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Artemisinin triggers a G1 cell cycle arrest of human Ishikawa endometrial cancer cells and inhibits Cyclin Dependent Kinase-4 promoter activity and expression by disrupting NF-kB transcriptional signaling

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Abstract

Relatively little is known about the anti-proliferative effects of Artemisinin, a naturally occurring anti-malarial compound from *Artemisia annua*, or sweet wormwood, in human endometrial cancer cells. Artemisinin induced a G1 cell cycle arrest in cultured human Ishikawa endometrial cancer cells and down regulated CDK2 and CDK4 transcript and protein levels. Analysis of CDK4 promoter-luciferase reporter constructs showed that the artemisinin ablation of CDK4 gene expression was accounted for by the loss of CDK4 promoter activity. Chromatin immunoprecipitation demonstrated that artemisinin inhibited nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) subunit p65 and p50 interactions with the endogenous Ishikawa cell CDK4 promoter. Coimmunoprecipitation revealed that artemisinin disrupts endogenous p65 and p50 nuclear translocation via increased protein-protein interactions with IκB-α, an NF-κB inhibitor, and disrupts its interaction with the CDK4 promoter, leading to a loss of CDK4 gene expression. Artemisinin treatment stimulated the cellular levels of IκB-α protein without altering the level of IκB-α transcripts. Finally, expression of exogenous p65 resulted in the accumulation of this NF-κB subunit in the nucleus of artemisinin treated and untreated cells, reversed the artemisinin down-regulation of CDK4 protein expression and promoter activity and prevented the artemisinin induced G1 cell cycle arrest. Taken together, our results demonstrate that a key event in the artemisinin anti-proliferative effects in endometrial cancer cells is the transcriptional down-regulation of CDK4 expression by disruption of NF-κB interactions with the CDK4 promoter.

Keywords

Endometrial cancer cells; Artemisinin anti-proliferative signaling; NF-κB; CDK4 gene expression

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Introduction

Endometrial carcinoma is the most common gynecological cancer in the United States and fourth most common cancer among women, after breast, lung, and colon cancer with 47,130 cases and 8,010 deaths reported in 2012 [1]. There are two distinct types of endometrial cancers based on histological and molecular characteristics. Type I tumors, referred to as the endometrioid type, are generally well-differentiated adenocarcinomas with or without squamous differentiation, whereas Type II, the nonendometrioid type, are uterine papillary serous and clear-cell carcinomas [2]. Type I tumors comprise approximately 80-90% of all endometrial cancers [3, 4]. Current treatment options for endometrial cancer involves the use of several types of chemotherapeutic agents, such as paclitaxel, cisplatin, and doxorubicin, that are efficacious at high doses, but also exhibit significant adverse side affects such as renal damage, gastrointestinal toxicity, and bone marrow suppression [5, 6]. Moreover, development of chemoresistance of endometrial cancer appears after 4-18 months [5]. Thus, there is a need to develop new classes of chemotherapeutic agents that exhibit increased efficacy along with lower toxicity profiles and reduced side effects. One such promising approach is to screen traditional indigenously used medicinal plants for potential anticancer properties [6-15].

Naturally occurring plant compounds represent a largely untapped source of potential chemotherapeutic molecules to control different types of cancers with very minimal side effects [16-18]. One such phytochemical is artemisinin, which is a sequiterpene lactone that was isolated from the aerial portions of the *Artemisia annua* plant (more commonly known as qinghaosu or sweet wormwood). For over 2000 years, Chinese traditional medicine practitioners have utilized this herb to treat a variety of illnesses, such as intestinal parasitic infections, hemorrhoids, and fever [19]. The compound was isolated from *A. annua* by Chinese chemists in 1970s, and since then, artemisinin and a number of its derivatives have been used to effectively treat forms of malaria in the past three decades [20]. Recent studies have demonstrated that artemisinin and its derivatives exhibit potent anticancer effects in a various human cancer cell model systems such as colon, melanoma, breast, ovarian, prostate, central nervous system, leukemic, and renal cancer cells [21, 22]. Additionally, dihydroartemisinin and artemisinin-derived trioxane dimers were shown to exhibit strong growth inhibitory and apoptotic effects of several types of human cancer cell lines without inducing cytotoxic effects on normal adjacent cells [23, 24]. Depending on the tissue type and experimental system, molecular, cellular, and physiological studies have demonstrated that the responses to artemisinin and its derivatives target a variety of cancer signaling pathways which can involve cell cycle arrest, apoptosis, inhibition of angiogenesis, and cell migration, as well as modulation of nuclear receptor responsiveness [25-27].

One proposed mechanism of the anti-cancer actions of artemisinin is based on the cleavage of its endoperoxide bridge that is catalyzed by high concentrations of ferrous iron, similar to what is observed in individuals infected with the malaria parasite due to proteolysis of host cell hemoglobin [28]. Peroxides are a known source of reactive oxygen species, such as hydroxyl radicals or superoxide, which can cause oxidative damage to cells, as well as iron depletion in the cells [29, 30]. However, previous experiments have shown that artemisinin's anti-cancerous effects do not depend on the generation of these toxic-free radicals [31]. In

addition, expression profiling and gene expression studies of several types of human cancer cells revealed that artemisinin treatment causes selective changes in expression of many oncogenes and tumor suppressor genes than can be accounted for by changes restricted only to genes responsible for iron metabolism [32-34]. These results indicate that the anticancer properties of artemisinin cannot be attributed solely to global toxic effects of oxidative damage.

There is only limited information on the mechanisms by which artemisinin and its derivatives regulate expression and activity of specific transcription factors. We previously demonstrated in prostate cancer cells that artemisinin arrests cell growth and proliferation by down-regulation of CDK4 expression via disruption of endogenous Sp1 transcription factor interactions with the CDK4 promoter [31]. We further observed that in human breast cancer cells, artemisinin treatment disrupted E2F1 transcription factor expression, which led to the inhibited expression of two G1-activing cell cycle regulators CDK2 and cyclin E [34]. These results suggest 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 Ishikawa human endometrial cancer cells is mediated by the inhibition of NF-κB transcription factor nuclear localization that leads to the disruption of CDK4 promoter activity and loss of gene transcription. Furthermore, we show that expression of exogenous NF-κB subunit p65 confers resistance to the antiproliferative effects of artemisinin, demonstrating the critical role of p65 expression mediated this artemisinin response in human endometrial cancer cells.

