein A-Sepharose beads (Amersham Biosciences) were added for 1 hour at 4°C to recover the immune complexes, which were then washed twice in modified radioimmunoprecipitation assay buffer, twice in Tris-saline [TN; 50 mmol/L Tris (pH 7.5) and 150 mmol/L NaCl], and resuspended in 2× Laemmli sample buffer before electrophoresis as described above.

Cell Proliferation Assays

MCF7/LCC1 and MCF7/LCC9 cells were seeded at a density of 1 to 2 × 10^4 cells per well in 24-well plates, and 24 hours later were treated with the indicated concentrations of drug in CCS-IMEM. Cells were incubated with the drugs for 7 days, and the media were changed on days 3 and 5. Cells were then trypsinized, resuspended in PBS (Biofluids), and counted using a Z1 Single Coulter Counter (Beckman Coulter, Miami, FL). At least three independent experiments were done in quadruplicate, and data were normalized to vehicle-treated cells. Data are presented as the mean ± SE for a representative experiment.

Transcriptional Reporter Assays

The estrogen response element-containing 3xERE-tk-luc reporter plasmid was purchased from Promega (Madison, WI). MCF7/LCC1 and MCF7/LCC9 cells were seeded into 12-well plates at a density of 7 to 8 × 10^4 cells per well. The following day, cells were transfected with 0.4 μg of luciferase reporter plasmid and 0.1 μg pCMV-*Renilla* (Promega) per well using the FuGENE 6 transfection reagent (Roche, Indianapolis, IN). Three hours post-transfection, media were changed and cells were treated with 100 nmol/L ICI 182,780 and/or 600 nmol/L parthenolide in CCS-IMEM for 24 hours. Subsequently, cells were lysed and activation of the luciferase constructs was measured using the Dual Luciferase Assay Kit (Promega). Luminescence was quantified using a Lumat LB 9501 luminometer (EG&G Berthold, Bundoora VIC, Australia). Luciferase values were normalized to *Renilla* luminescence, and four independent experiments were done each at least in quadruplicate. Data are presented as the mean ± SE for all experiments.

Cell Cycle Assays

Cells (n = 5 × 10^5) were seeded into 10-cm^2 dishes 1 day before treatment with 100 nmol/L ICI 182,780 and/or 600 nmol/L parthenolide in CCS-IMEM for 24 hours. Cells were then analyzed for alterations in cell cycle via fluorescence activated cell sorting, which was done by the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource according to the method of Vindelov et al. (28). Data are presented as the mean ± SE for three independent experiments.

Apoptosis Assays

Cells (n = 1 × 10^5) were seeded onto 18 × 18 mm glass coverslips in each well of a 6-well plate in duplicate and the following day were treated with 100 nmol/L ICI 182,780 and/or 600 nmol/L parthenolide in CCS-IMEM for 24 hours. Cells were then fixed with 3.7% formalin in PBS for 20 minutes at room temperature prior to Annexin V and propidium iodide staining using the Vybrant Apoptosis Assay Kit 3 purchased from Vector Laboratories (Burlingame, CA). Coverslips were then mounted on glass slides using VectaShield fluorescence mounting medium (Vector Laboratories). Cells were visualized on a Nikon E600 fluorescence microscope (provided by the Lombardi Comprehensive Cancer Center Microscopy Shared Resource), and several random fields (≥200 cells) were scored per treatment condition. The number of cells stained red (propidium iodide, indicating necrosis) was subtracted from the number of cells stained green (Annexin V-FITC, indicating apoptosis), and subsequently divided by the total number of cells seen by phase-contrast. Data are presented as the percentage of apoptotic cells and represent the mean ± SE for three independent experiments.

