Artemisinin selectively decreases functional levels of estrogen receptor-alpha and ablates estrogen-induced proliferation in human breast cancer cells

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MCF7 cells are an estrogen-responsive human breast cancer cell line that expresses both estrogen receptor (ER) α and ERβ. Treatment of MCF7 cells with artemisinin, an antimalarial phytochemical from the sweet wormwood plant, effectively blocked estrogen-stimulated cell cycle progression induced by either 17β-estradiol (E2), an agonist for both ERs, or by propyl pyrazole triol (PPT), a selective ERα agonist. Artemisinin strongly downregulated ERα protein and transcripts without altering expression or activity of ERβ. Transfection of MCF7 cells with ERα promoter-linked luciferase reporter plasmids revealed that the artemisinin downregulation of ERα promoter activity accounted for the loss of ERα expression. Artemisinin treatment ablated the estrogenic induction of endogenous progesterone receptor (PR) transcripts by either E2 or PPT and inhibited the estrogen stimulation of a luciferase reporter plasmid driven by consensus estrogen response elements (EREs). Chromatin immunoprecipitation assays revealed that artemisinin significantly downregulated the level of endogenous ERα bound to the PR promoter, whereas the level of bound endogenous ERβ was not altered. Treatment of MCF7 cells with artemisinin and the pure antiestrogen fulvestrant resulted in a cooperative reduction of EREs protein levels and enhanced G1 cell cycle arrest compared with the effects of either compound alone. Our results show that artemisinin switches proliferative human breast cancer cells from expressing a high ERα:ERβ ratio to a condition in which ERβ predominates, which parallels the physiological state linked to antiproliferative events in normal mammary epithelium.

Introduction

Breast cancer is the most common malignancy and the second leading cause of death among women in North America. Therapeutic options for women with breast cancer depend on many prognostic factors of which response to estrogens plays a central role (1). Sensitivity to estrogens is conferred by the presence of two distinct intracellular receptors, estrogen receptor (ER) α and ERβ that regulate the transcription of distinct as well as overlapping sets of target genes (2). The exact roles of ERβ and ERβ in breast carcinogenesis are not clear, although a high ERα:ERβ ratio correlates well with high levels of cellular proliferation, whereas high ERβ:ERα is generally linked to antiproliferative events (3–14).

The majority of breast cancers expressing ERα are estrogen sensitive and are clinically managed with mixed non-steroidal anti-estrogens such as tamoxifen, although detrimental side effects to long-term treatment with this antiestrogen include an increased endometrial cancer risk and eventual resistance (15–17). Pure steroid antiestrogens, such as fulvestrant (Ful), are promising therapeutics for hormone-responsive breast cancer (17). Estrogen-unresponsive breast cancers are thought to arise from estrogen-responsive precursors. The current options for treatment of estrogen-unresponsive breast cancer are surgical removal of the tumors, general chemotherapy and/or radiation therapy (1). Therapeutic strategies that ablate cellular sensitivity to estrogens with minimum side effects could effectively prevent tumor progression to a hormone refractory state.

Natural plant compounds provide a potential source of such chemotherapy agents that act on various types of human breast cancers. One such promising natural compound is artemisinin, a sesquiterpene lactone that was isolated from a Chinese plant Artemisia annua (commonly known as qinghaosu or sweet wormwood) that has been used by Chinese traditional medicine practitioners for at least 2000 years to treat fever (18). Artemisinin is a potent Food and Drug Administration-approved antimalarial agent that has been used in clinical management of malaria. Evidence that artemisinin and some of its active derivatives have antiproliferative effects in human cancer cells is beginning to emerge, although relatively little mechanistic information has been established (18–23). The Developmental Therapeutics Program of the National Cancer Institute, USA, which analyzed 55 human cancer cell lines, showed that artemesinate, the semisynthetic derivative of artemisinin, has anticancer activity against several types of cancers including leukemia, colon cancer cell lines, melanoma, breast, ovarian, prostate, central nervous system and renal cancer cell lines (24). Artemisinin also has inhibitory effects on the growth of certain cancer cells in culture and cell line-derived tumors in nude mouse xenografts. In rats exposed to the potent indirect mammalian carcinoma 7,12-dimethylbenz(a)anthracene, orally administered artemisinin inhibited the genesis of mammary cancers (25). Because these cancers are predominantly estrogen responsive (26), inhibition by artemisinin suggests that this phytochemical might be disrupting estrogenic promotion of the initiated rat mammary epithelial cells, by possibly interfering with proliferative signaling through ERα. However, nothing is known about the potential effects of artemisinin on ER expression and/or function in human breast cancer cells.