Materials and Methods

Materials

Artemisinin (90%) was purchased from Sigma (St Louis, Missouri, USA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA) and Cytoskeleton Inc (Denver, CO). All media-related reagents were purchased from Lonza (Walkersvilee, Maryland, USA). Reagents obtained elsewhere are indicated in text. The Ishikawa cells were obtained from American Type Culture Collection (Manassas, VA).

Cell culture

Ishikawa cells were grown in Dulbecco's modified Eagles Medium, supplemented with 10% fetal bovine serum (purchased from Mediatech, Henderson VA), 10 μg/ml insulin, 50 U/ml penicillin, 50 U/ml streptomycin, and 2 mmol/l L-glutamine from Sigma-Aldrich. The cells were maintained at subconfluency in a humidified air chamber at 37°C containing 5% CO_2. Stock solutions of artemisinin were dissolved in dimethyl sulfoxide (DMSO), and then diluted in the ratio 1: 1000 in media before culture plate application.

Flow cytometry

Ishikawa cells were plated onto Nunc six-well tissue culture (Nunc A/A, Denmark) and treated for the indicated time points in the specified manner. Cells were treated with various concentrations of artemisinin. Experiments were performed in triplicate. The medium was changed every 24 hours. Following treatment, the medium was aspirated, and the cells were

washed once with PBS and subsequently harvested in 1 mL of PBS. The cells were then hypotonically lysed in 300 μL of DNA staining solution (0.5 mg/mL propidium iodide, 0.1 % sodium citrate, and 0.05% Triton X 100). Propidium iodide-stained nuclei were analyzed using a detector with a 575 nm band pass filter on a Beckman-Coulter EPICS XL instrument with laser output adjusted to deliver 15 megawatts at 488 nm. Ten thousand nuclei were analyzed from each sample. The percentage of cells within the $G_{1,}$ S, $G_{2/M}$ phases of the cell cycle were determined by analyzing the histographic output with the multicycle computer program MPLUS, provided by Phoenix Flow Systems in the Cancer Research Laboratory, Flow Cytometry Facility of the University of California, Berkeley [31].

Immunoblotting

After the indicated treatments, western blots were harvested in 1 mL of PBS and pelleted by centrifugation. The cells were then resuspended in radioimmunoprecipitation assay buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (50 g/mL phenylmethylsulfonyl fluoride, 10 g/mL aprotin, 5 g/mL leupeptin, 0.1 g/mL NaF, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 0.1 g/mL NaF, ß-mercaptoethanol, 10% bromphenol blue, 3.13% 0.5 M Tris-HCl, and 0.4% SDS (pH 6.8) and fractionated on 10% polyacrylamide/0.1% SDS resolving gels by electrophoresis. The Spectra Multicolor Broad Range Protein Ladder from Fermentas Life Sciences was used as the molecular weight standard. Proteins were electrically transferred to nitrocellulose membranes (Micron Separations, Inc., Westboro, MA), and equal protein loading was confirmed by Ponceau S staining of blotted membranes. Membranes were blocked for one hour at room temperature with Western wash buffer-5% NFDM (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20, 5% nonfat dry milk). Protein blots were subsequently incubated overnight at 4°C with primary antibody in 10 mL of Western wash buffer and the working concentration for all antibodies was 1 μL/mL in Western wash buffer. Immunoreactive proteins were detected after incubation with horseradish peroxidase conjugated secondary antibody diluted to 0.2 μL/mL in Western wash buffer (goat anti-rabbit IgG and rabbit anti-mouse IgG (Bio-Rad). Blots were treated with enhanced chemiluminescence reagents (GE healthcare) and visualized on high performance chemiluminescence film (GE healthcare, UK). The following antibodies were diluted 1:1000 in TBST: mouse anti-CDK2 (sc-6248), rabbit anti-CDK4 (sc-749), rabbit anti-CDK6 (sc-177), mouse anti-cyclin D1 (sc-749), mouse anti-p21 (NA35, Calbiochem, Rockland, MA), mouse anti-c-fos (sc-447), rabbit anti-c-Jun (sc-44), rabbit anti-p50 (sc-53744), rabbit anti-p65 (sc-372), rabbit anit-phospho-p65 (sc-33020R), rabbit anti-IκB-α (sc-21) were purchased from Santa Cruz Biotechnology and diluted in the ratio 1:1000 in TBST. Rabbit HSP90 (#00240 BD Biosciences, San Diego, CA), and Rabbit antiactin (#AANO1 cytoskeleton Inc.) was diluted in the ratio 1:1000 in TBST and was used as a protein loading control.

Subcellular fraction

Cells were plated onto Nunc 10cm plates (Nunc A/A, Denmark) and treated with or without 300 μM artemisinin for 48 hours. Following the treatment, the medium was aspirated and the cells were washed once with PBS and harvested in 1 mL of PBS. Preparation of cytoplasmic and nuclear extraction of the Ishikawa cells treated with or without 300 μM

artemisinin was performed by using NE-PER Nuclear and Cytoplasmic extraction kit (Thermo Scientific, Rockford, IL) following the manufacturer's protocol. The cells were then stored in −80 °C until use and analyzed via western blot. To confirm the fidelity of the subcellular fraction, western blots of each fraction were examined for enrichment of the lamin nuclear marker protein or the tubulin cytoplasmic marker protein.

Reverse transcription and polymerase chain reaction

Ishikawa cells treated with indicated doses of artemisinin and duration were harvested in Trizol (Invitrogen, Calrsbad, 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 primers of the following sequences: CDK2 forward, 5' - TGGATGTCATTCA-3' and reverse 5' - CAGGGACTCCAAAAGCTCTG-3'; CDK4 were forward (5' -CTGAGAATGGCTACCTCTCGATATG-3') and reverse (5' - AGAGTGTAACAACCACGGGTGTAAG-3'); CDK6 were forward (5'-CCGAGTAGTGCATCGCGATCTAA-3') and reverse (5'-CTTTGCTAGTTCATCGATATC-3'); and glyceraldehyde-3-phophate dehydrogenase (GAPDH) forward, 5'-TGAACGTCGGAGTCAACGGATTTG-3' and GAPDH reverse, 5'-CATGTGGGCCATGAGGTCCACCAC-3'. Twenty microliters of this reaction was electrophoretically fractionated in a 1% agarose gel and visualized using an ultraviolet transilluminator. [35]

Plasmids and Transfections

Indicated plasmids (1μg) were transfected into Ishikawa cells using Superfect reagent (Qiagen, Germantown, Maryland, USA) according to the manufacturer's instructions. Cells were treated with 300 μM artemisinin 24 h post-transfection 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 CDK4 luciferase plasmids were kind gifts from Dr. Leonard Bjeldanes, University of California, Berkeley, California, USA. The pCMV-p65 vector was a kind gift from Dr. Kahryn Calames, Columbia University.

