Antiproliferative effects of artemisinin on human breast cancer cells requires the downregulated expression of the E2F1 transcription factor and loss of E2F1-target cell cycle genes

Antony S. Tin, Shyam N. Sundar, Kalvin Q. Tran, Anna H. Park, Kevin M. Poindexter and Gary L. Firestone

Artemisinin, a sesquiterpene phytolactone derived from *Artemisia annua*, is a potent antimalarial compound with promising anticancer properties, although the mechanism of its anticancer signaling is not well understood. Artemisinin inhibited proliferation and induced a strong G1 cell cycle arrest of cultured MCF7 cells, an estrogen-responsive human breast cancer cell line that represents an early-stage cancer phenotype, and effectively inhibited the in-vivo growth of MCF7 cell-derived tumors from xenografts in athymic nude mice. Artemisinin also induced a growth arrest of tumorigenic human breast cancer cell lines with preneoplastic and late stage cancer phenotypes, but failed to arrest the growth of a nontumorigenic human mammary cell line. Concurrent with the cell cycle arrest of MCF7 cells, artemisinin selectively downregulated the transcript and protein levels of the CDK2 and CDK4 cyclin-dependent kinases, cyclin E, cyclin D_1, and the E2F1 transcription factor. Analysis of CDK2 promoter-luciferase reporter constructs showed that the artemisinin ablation of CDK2 gene expression was accounted for by the loss of CDK2 promoter activity. Chromatin immunoprecipitation revealed that artemisinin inhibited E2F1 interactions with the endogenous MCF7 cell CDK2 and cyclin E promoters. Moreover, constitutive expression of exogenous E2F1 prevented the artemisinin-induced cell cycle arrest and downregulation of CDK2 and cyclin E gene expression. Taken together, our results demonstrate that the artemisinin disruption of E2F1 transcription factor expression mediates the cell cycle arrest of human breast cancer cells and represents a critical transcriptional pathway by which artemisinin controls human reproductive cancer cell growth. *Anti-Cancer Drugs* 23:370–379 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Breast cancer is the most common malignancy in nonsmoking women in the western hemisphere. Clinical management of breast cancer has been arduous owing to the complex heterogeneity in phenotypes as well as emergence of resistant states with current therapy options [1,2]. Breast cancer can manifest itself as a hormone-responsive disease marked by the presence of functional estrogen receptors or as a steroid-unresponsive phenotype that can involve the loss of estrogen receptor expression [3–5]. Determination of this trait is critical to prescribing appropriate therapy to the patient. Hormone-responsive breast cancer can be managed by the use of selective estrogen receptor modulators such as tamoxifen, and aromatase inhibitors such as letrozole [6]. However, patients receiving such therapy have a high recurrence rate within 5 years, and present resistant states or increased risk for other gynecological malignancies [7]. Hormone-unresponsive breast cancer patients undergo more drastic therapy that includes a possible combination of surgery, radiation, and immunotherapy, which exert deleterious side effects [8,9]. Currently, there is a strong demand for therapy that is effective against a wide variety of breast cancer phenotypes with minimum side effects.

Naturally occurring phytochemicals represent a largely untapped source of potential therapeutic molecules to control different types of cancers with very minimal side effects [10–12]. One such promising compound is artemisinin, a sequiterpene lactone that was isolated from the aerial portions of the *Artemisia annua* plant (more commonly known as qinghaosu or sweet wormwood). Artemisinin and a number of its derivatives have been used to effectively treat different forms of malaria in the past three decades [13]. More recently, artemisinin and its related compounds have been shown to exhibit potent anticancer effects in a variety of human cancer cell model systems including leukemic, colon, melanoma, breast, ovarian, prostate, central nervous system, and renal cancer cells [14–16]. Molecular, cellular, and physiological

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studies have demonstrated that, depending on the tissue type and experimental system, the responses to artemisinin and its derivatives in a variety of cancer cell lines and tumor xenografts can involve a cell cycle arrest, apoptosis, inhibition of angiogenesis, and cell migration, as well as modulation of nuclear receptor responsiveness and signal transduction pathways [17–20].

One proposed mechanism by which artemisinin targets cancer cells is the presence of relatively high concentrations of iron that induce cleavage of the endoperoxide bridge, thereby allowing the formation of free radical molecules such as reactive oxygen species and resulting in oxidative damage to the cells, as well as iron depletion in the cells [21,22]. This mechanism resembles the action of artemisinin in malarial parasites. However, expression profiling of several classes of tumor cells revealed that artemisinin treatment causes selective changes in expression of many more oncogenes and tumor suppressor genes than can be accounted for by changes restricted only to genes responsible for iron metabolism [23,24]. This result suggests that the anticancer properties of artemisinin cannot be explained only by the global toxic effects of oxidative damage. Mechanistic information on the effects of artemisinin and its derivatives on the expression and activity of specific transcription factors has been limited. We previously demonstrated that artemisinin did not alter general Sp1 transcription factor responsiveness or expression, but selectively disrupted the endogenous interactions of the Sp1 transcription factor with the cyclin-dependent kinase 4 (CDK4) promoter to inhibit CDK4 gene expression [25]. This result suggests that cell cycle gene-specific transcriptional responses to artemisinin may control cell cycle progression in different types of human cancer cells. In this study, we report that the artemisinin cell cycle arrest of MCF7 human breast cancer cells is mediated by the downregulated expression of the E2F1 transcription factor that leads to the disruption of CDK2 promoter activity and loss of gene transcription. We further show that exogenous expression of E2F1 confers resistance to the antiproliferative effects of artemisinin, demonstrating the critical importance of E2F1 expression mediated this artemisinin response in human breast cancer cells.