Statistical Analyses

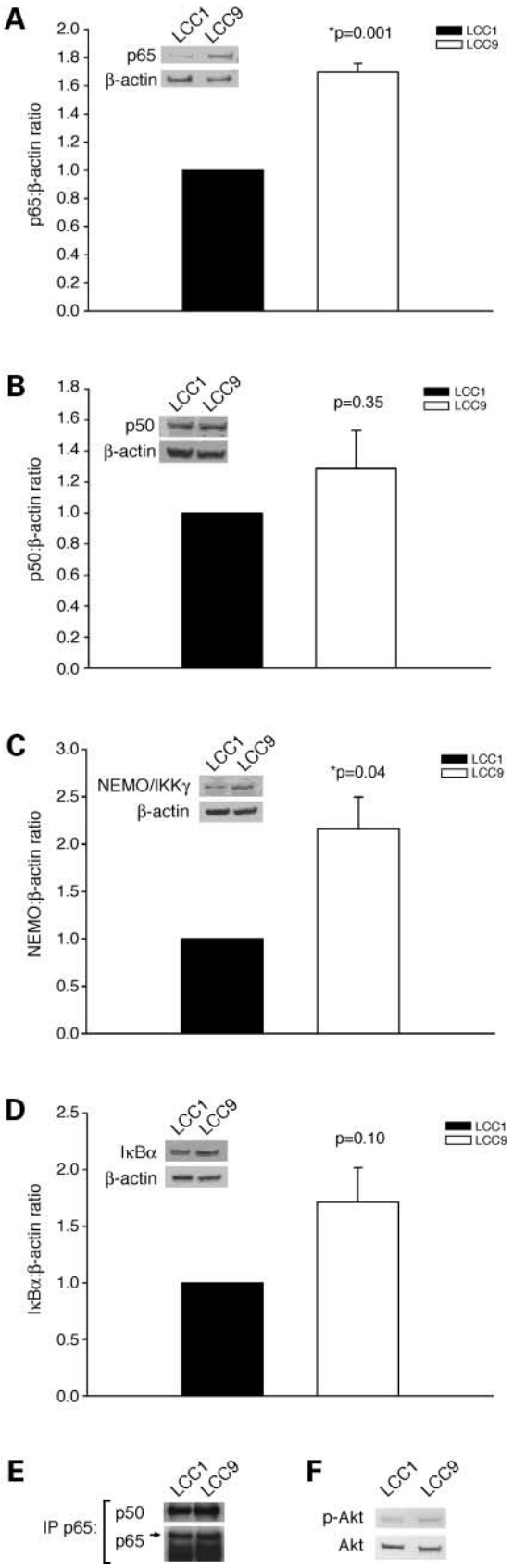
Two-tailed Student's t tests were used for the comparison of two groups for immunoblot, cell proliferation, and apoptosis assays as indicated. For luciferase reporter assays, Dunn's post hoc t test was used to compare all treatment groups following one-way ANOVA. Defining the nature of the interaction between Faslodex and parthenolide was done by determining the R index (RI; ref. 29). RI values were obtained by calculating the expected cell survival (S_{exp}; the product of survival obtained with drug A alone and the survival obtained with drug B alone) and dividing S_{exp} by the observed cell survival in the presence of both drugs (S_{obs}). $S_{exp}/S_{obs} > 1.0$ indicates a synergistic interaction. This method is an appropriate way to define synergy in this case because clinically relevant concentrations of Faslodex are ineffective on cell proliferation or apoptosis in the resistant MCF7/LCC9 cells when given as a single agent (29).

Results

p65/RelA and NEMO/IKKγ Are Up-Regulated in Antiestrogen-Resistant Cells

Our previous studies identified a 2-fold up-regulation of p65/RelA mRNA in Faslodex-resistant MCF7/LCC9 cells by expression microarray analysis (10). To confirm altered expression of p65 at the protein level and to examine other NFκB family members and regulatory molecules, whole cell lysates were prepared from MCF7/LCC9 cells and antiestrogen-sensitive MCF7/LCC1 cells and subjected to SDS-PAGE and immunoblot analysis. Similar to mRNA levels, p65/RelA protein is increased ~2-fold in the MCF7/LCC9 cells (Fig. 1A, P = 0.001). In contrast, expression of the p50 subunit of the NFκB heterodimer (Fig. 1B, P = 0.35) or of p52 NFκB2 (data not shown) is not different between the cell lines.