Employing MCF7 cells, an estrogen-responsive human breast cancer cell line, we report that artemisinin selectively downregulated ERα expression by attenuating its promoter activity without altering ERβ levels and disrupted ERα-responsive growth and gene expression. Our results show that artemisinin switches highly proliferative human breast cancer cells from expressing a high ERα:ERβ ratio to a growth-arrested state in which expression of ERβ is significantly greater to that of ERα, similar to a state linked to antiproliferative events in both normal mammary epithelium and in breast cancer cells.

Materials and methods

Materials

Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), calcium- and magnesium-free phosphate-buffered saline, l-glutamine and trypsin-versene mixtures were purchased from Biowhittaker (Walkersville, MD). Insulin (bovine), 17β-estradiol (E2), Ful and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO). Propyl pyrazole triol (PPT) was obtained from Tocris (Ellisville, MO). Artemisinin was purchased from Aldrich (Milwaukee, WI). The sources of other reagents are either listed below or were of the highest purity available. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

MCF7 human breast adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA). MCF7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 µg/ml bovine insulin and 100 U penicillin/streptomycin at 37°C in a chamber with 5% carbon dioxide. Artemisinin (99.9% high-performance liquid chromatography grade) was dissolved in appropriate concentrations; stock solutions were a 1000-fold higher
than the desired concentration in the medium. E2 and PPT were also dissolved in DMSO the same way at 1000-fold higher concentration than desired. DMSO was used as vehicle control for all experiments. Phenol red-free media supplemented with dextran charcoal-stripped serum (Gemini Bio-Products, West Sacramento, CA) was employed for all estrogen sensitivity assays.

ERα gene promoter plasmid transfection and luciferase activity assays
The longest promoter construct (−3561) was a kind gift of Lisa McPherson at Stanford University. The −1892 fragment was amplified from purified genomic DNA isolated from LNCaP prostate cancer cells using forward primer 5′-TGCCATCCACCACCAAACATC-3′ with an Mlu restriction enzyme cut site at the 5′ end and reverse primer 5′-TAAGATCTGGTCTCCCGA-3′ with a BglII restriction enzyme cut site on the 5′ end and was amplified using the VENT polymerase (NEB, Ipswich, MA). This polymerase chain reaction (PCR) employed an elongation time of 2.5 min and 38 cycles. After purification by Qiagen Gel Extraction Kit (Qiagen, Valencia, CA), this was inserted into the pgl3 reporter plasmid. This was achieved by digestion of reporter plasmid with MluI and BglII (NEB). The fragment and vector were purified of restriction enzymes using PCR purification kit (Promega) followed by overnight ligation using T4 ligase (NEB), grown in TOP10 cloning bacteria (Invitrogen, Carlsbad, CA) and verified by internal digest and sequencing.

The pELRa-958 construct was amplified using forward primer 5′-ATGTGTTGTTGATGGTCTG-3′ with an MluI restriction enzyme cut site and reverse primer 5′-AAAGACACACGCGCCAGTGA-3′ with a BglII cut site inserted on the 5′ end, amplified by (Promega, Madison, WI) GoTaq Green polymerase using LNCaP genomic DNA again. This fragment was amplified and plasmid was generated as described above. This plasmid was verified by internal digestion using NsiI. Transfection was performed in serum-supplemented media using Fugene 6 (Roche, Pleasanton, CA) according to the manufacturer’s instructions. Twenty-four hours post-transfection, cells were treated with DMSO or 300 μM artemisinin for 24 h. Cells were lysed and relative luciferase activity was evaluated using Promega luciferase assay kit (Promega) and a luminometer. Relative luciferase activities were normalized to the protein input with standard error. Three replicates per treatment were performed.