Materials and methods

Materials

Artemisinin (99%) was purchased from Sigma (St Louis, Missouri, USA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA) and Cytoskeleton Inc (Denver, Colorado, USA). All media-related reagents were purchased from Lonza (Walkersvilee, Maryland, USA). Reagents obtained elsewhere are indicated in the text. The MCF7 and MDA-MB-231 cell lines were obtained from American type culture collection (Manassas, Virginia, USA). MCF10AT cells and MCF10A cells were kind gifts from Dr Fred Miller at the Barbara Ann Karmanos Cancer Center (Wayne State University).

Cell culture

MCF7 cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum (purchased from Mediatech, Henderson, Virginia, USA), 10 μg/ml insulin, 50 U/ml penicillin, 50 U/ml streptomycin, and 2 mmol/l L-glutamine from Sigma-Aldrich. MDA-MB-231 cells were cultured similarly to MCF7 cells except with the use of Iscove's Modified Dulbecco's Medium and without supplementation of insulin. MCF10AT and MCF10A cells were cultured in DMEM/F-12, 10% fetal bovine serum, 50 U/ml penicillin, 50 U/ml streptomycin, (purchased from Lonza), 0.02 μg/ml epidermal growth factor (purchased from Promega, Madison, Wisconsin, USA), 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, and 0.1 μg/ml cholera toxin (purchased from Sigma-Aldrich). Cells were grown to subconfluency in a humidified chamber at 37°C containing 5% CO_2. A 300 mmol/l stock solution of artemisinin was dissolved in dimethyl sulfoxide (DMSO), and then diluted in the ratio 1 : 1000 in media before culture plate application.

Flow cytometry

To monitor the cell population DNA content, cultured cell lines were treated with the indicated concentrations and durations of artemisinin, lysed in hypotonic propidium iodide, and subjected to flow cytometric analysis as previously described [25].

Immunoblotting

After the indicated treatments, western blots were performed as previously indicated [25]. Briefly, cells were harvested in ice-cold PBS and lysed using radioimmunoprecipitation assay buffer. After centrifugation, total protein in the lysate was estimated using the Protein Quantification Kit (Bio-Rad, Hercules, California, USA). Cell lysates were electrophoretically fractionated using SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk for an hour at room temperature and incubated with primary antibodies overnight at 4°C. Immunoreactive proteins were detected after 1-h incubation with horseradish peroxidase-conjugated secondary antibodies diluted 3×10^{-4} in 1% nonfat dry milk in tris-buffered saline and Tween 20 (TBST). Blots were then treated with enhanced chemiluminescence reagents (Eastman Kodak, Rochester, New York, USA) for visualization on film. The primary antibodies, rabbit anti-E2F1 (sc-193), mouse anti-Sp1 (sc-420), mouse anti-CDK2 (sc-6248), rabbit anti-CDK4 (sc-749), mouse anti-cyclin E (sc-248), mouse anti-cyclin D1 (sc-6281), mouse anti-c-Fos (sc 447), rabbit anti-c-Jun (sc44), and goat anti-p21 (sc397) were purchased from Santa Cruz Biotechnology and diluted in the ratio 1 : 1000 in TBST. Rabbit antiactin (#AANO1

Cytoskeleton Inc.) was diluted in the ratio 1 : 1000 in TBST and was used as a protein loading control.

Reverse transcription and polymerase chain reaction

MCF7 cells treated with the indicated doses of artemisinin and duration were harvested in Trizol (Invitrogen, Carlsbad, California, USA), and total RNA was extracted according to the manufacturer's protocol. This was quantified and 1 μg of total RNA was used for reverse transcription (RT) using Mu-MLV reverse transcriptase (Invitrogen) and random hexamers according to the manufacturer's protocol. The cDNA pool was used (2 μl) in polymerase chain reaction (PCR) with specific primers. Twenty microliters of this reaction was electrophoretically fractionated in a 1% agarose gel and visualized using an ultraviolet transilluminator.

Plasmids and transfections

Indicated plasmids (1 μg) were transfected into MCF7 cells using Superfect reagent (Qiagen, Germantown, Maryland, USA) according to the manufacturer's instructions. Cells were treated with 300 μmol/l artemisinin 24 h posttransfection and harvested in ice-cold PBS. The cells were subjected to immunoblotting as described, or to luciferase assays with the luciferase assay kit (Promega). The CDK2 luciferase plasmids were kind gifts from Dr Leonard Bjeldanes, University of California, Berkeley, California, USA. The CMV-E2F1 expression vector was a kind gift from Dr Kahryn Calames, Columbia University.

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed as previously described [26,27]. Briefly, cross-linking of proteins to DNA was obtained by the addition of formaldehyde at 1% of the final concentration for 5 min at room temperature to cultured cells. Fixation was quenched with glycine for a final concentration of 125 mmol/l for 5 min. Harvested cells were sonicated and the chromatin was immunoprecipitated overnight with 15 μl antibodies recognizing E2F1 (Santa Cruz Biotechnology). Each binding site was amplified by PCR. Cyclin E (forward: 5′-CCAATGCACTGACGG ATGAAT-3′; reverse: 5′-AAATCCCTGCGCGCGGAAC CG -3′) was cycled 36 times (95°C, 30 s/55°C 50 s/72°C, 30 s) with a 72°C, 10 min extension and a 95°C 5 min hot start. The same settings were used for CDK2 (forward: 5′-CATCCCAAGAGGAGA GGATT-3′, reverse: 5′-TGGC ACTGATTCTGAGGCTT-3′) and E2F (forward: 5′-CC AAGGCCATGACGCTCAC-3′, reverse: 5′-GCGGGTT AAAGCCAATAGGAA-3′), except the annealing time was 30 s. These primers frame a 178 bp promoter fragment spanning from −1064 to −1142 upstream of the CDK2 gene transcription start site, a 682 bp promoter fragment spanning from −1289 to −1971 upstream of the cyclin E gene transcription start site, and a 255 bp promoter fragment spanning from +10 to −245 upstream of the E2F1 gene transcription start site. The PCR products were electrophoretically fractionated in a 1.5% agarose gel and visualized using a transilluminator.