Transcriptional activity of the p65/p50 heterodimer is modulated by the inhibitor IκB, which is in turn negatively regulated by the IKK complex comprised of IKKα, IKKβ,



and the scaffolding protein NEMO/IKKγ (30). To determine whether MCF7/LCC9 cells exhibit changes in these regulatory molecules, lysates were immunoblotted for NEMO/IKKγ and IκBα (Fig. 1C and D). Whereas there is no significant change in IκBα expression ($P = 0.10$), a significant 2-fold increase in the level of NEMO/IKKγ is apparent in MCF7/LCC9 cells ($P = 0.04$). NEMO/IKKγ is required for activity of the IKK complex and the inhibitory phosphorylation of IκB in response to inflammatory stimuli that activate NFκB (31), and dysregulation of NEMO is linked to several human pathologies (32). These data suggest that NEMO may also play a role in the response of breast cancer to antiestrogens.

To examine whether the binding of p65 and p50 was altered in antiestrogen-resistant cells, cell lysates were immunoprecipitated with p65 antibodies and immune complexes were captured and subjected to SDS-PAGE as described above (Fig. 1E). No clear differences in p65/p50 complex formation were found between MCF7/LCC1 and MCF7/LCC9 cells.

Independent of the IKK-IκB signaling pathway, NFκB can also be activated by phosphatidylinositol 3-kinase (PI3K); PI3K-mediated activation of Akt can enhance NFκB transcriptional activity without the degradation of IκB (33). Because overexpression of active Akt has also been shown to induce resistance to antiestrogens and cytotoxic drugs (34), MCF7/LCC1 and MCF7/LCC9 cell lysates described above were immunoblotted for phospho-Serine 473 Akt (Fig. 1F). No difference in the level of activated phospho-Akt is observed in the antiestrogen-resistant MCF7/LCC9 cells, suggesting that Akt → NFκB signaling is not the only pathway through which cells can modulate NFκB activation and acquire resistance to Faslodex.

Inhibition of NFκB by Parthenolide Restores Faslodex Sensitivity to MCF7/LCC9 Cells

We have previously reported that MCF7/LCC9 cells are more sensitive than MCF7/LCC1 cells to growth inhibition by parthenolide, suggesting that these cells, in which p65/RelA is up-regulated, are more dependent on NFκB-driven cell growth (10). Faslodex (100 nmol/L) approximates the IC_{50} for proliferation in antiestrogen-sensitive MCF7/LCC1 cells but is ineffective in MCF7/LCC9 cells (Fig. 2A, $P = 0.01$). To determine whether

Figure 1. Expression of NFκB family members and upstream regulatory molecules. **A–D,** quantitiation and representative immunoblots of p65/RelA, p50 NFκB, NEMO/IKKγ, and IκBα levels in MCF7/LCC1 and MCF7/LCC9 cells. Lysates (20 μg) were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted. *β-actin*, loading control. *Columns,* mean of at least 3 independent experiments; *bar,* ±SE. *P*s were calculated by Student's *t* test. **E,** coimmunoprecipitation of p65 and p50. Lysates (400 μg) were immunoprecipitated with polyclonal anti-p65 antibodies; immune complexes were isolated and separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted. **F,** Akt activity is not altered in antiestrogen-resistant MCF7/LCC9 cells. Lysates (20 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies specific for phospho-Ser473 of Akt. The membrane was then stripped and reprobed for total Akt.