Estrogen response element-luciferase assays
Plasmid containing the consensus vitellogenin estrogen response element (ERE) in pgll2 vector was transfected according to the manufacturer’s instructions using Fugene 6 (Roche). Twenty-four hours later, the media was replaced with phenol red-free media containing 10% dextran charcoal-stripped FBS. Twenty-four hours later, the cells were treated with DMSO or 300 μM artemisinin for 48 h. The cells were then treated with DMSO, 10 nM E2 or 100 nM PPT. After 24 h of treatment, cells were lysed and subjected to luciferase activity assays using the luciferase assay system kit (Promega). The amount of protein was determined using the Lowry method, and the relative light units were normalized to protein input. Three replicates per treatment were employed.

Western blotting for ERα and ERβ
After the indicated treatments, cells were harvested in the media, pelleted by centrifugation at 8000 r.p.m. for 5 min, resuspended in 1 ml phosphate-buffered saline and pelleted again by centrifugation. Thirty microgram of protein extract was then subjected to electrophoresis and separation, transfer and immunoblotting using specific antibodies as described (13). Specificity of antibodies was verified using recombinant proteins (Santa Cruz Biotechnology).

Reverse transcription–PCR
MCF7 cells were harvested in Trizol Reagent (Invitrogen), and the recommendation protocol was followed to extract total RNA. RNA was quantified using a spectrophotometer and the quality of RNA was confirmed using A260/A280 and by electrophoresis on 1% agarose gels. Two microgram of total RNA was subjected to reverse transcription (RT) using Mu-MLV reverse transcriptase (Invitrogen) with random hexamers, deoxynucleoside triphosphates and RNase inhibitor (Invitrogen). Four microliter of complementary DNA was then subjected to PCR using primers specific to ERα, ERβ, progesterone receptor (PR) and glyceraldehyde phosphate dehydrogenase as described (27). The PCR products were run on 1.5% agarose gels along with a 1 kb Plus DNA Ladder (Invitrogen).

Affinity chromatography for ER–ERE binding
This assay was performed as described elsewhere (13). Lysates from DMSO- or artemisinin-treated MCF7 cells were employed in this assay.

Flow cytometric analyses of DNA content
MCF7 cells were plated onto six-well tissue culture dishes and grown in phenol red-free media containing dextran charcoal-stripped 10% FBS. Cells were treated with 10 nM E2 or 100 nM PPT in the presence or absence of 300 μM artemisinin. Cells were exposed to indicated treatments for 48 h and hypotonically lysed in 0.5 ml of DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate and 0.05% Triton X-100). The nuclei were subjected to flow cytometric analysis as described (13).

Chromatin immunoprecipitation assay
MCF7 cells were grown to subconfluence and treated for 48 h with 300 μM artemisinin or DMSO vehicle control. Cross-linking of DNA to bound protein was done with formaldehyde (1% final concentration), which was added directly to the media and quenched with 2.5 M glycine. Cells were lysed with chromatin immunoprecipitation (ChIP) lysis buffer (50 mM N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid, pH 7.5, 140 mM NaCl, 1% Triton X-100 and 0.1% sodium deoxycholate) and protease inhibitors described previously (13). Cells were sonicated, supernatants were standardized based on protein content using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). One milligram of protein was used for each immunoprecipitation. Two milligram of the ERα-specific antibody sc-8005X or the ERβ-specific antibody sc-6820X (Santa Cruz Biotechnology) was used for immunoprecipitation of protein–DNA complexes. Complexes were precipitated using Sepharose-G beads (GE Healthcare, Piscataway, NJ), followed by 2 × ChIP lysis buffer, 2 × ChIP wash buffer (10 mM Tris, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate and 1 mM ethylenediaminetetraacetic acid) and 2 × Tris-ethanolamine. Immunoprecipitated DNA was amplified using specific primers and PCR mix (1 U Taq polymerase (NEB), 1.5 mM MgCl2, 0.2 μM deoxynucleoside triphosphates) as follows: ERα and ERβ (1 min at 94°C hotstart, 30s/94°C, 30s/58°C, 30s/72°C, 37 cycles) and 1% input control (1 min at 94°C hotstart, 30s/94°C, 30s/58°C, 30s/72°C, 30 cycles). Primers for PR are as listed previously (28). Products were visualized on a 1.5% agarose gel stained with 0.1% ethidium bromide.

