

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

Shyam N.Sundar, Crystal N.Marconett, Victor B.Doan,
Jamin A.Willoughby Sr and Gary L.Firestone*

Department of Molecular and Cell Biology and the Cancer Research
Laboratory, University of California at Berkeley, Berkeley, CA 94720, USA

*To whom correspondence should be addressed. Department of Molecular and
Cell Biology, 591 LSA, University of California at Berkeley, Berkeley, CA
94720-3200, USA. Tel: +1 510 642 8319; Fax: +1 510 643 6791;
Email: glfire@berkeley.edu

MCF7 cells are an estrogen-responsive human breast cancer cell line that expresses both estrogen receptor (ER) α and ER β . Treatment of MCF7 cells with artemisinin, an antimalarial phytochemical from the sweet wormwood plant, effectively blocked estrogen-stimulated cell cycle progression induced by either 17 β -estradiol (E_2), an agonist for both ERs, or by propyl pyrazole triol (PPT), a selective ER α agonist. Artemisinin strongly downregulated ER α protein and transcripts without altering expression or activity of ER β . Transfection of MCF7 cells with ER α promoter-linked luciferase reporter plasmids revealed that the artemisinin downregulation of ER α promoter activity accounted for the loss of ER α expression. Artemisinin treatment ablated the estrogenic induction of endogenous progesterone receptor (PR) transcripts by either E_2 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 ER α bound to the PR promoter, whereas the level of bound endogenous ER β was not altered. Treatment of MCF7 cells with artemisinin and the pure antiestrogen fulvestrant resulted in a cooperative reduction of ER α 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 ER α :ER β ratio to a condition in which ER β 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 ER β that regulate the transcription of distinct as well as overlapping sets of target genes (2). The exact roles of ER α and ER β in breast carcinogenesis are not clear, although a high ER α :ER β ratio correlates well with high levels of cellular proliferation, whereas high ER β :ER α is generally linked to antiproliferative events (3–14).

The majority of breast cancers expressing ER α 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

Abbreviations: ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; ER, estrogen receptor; ERE, estrogen response element; E_2 , 17 β -estradiol; FBS, fetal bovine serum; Ful, fulvestrant; pCMV, cytomegalovirus promoter-containing plasmid; PCR, polymerase chain reaction; PPT, propyl pyrazole triol; PR, progesterone receptor; RT, reverse transcription.

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 ER α . 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 ER α expression by attenuating its promoter activity without altering ER β levels and disrupted ER α -responsive growth and gene expression. Our results show that artemisinin switches highly proliferative human breast cancer cells from expressing a high ER α :ER β ratio to a growth-arrested state in which expression of ER β is significantly greater to that of ER α , 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), 17 β -estradiol (E_2), 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 μ g/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.

ER α 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′-TGCCATTCCACGCACAAACACATC-3′ with an MluI restriction enzyme cut site at the 5′ end and reverse primer 5′-TAAGTACTGGTCTCCCGA-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 BglII (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′-ATGTGTGTGTATGTGCGTGT-3′ with an MluI restriction enzyme cut site and reverse primer 5′-AAAGAGCACAGCCCGAGGTTAGA-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 NsiI. 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 μ M 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 μ M 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 ER α and ER β

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 ER α , ER β , 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 μ M 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 μ M 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 ER α -specific antibody sc-8005X or the ER β -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 \times ChIP lysis buffer, 2 \times 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 \times 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 μ l using specific primers and PCR mix (1 U *Taq* polymerase (NEB), 1.5mM MgCl₂, 0.2 μ M deoxynucleoside triphosphates) as follows: ER α and ER β (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.

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, ER α and ER β , and is highly estrogen responsive. Addition of E₂ or the ER α -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 μ M 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 μ M 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 ER α .