Immunoprecipitation

Ishikawa cells were cultured on growth medium with artemisinin for the indicated times and then rinsed twice with PBS, harvested, and stored as dry pellets at −70°C. Cells were lysed for 15 min in IP buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Triton X-100) containing protease, 10 μg/ml aprotonin, 5 μg/ml leupeptin, 0.1 μg/ml NaF, 10 μg/ml β-glycerophosphate, and 0.1 mM sodium orthovanadate). Samples were diluted to 1 mg of protein in 1 ml of IP buffer. Samples were diluted to 1 mg of protein in 1 ml of IP buffer. Samples were precleared for 30 min at 4°C with 40 μl of a 1:1 slurry of protein GSepharose beads (GE health BioSciences AB). After a brief centrifugation to remove precleared beads, 0.5 μg of rabbit anti-p65 or rabbit anti-p50 (Santa Cruz Biotechnology) antibody was added to each sample for one set of samples. The beads were then washed three times with IP buffer. Half of the immunoprecipitated sample was checked by Western blot analysis to

confirm the immunoprecipitation, and the IκB-α levels were examined by Western blot using the second half of the samples.

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed as previously described [36, 37]. Briefly, cross-linking of proteins to DNA was obtained by the addition 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 5min. Harvested cells were sonicated and the chromatin was immunoprecipitated overnight with 15μl antibodies recognizing NF-κB (Santa Cruz Biotechnology). Each binding site was amplified by PCR. CDK4 NF-κB binding site (forward: 5'-TCTTTATCTCCCAGCCCTTTGG-3; reverse: 5'-AGTGGAAGGAGTGGCAAATAGGGT-3') was cycled 36 times (95C, 30 s/55C 50 s/72C, 30 s) with a 72°C, 10 min extension and a 95°C 5 min hot start. The same settings were used for CDK4 Sp1 binding site (forward: 5'-ACAGATGCCTCCTCTTTGCATCCT-3', reverse: 5'-AGAAGCCTTACAAAGCACCCACC-3') and CDK4 AP-1 binding site (forward: 5'-ACCCTATTTGCCACTCCTTCCACT-3', reverse: 5'-GGCCCAGGGCTACAAGTAT-3'), except the annealing time was 30s. These primers frame a 228 bp promoter fragment spanning from – 1506 to – 1278 upstream of the CDK4 gene transcription start site. The PCR products were electrophorically fractionated in a 1.5% agarose gel and visualized using a transilluminator [31].

Results

Artemisinin inhibits the proliferation of Ishikawa human endometrial cancer cells by inducing a G1 cell cycle arrest

The potential effects of artemisinin on proliferation of the Ishikawa human endometrial cancer cell line was initially examined by treating cells with increasing concentrations of artemisinin up to 400 μM and then quantifying cell number after 48 h treatments. As shown in Figure 1A, artemisinin dose dependently inhibited cell number, and by 300 μM artemisinin there was a maximal inhibition of cellular proliferation. To determine effects of artemisinin on the cell cycle distribution, Ishikawa cells were treated without or with 300 μM artemisinin or 400 μM artemisinin for 48 h and the propidium iodide stained nuclear DNA was quantified by flow cytometry. Vehicle control treated cells proliferate as an asynchronous population with cells in all stages of the cell cycle, whereas, both concentrations of artemisinin treatment induced a marked increase in the percentage of cells in the G1 phase with a concomitant decrease in S and G2/M phase cells (Fig 1B). Compared to vehicle control treated cells, there was no increase in the level of sub-G1 content, which is indicative of the absence of any apoptotic effects of artemisinin. Coupled with the cell proliferation assay, the quantification of cellular DNA content revealed that the increased number of G1 phase arrested Ishikawa cells (Fig 1B, lower right panel histograms) correlated with the inhibition of cell proliferation (Fig 1A), and that 300 μM artemisinin was the lowest concentration that induced the maximal effect without any effects on apoptosis. Therefore, this concentration of artemisinin was used in all experiments unless otherwise specified.

Time course of artemisinin-induced G1 cell cycle arrest of Ishikawa human endometrial cancer cells and regulated expression of G1 acting cell cycle components

To determine the kinetics of the artemisinin-induced G1 cell cycle arrest, Ishikawa cells were treated with or without 300 μM artemisinin over a 72 h time course. Nuclear DNA was stained with propidium iodide, and DNA content of the cell populations was analyzed by flow cytometry. Within 24 h, there was a small increase in level of G1 phase cells (Fig 2A). By 48 h treatment, artemisinin induced a robust accumulation of cells with a G1 DNA content that had a proportional decline in the number of cells in the S and G2/M phase, which was maintained through 72 h (Fig. 2A). To further characterize the artemisinin-induced G1 cell cycle arrest, Ishikawa cells were treated with or without 300 μM artemisinin over a 72 h time course, total cell lysates were electrophoretically fractionated and western blots probed with specific antibodies for several key G1-acting proteins such as the three cyclin-dependent kinases (CDK2, CDK4 and CDK6), cyclin D1 and the p21 CDK inhibitor. Consistent with the observed G1 cell cycle arrest, artemisinin strongly down-regulated the protein levels of CDK2, CDK4 and CDK6, with no significant change in the expression of cyclin D1 or $p21^{Waf/Cip1}$ proteins (Fig 2B).

Other than our previous studies using human prostate and breast cancer cells [34, 35], relatively little is known about the artemisinin regulation of CDK gene expression. Therefore, to assess whether the artemisinin down-regulation CDK2, CDK4 and/or CDK6 protein are due to changes in CDK transcript levels, Ishikawa cells were cultured in the presence or absence of 300 μM artemisinin for a 72 h time course and the levels of CDK2, CDK4 and CDK6 transcripts determine by reverse transcription-PCR analysis. As shown in Figure 2C, artemisinin treatment significantly down-regulated expression of CDK2 and CDK4 transcripts by 48 h, with the maximal artemisinin effects observed by 72 h. The decreased CDK2 and CDK4 transcripts accounts for the loss of the corresponding proteins, whereas, in contrast, there were no apparent changes in CDK6 transcript levels suggesting a selective loss of CDK6 protein.