In-vivo tumor xenografts

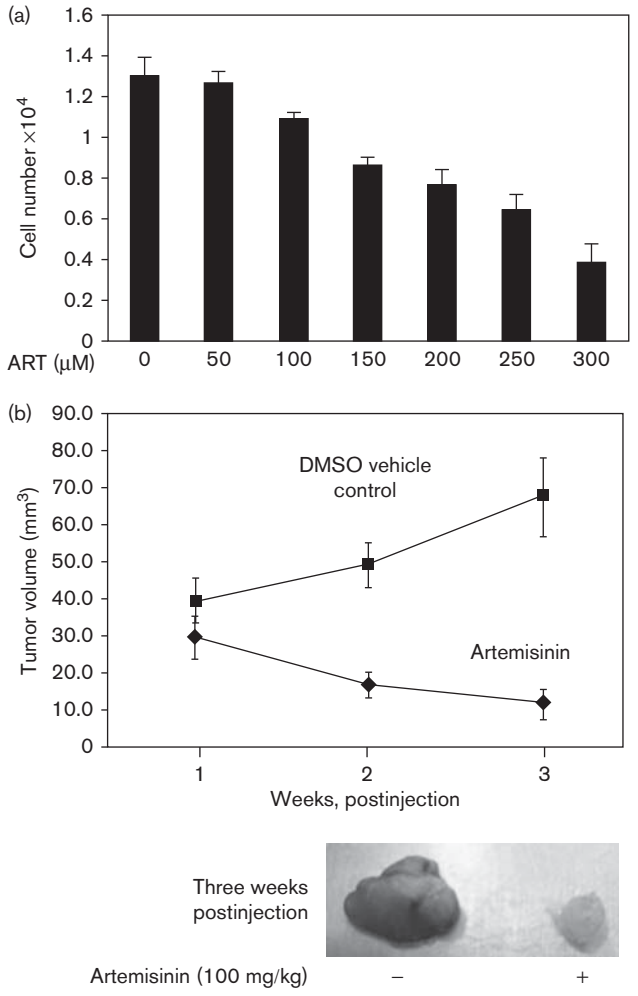
All animals were housed in our facility and treated in strict adherence to our Animal Use and Care Committee-approved protocols. Immunocompromised athymic 40-day-old female National Institutes of Health III (NIH III) nude mice were subcutaneously implanted with a mixture of 5×10^6 MCF7 cells and matrigel (Becton Dickinson, San Jose, California, USA) in the ratio of 1 : 1 by volume at two distinct sites per animal. The mice received 17β-estradiol as daily subcutaneous injections (1 mg), and were palpated for 3 weeks until tumors developed. The mice were randomized (five per group with a total of 10 tumor sites per group) and treated with 100 mg/kg body mass of artemisinin suspended in sterile DMSO and PBS or with sterile DMSO/PBS vehicle control in drinking water with replacement of drug containing water every 2 days. Tumor volumes were calculated based on the formula $(P/6 \times L \times W \times H)$ [28].

Results

Artemisinin inhibits the proliferation and in-vivo tumor growth of MCF7 human breast cancer cells

The cellular and in-vivo antiproliferative effects of artemisinin were initially examined in the MCF7 human breast cancer cell line that has an estrogen-responsive and tumorigenic, but poorly invasive, phenotype representative of an early stage breast cancer [29,30]. Cells were treated with increasing concentrations of artemisinin for 48 h, and an analysis of cell number revealed that 300 μmol/l artemisinin was the most effective dose that inhibits cellular proliferation in the absence of any apoptosis (Fig. 1a). A similar dose response was observed at 72 h of artemisinin treatment (data not shown). This effective concentration of artemisinin is generally consistent with what has been reported in other cell systems to induce a growth arrest without any apoptotic response [31], and likely reflects a combination of cellular transport, stability, and target protein interactions. The efficacy of artemisinin toward inhibition of MCF7 cell growth *in vivo* was investigated in tumor xenografts in nude athymic NIH III mice. The tumor xenografts were allowed to grow to an average size of approximately 35 mm^3 and then the mice were injected subcutaneously with either artemisinin (100 mg/kg/day) or the DMSO vehicle control. As shown in Fig. 1b, administration of artemisinin effectively inhibited the growth of MCF7 cell-derived tumor xenografts. In addition, in artemisinin-treated animals, the tumors appeared to show a robust inhibition of angiogenesis as indicated by the significantly reduced coloring of the tumors at the 3-week postinjection time point (Fig. 1b, tumor pictures). In other xenograft models, the anti-angiogenic effect of artemisinin has been attributed to

Fig. 1



Artemisinin inhibits the proliferation of cultured MCF7 human breast cancer cells and growth of MCF7 tumor xenografts *in vivo*. (a) Equal numbers (~300 000) of MCF7 cells were treated with the indicated concentrations of artemisinin for 72 h and the final cell number determined by counting the trypsin-releasable cells in a hemocytometer. Cell numbers were analyzed in triplicate-independent cultures of each tested concentration of artemisinin. (b) Palpable MCF7 cell-derived tumor xenografts were formed in athymic NIH III nude mice as described in the 'Materials and methods' section. Animals were treated orally with either 100 mg/kg of artemisinin or the DMSO vehicle control and tumor volumes monitored with a caliper and volume calculated as described in the 'Materials and methods' section. Ten tumor xenografts were formed for each condition using two tumor sites per animal. Three weeks postinjection representative tumors were excised and shown in the bottom panel. CDK, cyclin-dependent kinase.