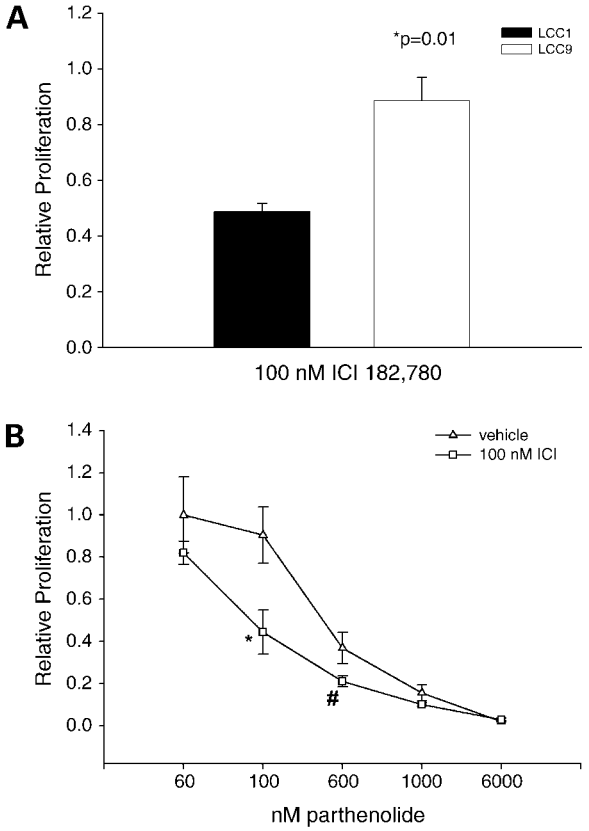


Figure 2. Parthenolide inhibits the proliferation of antiestrogen-resistant cells and partially restores Faslodex sensitivity. **A,** MCF7/LCC9 cells are unresponsive to Faslodex. Cells were seeded in quadruplicate and treated with 100 nmol/L Faslodex in CCS-IMEM for 7 d before counting. *Columns,* mean from a single representative experiment of relative proliferation (relative to vehicle-treated control); *bar,* ±SE. *P* was calculated by Student's *t* test. The experiment was independently done at least thrice. **B,** Faslodex and parthenolide synergistically inhibit MCF7/LCC9 cell proliferation. Cells were seeded in quadruplicate and treated with 0 to 6,000 nmol/L parthenolide in the presence or absence of 100 nmol/L Faslodex in CCS-IMEM for 6 d. *Points,* mean of relative proliferation; *bars,* ±SE. *, *P* = 0.034 versus 100 nmol/L parthenolide without Faslodex by Student's *t* test; RI = 1.82. #, *P* = 0.05 versus 600 nmol/L parthenolide without Faslodex; RI = 1.48.

inhibition of NFκB activity could restore Faslodex sensitivity, MCF7/LCC9 cells were treated with increasing concentrations of parthenolide in the presence or absence of 100 nmol/L Faslodex. In the absence of Faslodex, parthenolide effectively inhibits MCF7/LCC9 cell proliferation with an IC_{50} of 500 to 600 nmol/L. However, the addition of Faslodex generates a significant nearly 5-fold sensitization, where 50% growth inhibition occurs at a concentration of 100 nmol/L parthenolide (Fig. 2B; *P* = 0.034 for parthenolide plus Faslodex compared with parthenolide alone).

The interaction of Faslodex and parthenolide is synergistic in MCF7/LCC9 cells, generating an RI value of 1.82. Treatment with 100 nmol/L Faslodex and 600 nmol/L parthenolide also produces a greater than additive inhibition of cell proliferation (*P* = 0.05, RI = 1.48). These data strongly suggest that the up-regulated NFκB activity present in MCF7/LCC9 cells is a major contributor to the antiestrogen resistance phenotype.

Parthenolide and Faslodex Synergistically Increase Apoptosis

We subsequently sought to define the mechanism by which parthenolide and Faslodex synergistically inhibit the growth of MCF7/LCC9 cells. A primary action of antiestrogens is to antagonize endogenous estrogen and block ER function; Faslodex can achieve this by affecting receptor turnover (5). We asked whether parthenolide can restore Faslodex-mediated inhibition of ER-dependent transcriptional activity (Fig. 3). MCF7/LCC1 and MCF7/LCC9 cells were cotransfected with an ERE-tk-luciferase reporter vector and the pCMV-*Renilla* control vector. Three hours post-transfection, cells were treated with estradiol, Faslodex, and/or parthenolide for 24 hours before performing dual-luciferase promoter-reporter assays.