Artemisinin inhibits CDK4 promoter activity

Because of the importance of CDK4 in controlling progression through the G1 phase of the cell cycle and the near ablation of CDK4 transcript levels by artemisinin, the effects of artemisinin on CDK4 promoter activity was further evaluated. To determine the region in the CDK4 promoter that confers artemisinin responsiveness, serial 5' deletion constructs of the 2120-bp CDK4 promoter were constructed and cloned into PGL3 luciferase reporter vectors. Each of the CDK4 promoter-luciferase reporter plasmids were transiently transfected into Ishikawa cells, and luciferase activity assayed in cells treated with or without artemisinin for 48 h. As shown in Figure 3, artemisinin strongly inhibited luciferase activity in cells transfected with the luciferase reporter plasmids driven by the – 2120 bp and – 1506 bp CDK4 promoter fragments, but not in cells transfected with the – 1278 bp and – 867 bp CDK4 luciferase reporter plasmids. These results suggest there is a 228-bp region of the CDK4 promoter between – 1278 bp and – 1506 bp that confers artemisinin responsiveness.

Artemisinin disrupts NF-κB binding to the artemisinin responsive region of the CDK4 promoter

Analysis of consensus transcription factor binding sites within the artemisinin-regulated region of the CDK4 promoter (– 1506 bp to – 1278 bp) using the Alibaba 2.1 transcription factor-binding site computer program revealed the presence of several transcription factor consensus binding sites, including the Nuclear Factor-Kappa B (NF-κB) subunit p50 and p65 binding site at – 1411 bp, Specificity Protein-1 (Sp1) binding site at – 1422 bp, and Activating Protein-1 (AP-1) binding site at – 1475 bp. To determine if one or more of these sites plays a role in conferring artemisinin responsiveness to the CDK4 promoter, chromatin immunoprecipitation was used to directly determine whether artemisinin disrupts the endogenous interactions of Sp1, AP-1, p50, and p65 with the CDK4 promoter. Ishikawa cells were treated with or without 300 μM artemisinin for 48 h, and the genomic fragments cross-linked to protein were immunoprecipitated with anti-Sp1, anti-AP-1, anti-p65, and anti-p50 antibodies, or with an IgG control antibody. As shown in Figure 4A, primers specific to each of the tested promoter binding sites revealed that artemisinin nearly ablated the endogenous binding of NF-κB to its binding site on the on CDK4 promoter (– 1411 bp from start site). In contrast, artemisinin had no effect on endogenous binding of Sp1 and AP-1 (Figs. 4B and 4C) to its binding site on CDK4 promoter (– 1422 bp, – 1475 bp from start site). One percent input was used as a loading control. Interestingly, our lab previously reported that artemisinin down regulates CDK4 expression in human prostate cancer cells by disruption of Sp1 interactions with the CDK4 promoter [31], which shows that the cell cycle effects of artemisinin and control of CDK gene expression can be highly tissue specific.

Artemisinin disrupts NF-κB subunit p65 and p50 localization into the cell nuclei and promotes p65-IκB-α and p50-IκB-α interaction

It is well established that NF-κB is a crucial transcription factor associated with several cancer types including endometrial cancer, and controls multiple genes involved in tumor progression such as cell proliferation and survival [38, 39]. Therefore, given the observed effects of artemisinin disrupting the binding of NF-κB subunits p65 and p50 to the CDK4 promoter, we investigated whether artemisinin disrupts NF-κB localization into the cell nuclei. The localization of both p65 and p50 was initially examined in cells treated with or without 300 μM artemisinin and then biochemically fractionated into nuclear and cytoplasmic extracts. As shown in Figure 5A, western blots analysis showed that artemisinin treatment causes the relocalization of p65 and p50 from both cytoplasmic and nuclear fractions into predominantly the cytoplasmic fraction, but there was no change in the total protein levels of p65 and p50. The fidelity of the fractionation was shown by the selective enrichment of lamin in the nuclear fractionation and the enrichment of tubulin in the cytoplasmic fraction. Therefore, artemisinin disruption of NF-κB binding to CDK4 promoter can be attributed to the artemisinin inhibition of NF-κB nuclear translocation.

The NF-κB p65/p50 heterodimer contains the transcriptional activation domain and is sequestered in the cytoplasm as an inactive complex by binding to IκB-α, an inhibitor of NF-κB [40, 41]. To determine whether artemisinin regulates NF-κB protein-protein interactions, the NF-κB subunit p65 was immunoprecipitated from 48 h artemisinin treated or untreated Ishikawa cells and IκB-α binding was detected by western blot analysis of

electrophoretically fractionated p65. Artemisinin treatment strongly enhanced p65 interactions with total IκB-α protein (Fig 5B, left panel) without altering the total level of p65 protein (Fig 5B, right panel). Intriguingly, Artemisinin significantly up-regulated total IκB-α protein levels, which may account for the enhanced binding of IκB-α to p65 (Fig 5B, right panel). Artemisinin had no effect on IκB-α transcript levels (Fig 2C) suggesting a selective effect on IκB-α protein stability. Co-immunoprecipitation analysis, in which immunoprecipitated p50 was examined for its interactions with IκB-α by western blots, showed that artemisinin treatment significantly increased p50-IκB-α interactions under conditions in which there were no changes in p50 protein levels protein (Fig 5C, right panel). Thus, artemisinin inhibition of p65 and p50 nuclear translocation is likely due to the increased interactions with its inhibitory protein IκB-α. This observed effect, conceivably can be attributed to the significant artemisinin stimulation in total IκB-α protein production.

Expression of exogenous NF-κB subunit p65 reverses the artemisinin G1 cell cycle arrest and effects on CDK4 protein expression and promoter activity

To functionally test the cellular significance of artemisinin inhibited interaction of the p65 NF-κB subunit with the CDK4 promoter, Ishikawa cells were transfected with either a constitutive p65 expression plasmid (CMV-p65) or an empty expression vector (CMVNeo) as a transfection control, and the cells were then treated with or without 300 μM artemisinin for 48 h. Western blot analysis confirmed that the cells receiving the CMV-p65 expression vector produced significantly higher levels of p65 protein compared to the cells transfected with the CMV-Neo empty expression vector (Fig 6A). Importantly, artemisinin failed to down-regulate CDK4 protein levels in cells expressing the exogenous p65, whereas in the empty vector transfected cells, artemisinin strongly inhibited production of CDK4 (Figure 6A).