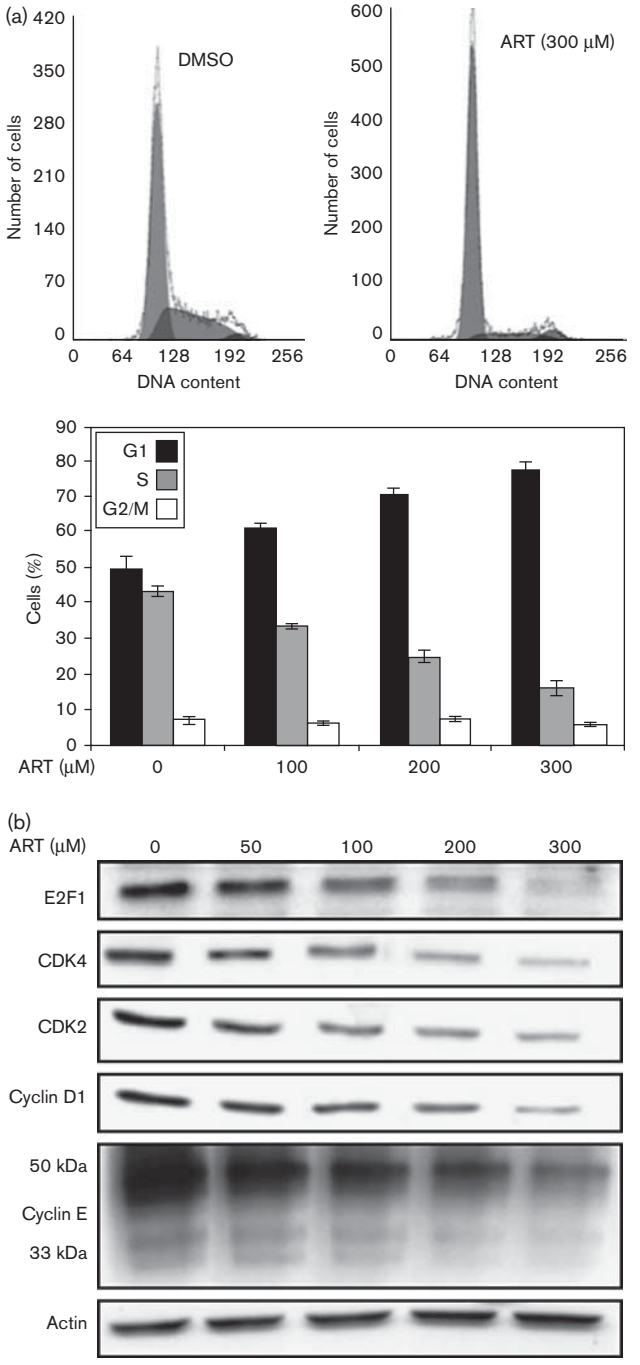
the inhibition of epidermal growth factor and vascular endothelia growth factor receptor expressions [32,33].

Artemisinin-induced G1-cell cycle arrest of MCF7 human breast cancer cells.

The artemisinin-mediated inhibition of cellular and in-vivo MCF7 cell proliferation suggested that this phytochemical has selective effects on the human breast cancer cell cycle. To determine the dose-dependent effects of artemisinin on the cell cycle distribution, 48-h-treated cells were harvested, stained with propidium iodide, and then subjected to flow cytometry to determine the DNA content of each cell population. As shown in Fig. 2a (bar graphs), artemisinin caused a robust accumulation of cells with a G1 DNA content with a proportional decline in the number of cells in the S phase. The flow cytometry profiles of MCF7 cells treated with or without 300 μmol/l artemisinin showed that in growing untreated conditions, the DNA content of the cells was dispersed throughout the cell cycle, whereas, after artemisinin treatment, the majority of the cell population was arrested with a G1 DNA content. Consistent with the cellular proliferation analysis in Fig. 1, 300 μmol/l artemisinin induced a G1-cell cycle arrest without any observed accumulation of sub-G1-DNA, which is typically indicative of apoptosis. Therefore, this concentration of artemisinin was used in all experiments unless otherwise specified. We also observed that artemisinin arrested the proliferation of several types of tumorigenic human breast cancer cells representing various clinical stages of disease manifestation (Table 1). In addition to MCF7 cells, artemisinin induced a G1 cell cycle arrest of MDA-MB-231 breast cancer cells, which do not express estrogen receptor-α (ERα) and represent a late stage cancer phenotype (Table 1). Interestingly, nontumorigenic MCF10A human mammary epithelial cells were resistant to artemisinin-induced cell cycle arrest, whereas artemisinin triggered a G1 cell cycle arrest in the MCF10AT preneoplastic cell line variant of MCF10A cells (Table 1).

To further characterize the artemisinin-induced G1 cell cycle arrest, MCF7 cells were treated with varying concentrations of artemisinin for 48 h and total cell lysates were electrophoretically fractionated and western blots probed with specific antibodies for several key G1-acting cell cycle gene products. As shown in Fig. 2b, artemisinin strongly downregulated the protein levels of the G1-acting CDKs, CDK2 and CDK4, both the 50 kDa and 33 kDa forms of cyclin E protein, cyclin D1, and the E2F1 transcription factor, which is a CDK2 and CDK4 substrate. Under these conditions, the levels of the CDK6, p21, and p27 cell cycle genes, and the Sp1, c-fos, and c-jun transcription factors did not significantly change (data not shown, and see time course studies). The dose-dependent effects of artemisinin on the G1-acting cell cycle genes approximated those of induced G1 cell cycle arrest, suggesting that the artemisinin-triggered loss of one or more of these cell cycle gene products is associated with the antiproliferative response. In addition, co-treatment of MCF7 with effective concentrations of dithiothreitol or ascorbic acid, two potent antioxidants, failed to reverse the artemisinin-mediated cell cycle arrest and downregulation of cell cycle genes (data not shown), indicating that the antiproliferative effects are not due to oxidative effects of artemisinin leading to apoptosis.

Fig. 2



Artemisinin (ART)-induced cell cycle arrest of MCF7 breast cancer cells. (a) MCF7 cells were treated with indicated concentrations of artemisinin for 48 h and the cell population DNA contents quantified by flow cytometry. The bar graphs show the average DNA content corresponding to the cell cycle phases in three independent experiments. A representative flow cytometry histogram of cells treated with or without 300 μmol/l artemisinin for 48 h is shown in the upper panels. (b) After treatment of MCF7 cells with the indicated concentrations of artemisinin for 48 h, total cell extracts were electrophoretically fractionated and western blots probed for the indicated G1-acting cell cycle genes as described in the 'Materials and methods' section. Analysis of actin protein levels was used as a gel loading control. CDK, cyclin-dependent kinase.