MCF7/LCC1 cells exhibit a basal ERE-luciferase activity that is enhanced 8-fold by estradiol treatment and almost abolished by Faslodex. In contrast, MCF7/LCC9 cells express a higher basal ERE-luciferase activity that is slightly enhanced by estradiol but is not inhibited by Faslodex treatment. Whereas transcription from an NFκB-dependent reporter is inhibited by 600 nmol/L parthenolide in MCF7/LCC9 cells (data not shown), parthenolide either alone or in combination with Faslodex has no statistically significant effect on ERE-luciferase activity in MCF7/LCC9 cells, suggesting that the mechanism of their antiproliferative synergy does not involve the regulation of ER-dependent transcriptional events.

Treatment with antiestrogens such as Faslodex can have a cytostatic effect on cell growth, typically manifested as

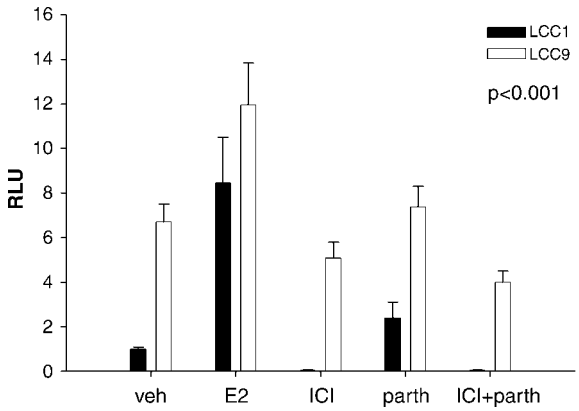


Figure 3. Combined treatment with Faslodex and parthenolide does not inhibit ER-dependent transcriptional activity. MCF7/LCC1 and MCF7/LCC9 cells were transfected in quadruplicate with ERE-tk-luciferase and pCMV-*Renilla* constructs prior to treatment with 10 nmol/L estradiol, 100 nmol/L Faslodex, and 600 nmol/L parthenolide singly or in combination (or ethanol vehicle) in CCS-IMEM for 24 h. *Columns,* mean of the ratio of luciferase-to-Renilla activity (relative light units) for four independent experiments; *bars,* ±SE. *P* < 0.001 for all treatment groups by one-way ANOVA.

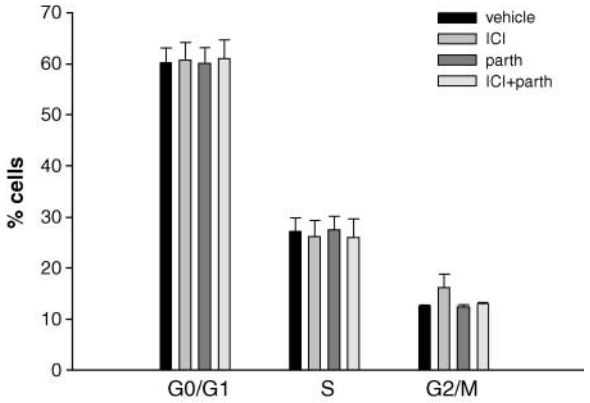


Figure 4. Combined treatment with Faslodex and parthenolide has no effect on the cell cycle profile of MCF7/LCC9 cells. Cells were treated with 100 nmol/L Faslodex, 600 nmol/L parthenolide, Faslodex + parthenolide, or ethanol vehicle in CCS-IMEM for 24 h before cell cycle analysis. *Columns,* mean for three independent experiments (% total cells); *bars,* ±SE.

an accumulation of cells in the G_0-G_1 phase of the cell cycle (1, 8). In some cell systems, parthenolide can arrest cells at the G_2-M phase transition (35). To test whether parthenolide restored the cytostatic activities of Faslodex or induced a G_2-M blockade, MCF7/LCC9 cells were treated with Faslodex ± parthenolide or ethanol vehicle for 24 hours prior to cell cycle analysis (Fig. 4). Parthenolide alone or in combination with Faslodex does not alter the MCF7/LCC9 cell cycle profile, indicating that a block in cell cycle progression does not explain the synergistic reduction in cell growth.