Artemisinin selectively downregulates the level of the ER α 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 μ M artemisinin, 300 μ M artemisinin or with the DMSO vehicle control for 24, 48 or 72 h, and the electrophoretically fractionated total cell lysates

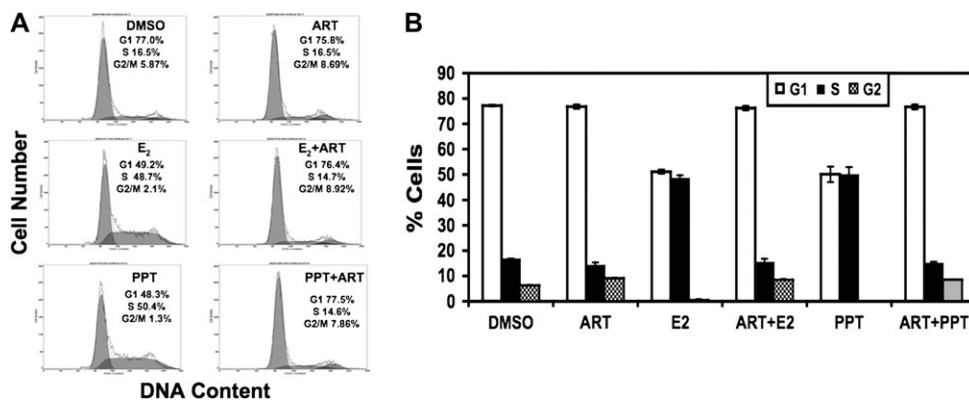


Fig. 1. Effects of artemisinin on estradiol and ER α -specific agonist PPT-induced proliferation of MCF7 breast cancer cells. (A) MCF7 cells were grown in steroid-deficient media supplemented with 10% dextran charcoal-stripped FBS. Cells were pretreated with DMSO or 300 μ M artemisinin for 24 h and then treated with DMSO or 300 μ M 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.

were analyzed by immunoblotting using specific antibodies for ER α or ER β . As shown in Figure 2A, treatment with 300 μ M artemisinin down-regulated ER α protein levels in MCF7 cells as early as 24 h, with a maximal effect observed by 48 h of phytochemical treatment. In contrast, ER β 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 ER α was 300 μ M 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 ER α 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 μ M MG132, a 26S proteasome inhibitor, for 4 h. Immunoblot analysis showed that although treatment with MG132 caused an accumulation of ER α protein, artemisinin still was able to strongly downregulate ER α 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 ER α protein, which suggests that artemisinin does not alter ER α protein degradation. Consistent with this concept, artemisinin was unable to downregulate ER α in MCF7 cells transfected with a constitutive cytomegalovirus promoter-containing plasmid (pCMV)-ER α expression vector compared with cells transfected with a control pCMV-Neo expression vector (Figure 2C). Ectopic elevated expression of ER α did not alter the cell cycle kinetics of MCF7 cells consistent with previous observations (14).

Artemisinin downregulates ER α transcript levels by attenuating promoter activity

To examine the effects of artemisinin on ER α transcript levels, MCF7 cells were treated with 100 μ M artemisinin, 300 μ M artemisinin or with the DMSO vehicle control for 24, 48 and 72 h, and the level of ER α transcripts was monitored by RT-PCR analysis of total RNA using specific oligonucleotide primers. As shown in Figure 3A, 300 μ M artemisinin treatment decreased ER α messenger RNA levels as early as 24 h of exposure in a dose- and time-dependent fashion. The messenger RNA levels of ER β and glyceraldehyde phosphate dehydrogenase remained unaltered throughout the time course, and the latter gene product was used as a control for RNA input.