To begin to assess potential functional relationships between the artemisinin downregulation of the E2F1 transcription factor and altered expression of the cell cycle genes, the kinetics of expression was investigated in cells exposed to 300 μmol/l artemisinin. At the indicated treatment times, the levels of E2F1, Sp1, c-Fos, c-Jun, CDK2, CDK4, cyclin D1, cyclin E, and p21 protein were determined by western blot analysis of total cell extracts. As shown in Fig. 3 (upper panel: 24–72 h time course; lower panel: 6–24 h time course), by 12 h the artemisinin downregulation of E2F1 levels was mostly complete, which was the most rapid response to this phytochemical. The downregulation of CDK2 protein was not detectable at 12 h; however, in the following 12 h the level of CDK2 protein was reduced by nearly 90%. Closely following CDK2 was the loss of cyclin D1 protein and at a somewhat later time point the downregulation of CDK4 and cyclin E were clearly observed. No changes were observed in the levels of the Sp1, C-fos, or C-Jun transcription factors or in the levels of the CDK inhibitor gene $p21^{Waf/Cip1}$. In addition, no changes were observed in the level of p53 or in its various phosphorylated states (data not shown). These kinetic results implicate the rapid downregulation of the E2F1 transcription factor as a potentially important regulatory event in the artemisinin cell cycle arrest of MCF7 human breast cancer cells.

Artemisinin downregulates CDK2 gene expression, inhibits its promoter activity, and disrupts E2F1 promoter interactions

RT-PCR analysis of total RNA isolated from artemisinin-treated and artemisinin-untreated cells revealed that artemisinin induced a significant decline in the levels of CDK2, CDK4, cyclin D1, cyclin E, and E2F1 transcripts (Fig. 4a), which quantitatively accounts for the loss of the corresponding proteins. The most rapid response was the loss of transcripts encoding the E2F1 transcription factor, which was noticeably reduced by 12 h of artemisinin treatment. Because of the importance of CDK2 in controlling progression through the G1 phase of the cell cycle and the nearly complete ablation of CDK2 transcript levels by artemisinin, we further evaluated whether the artemisinin had an effect on CDK2 promoter activity. To test this possibility, MCF7 cells were transfected with pGL2 plasmids containing either of two CDK2 gene promoter regions (−2400 or −800 bp) fused to the luciferase reporter, and transfected cells were treated with or without 300 μmol/l artemisinin for 24 h before assaying luciferase activity in the isolated cell extracts. As shown in Fig. 4b, artemisinin strongly inhibited luciferase activity in cells transfected with the reporter plasmid driven by the −2400 CDK2 promoter, but not in cells transfected with the −800 bp CDK2-luciferase reporter plasmid.

Analysis of consensus transcription factor binding sites within the relatively large artemisinin-regulated region of

Table 1 Flow cytometry analysis of artemisinin cell cycle effects on human breast cancer cell lines with distinct phenotypes

		No treatment			Artemisinin		
Cell line	Phenotype	G1 (%)	S (%)	G2/M (%)	G1 (%)	S (%)	G2/M (%)
MCF7	Estrogen-responsive growth Tumorigenic poorly invasive wildtype p53	50	42	8	77	12	11
MDA-MB-231	Estrogen-independent growth Tumorigenic highly invasive Mutant p53	57	31	12	75	18	7
MCF 10AT	Estrogen-dependent growth Preneoplastic poorly Tumorigenic wildtype p53	52	41	7	74	11	15
MCF 10A	Estrogen-independent growth Nontumorigenic wildtype p53	71	20	9	73	17	10

the CDK2 promoter (−2400 to −800) using the Transfac program revealed the presence of several transcription factor consensus binding sites within the artemisinin-regulated region of the CDK2 promoter, including a single E2F binding site at −1117 bp from the transcriptional start. The relatively rapid artemisinin downregulation of E2F1 gene expression suggested that the control of E2F1 levels could potentially play an important role in the artemisinin inhibition of CDK2 promoter activity. In addition, both the cyclin E promoter and the E2F1 promoter itself contain consensus sites for the E2F1 transcription factor. Chromatin immunoprecipitation was used to directly determine whether artemisinin disrupts the endogenous interactions of E2F1 with the CDK2, cyclin E, and E2F1 promoters. MCF7 cells were treated with or without 300 µmol/l artemisinin for 48 h, and the genomic fragments cross-linked to protein were immunoprecipitated with anti-E2F1 antibodies, or with an IgG control antibody. Primers specific to each of the tested promoters revealed that artemisinin nearly ablated the endogenous binding of E2F1 to its binding sites on the CDK2 promoter (−1117 bp from start site), cyclin E promoter (−1614 bp and −1659 from start site), and E2F1 promoter (−15 bp from start site). Ten percent input was used as a loading control.

