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

Abstract

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 estrogenic 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 endogeneous ERβ was not altered. Treatment of MCF7 cells with artemisinin and the pure antiestrogen fulvestrant resulted in a cooperative reduction of ERα 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.
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 ERα 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 antiestrogens 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 steroidal 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 chemotherapeutic 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 artesunate, 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 mammary carcinogen 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′–TGCCATTCCACGCACAACACATC–3′ with an MluI restriction enzyme cut site at the 5′ end and reverse primer 5′–TAAGTACTGTGTTCCCCGA–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 Qiaquick 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 BglIII (NEB). The fragment and vector were purified of restriction enzymes using PCR purification kit (Qiagen), followed by overnight ligation using T4 ligase (NEB), grown in TOPO10 cloning bacteria (Invitrogen, Carlsbad, CA) and verified by internal digest and sequencing. The pERAlpha−985 construct was amplified using forward primer 5′–ATGTGTGTGTGTGTTGCT–3′ with an Mlu restriction enzyme cut site and reverse primer 5′–AAAGGACACACGCCCAGGT–3′ with a BglII cut site inserted on 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 Nsil. Transfection was performed in serum–supplemented media using Fugene 6 (Roche, Pleasanton, CA) as per 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 pgl2 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 electrophoretic 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 recommended protocol was followed to extract total RNA. RNA was quantified using spectroscopy 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% 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 subconfluency 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-hydroxyethylpiperazine-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 immunoprecipitaiton 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. Immunoprecipitation and input samples were eluted 65°C/18 h in elution buffer (50 mM Tris, pH 8.0, 1% sodium dodecyl sulfate and 10 mM ethylenediaminetetraacetic acid). PCR amplification was carried out in a total volume of 50 μl using specific primers and PCR mix (1 U Taq polymerase (NEB), 1.5mM 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 0.01% ethidium bromide.

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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 growth 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.

Fig. 1.
Effects of artemisinin on estradiol and ERα-specific agonist PPT-induced proliferation of MCF7 breast cancer cells. (A) MCF7 cells were grown in steroid-deficient media supplemented with 10% dextran charcoal-stripped FBS. Cells were pretreated with DMSO or 300 μM artemisinin for 24 h and then treated with DMSO or 300 μM artemisinin in the presence or absence of 10 nM E₂ or 100 nM PPT for 24 h. Cells were harvested in phosphate-buffered saline and stained with a hypotonic solution containing propidium iodide. Stained nuclei were subjected to flow cytometry analysis as described in ‘Experimental Procedures’. (B) The flow cytometry results from three independent experiments were quantified. Bar graph indicates percent cells in G₁, S and G₂ phases with standard error bars.

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 were analyzed by immunoblotting using specific antibodies for ERα or ERβ. As shown in Figure 2A, treatment with 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.

![Fig. 2.](image)

**Fig. 2.**
Dose- and time-dependent effects of artemisinin on the production of ERα and ERβ protein in MCF7 human breast cancer cells. (A) MCF7 cells were treated with the indicated concentrations of artemisinin for 24, 48 and 72 h. Isolated protein extracts were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretically 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 electrophoretically transferred to nitrocellulose and immunoblotted with antibodies specific for ERα or β–Actin.

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 the 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 100 μM artemisinin, 300 μM artemisinin or with the DMSO vehicle control for 24, 48 and 72 h, and the level of ERα transcripts was monitored by RT–PCR analysis of total RNA using specific oligonucleotide primers. As shown in Figure 3A, 300 μM artemisinin treatment decreased ERα messenger RNA levels as early as 24 h of exposure in a dose– and time–dependent fashion. The messenger RNA levels of ERβ and glyceraldehyde phosphate dehydrogenase remained unaltered throughout the time course, and the latter gene product was used as a control for RNA input.
Fig. 3.
Artemisinin downregulates ERα transcript levels by decreasing ERα promoter activity at start site ‘B’. (A) MCF7 cells were treated with indicated concentrations of artemisinin for indicated duration. Total RNA was isolated and 2 μg of isolated total RNA was reverse transcribed with random primers and Mu–MLV reverse transcriptase. Four hundred nanogram of complementary DNA was employed in the PCR using specific primers for ERα (300 bp product), ERβ (250 bp product) or glyceraldehyde phosphate dehydrogenase (GAPDH) (122 bp product). The PCR products were electrophoretically fractionated in 1% agarose gels, glyceraldehyde phosphate dehydrogenase levels served as a loading control. (B) MCF7 cells were grown in six–well plates, transfected with plasmids containing 5′ deletion constructs of the ERα promoter and treated with DMSO or 300 μM artemisinin for 24 h. Cells were harvested and relative luciferase activity was determined, normalized to protein input and presented as a bar graph with standard error. Triplicate samples were used per treatment condition. (C) Artemisinin–responsive region in the ERα promoter indicates the predicted transcription factor–binding sites within this region.