Given that artemisinin downregulated ER α messenger RNA levels, its effect on ER α gene promoter activity was evaluated by transfection of MCF7 cells with series of luciferase reporter plasmids

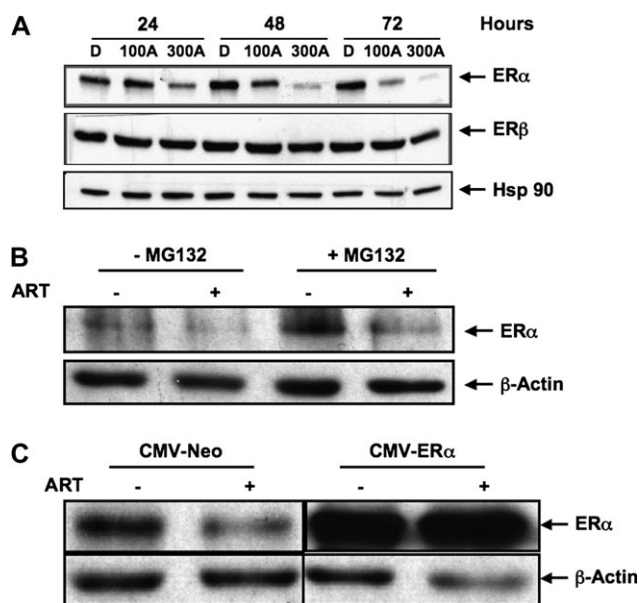


Fig. 2. Dose- and time-dependent effects of artemisinin on the production of ER α and ER β protein in MCF7 human breast cancer cells. (A) MCF7 cells were treated with the indicated concentrations of artemisinin for 24, 48 and 72 h. Isolated protein extracts were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose membranes and immunoblots were probed with antibodies specific for ER α and ER β . Heat shock protein 90 (Hsp 90) was used as a protein loading control. (B) MCF7 cells were treated with DMSO or 300 μ M artemisinin for 48 h. Treated cells were exposed to DMSO or 20 μ M MG132, a proteasome inhibitor for 4 h. Cells were lysed and 30 μ g total protein was subjected to immunoblot analysis with antibodies specific for ER α or β -Actin. (C) MCF7 cells were transfected with pCMV-Neo or pCMV-ER α . Twenty-four hours later, transfected cells were treated with DMSO or 300 μ M 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 ER α or β -Actin.

containing specific regions of the ER α promoter. ER α gene transcription is initiated from several promoter proximal regions (32). Luciferase reporter plasmids containing 5' deletion constructs of the ER α promoter (Figure 3B) were individually transfected into MCF7 cells and treated with 300 μ M artemisinin or the DMSO vehicle