Expression of exogenous E2F1 reverses the effects of artemisinin on the expression of CDK2 and cyclin E and G1 cell-cycle arrest

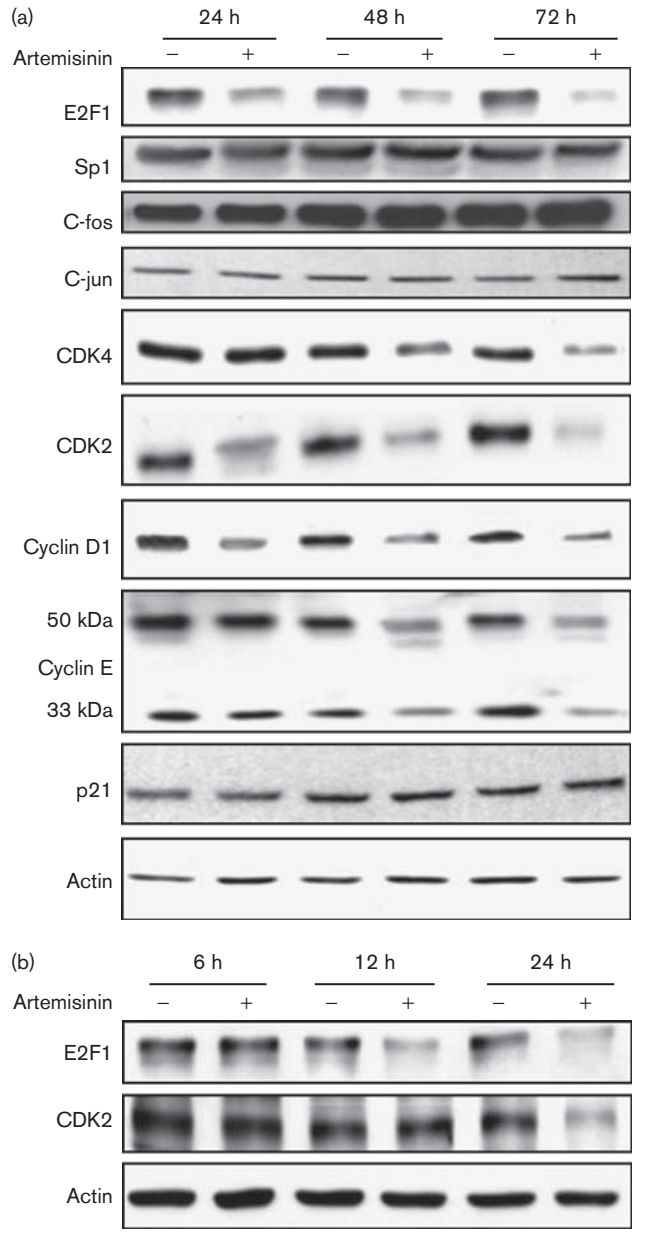
The chromatin immunoprecipitation analysis suggests that the disruption of E2F1 interactions with the CDK2 and cyclin E promoters leads directly to the loss of CDK2 and cyclin E gene expression, resulting in a G1 cell cycle arrest. To functionally test the significance of artemisinin downregulation of E2F1 gene expression, MCF7 cells were transfected with either a constitutive E2F1 expression plasmid (pcmv-E2F1) or an empty expression vector (pcmv-neo) as a transfection control, and the cells were then treated with or without 300 µmol/l artemisinin for 48 h. Western blot analysis revealed that in MCF7 cells transfected with the constitutive E2F1 expression vector, the total levels of E2F1 protein remained unchanged in the presence or absence of artemisinin. Importantly, artemisinin failed to downregulate CDK2 and cyclin E in cells expressing the exogenous E2F1, whereas in the empty vector transfected cells, artemisinin strongly inhibited production of CDK2, cyclin E, and E2F1 (Fig. 5a). These results demonstrate that the artemisinin downregulation of E2F1 levels is required for the artemisinin downregulation of CDK2 and cyclin E levels.

To examine effects on cell cycle progression, the cell population DNA contents of transfected cells treated with or without artemisinin were analyzed by flow cytometry. As shown in Fig. 5b, control transfected cells (Neo) showed a robust artemisinin-mediated cell cycle arrest that displayed a significant increase in the number of cells in the G_1 phase. In contrast, in cells constitutively expressing exogenous E2F1, artemisinin treatment failed to induce a G1 cell cycle arrest. In transfected cells ectopically expressing elevated levels of E2F1, both artemisinin-treated and artemisinin-untreated cells, the number of cells with a G1 DNA content remained at approximately 40%, while in both conditions a significant fraction of the cell population displayed an S phase DNA content (Fig. 5b) that is indicative of rapidly proliferating cells. These results show that the downregulation of E2F1 transcription factor levels is required for the artemisinin-induced G1 cell cycle arrest of MCF7 human breast cancer cells, which has uncovered a new transcriptional pathway that is targeted by this phytochemical.

Discussion and conclusion

Artemisinin, an antimalarial phytosesquiterpene lactone, has been shown to have antiproliferative effects on a variety of cellular and animal models of cancer [17,34], and on several of the tested systems, artemisinin or its derivatives were shown to modulate transcriptional and/or cell signaling pathways [35]. We have observed that artemisinin exerts a potent G1 cell cycle arrest of cultured MCF7 human breast cancer cells and strongly inhibits the growth of MCF7 cell-derived tumor xenografts *in vivo*. One of the earliest responses to artemisinin treatment was the decline in the levels of the E2F1 transcription factor, which was followed by the loss in expression of several key G1-acting cell cycle genes. Artemisinin was shown to disrupt the binding of E2F1 to the CDK2 and cyclin E promoters, resulting in the loss of expression of both cell cycle genes and triggering a G1 block in cell cycle progression. The constitutive expression of exogenous E2F1 prevented the artemisinin downregulation of both CDK2 and cyclin E expression and precluded the G1 cell cycle arrest, which functionally demonstrates the critical role of E2F1 as a downstream target of artemisinin antiproliferative signaling in human breast cancer cells. The CDK2–cyclin E complex is