Faslodex and other antiestrogens actively promote apoptosis, and parthenolide has been shown to cooperatively enhance apoptosis induced by other cytotoxic agents such as paclitaxel and 4-hydroxyphenylretinamide (19, 20). Therefore, we measured the effects of Faslodex ± parthenolide or ethanol vehicle on apoptosis as detected by immunostaining for FITC-conjugated Annexin V and propidium iodide staining (Table 1). Approximately 3% of vehicle-treated and 4% of Faslodex-treated MCF7/LCC9 cells undergo apoptosis. In contrast, parthenolide treatment increases the apoptotic fraction to nearly 10%; upon cotreatment with Faslodex and parthenolide, 18% of the cells undergo apoptosis. Importantly, the level of apoptosis seen in the presence of the Faslodex/parthenolide combination was essentially identical to that induced by Faslodex alone in the antiestrogen-sensitive LCC1 cells (Table 1). The strong induction of apoptosis in MCF7/LCC9 cells seen in the presence of both drugs is statistically significant compared with either Faslodex or parthenolide alone ($P = 0.001$ and $P = 0.01$, respectively). The calculated RI = 2.28 for the parthenolide/Faslodex interaction indicates synergistic induction of apoptosis in antiestrogen-resistant MCF7/LCC9 cells.

Parthenolide stabilizes the inhibitor IκB, leading to the retention of p65 in the cytoplasm in an inactive state (36). Therefore, we measured the effects of Faslodex ± parthenolide or ethanol vehicle on IκBα expression (Fig. 5). Since protein levels of IκBα were unchanged in MCF7/LCC9 cells regardless of treatment, parthenolide may be acting through other alternative mechanisms to synergize with Faslodex and restore the apoptotic response to antiestrogen-resistant MCF7/LCC9 cells.

Discussion

Our previous studies reported the p65/RelA subunit of NFκB as being up-regulated in MCF-7–derived MCF7/LCC9 breast cancer cells that had acquired resistance to Faslodex (10, 11). We have now identified additional changes in the expression of NFκB pathway members in these cells and showed that pharmacologic inhibition of NFκB restores Faslodex sensitivity by markedly enhancing apoptosis. Because the NFκB inhibitor parthenolide is currently being investigated in clinical trials (21), these findings have direct clinical relevance and provide support for the design of clinical studies combining antiestrogens and NFκB inhibitors such as parthenolide in ER+ breast cancer.

Protein expression of the p65/RelA subunit of NFκB is increased ~2-fold in MCF7/LCC9 cells when compared with antiestrogen-sensitive MCF7/LCC1 cells; this agrees with the up-regulation in mRNA levels previously observed (10). However, NFκB-dependent transcriptional activity is elevated almost 10-fold in MCF7/LCC9, implying that other elements of the NFκB signaling pathways are activated in these cells. We found no changes in p50 expression or association with p65; there were also no alterations in expression of p52 NFκB2 (data not shown) or the NFκB negative regulator IκBα. PI3K-dependent signaling can also activate NFκB and Akt activation, a primary downstream target of PI3K, has been implicated in antiestrogen resistance. However, we found no differences the levels of phospho-Akt, indicating that this pathway also is unlikely to account for the increased NFκB activity.