Given that artemisinin downregulated ERα messenger RNA levels, its effect on ERα gene promoter activity was evaluated by transfection of MCF7 cells with series of luciferase reporter plasmid containing specific regions of the ERα promoter. ERα gene transcription is initiated from several promoter proximal regions (32). Luciferase reporter plasmids containing 5′ deletion constructs of the ERα promoter (Figure 3B) were individually transfected into MCF7 cells and treated with 300 μM artemisinin or the DMSO vehicle control for 24 h. The relative luciferase activity (relative light unit) was monitored in total cell extracts. As shown in Figure 3B, artemisinin treatment downregulated ERα promoter activity in cells transfected with the portions of the ERα promoter containing the region between −1892 and −985 bp upstream of the transcription start site. Thus, the artemisinin–responsive region in the ERα promoter was identified as shown in Figure 3C. Figure 3C shows the predicted transcription factor–binding sites in this region, notably three GATA–binding elements and one CCAAT/enhancer binding protein and one erythroblastosis gene consensus–binding sites. It is well established that GATA–3 and ERα are coexpressed in a majority of primary breast cancers, and the molecular mechanism of this positive regulatory 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 ERα gene transcription. Mutagenesis of these specific sites as well as ChIP experiments will reveal the precise mechanism by which artemisinin downregulates ERα 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 ERα 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 ERα expression by attenuating ERα promoter activity that leads to a significant downregulation of ERα transcript and protein levels.
Artemisinin disrupts the estradiol or PPT activation of consensus ERE-driven reporter plasmid and results in decreased ERE-bound ERα

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 ERα may also result in inhibition of ERα ligand–independent activity. Levels of basal ERE activity in MCF7 cells were high possibly due to the stimulation of ERα phosphorylation 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 ERα (2).

Fig. 4.
Artemisinin inhibits total ERE activity induced by estradiol or PPT. MCF7 cells transfected with pgl2 reporter plasmid containing a tandem array of vitellogenin–3xERE or with an empty pGL2 vector were grown in steroid–deficient media supplemented with 10% dextran charcoal–stripped FBS. Cells were then pretreated with either DMSO or 300 μM artemisinin for 24 h and then incubated for 24 h with the indicated combinations of 300 μM artemisinin, 10 nM E2 and/or 100 nM PPT. Cell lysates were evaluated for relative luciferase activity using the Promega luciferase assay system. Bar graphs indicate relative luciferase activity as measured in relative light units (RLUs) normalized to protein input with standard error. The experiment was performed three times with triplicate samples per treatment. (B) MCF7 breast cancer cells were treated with DMSO or 300 μM artemisinin for 48 h, and
2 mg of protein lysates were subjected to affinity column chromatography using biotinylated ERE conjugated to streptavidin agarose beads. Following elution with a 400 mM NaCl solution, eluates were subjected to immunoblot analysis for ERα and ERβ. Band intensities were quantified using the National Institutes of Health Imager program, and bar graphs indicate relative amounts of ERβ to ERα bound to ERE.

The relative binding of ERα 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 ERα, 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 ERα−/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 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 blocks estradiol or PPT activation of target gene expression by decreasing levels of promoter-bound ERα**

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 glyceraldehyde 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.
Fig. 5.
Artemisinin disrupts E$_2$ and PPT stimulation of progesterone receptor expression and decreases levels of promoter-bound ER$\alpha$ in vivo. (A) MCF7 cells were grown in phenol red–free steroid–deficient media supplemented with 10% dextran charcoal–stripped FBS. Cells were pretreated with DMSO or 300 μM artemisinin for 24 h. Cells were then treated with DMSO and 300 μM artemisinin in the presence and absence of 10 nM E$_2$ or 100 nM PPT for 24 h. Cells were harvested in Trizol and 2 μg of total RNA were subject to RT using random primers and Mu–MLV reverse transcriptase. Four hundred nanogram of this complementary DNA was subjected to PCR with primers specific for progesterone receptor (122 bp product). Glyceraldehyde phosphate dehydrogenase transcript levels served as loading control. (B) MCF7 cells were treated with DMSO or 300 μM artemisinin for 48 h and lysates were subjected to ChIP analysis using specific antibodies as described in Materials and Methods.