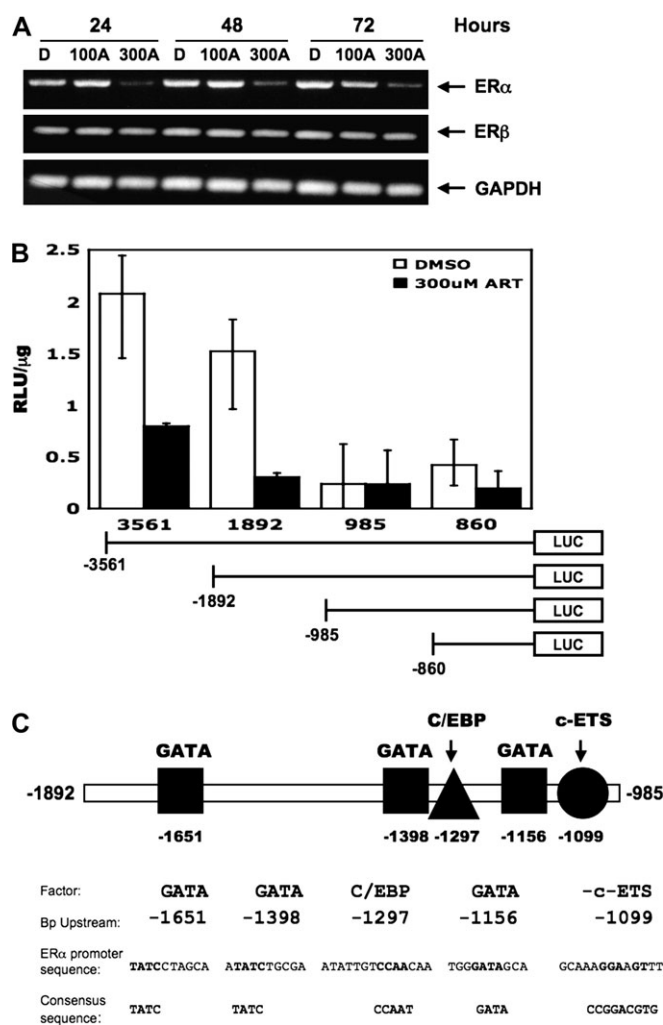


Fig. 3. Artemisinin downregulates ER α transcript levels by decreasing ER α promoter activity at start site 'B'. (A) MCF7 cells were treated with indicated concentrations of artemisinin for indicated duration. Total RNA was isolated and 2 μ g of isolated total RNA was reverse transcribed with random primers and Mu-MLV reverse transcriptase. Four hundred nanogram of complementary DNA was employed in the PCR using specific primers for ER α (300 bp product), ER β (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 ER α promoter and treated with DMSO or 300 μ M 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 ER α promoter indicates the predicted transcription factor-binding sites within this region.

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 ER α promoter activity in cells transfected with the portions of the ER α promoter containing the region between -1892 and -985 bp upstream of the transcription start site. Thus, the artemisinin-responsive region in the ER α 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 ER α 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 ER α gene transcription. Mutagenesis of these specific sites as well as ChIP experiments will reveal the precise mechanism by which artemisinin downregulates ER α 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 ER α 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 ER α expression by attenuating ER α promoter activity that leads to a significant downregulation of ER α transcript and protein levels.

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

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 ER α agonists 10 nM E $_2$ 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 E $_2$ - 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 ER α may also result in inhibition of ER α ligand-independent activity. Levels of basal ERE activity in MCF7 cells were high possibly due to the stimulation of ER α phosphorylation by insulin and other growth factor signaling pathways. Because E $_2$, an ER α and ER β agonist, as well as PPT, an ER α -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 ER α (2).

The relative binding of ER α and ER β 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 ER α , which is consistent with the artemisinin downregulation of ER α expression. In contrast, the levels of ERE-bound ER β remain unaltered. A similar result with ERE-bound ER β was also observed by the same technique in ER α -/ER β + 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 ER α directly lead to the decrease in ERE-bound ER α , which in turn causes the loss of ER α -stimulated gene expression in MCF7 cells. The relative amounts of functional ERE-bound ER β found in artemisinin-treated cells is much higher than ER α 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 ER α

Artemisinin's ability to disrupt the regulated expression of the endogenous PR, a primary estrogen-responsive gene, was studied by