Subcelluar fractionation revealed that the exogenous p65 NF-κB subunit that is produced from the p65-CMV expression vector accumulated in the nucleus of artemisinin treated and untreated cells to approximately the same extent (Fig 6B) and therefore overrides the artemisinin disruption of endogenous p65 nuclear accumulation. Under these conditions, the nuclear marker protein lamin is only detected in the nuclear fractions, whereas, the cytoplasmic marker tubulin is selectively enriched in the cytoplasmic fraction. To assess the effects of exogenous p65 expression on the artemisinin inhibition of CDK4 promoter activity, Ishikawa cells were co-transfected with the – 1506 CDK4 promoter luciferase reporter plasmid in the presence of either CMV-p65 constitutive expression vector or the CMV-Neo control vector. As shown in Figure 6C, expression of exogenous p65 reversed the artemisinin-mediated down-regulation of CDK4 promoter activity, demonstrating the central role of the p65 transcription factor in attenuation of CDK4 transcription by artemisinin. These results further demonstrate that the artemisinin inhibition of NF-κB subunit p65 nuclear translocation is required for the artemisinin down-regulation of CDK4 gene expression.

To examine effects of expressing exogenous p65 on the artemisinin induced G1 cell cycle arrest, Ishikawa cells transfected with either the CMV-p65 or CMV-Neo expression vectors were treated with or without artemisinin and the DNA content of the cell populations

evaluated by flow cytometry of propidium iodide stained nuclear DNA. As shown in Figure 7, control transfected cells (CMV-Neo) showed a robust artemisinin-mediated G1 cell cycle arrest with a significant increase in the number of G1 phase cells. In contrast, in cells constitutively expressing exogenous p65 (CMV-p65), artemisinin treatment failed to induce a G1 cell cycle arrest and the cell population remained proliferating and in both artemisinin-treated and artemisinin-untreated cells, the number of cells with a G1, S or G2/M DNA content remained unchanged (Figure 7). These results show that the inhibition of p65 transcription factor nuclear translocation is required for the artemisinin-induced G1 cell cycle arrest of Ishikawa endometrial cancer cells, which has uncovered a new transcriptional pathway that is targeted by this phytochemical.

Discussion

The G_1 phase of the cell cycle can be exquisitely regulated in normal and transformed mammalian cells [42], and has been targeted in certain therapeutic strategies that arrest cell proliferation. Most anti-cancer agents that induce a G_1 arrest in human cancer cells typically do so through decreased enzymatic activity of the G_1-acting CDKs through the increased expression of cyclin-dependent kinase inhibitors, decreased expression of cyclins and/or expression of different isoforms of cyclins that alter CDK activity [43-46]. Our results demonstrated that artemisinin induces a G1 cell cycle arrest of human Ishikawa endometrial cancer cells by disrupting the nuclear localization and transcriptional activity of the NF-kB transcription factor, which directly resulted in the loss of NF-κB interactions with the endogenous CDK4 promoter activity and inhibition of CDK4 gene expression. A key functional test of the role of artemisinin disrupted transcriptional signaling in the anti-proliferative response was that expression exogenous p65 subunit of NF-kB reversed the artemisinin induced G1 cell cycle arrest, prevented the loss of nuclear p65 and reversed the artemisinin inhibition CDK4 promoter activity and expression. Artemisinin treatment strongly impeded production of all three G1-acting CDKs although at least two distinct mechanisms target these cell cycle genes. Artemisinin down regulated CDK4 and CDK2 transcript expression, which accounted for the loss of their corresponding proteins, whereas, CDK6 protein levels were decreased in the absence of any changes in transcript levels, suggesting that this phytochemical triggers the degradation of CDK6 protein. We are currently attempting to determine identify the artemisinin-regulated transcription factor(s) that account for the loss of CDK2 gene expression and uncover the protein degradation components acting on CDK6. Regardless of the precise mechanisms, the artemisinin-mediated loss of CDK4, CDK2 and CDK6 gene products directly supports the stringent G1 cell cycle arrest of endometrial cancer cells. The loss of production of all three G1-acting CDKs is rarely associated with the cell cycle arrest of human cancer cells and has not been previously reported for human endometrial cancer cells.

The NF-κB transcription factor controls the expression of many genes involved in cancer cell proliferation, inflammation, apoptosis, survival, neo-plastic transformation, and chemoresistance [47, 48], and can regulate the proliferation of endometrial cancer cells [39, 49, 50]. Our results provide direct evidence that artemisinin disrupts interactions of the p50 and p65 subunits of NF-kB with a critical cell cycle target gene promoter to mediate an anti-proliferative response in human endometrial cancer cells. NF-κB resides in the cytoplasm as

an inactive heterodimeric protein complex comprised of both NF-κB subunits p50 and p65 bound to its inhibitor protein IκB-α. For activation of NF-κB, IκB-α is phosphorylated, ubiquitinated, and then degraded by the 26S proteasome, therefore allowing nuclear translocation of NF-κB and subsequent regulated expression of specific sets of target genes [51, 52]. In Ishikawa endometrial cancer cells, we observed that artemisinin treatment up-regulated the total levels of the inhibitory IκB-α protein, which resulted in the enhanced interactions of p50 and p65 with IκB-α. A key consequence of artemisinin stimulated binding of IκB-α to both NF-κB subunits is the sequestering of the NF-κB protein complex (p50-p65) in the cytoplasm, which prevented the accessibility of NF-κB to the nucleus and binding to the CDK4 promoter. As a functional test of this mechanism, expression of high levels of exogenous p65 reversed the artemisinin-mediated decrease in CDK4 promoter activity and gene expression as well as precluded artemisinin-induced G1 cell cycle arrest. Artemisinin had no effect on the level of IκB-α transcripts suggesting that the increase in IκB-α protein is likely due to an effect of artemisinin on protein stabilization.

The disruption of NF-κB transcriptional signaling by artemisinin has been shown to be associated with the inhibition of pro-inflammatory responses in several systems. For example, in phorbal-12-myristate-13-acetate induced THP-1 monocytes, artemisinin inhibited expression of several pro-inflammatory cytokines, including tumor necrosis factor-alpha and interleukin-6, was accompanied by the loss of IκB-α phosphorylation and stabilization of the IκB-α protein, which correlated with the impeded nuclear translocation of NF-κB [53]. Although the direct effects of artemisinin on gene transcription were not evaluated, our results suggest that it is highly likely that artemisinin disrupts NF-κB interactions with the endogenous cytokine gene promoters. In microglia cells, artemisinin treatment attenuated lipopolysaccharide-induced production by promoting the formation of the inactive p65-p50 heterodimeric NF-κB protein complex that requires the presence of IκB-α [54]. The disruption of NF-κB signaling has also been shown to be involved with the artemisinin inhibition of rat cardiac hypertrophy [55] and is associated with therapeutic effects in lupus nephritis mice [56]. In pancreatic cancer cells, the artemisinin derivative dihydroartemisinin induced a G1 cell cycle arrest and inactivated NF-κB function, although the NF-κB target genes critical for this response were not functionally characterized [57]. Taken together, an emerging concept is that NF-κB plays a central role in artemisinin transcriptional signaling and biological effects in a range of responses and tissue types.