Fig. 3



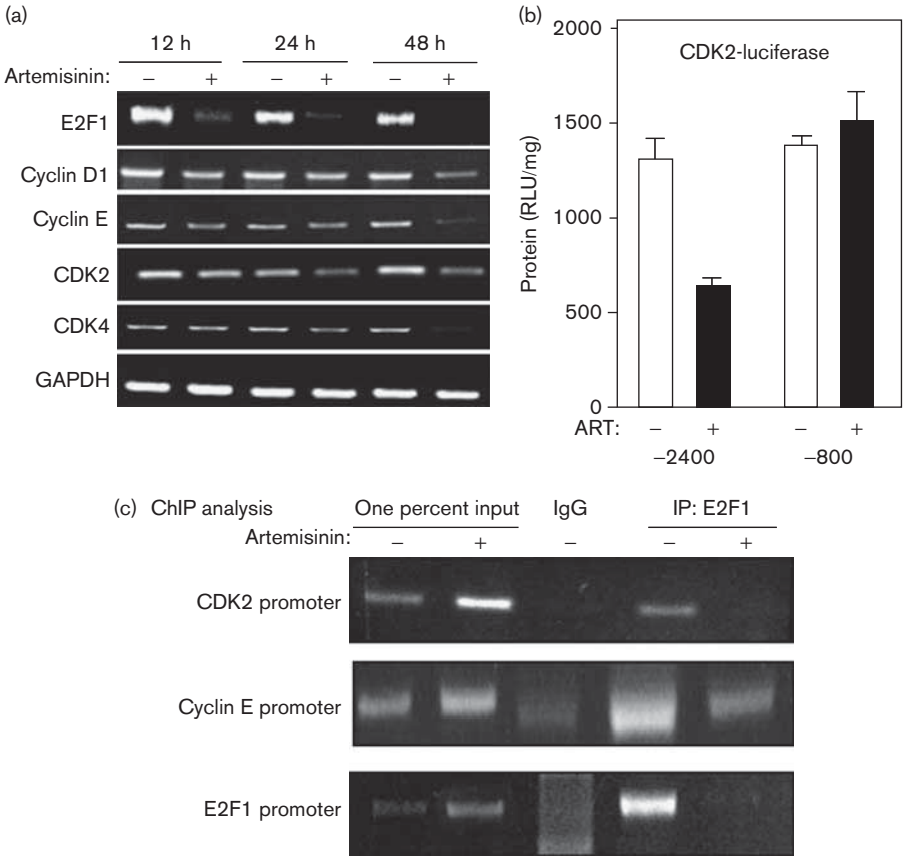
Time course of artemisinin-regulated production of transcription factors and cell cycle-regulated genes. MCF7 cells were treated with 300 μmol/l artemisinin for indicated durations, total cell extracts electrophoretically fractionated, and the protein levels of the indicated transcription factors or cell cycle-regulated gene analysis by western blot analysis. Analysis of actin protein levels was used as a gel loading control. The upper panel displays the 24–72 time course and the lower panel displays the 6–24 h time course. CDK, cyclin-dependent kinase.

known to hyperphosphorylate retinoblastoma leading to release of E2F1, which then increases transcription of CDK2 and cyclin E [36]. In addition, the hyperphosphorylated retinoblastoma leads to an increase in E2F1 gene expression because the E2F1 promoter binds preferentially to the E2F1/E2F3 members of this transcription factor family, which drives transcription of the gene [37]. Because artemisinin rapidly downregulates E2F1 expression and its CDK2 and cyclin E target genes, we propose that artemisinin mediates its antiproliferative response by disrupting an E2F1 autoregulatory loop that reduces E2F1 levels and triggers a G1 block in cell cycle progression.

In MCF7 breast cancer cells, the most significant effects of artemisinin on expression of cell cycle genes was the strong downregulation of CDK2, CDK4, cyclin E, cyclin D1, whereas, expression of the other main G1-acting cell cycle genes remained relatively unchanged. The disrupted expression of this set of cell cycle genes is likely critical for the anticancer effects of artemisinin and related compounds. Expressions of CDK2, CDK4, cyclin D1, and cyclin E have been strongly associated with increased breast cancer recurrence, and overexpression of CDK2 correlated positively with lymph node metastasis [38]. Other studies have implicated CDK2 enzymatic activity in mediating Sp1 phosphorylation and consequently matrix metalloproteinase 2 expression [39]. Similar effects have been reported with another potent anticancer phytochemical indole-3-carbinol, which attenuates CDK2 activity and matrix metalloproteinase 2 expression [40,41]. Elevated expression of cyclin E has also been shown to correlate with poorer prognosis, increased genomic instability, as well as higher grade of tumors [42]. In addition, the percentage of E2F1-positive cells increased positively with increasing stages of breast cancer [43], which likely controls the expression levels of CDK2 and cyclin E in late stage cancers.

We uncovered direct evidence that the loss of CDK2 and cyclin E expression was due to the disruption of E2F1 binding to both promoters, and we are currently attempting to identify the artemisinin-regulated transcription factor(s) that account for the loss of cyclin D1 and CDK4 expression. In this regard, cyclin D1 expression is maintained in an ERα-dependent manner in estrogen-responsive breast cancer cells, and artemisinin downregulated ERα promoter activity [26], suggesting that the loss of cyclin D1 expression is likely linked to the downregulation of ERα expression. We also previously reported that the artemisinin-induced G1 cell cycle arrest of LNCaP prostate cancer cells was controlled by the ablation of CDK2 and CDK4 expression and that downregulation of CDK4 promoter activity was due to the artemisinin-mediated inhibition of cellular Sp1 activity [25]. Consistent with our results, artemisinin has been shown to induce a G1 cell cycle arrest of cultured liver cancer cells by downregulating CDK2, CDK4, cyclin D1, and cyclin E [24]; however, the mechanism of downregulation of these cell cycle genes was not investigated. In pancreatic cancer cells [44], human colon cancer cells [45], and in leukemia [46] the artemisinin-mediated growth arrest is associated with the