Results
Artemisinin blocks the estrogenic stimulation of MCF7 breast cancer cell proliferation
MCF7 is a human breast cancer cell line that expresses both the ER subtypes, ERα and ERβ, and is highly estrogen responsive. Addition of E2 or the ERα-selective agonist PPT to MCF7 cells cultured in steroid-deficient media leads to a robust increase in cell proliferation and stimulation of cell cycle progression (29). To assess the effect of artemisinin on the sensitivity of MCF7 cells to estrogenic stimulation, MCF7 cells cultured in steroid-deficient phenol red-free media were treated with or without 300 μM artemisinin for 48 h in the presence or absence of E2 or PPT. For the vehicle control, cells were incubated with DMSO and under steroid-deficient conditions predictably exhibited a growth arrest characterized by most cells blocked in G1 phase of the cell cycle and a very small number of cells in S phase. Cell cycle progression was examined by flow cytometry of propidium iodide-stained cell nuclei. As shown in Figure 1A, MCF7 cells treated with 10 nM E2 or 100 nM PPT in comparison with DMSO-treated cells showed the expected robust increase in proportion of cells in S phase (16.5% DMSO versus 48.7% E2 and 50.4% PPT) that was accompanied by a proportional decrease of population of cells in G1 phase of the cell cycle (77% DMSO versus 49.2% E2 and 48.3% PPT). In the presence of 300 μM artemisinin, the E2- or PPT-stimulated increases in the S phase population and decreases in G1 phase population of MCF7 cells were ablated (Figure 1A). As shown in Figure 1B, quantification of the flow cytometry experiments from three independent experiments revealed that the overall DNA content profiles of MCF7 cells treated with combinations of artemisinin and E2 or artemisinin and PPT resembled the profile of MCF7 cells not exposed to estrogens. Because artemisinin effectively blocks PPT-induced proliferation of MCF7 cells, our results suggest that artemisinin disrupts estrogen-responsive signaling through ERα.

Artemisinin selectively downregulates the level of the ERα receptor subtype in MCF7 cells
The effects of artemisinin on the levels of the ER subtypes were examined through a 72 h time course. MCF7 cells were treated with 100 μM artemisinin, 300 μM artemisinin or with the DMSO vehicle control for 24, 48 or 72 h, and the electrophoretically fractionated total cell lysates

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using specific oligonucleotide primers. As shown in Figure 3A, 300 µM artemisinin downregulated ERα protein levels in MCF7 cells as early as 24 h, with a maximal effect observed by 48 h of phytochemical treatment. In contrast, ERβ production in MCF7 cells was not altered in response to artemisinin treatment. Heat shock protein 90 levels were not altered with artemisinin treatment and were used as a control for protein loading. The optimal dose of artemisinin for maximal downregulation of ERα was 300 µM and this concentration of artemisinin was used for the remainder of this study unless specifically mentioned otherwise.

In a variety of systems, including human breast cancer cells, ER protein levels in breast cancer cells can be regulated by modulation of proteasome-mediated degradation as well as by changes in transcriptional regulation of the ER genes (30,31). To determine whether the artemisinin downregulation of ERα is due to the proteasome degradation pathway, MCF7 cells were treated with or without artemisinin for 48 h. In the presence or absence of 20 µM MG132, a 26S proteasome inhibitor, for 4 h. Immunoblot analysis showed that although treatment with MG132 caused an accumulation of ERα protein, artemisinin still was able to strongly downregulate ERα protein levels (Figure 2B). β-Actin was used as a gel loading control for this experiment. Thus, MG132 treatment was unable to reverse the artemisinin-mediated downregulation of ERα protein, which suggests that artemisinin does not alter ERα protein degradation. Consistent with this concept, artemisinin was unable to downregulate ERα in MCF7 cells transfected with a constitutive cytomegalovirus promoter-containing plasmid (pCMV)-ERα expression vector compared with cells transfected with a control pCMV-Neo expression vector (Figure 2C). Ectopic elevated expression of ERα did not alter the cell cycle kinetics of MCF7 cells consistent with previous observations (14).