We recently reported that the artemisinin-induced G1 cell cycle arrest of MCF7 human breast cancer cells was controlled by artemisinin disruption of E2F1 transcription factor expression, which leads to the ablation of key G1 cell cycle regulators CDK2 and cyclin E expression [34]. In addition, we also previously observed that the artemisinin-induced G1 cell cycle arrest of LNCAP prostate cancer cells was caused by strong transcriptional down-regulation of CDK2 and CDK4 expression and that decreased CDK4 promoter activity that was due to the artemisinin-mediated disruption of Sp1 transcription factor interactions with the CDK4 promoter [31], suggesting that transcriptional effects of artemisinin are highly tissue specific. Based on our previous and current studies, we propose that artemisinin can induce a G1 cycle arrest in different types of reproductive cancer cells through the regulation of distinct cell signaling pathways that ultimately control the activities of specific sets of transcription factors. For example, artemisinin down regulates CDK4 promoter

activity through the loss of Sp1 activity in prostate cancer cells [31] and through the loss of NF-κB activity in endometrial cancer cells. Because Sp1 is ubiquitously expressed in normal and transformed human cells, the interactions of Sp1 with other transcription factor binding partners in target gene promoters such as CDK4 may determine whether artemisinin triggers the loss of CDK4 promoter activity through Sp1 or through other transcription factors such as NF-κB. Consistent with our results, artemisinin was shown to induce a G1 cell cycle arrest of human hepatoma cancer cells by down-regulating the levels of CDK2, CDK4, cyclin D1, and cyclin E [33]; however, the mechanism of down regulation was not examined. In human osteosarcoma cells, as well as in several other human cancer cell lines, the artemisinin-derived derivative artesunate induced a G2/M cell cycle arrest [58], suggesting that depending on the cell type artemisinin-based compounds can have distinct cell cycle effects.

Our results strongly reinforce the view that artemisinin is a promising anti-cancer compound that could potentially be developed as a potent therapeutic strategy for endometrial carcinomas. Further examination of the NF-κB interactions with the CDK4 promoter will be necessary to determine whether artemisinin controls the function of other transcription factors in addition to NF-κB and whether induced changes in transcription factor protein-protein interactions and/or co-regulators are selectively altered by the loss of nuclear NF-κB. An important future direction will be to identify the direct cellular targets of artemisinin upstream of NF-κB that triggers the sequestration of inactive NF-κB away from the nucleus. Collectively, our study provides direct mechanistic support for the concept that artemisinin-based compounds can mediate anti-cancer responses through the transcriptional down-regulation of genes that are directly involved in cellular proliferation and/or cell survival.

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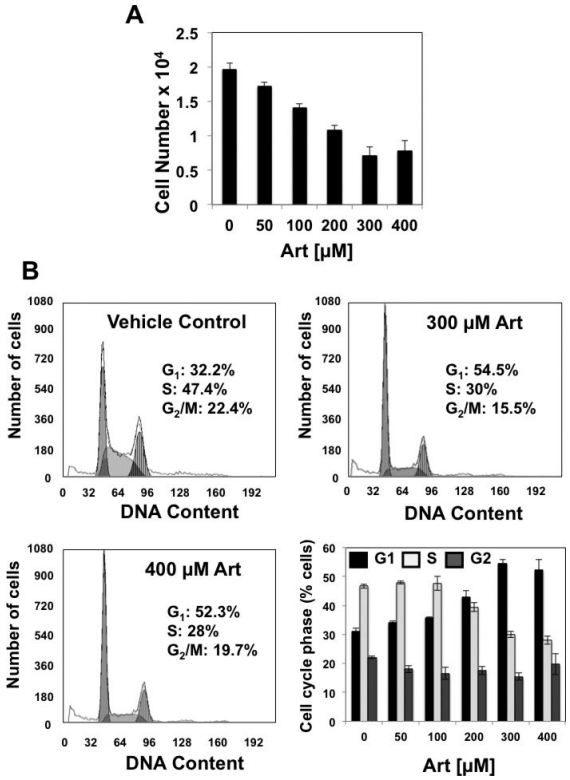

FIGURE 1. Artemisinin inhibits proliferation of cultured Ishikawa human endometrial cancer cells via artemisinin-induced G1 cell cycle arrest

(A) Equal numbers (~300,000) of Ishikawa cells were treated with the indicated concentrations of artemisinin for 48 h and the final cell number was determined by counting the trypsinreleasable cells in a hemocytometer. Cell numbers were analyzed in triplicate-independent cultures of each tested concentration of artemisinin (Art). (B) Ishikawa cells were treated with indicated concentrations of artemisinin (Art) for 48 h and the cell population DNA contents were quantified by flow cytometry. The bar graphs show the average DNA content corresponding to the cell cycle phases in three independent experiments. Representative flow histograms of cells treated with or without 300 μM or 400 μM artemisinin for 48 h are shown.

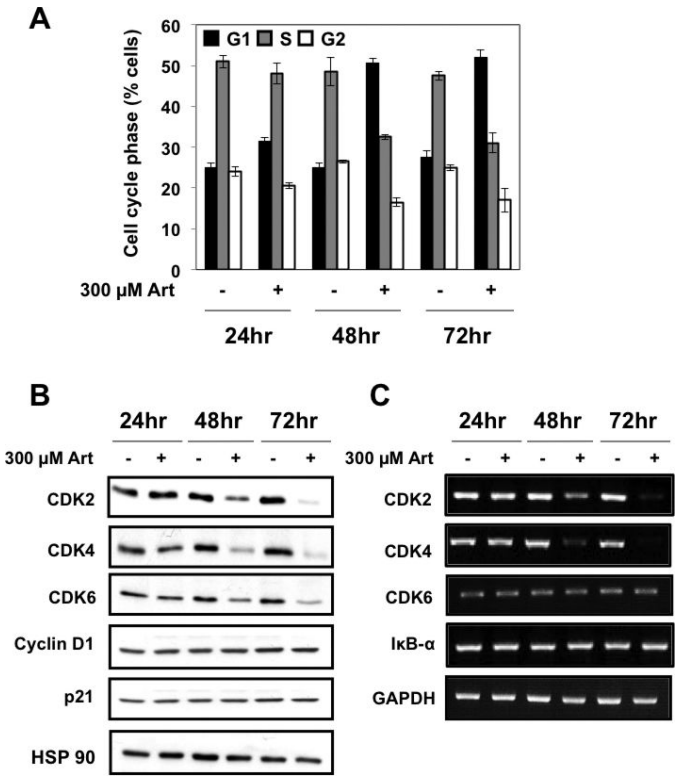

FIGURE 2. Time course of artemisinin down-regulated expression of G1-acting cell cycle genes