In contrast, MCF7/LCC9 cells express ~2-fold higher levels of NEMO/IKKγ. NEMO binds to IKKβ and controls the formation of the IKK complex (37); this is required for the activation of NFκB in response to external stimuli such as tumor necrosis factor α (31, 38). Up-regulation of NEMO

Table 1. Faslodex and parthenolide synergistically enhance apoptosis in MCF7/LCC9 cells

Cell line/drug	% Apoptosis ± SE	*P*
LCC1 vehicle	4.22 ± 0.98	—
LCC1 ICI	19.96 ± 4.43	0.03*
LCC9 vehicle	3.20 ± 1.96	—
LCC9 ICI	4.41 ± 0.90	0.61*
LCC9 parthenolide	9.95 ± 1.21	0.04*
LCC9 ICI + parthenolide	18.34 ± 1.45	0.003*, 0.001†, 0.01‡

NOTE: RI = 2.28 for combination of ICI and parthenolide.
*Versus vehicle.
†Versus ICI.
‡Versus parthenolide.

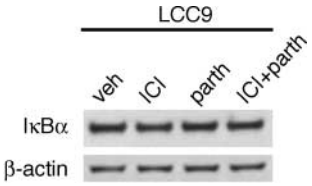


Figure 5. Combined treatment with Faslodex and parthenolide has no effect on the stability of IκBα expression. LCC9 cells were treated with 100 nmol/L Faslodex, 600 nmol/L parthenolide, Faslodex + parthenolide, or ethanol vehicle in CCS-IMEM for 24 h before cell lysis. Lysates (20 μg) were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted. *β-actin*, loading control.

in MCF7/LCC9 cells would enhance the kinase activity of IKK and likely adds to the elevated levels of p65 to further increase basal NFκB activation.

Constitutive NFκB activity is known to arise as breast cancer cells progress to an estrogen-independent (26, 27) and antiestrogen-resistant state (10). However, this is the first report implicating NEMO/IKKγ in these events. Regulatory control of NEMO is complex, involving sequential small ubiquitin-like modifier and ubiquitin modification occurring in both the cytoplasm and nucleus (39). Whether the hormonal regulation of NEMO is altered in the MCF7/LCC9 cells has not been determined, but is currently being pursued to clarify further the mechanism by which NFκB activity is elevated in breast cancer cells with acquired antiestrogen resistance.

The NFκB inhibitor parthenolide strongly represses the proliferation of MCF7/LCC9 cells (100 nmol/L, ineffective; IC_{50}= 600 nmol/L) and restores their sensitivity to Faslodex. For example, whereas treatment with 100 nmol/L Faslodex alone is ineffective, 50% growth inhibition is achieved in the presence of only 100 nmol/L parthenolide. This interaction between Faslodex and parthenolide, which generates an estimated RI = 1.82, is synergistic (29). The restoration of Faslodex sensitivity by parthenolide is a significant finding and directly supports our hypothesis that the up-regulated NFκB activity present in MCF7/LCC9 cells is a major contributor to the antiestrogen resistance phenotype.

We have explored several mechanisms through which parthenolide may restore Faslodex sensitivity in antiestrogen-resistant cells. For example, NFκB inhibition could rescue the ability of Faslodex to block ER-dependent transcriptional activity. Expression of several well-characterized estrogen-regulated genes, including *progesterone receptor*, *pS2*, and *cathepsin D* (10), is increased in MCF7/LCC9 cells. This may reflect the 7-fold higher basal levels of ER-dependent transcription in vehicle-treated MCF7/LCC9 cells, relative to that seen in MCF7/LCC1 cells. Faslodex completely inhibits transcription of an ERE-luciferase construct in MCF7/LCC1 cells (antiestrogen sensitive) but has no effect on ERE-mediated transcription in MCF7/LCC9 cells (antiestrogen resistant). Whereas exogenous expression of p65/RelA NFκB can repress ER transcriptional activity *in vitro* (40), treatment with parthenolide does not restore the ability of Faslodex to affect ER transcriptional function in MCF7/LCC9 cells. Thus, the elevated basal ER-dependent transcriptional activity in these cells is likely not due to NFκB up-regulation. Parthenolide may rescue traditional Faslodex-mediated effects in these cells downstream of ER activity and/or in nonclassic ER pathways.