**Artemisinin downregulates ERα transcript levels by attenuating promoter activity**

To examine the effects of artemisinin on ERα transcript levels, MCF7 cells were treated with or without 300 µM artemisinin or with the DMSO vehicle for 24, 48 and 72 h, isolated protein extracts were fractionated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electropherotherically transferred to nitrocellulose membranes and immunoblots were probed with antibodies specific for ERα and ERβ. Heat shock protein 90 (Hsp 90) was used as a protein loading control. (B) MCF7 cells were treated with DMSO or 300 µM artemisinin for 48 h. Treated cells were exposed to DMSO or 20 µM MG132, a proteasome inhibitor for 4 h. Cells were lysed and 30 µg total protein was subjected to immunoblot analysis with antibodies specific for ERα or β-Actin. (C) MCF7 cells were transfected with pCMV-Neo or pCMV-ERα. Twenty-four hours later, transfected cells were treated with DMSO or 300 µM artemisinin. After 48 h of treatment, cells were lysed and total protein was electrophoresed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were electrotherotherically transferred to nitrocellulose and immunoblotted with antibodies specific for ERα or β-Actin.
Artemisinin inhibits ERα expression and function in breast cancer cells

feedback loop is known (32). The regulatory role of CCAAT/enhancer binding protein alpha and/or beta along with erythroblastosis gene has been well characterized in other gene promoters (33–35). It is possible that GATA family of transcription factors are repressed at the post-transcriptional or post-translational level by artemisinin. CCAAT/enhancer binding protein family of transcription factors and erythroblastosis gene might be activated by artemisinin, leading to repression of ERαl gene transcription. Mutagenesis of these specific sites as well as ChIP experiments will reveal the precise mechanism by which artemisinin downregulates ERαl promoter activity. Artemisinin had no effect on the promoter activity of MCF7 cells transfected with the constitutively active pCMV-luciferase reporter plasmid (data not shown), and the activity of the ERαl promoter containing reporter plasmids were at least a 100-fold higher than the empty vector-transfected cells. Taken together, these data demonstrate that artemisinin downregulates ERαl expression by attenuating ERαl promoter activity that leads to a significant downregulation of ERαl transcript and protein levels.

Artemisinin disrupts the estradiol or PPT activation of consensus ERE-driven reporter plasmid and results in decreased ERE-bound ERαl

To confirm that artemisinin treatment results in the disruption of ER-stimulated gene transcription, the effects of artemisinin on the estrogen stimulation of a reporter plasmid containing a vitellogenin consensus ERE fused to the luciferase gene were tested. Transfected MCF7 cells were cultured in steroid-deficient phenol red-free media supplemented with bovine insulin, treated with or without artemisinin for 48 h, incubated with the ERα agonists 10 nM E2 or 100 nM PPT in the presence or absence of artemisinin for 24 h and then the total luciferase activity was then assayed in cell extracts. As shown in Figure 4A, artemisinin effectively blocked either the E2- or PPT-induced reporter plasmid activity. Interestingly, artemisinin treatment decreased the total ERE activity of MCF7 cells in the absence of estrogens, suggesting that disruption of ERαl may also result in inhibition of ERαl ligand-independent activity. Levels of basal ERE activity in MCF7 cells were high possibly due to the stimulation of ERα phosphorlylation by insulin and other growth factor signaling pathways. Because E2, an ERα and ERβ agonist, as well as PPT, an ERα-specific agonist, induced the ERE-driven reporter plasmid to the same extent, these results are consistent with the concept that in cells with both receptor subtypes, most of the estrogen-induced transcriptional activity is mediated through the activation of ERαl (2).

The relative binding of ERαl and ERβ to a consensus ERE in vitro was evaluated in artemisinin-treated and -untreated cells using affinity chromatography. Briefly, total cellular protein lysates from cells treated with artemisinin or with the DMSO vehicle control were passed through columns containing streptavidin beads conjugated to biotinylated ERE. Bound proteins were eluted using a high-salt buffer and eluates containing ERE-bound proteins were analyzed by immunoblotting of electrophoretically fractionated samples. As shown in Figure 4B, artemisinin treatment of cells significantly inhibited the level of ERE-bound ERαl, which is consistent with the artemisinin downregulation of ERα expression. In contrast, the levels of ERE-bound ERβ remain unaltered. A similar result with ERE-bound ERβ was also observed by the same technique in ERαl/ERβ+ MDA-MB-231 breast cancer cells, where artemisinin did not affect ERE- or activator protein-1-controlled luciferase activity (data not shown). Taken together, these results demonstrate that artemisinin-mediated decreases in levels of ERαl directly lead to the decrease in ERE-bound ERαl, which in turn causes the loss of ERα-stimulated gene expression in MCF7 cells. The relative amounts of functional ERE-bound ERβl found in artemisinin-treated cells is much higher than ERαl levels, correlating well with artemisinin-induced growth arrest of MCF7 cells.