(A) Ishikawa endometrial cancer cells were treated with or without 300 μM artemisinin for indicated durations and cell population DNA contents were quantified by flow cytometry. The bar graphs show the average DNA content corresponding to the cell cycle phases in three independent experiments. (B) Ishikawa cells were treated either with 300 μM artemisinin for the indicated durations, total cell extracts were electrophoretically fractionated and western blots were probed for the indicated G1-acting cell cycle genes as described in the 'Materials and Methods' section. Analysis of HSP90 protein levels was used as a gel loading control. (C) Ishikawa cells were treated with or without 300 μM artemisinin for the indicated times and total RNA was isolated. The transcript levels of CDK2, CDK4, CDK6, IkB-α, and GAPDH were determined by reverse transcription-polymerase chain reaction using specific primers, and the products were fractionated by electrophoresis on a 1.5% agarose gel and visualized on an ultraviolet transilluminator. GAPDH served as a loading control.

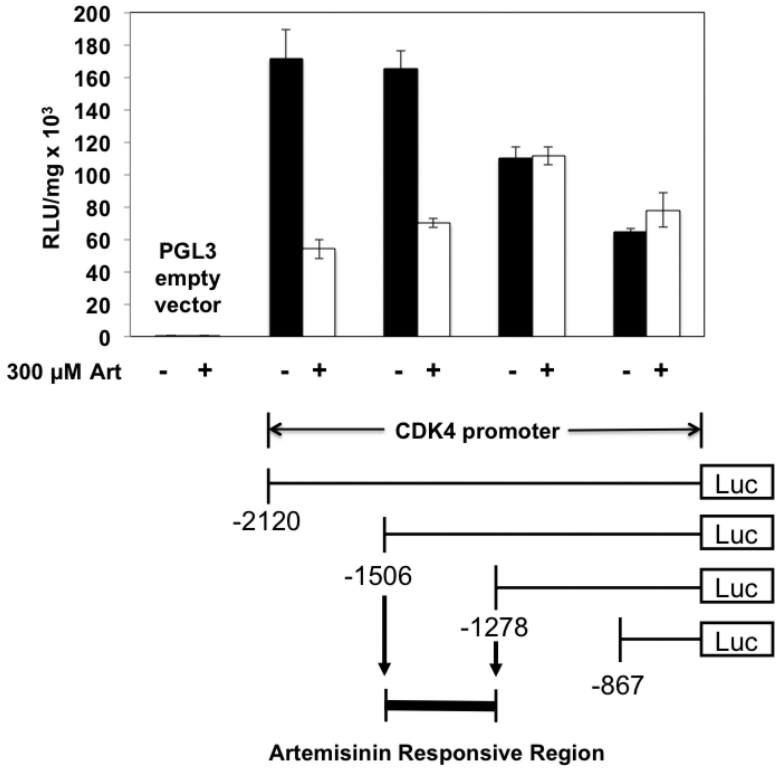

FIGURE 3. Identification of the artemisinin-responsive region of the CDK4 promoter

Ishikawa endometrial cancer cells were transfected with a series of CDK4-luciferase reporter plasmids that contain the indicated 5' deletions of the CDK4 promoter. Cells were treated with DMSO vehicle control or 300 μM artemisinin for 48 h. Cells were harvested and relative light units (*RLU*) were determined as described in the 'Materials and Methods' section, and normalized to protein concentration of the same sample. Data are the mean of triplicate experiments, and *error bars* are standard deviation.

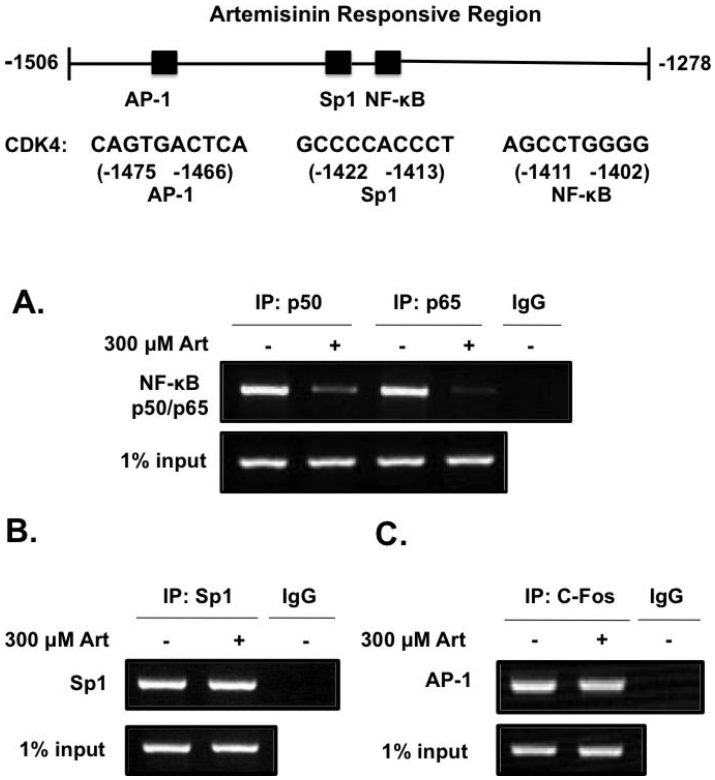

FIGURE 4. Artemisinin inhibits NF-κB binding to the artemisinin-responsive region of the CDK4 promoter

The diagram shows the locations of consenesus DNA binding elements in the artemisinin responsive region of the CDK4 promoter for transcription factors associated with the control of cancer cell prolieration. Chromatin immunoprecipitation (ChIP): Ishikawa endometrial cancer cells were treated with or without 300 μM artemisinin for 48 h, fixed and subjected to chromatin immunoprecipitation with NF-κB (p50 and p65) antibodies (Panel A), Sp1 antibodies (Panel B) and AP-1 antibodies (Panel C), followed by PCR of the consensus binding sites of NF-κB, Sp1, and AP-1 in the CDK4 gene promoter. One percent input served as the loading control. IgG, immunoglobulin G. IP, immunoprecipitated.