Faslodex induces G_0-G_1 arrest in MCF7/LCC1 cells treated with 100 nmol/L Faslodex (data not shown), but neither Faslodex nor parthenolide has any effect on MCF7/LCC9 cell cycle distribution. Parthenolide-mediated G_2 arrest has been observed at 10-fold higher concentrations than were used in this study (35); therefore, the possibility that MCF7/LCC9 cell cycle progression is inhibited by much higher concentrations of parthenolide cannot be excluded. Indeed, several studies with parthenolide and other sesquiterpene lactones used 1 to 10 μmol/L or greater concentrations to achieve 50% inhibition of cell growth, whereas our cells required up to 10-fold lower concentrations to achieve the same results.

Our results show that the inhibition of cell growth by Faslodex and parthenolide is not primarily cytostatic in nature. In marked contrast, a combination of Faslodex and parthenolide synergistically promotes programmed cell death (RI = 2.28). Importantly, the proportion of apoptotic cells observed in the presence of both drugs (19%) is comparable to that seen when MCF7/LCC1 cells are treated with the same dose of Faslodex (19.9%). Parthenolide can enhance the apoptotic activities of taxanes and retinoids (19, 20), and we now show that it can also potentiate the death of antiestrogen-resistant breast cancer cells by restoring their sensitivity to Faslodex.

DeGraffenried et al. (41) have recently reported that NFκB inhibition by parthenolide increased breast cancer cell sensitivity to tamoxifen. However, these investigators used MCF-7 cells genetically engineered to overexpress activated Akt; these cells exhibit tamoxifen resistance and NFκB activation that is entirely dependent on Akt-mediated pathways. In our cell system, which was derived by selection in the presence of Faslodex rather than by genetic engineering of the cells, NFκB up-regulation does not correlate with enhanced Akt activity.

Sesquiterpene lactones in general, and parthenolide in particular, can prevent the degradation of IκB, block activation of IKK, alkylate cysteine-38 in p65/RelA to prevent DNA binding, and inhibit inducible nitric oxide synthase (36, 42, 43). We found no evidence of IκBα stabilization when MCF7/LCC9 cells were treated with parthenolide either in the absence or presence of Faslodex. Parthenolide can also inhibit p42/44 mitogen-activated protein kinase activity (42) but we observed no reduction in the levels of phospho-mitogen-activated protein kinase in our cells upon parthenolide treatment (data not shown). Interestingly, Nakshatri et al. (44) have recently shown that parthenolide can reverse breast cancer cell resistance to tumor necrosis factor-related apoptosis-inducing ligand by enhancing the activation of c-Jun NH_2-terminal kinase. Whether parthenolide-induced c-Jun NH_2-terminal kinase activity plays a role in its restoration of Faslodex sensitivity in MCF7/LCC9 cells has yet to be determined.

Our studies clearly show that treatment with the NFκB inhibitor parthenolide is a viable approach to restoring Faslodex-induced apoptosis in breast cancer cells that have acquired resistance. Several preclinical studies have shown that parthenolide also is effective in the treatment or chemoprevention of cancer cell growth (45). A phase I study of feverfew in cancer patients was recently completed and reported no significant toxicity observed at the doses tested (21). Other direct or indirect inhibitors of the NFκB pathway also show promise as antiproliferative agents (16) and include some nonsteroidal anti-inflammatory drugs, antioxidants, immunosuppressants, proteasome inhibitors, and glucocorticoids. Our work now shows that inhibition of NFκB may also be useful for the treatment of ER-positive breast cancers that have acquired resistance to antiestrogen therapy, thus restoring the activity of one of the most active and least toxic modalities available in the treatment of endocrine-dependent breast cancer.

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Rebecca B. Riggins, Alan Zwart, Ruchi Nehra, et al.

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