Artemisinin blocks estradiol or PPT activation of target gene expression by decreasing levels of promoter-bound ERαl

Artemisinin’s ability to disrupt the regulated expression of the endogenous PR, a primary estrogen-responsive gene, was studied by...
RT-PCR. MCF7 cells cultured in steroid-deficient phenol red-free media were treated with combinations of 300 μM artemisinin and either 10 nM E₂ or 100 nM PPT for 24 h, and level of PR transcripts was examined by RT-PCR. As shown in Figure 5A, artemisinin treatment ablated the robust E₂ or PPT stimulation of PR transcripts compared with gliceraldehyde phosphate dehydrogenase, the loading control. In vivo analysis using ChIP shows that ERα binding to endogenous chromatin, specifically at the estrogen-responsive composite site in the PR promoter, was also decreased in the presence of artemisinin, as shown in Figure 5B. Taken together, these results demonstrate that the artemisinin-mediated decrease in levels of ERα directly lead to the decrease in ERE-bound ERα, which in turn causes the loss of ERα-stimulated gene expression in MCF7 cells. The relative amounts of functional ERE-bound ERβ found in artemisinin-treated cells is much higher than ERα levels, correlating well with artemisinin-induced growth arrest of MCF7 cells.

Artemisinin cooperates with Ful, a pure antiestrogen, in causing G₁ cell cycle arrest and ERα downregulation in MCF7 human breast cancer cells

Ful, a steroidal pure antiestrogen, has been shown to cause a cell cycle arrest and to induce degradation of the ERα protein in MCF7 breast cancer cells (30). Because Ful treatment also results in decreased ERα levels, we tested whether suboptimal concentrations of these two molecules could cooperate in their cell cycle and ER expression effects. The suboptimal doses of Ful and artemisinin in MCF7 cells were determined by independent dose response experiments (data not shown) and represent concentrations that display only a mild cell cycle effect. MCF7 cells were treated under proliferative conditions in estrogen-rich media (10% FBS supplemented) for 48 h with 1 nM Ful, 50 μM artemisinin, a combination of both molecules or with the DMSO vehicle control. Cell nuclei were stained with propidium iodide and analyzed for DNA content using flow cytometry. As shown in Figure 6A and B, treatment with 50 μM artemisinin caused a modest increase in the number of cells in G₁ (44.1% DMSO versus 56.4% artemisinin), whereas treatment with 0.5 nM Ful leads to a small increase in proportion of cells in G₁ (44.1% DMSO versus 59.4% Ful). However, treatment of MCF7 cells with a combination of suboptimal doses of artemisinin and Ful led to a striking increase in number of cells in G₁ (44.1% DMSO versus 74.8% Ful + artemisinin) with a proportional decrease in S phase (49.1% DMSO versus 23.6% Ful + artemisinin). As shown in Figure 6A, proportion of cells in G₂/M was also significantly decreased (6.9% DMSO versus 1.5% Ful + artemisinin).

Cells treated with the above mentioned doses of artemisinin and Ful were analyzed for the levels of the hyperphosphorylated retinoblas toma protein (a marker for G₁ to S cell cycle progression) and of both ER subtypes by immunoblotting with specific antibodies. As shown in Figure 6B, the enhanced G₁ cell cycle arrest observed with a combination of suboptimal concentrations of artemisinin and Ful was also accompanied by an ablation of cellular levels of hyperphosphorylated retinoblastoma protein levels. Treatment with suboptimal doses of either artemisinin or Ful for 48 h has a minimal effect on the level of ERα protein. However, a combination of the two caused a marked
downregulation of ERα protein levels (Figure 6B). In contrast, ERβ levels remained unaltered.