Fig. 4

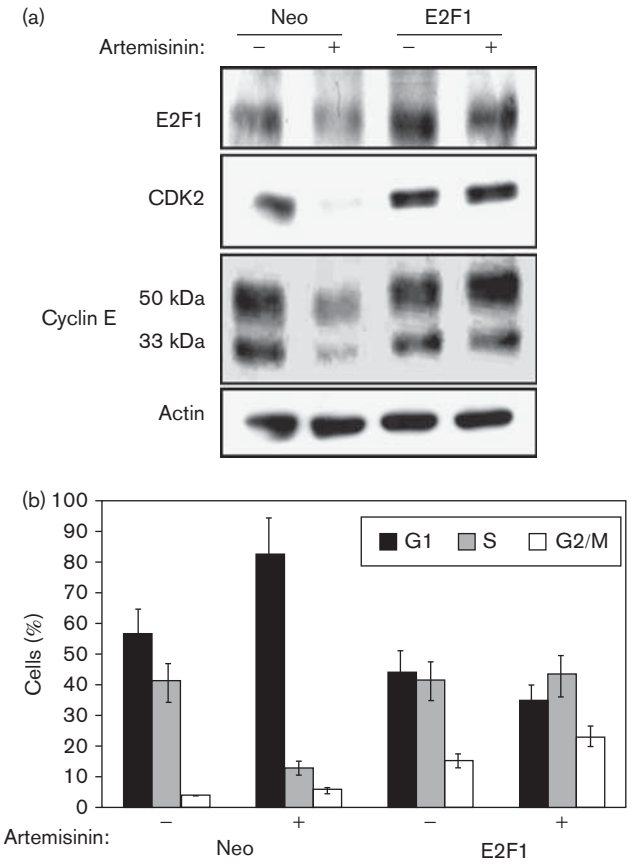


Artemisinin inhibits expression of E2F1, cyclin-dependent kinase 2 (CDK2), and cyclin E transcripts and disrupts CDK2 promoter activity and E2F1 interactions with the CDK2 promoter. (a) MCF7 cells were treated with or without 300 μmol/l artemisinin for the indicated times and total RNA isolated. The transcript levels of E2F1, cyclin D, cyclin E, CDK2, CDK4, and GAPDH were determined by reverse transcription-polymerase chain reaction using specific primers, and the products fractionated by electrophoresis on a 1.5% agarose gel and visualized on an ultraviolet transilluminator. GAPDH served as a gel loading control. (b) MCF7 cells were transfected with either of the two indicated CDK2 promoter luciferase reporter plasmids (5′ ends of either −2400 bp or −800 bp), treated with or without 300 μmol/l artemisinin for 24 h, and assayed for luciferase activity as described in the 'Materials and methods' section. The assay baseline was determined by the level of luciferase activity observed in the presence of 1 μg/ml of the general polymerase II transcription inhibitor actinomycin D (1 μg/ml). (c) Chromatin immunoprecipitation (ChIP): MCF7 cells were treated with or without 300 μmol/l artemisinin for 24 h, and fixed and subjected to chromatin immunoprecipitation with E2F1 antibody followed by PCR of the E2F1 consensus binding sites in the CDK2, cyclin E, and E2F1 gene promoters. One percent input served as the loading control. IgG, immunoglobulin G. IP, immunoprecipitated.

downregulation of the nuclear factor-κB transcription factor, although the critical target genes were not functionally characterized. In prostate cancer, as well as in other cancer cell lines, artemisinin-derived drugs such as artesunate are able to induce a G2/M cell cycle arrest [47,48], suggesting that the effects of artemisinin and its derivatives may be tissue specific. Taken together, an emerging concept is that artemisinin-based compounds mediate key features of their anticancer responses through the transcriptional downregulation of genes directly involved in cellular proliferation and/or cell survival.

Artemisinin was more effective in triggering a proliferative arrest of tumorigenic breast cancer cell lines (MCF7, MDA-MB-231, and MCF10AT) compared with a nontumorigenic cell line (MCF10A) that was resistant to artemisinin. The MCF7 breast cancer cells represent a relatively early stage estrogen-responsive breast cancer phenotype, whereas MDA-MB-231 cells represent a steroid-independent late stage cancer phenotype [49]. This observation showed that the efficacy of artemisinin did not depend on the cellular expression of ERα because MDA-MB-231 cells have a silenced ERα expression due to promoter hypermethylation [50]. The artemisinin-sensitive MCF10AT cells, which have a preneoplastic phenotype that can form tumors at a low rate *in vivo* [51], were derived from the artemisinin-resistant MCF10A cells, which are a nontumorigenic pseudodiploid breast cell line [52]. The selectivity of artemisinin to cancerous cells has been observed in other tissue types. For example, the leukemic cell line Molt4 is sensitive to

Fig. 5



Constitutive expression of E2F1 ablates artemisinin-mediated downregulation of cyclin E and CDK, cyclin-dependent kinase 2 (CDK2) prevents the G1 cell cycle arrest. (a) MCF7 cells were transfected with the empty expression vector (Neo) or constitutive expression vector for E2F1 (E2F1), and treated with or without 300 μmol/l artemisinin for 48 h. The total cell extracts were electrophoretically fractionated and the levels of E2F1, CDK2, and cyclin E analyzed by western blots. The level of actin protein was used as a gel loading control. (b) The cell population DNA content and cell cycle phase of MCF7 cells transfected with the empty expression vector (Neo) or constitutive expression vector for E2F1 (E2F1) were analyzed after treatment with 300 μmol/l artemisinin for 48 h. Breast cancer cells were treated with or without 300 mmol/l artemisinin for 48 h, and the cell population DNA contents quantified by flow cytometry after staining with propidium iodide. The results are an average of independent triplicate results. The average error was approximately between ±1% and ±2% for each of the results.

artemisinin, but normal primary lymphocytes are resistant to this phytochemical [53]. Similar differences in artemisinin sensitivity were observed with ovarian cancer cells compared with normal ovarian epithelial cells [54]. The selectivity that artemisinin exhibits in exerting a growth arrest is a major asset to its potential as a cancer management strategy. This study also presents artemisinin as an effective mitostatic phytochemical in early breast cancer preneoplastic cell lines, providing a promising candidate for breast cancer prevention. Thus, artemisinin and its related derivatives represent promising anticancer therapeutic molecules for a variety of human cancer cell types because of their selective modulation of expression and activity of key transcription factors that affect expression of specific cell cycle regulators.

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Conflicts of interest

There are no conflicts of interest.

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