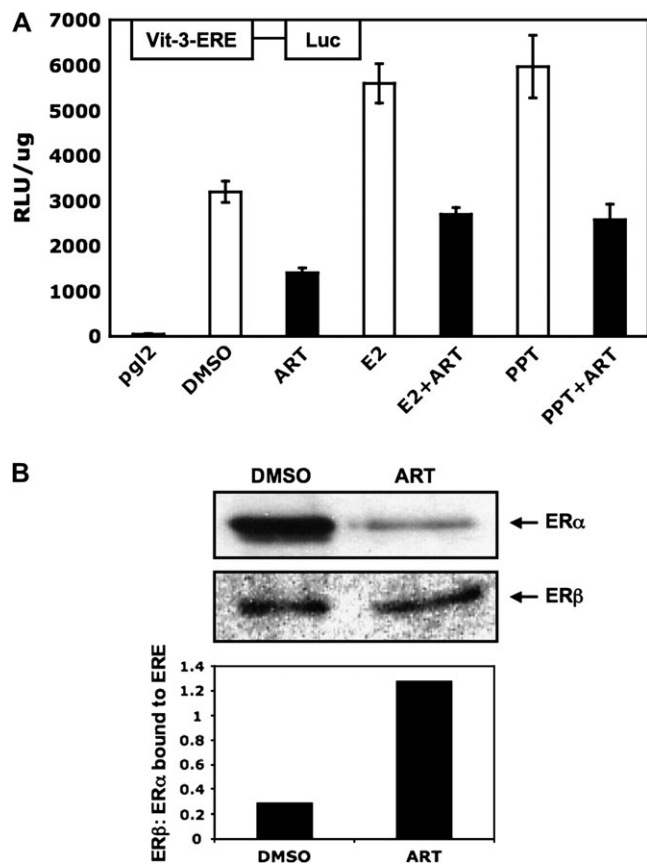


Fig. 4. Artemisinin inhibits total ERE activity induced by estradiol or PPT. MCF7 cells transfected with pGL2 reporter plasmid containing a tandem array of vitellogenin-3xERE or with an empty pGL2 vector were grown in steroid-deficient media supplemented with 10% dextran charcoal-stripped FBS. Cells were then pretreated with either DMSO or 300 μ M artemisinin for 24 h and then incubated for 24 h with the indicated combinations of 300 μ M artemisinin, 10 nM E_2 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 μ M 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 ER α and ER β . Band intensities were quantified using the National Institutes of Health Imager program, and bar graphs indicate relative amounts of ER β to ER α bound to ERE.

RT-PCR. MCF7 cells cultured in steroid-deficient phenol red-free media were treated with combinations of 300 μ M artemisinin and either 10 nM E_2 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 E_2 or PPT stimulation of PR transcripts compared with glyceraldehyde phosphate dehydrogenase, the loading control. *In vivo* analysis using ChIP shows that ER α 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 ER α directly lead to the decrease in ERE-bound ER α , which in turn causes the loss of ER α -stimulated gene expression in MCF7 cells. The relative amounts of functional ERE-bound ER β found in artemisinin-treated cells is much higher than ER α levels, correlating well with artemisinin-induced growth arrest of MCF7 cells.

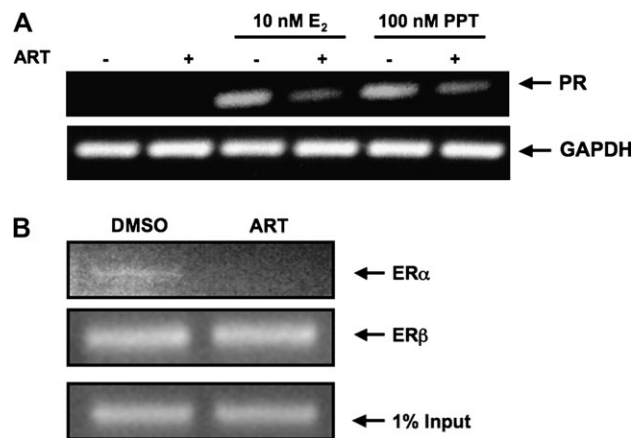


Fig. 5. Artemisinin disrupts E_2 and PPT stimulation of progesterone receptor expression and decreases levels of promoter-bound ER α *in vivo*. **(A)** MCF7 cells were grown in phenol red-free steroid-deficient media supplemented with 10% dextran charcoal-stripped FBS. Cells were pretreated with DMSO or 300 μ M artemisinin for 24 h. Cells were then treated with DMSO and 300 μ M 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 μ g of total RNA were subjected 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 μ M 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 α 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 α protein in MCF7 breast cancer cells (30). Because Ful treatment also results in decreased ER α 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 μ M 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 μ M 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).

Cells treated with the above mentioned doses of artemisinin and Ful were analyzed for the levels of the hyperphosphorylated retinoblastoma protein (a marker for G_1 to S cell cycle progression) and of both ER subtypes by immunoblotting with specific antibodies. As shown in Figure 6B, the enhanced G_1 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 ER α protein. However, a combination of the two caused a marked