Discussion

A direct cellular consequence of the artemisinin-mediated downregulation of ERα expression and disruption of ERα responsiveness in the absence of any effects on ERβ expression is a significant change in ratio of functional ERα:ERβ in MCF7 human breast cancer cells. An emerging concept concerning estrogen-responsive breast cancers is that the relative levels of ERα and ERβ play an important role in controlling estrogen-regulated proliferative and differentiation properties in estrogen-responsive cells. A high ratio of ERα:ERβ is associated with an increased proliferative state of estrogen-responsive cells (7). Reversal of this ratio resulting in lower intracellular levels of ERβ:ERα correlates with an inhibition of proliferation and induction of terminal differentiation in certain estrogen-responsive breast cancer cells (6,9,12,14). We have shown that artemisinin switches highly proliferative human breast cancer cells from expressing a high ERα:ERβ ratio to a condition in which expression of ERβ is significantly greater to that of ERα, which parallels the physiological state linked to antiproliferative events in both normal mammary epithelium and in breast cancer. We propose that the artemisinin-regulated reversal of the functional levels of the ER subtypes is responsible for the artemisinin-induced inhibition of estrogen-responsive growth. This artemisinin-mediated disruption of estrogen responsiveness is observed in the loss of estrogen stimulation of PR expression and ERE-driven reporter plasmid transcription, as well as the selective loss of ERα that can bind its corresponding ERE. The dose of artemisinin used was the minimum dose of artemisinin that resulted in a maximum G1 cell cycle arrest as assessed by flow cytometric analysis. This dose of artemisinin cannot be translated to the daily oral dose employed in humans against malaria (up to 1000 mg). Artemisinin is delivered orally to mice and harvested in phosphate-buffered saline and stained with a hypotonic solution containing propidium iodide. Stained nuclei were subjected to flow cytometric analysis. This dose of artemisinin was shown that production of high levels of functional ERβ leads to increased formation of both ERβ homodimers and of ERα/ERβ heterodimers, which would be predicted to alter the steroid-regulated gene expression profile (36).

Ectopic expression of ERβ in highly proliferating MCF7 cells or in T47D breast cancer cells leads to cell cycle arrest (12,14). Ectopic overexpression of ERβ in estrogen-responsive breast cancer cell lines also results in the growth arrest of nude mouse xenografts. The precise ERβ-regulated target genes that mediate the growth inhibition response has not been elucidated, although it has been shown that production of high levels of functional ERβ leads to increased formation of both ERβ homodimers and of ERα/ERβ heterodimers, which would be predicted to alter the steroid-regulated gene expression profile (36).

Estrogen-induced proliferation of mammary epithelial cells has been shown to be necessary for development of both estrogen-responsive and -unresponsive human and rodent mammary cancers. Studies in rodents showed that ovariectomy caused significant retraction of the mammary glands to developing mammary cancer
(37). Deregulated expression of ER\textsubscript{\alpha} led to development of high-risk premalignant lesions in the mammary gland (7). Further strengthening this concept, antiestrogens such as tamoxifen have been shown to display strong chemopreventive properties (16). However, chronic treatment with relatively high concentrations of tamoxifen can cause undesirable systemic side effects in the patient, which can limit the use of tamoxifen (17). Majority of the breast cancers that express functional ER\textsubscript{\alpha} are estrogen sensitive and can be clinically managed with mixed non-steroidal antiestrogens such as tamoxifen. Our studies suggest that artemisinin treatment could potentially slow down ER\textsubscript{\alpha}-induced proliferative signaling in low- and high-risk premalignant lesions and possibly the overall process of promotion of initiated cells to clinical breast cancer. As such, artemisinin could potentially be used in combinational therapies with well-established antiestrogens. In this regard, Ful, a steroidal pure antiestrogen, has been shown to cause a cell cycle arrest and to induce degradation of the ER\textsubscript{\alpha} protein in MCF7 breast cancer cells (30). We have shown in MCF7 cells that a combination of suboptimal concentrations of Ful and artemisinin cooperate to decrease ER\textsubscript{\alpha} protein levels, leading to attenuation of estrogen-mediated proliferative signaling in breast cancer cells. Thus, we propose that artemisinin has the potential to be a strong candidate for adjuvant therapy with Ful and could be extended to other breast cancer therapies such as tamoxifen. Patients could also benefit from lowering the systemic exposure of the patient to antiestrogens and minimizing undesirable side effects due to artemisinin–antiestrogen cooperativity.

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