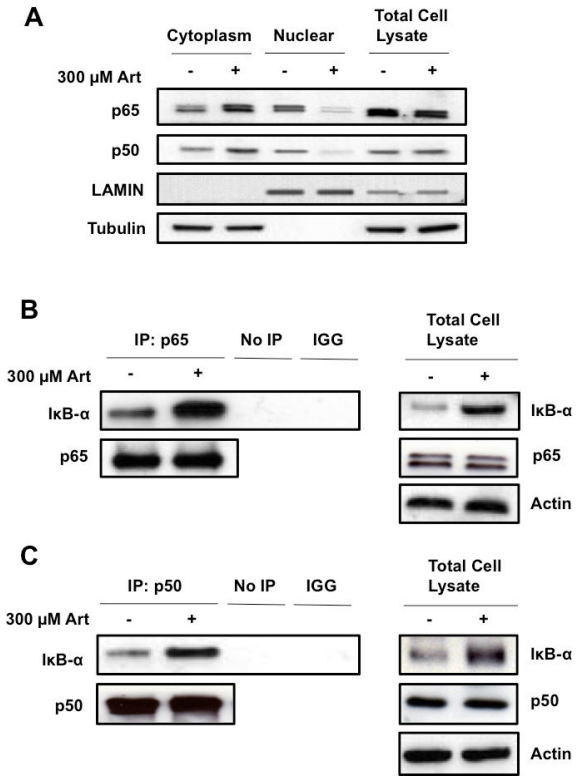

FIGURE 5. Artemisinin inhibits NF-κB translocation into the nucleus via increased interactions with its inhibitor IκB-α

(A) Ishikawa endometrial cancer cells were treated with or without 300 μM for 48 h, and cell extracts were biochemically separated into nuclear enriched and cytoplasmic fractions, electrophoretically fractionated, and western blots probed with antibodies specific for p65 and p50, the cytoplasmic marker tubulin and the nuclear marker lamin. (B) Ishikawa cells were treated with or without 300 μM artemisinin for 48 h. Total cell extracts were immunoprecipitated with either p65 (B) or p50 (C) antibodies. Control conditions used non-immune antibodies (IgG) for the immunoprecipitations or the samples were not immunoprecipitated (No IP). All extracts were electrophoretically fractionated and probed by western blot analysis using antibodies specific to either total IκB-α or total p65 (B) or using antibodies specific to total IκB-α or total p50 (C).

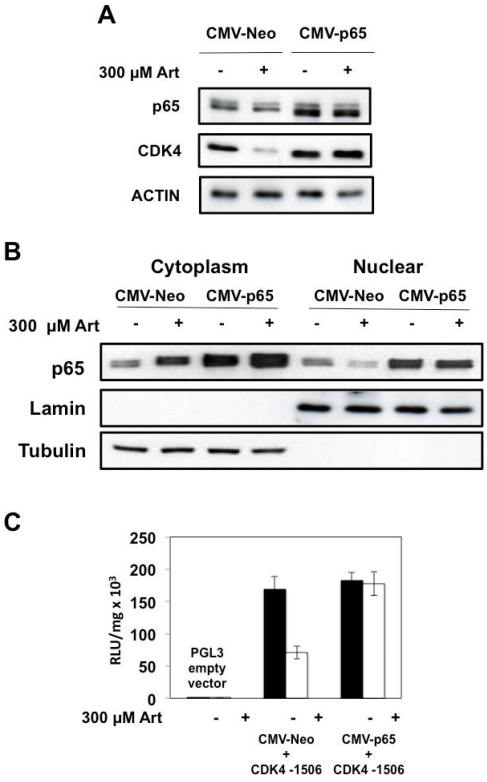

FIGURE 6. Constitutive expression of exogenous p65 reverses the artemisinin-mediated down-regulation of CDK4 gene expression and overrides the loss of nuclear p65

(A) Ishikawa cells were transfected with the empty expression vector CMV-Neo or constitutive expression vector for CMV-p65, and treated with or without 300 μM artemisinin for 48 h. The total cell extracts, were electrophoretically fractionated and the levels of p65 and CDK4 analyzed by western blots. The level of actin protein was used as a gel loading control. (B) Ishikawa endometrial cancer cells transfected with either CMVNeo or CMV-p65 were treated with or without 300 μM for 48 h, and cell extracts were biochemically separated into nuclear enriched and cytoplasmic fractions, electrophoretically fractionated, and western blots probed with antibodies specific for p65, the cytoplasmic marker tubulin and the nuclear marker lamin. (C) Ishikawa cells were cotransfected with artemisinin-responsive –1506 bp CDK4 promoter-luciferase reporter plasmid with either the CMV-p65 constitutive expression plasmid or with the CMV-Neo control vector. Luciferase activity was assayed in cells treated with or without 300 μM artemisinin for 48 h. Data are the mean of triplicate experiments, and *error bars* are standard deviation ($p < 0.01$).

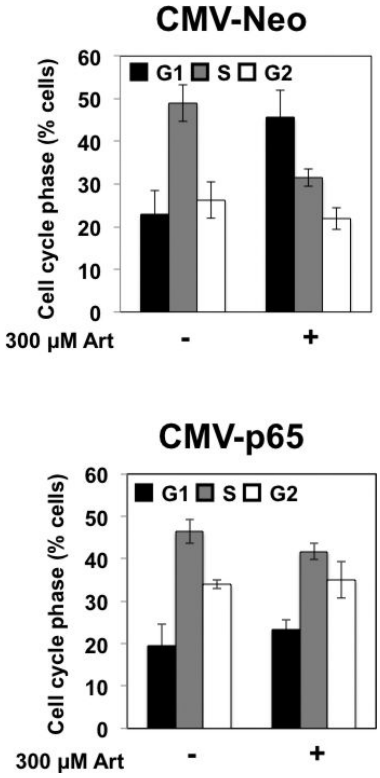

FIGURE 7. Constitutive expression of exogenous p65 prevents the artemisinin mediated G1 cell cycle arrest

The cell population DNA content and cell cycle phase of Ishikawa cells transfected with the empty expression vector CMV-Neo or constitutive expression vector CMV-p65 were analyzed after treatment with 300 μM artemisinin for 48 h. Endometrial cancer cells were treated with or without 300 μM 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 *bar graph* with standard error represents results from this analysis.