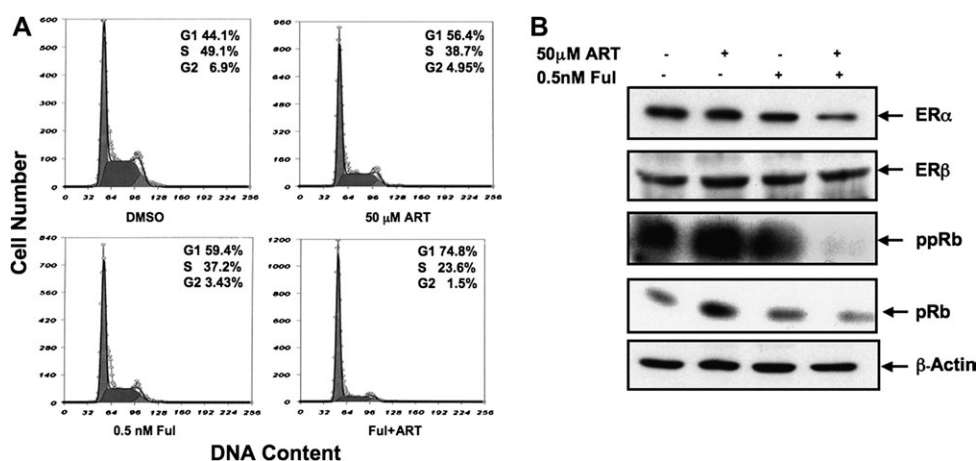


Fig. 6. Cooperative effects of artemisinin and the pure antiestrogen, Ful on proliferation and ER α levels in MCF7 breast cancer cells. (A) MCF7 cells were grown in media supplemented with 10% FBS and treated with DMSO, 50 μ M artemisinin (A), 1 nM Ful or a combination of Ful and artemisinin for 48 h. 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.

downregulation of ER α protein levels (Figure 6B). In contrast, ER β levels remained unaltered.

Discussion

A direct cellular consequence of the artemisinin-mediated downregulation of ER α expression and disruption of ER α responsiveness in the absence of any effects on ER β expression is a significant change in ratio of functional ER α :ER β in MCF7 human breast cancer cells. An emerging concept concerning estrogen-responsive breast cancers is that the relative levels of ER α and ER β play an important role in controlling estrogen-regulated proliferative and differentiation properties in estrogen-responsive cells. A high ratio of ER α :ER β is associated with an increased proliferative state of estrogen-responsive cells (7). Reversal of this ratio resulting in higher intracellular levels of ER β :ER α 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 ER α :ER β ratio to a condition in which expression of ER β is significantly greater to that of ER α , 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 ER α 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 ER α *in vivo* needs to be determined. Use of an ER α -specific agonist, PPT, revealed that majority of ERE activity induced by E₂ is attributable to ER α . Therefore, artemisinin-induced decrease in estrogen sensitivity in MCF7 cells is due to its downregulation of the ER α receptor subtype in MCF7 breast cancer cells.

The exact roles of ER α and ER β in normal mammary gland proliferation and in breast carcinogenesis are not clear, although an increased ER β :ER α 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. ER α 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 ER β . Also, elevated expression of ER α also occurs in high-grade proliferative precancerous lesions and in breast neoplasms, whereas ER β expression is usually lost in these disease states. This observation suggests that estrogen signaling through ER α mediates the proliferative growth effects in the gland and dominance of ER β correlates with cessation of epithelial proliferation in the differentiated gland and in transformed mammary epithelial cells. Consistent with these findings, ER α -null mammary gland, which produces normal levels of functional ER β , 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 ER β does not mediate proliferative effects of estrogens.

ER β -null mammary gland does not show any gross effects in a non-pregnant mammary gland; however, ER β -null mammary epithelial tree shows defects during lactation. Lactating wild-type glands show very low levels of proliferation, whereas proliferation is high in lactating ER β -null mice. This observation suggests that estrogen's effects on terminal mammary differentiation might be mediated by growth cessation effects of ER β signaling (6). This hypothesis is further strengthened by studies on cancer cells in culture. Ectopic expression of ER β in highly proliferating MCF7 cells or in T47D breast cancer cells leads to cell cycle arrest (12,14). Ectopic overexpression of ER β in estrogen-responsive breast cancer cell lines also results in the growth arrest of nude mouse xenografts. The precise ER β -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 ER β leads to increased formation of both ER β homodimers and of ER α /ER β 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 refractoriness of the mammary glands to developing mammary cancer

(37). Deregulated expression of ER α 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 α 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 α -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 α 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 α 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.