Artemisinin cooperates with Ful, a pure antiestrogen, in causing G$_1$ cell cycle arrest and ER$\alpha$ 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 ER$\alpha$ protein in MCF7 breast cancer cells (30). Because Ful treatment also results in decreased ER$\alpha$ 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 G$_1$ (44.1% DMSO versus 56.4% artemisinin), whereas treatment with 0.5 nM Ful leads to a small increase in proportion of cells in G$_1$ (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 G$_1$ (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 G$_{2}$/M was also significantly decreased (6.9% DMSO versus 1.5% Ful + artemisinin).

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Fig. 6.
Cooperative effects of artemisinin and the pure antiestrogen, Ful on proliferation and ERα levels in MCF7 breast cancer cells. (A) MCF7 cells were grown in media supplemented with 10% FBS and treated with DMSO, 50 μM artemisinin (A), 1 nM Ful or a combination of Ful and artemisinin for 48 h. Cells were harvested in phosphate-buffered saline and stained with a hypotonic solution containing propidium iodide. Stained nuclei were subjected to flow cytometry analysis as described in Materials and Methods. (B) MCF7 cells were treated with the indicated drugs for 48 h, subject to lysis, electrophoresis and immunoblotting with specific antibodies as described in Materials and Methods.

Cells treated with the above mentioned doses of artemisinin and Ful were analyzed for the levels of the hyperphosphorylated retinoblastoma 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 higher 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 malaria patients and the local bioavailability in the breast is unknown. Hence, the dose of artemisinin effective at downregulating ERα in vivo needs to be determined. Use of an ERα–specific agonist, PPT, revealed that majority of ERE activity induced by E2 is attributable to ERα. Therefore, artemisinin–induced decrease in estrogen sensitivity in MCF7 cells is due to its downregulation of the ERα receptor subtype in MCF7 breast cancer cells.
The exact roles of ERα and ERβ in normal mammary gland proliferation and in breast carcinogenesis are not clear, although an increased ERβ:ERα ratio has been shown to lead to decreased proliferation of both normal and neoplastic mammary epithelium. Epithelial cell proliferation in the normal mammary gland is maximal during the prepubertal phase and minimal during lactation. ERα expression predominates during the proliferative prepubertal phase and during lactation, a phase characterized by lowest levels of mammary epithelial proliferation, and the dominant ER subtype is ERβ. Also, elevated expression of ERα also occurs in high-grade proliferative precancerous lesions and in breast neoplasms, whereas ERβ expression is usually lost in these disease states. This observation suggests that estrogen signaling through ERα mediates the proliferative growth effects in the gland and dominance of ERβ correlates with cessation of epithelial proliferation in the differentiated gland and in transformed mammary epithelial cells. Consistent with these findings, ERα–null mammary gland, which produces normal levels of functional ERβ, is characterized by a severely underdeveloped gland that resembles a prepubertal gland and very low to undetectable levels of cellular proliferation, as well as ablated mammary tumor development (3,4,8). This phenotype is not rescued by administering pharmacological doses of estrogen, suggesting that E2 signaling through ERβ does not mediate proliferative effects of estrogens.

ERβ–null mammary gland does not show any gross effects in a non–pregnant mammary gland; however, ERβ–null mammary epithelial tree shows defects during lactation. Lactating wild–type glands show very low levels of proliferation, whereas proliferation is high in lactating ERβ–null mice. This observation suggests that estrogen’s effects on terminal mammary differentiation might be mediated by growth cessation effects of ERβ signaling (6). This hypothesis is further strengthened by studies on cancer cells in culture. 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 refactoriness of the mammary glands to developing mammary cancer (37). Deregulated expression of ERα 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 ERα 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 ERα–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 ERα 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 ERα 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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Footnotes

- Abbreviations:
  
  Abbreviations
  ChIP
  chromatin immunoprecipitation
  DMSO
  dimethyl sulfoxide
  ER
  estrogen receptor
  ERE
  estrogen response element
  E2
  17β-estradiol
  FBS
  fetal bovine serum
  Ful
  fulvestrant
  pCMV
  cytomegalovirus promoter–containing plasmid
  PCR
  polymerase chain reaction
  PPT
  propyl pyrazole triol
  PR
progesterone receptor

reverse transcription

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