Funding

California Breast Cancer Research Program (12IB-0166); predoctoral fellowship supported by National Institutes of Health National Research Service Grant (CA09041 to S.N.S.).

Acknowledgements

We thank the other members of the Firestone laboratory for their helpful comments and suggestions during the course of this study.

Conflict of Interest Statement: None declared.

References

- Draper, L. (2006) Breast cancer: trends, risks, treatments, and effects. *AAOHN J.*, **54**, 445–453.
- Kian Tee, M. *et al.* (2004) Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors alpha and beta. *Mol. Biol. Cell.*, **15**, 1262–1272.
- Bocchinfuso, W.P. *et al.* (1997) Mammary gland development and tumorigenesis in estrogen receptor knockout mice. *J. Mammary Gland Biol. Neoplasia*, **2**, 323–334.
- Bocchinfuso, W.P. *et al.* (2000) Induction of mammary gland development in estrogen receptor-alpha knockout mice. *Endocrinology*, **141**, 2982–2994.
- Cordera, F. *et al.* (2006) Steroid receptors and their role in the biology and control of breast cancer growth. *Semin. Oncol.*, **33**, 631–641.
- Forster, C. *et al.* (2002) Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium. *Proc. Natl Acad. Sci. USA*, **99**, 15578–15583.
- Frech, M.S. *et al.* (2005) Deregulated estrogen receptor alpha expression in mammary epithelial cells of transgenic mice results in the development of ductal carcinoma *in situ*. *Cancer Res.*, **65**, 681–685.
- Hewitt, S.C. *et al.* (2002) Lack of ductal development in the absence of functional estrogen receptor alpha delays mammary tumor formation induced by transgenic expression of ErbB2/neu. *Cancer Res.*, **62**, 2798–2805.
- Lazennec, G. *et al.* (2001) ER beta inhibits proliferation and invasion of breast cancer cells. *Endocrinology*, **142**, 4120–4130.
- Saji, S. *et al.* (2001) Quantitative analysis of estrogen receptor proteins in rat mammary gland. *Endocrinology*, **142**, 3177–3186.
- Shyamala, G. *et al.* (2002) Cellular expression of estrogen and progesterone receptors in mammary glands: regulation by hormones, development and aging. *J. Steroid Biochem. Mol. Biol.*, **80**, 137–148.
- Strom, A. *et al.* (2004) Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc. Natl Acad. Sci. USA*, **101**, 1566–1571.
- Sundar, S.N. *et al.* (2006) Indole-3-carbinol selectively uncouples expression and activity of estrogen receptor subtypes in human breast cancer cells. *Mol. Endocrinol.*, **20**, 3070–3082.
- Paruthiyil, S. *et al.* (2004) Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Res.*, **64**, 423–428.
- Ariazi, E.A. *et al.* (2006) Estrogen receptors as therapeutic targets in breast cancer. *Curr. Top. Med. Chem.*, **6**, 181–202.
- Osborne, M.P. (1999) Chemoprevention of breast cancer. *Surg. Clin. North Am.*, **79**, 1207–1221.
- Gielen, S.C. *et al.* (2005) Analysis of estrogen agonism and antagonism of tamoxifen, raloxifene, and ICI182780 in endometrial cancer cells: a putative role for the epidermal growth factor receptor ligand amphiregulin. *J. Soc. Gynecol. Investig.*, **12**, e55–e67.
- Meshnick, S.R. (2003) Artemisinin and heme. *Antimicrob. Agents Chemother.*, **47**, 2712; author reply 2712–2713.
- Paik, I.H. *et al.* (2006) Second generation, orally active, antimalarial, artemisinin-derived trioxane dimers with high stability, efficacy, and anti-cancer activity. *J. Med. Chem.*, **49**, 2731–2734.
- Dell'Eva, R. *et al.* (2004) Inhibition of angiogenesis *in vivo* and growth of Kaposi's sarcoma xenograft tumors by the anti-malarial artesunate. *Biochem. Pharmacol.*, **68**, 2359–2366.
- Efferth, T. *et al.* (2004) Enhancement of cytotoxicity of artemisinins toward cancer cells by ferrous iron. *Free Radic. Biol. Med.*, **37**, 998–1009.
- Nam, W. *et al.* (2007) Effects of artemisinin and its derivatives on growth inhibition and apoptosis of oral cancer cells. *Head Neck*, **29**, 335–340.
- Berman, P.A. *et al.* (1997) Artemisinin enhances heme-catalysed oxidation of lipid membranes. *Free Radic. Biol. Med.*, **22**, 1283–1288.
- Efferth, T. *et al.* (2001) The anti-malarial artesunate is also active against cancer. *Int. J. Oncol.*, **18**, 767–773.
- Lai, H. *et al.* (2006) Oral artemisinin prevents and delays the development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced breast cancer in the rat. *Cancer Lett.*, **231**, 43–48.
- Chan, M.M. *et al.* (2005) Gene expression profiling of NMU-induced rat mammary tumors: cross species comparison with human breast cancer. *Carcinogenesis*, **26**, 1343–1353.
- de Cremoux, P. *et al.* (2002) Quantitation of estradiol receptors alpha and beta and progesterone receptors in human breast tumors by real-time reverse transcription-polymerase chain reaction. Correlation with protein assays. *Biochem. Pharmacol.*, **64**, 507–515.
- Petz, L.N. *et al.* (2004) Differential regulation of the human progesterone receptor gene through an estrogen response element half site and Sp1 sites. *J. Steroid Biochem. Mol. Biol.*, **88**, 113–122.
- Wiese, T.E. *et al.* (1992) Optimization of estrogen growth response in MCF-7 cells. *In Vitro Cell. Dev. Biol.*, **28A**, 595–602.
- Long, X. *et al.* (2006) Fulvestrant (ICI 182,780)-dependent interacting proteins mediate immobilization and degradation of estrogen receptor-alpha. *J. Biol. Chem.*, **281**, 9607–9615.
- Madureira, P.A. *et al.* (2006) The Forkhead box M1 protein regulates the transcription of the estrogen receptor alpha in breast cancer cells. *J. Biol. Chem.*, **281**, 25167–25176.
- Eeckhoutte, J. *et al.* (2007) Positive cross-regulatory loop ties GATA-3 to estrogen receptor alpha expression in breast cancer. *Cancer Res.*, **67**, 6477–6483.
- Chakrabarty, A. *et al.* (2007) Ets-2 and C/EBP-beta are important mediators of ovine trophoblast Kunitz domain protein-1 gene expression in trophoblast. *BMC Mol. Biol.*, **8**, 14.
- Shimokawa, T. *et al.* (2003) C/EBP alpha and Ets protein family members regulate the human myeloid IgA Fc receptor (Fc alpha R, CD89) promoter. *J. Immunol.*, **170**, 2564–2572.
- Park, J.S. *et al.* (2000) A role for both Ets and C/EBP transcription factors and mRNA stabilization in the MAPK-dependent increase in p21 (Cip-1/WAF1/mda6) protein levels in primary hepatocytes. *Mol. Biol. Cell*, **11**, 2915–2932.
- Lindberg, M.K. *et al.* (2003) Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a “ying yang” relationship between ERalpha and ERbeta in mice. *Mol. Endocrinol.*, **17**, 203–208.
- Rees, E.D. *et al.* (1968) Influence of five anti-cancer drugs on the induction and growth of experimental mammary cancers: comparison with ovariectomy. *Cancer*, **21**, 1029–1041.

Received April 21, 2008; revised September 2, 2008;
accepted September 6, 2008