

Artemisinin Blocks Prostate Cancer Growth and Cell Cycle Progression by Disrupting Sp1 Interactions with the Cyclin-dependent Kinase-4 (CDK4) Promoter and Inhibiting CDK4 Gene Expression*

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Artemisinin, a naturally occurring component of *Artemisia annua*, or sweet wormwood, is a potent anti-malaria compound that has recently been shown to have anti-proliferative effects on a number of human cancer cell types, although little is known about the molecular mechanisms of this response. We have observed that artemisinin treatment triggers a stringent G₁ cell cycle arrest of LNCaP (lymph node carcinoma of the prostate) human prostate cancer cells that is accompanied by a rapid down-regulation of CDK2 and CDK4 protein and transcript levels. Transient transfection with promoter-linked luciferase reporter plasmids revealed that artemisinin strongly inhibits CDK2 and CDK4 promoter activity. Deletion analysis of the CDK4 promoter revealed a 231-bp artemisinin-responsive region between -1737 and -1506. Site-specific mutations revealed that the Sp1 site at -1531 was necessary for artemisinin responsiveness in the context of the CDK4 promoter. DNA binding assays as well as chromatin immunoprecipitation assays demonstrated that this Sp1-binding site in the CDK4 promoter forms a specific artemisinin-responsive DNA-protein complex that contains the Sp1 transcription factor. Artemisinin reduced phosphorylation of Sp1, and when dephosphorylation of Sp1 was inhibited by treatment of cells with the phosphatase inhibitor okadaic acid, the ability of artemisinin to down-regulate Sp1 interactions with the CDK4 promoter was ablated, rendering the CDK4 promoter unresponsive to artemisinin. Finally, overexpression of Sp1 mostly reversed the artemisinin down-regulation of CDK4 promoter activity and partially reversed the cell cycle arrest. Taken together, our results demonstrate that a key event in the artemisinin anti-proliferative effects in prostate cancer cells is the transcriptional down-regulation of CDK4 expression by disruption of Sp1 interactions with the CDK4 promoter.

Prostate cancer is the most diagnosed cancer and the second leading cause of cancer death among men in the United States (1). One third of all cancer cases reported in men are prostate cancer, and one out of every six men will be diagnosed with prostate cancer at some point in their lifetimes (1). The primary treatment for patients diagnosed with prostate cancer is androgen ablation therapy, which consists of administering anti-androgens and chemical castration to decrease the levels of circulating androgens, such as testosterone, in the body (2). Given that prostate cancers initially develop as androgen-responsive, this ablation therapy is particularly effective early on in the course of treatment (3). However, androgen ablation treatment is associated with a 70–80% progression rate into androgen-independent prostate tumors within 1–3 years so despite the initial success of this therapy, in most cases, the cancer will relapse as an incurable hormone-refractory condition in which the overall survival is ~15–16 months (4, 5). The lack of therapeutics that are highly effective against all types of prostate cancer is a critical problem in the field.

Naturally occurring plant compounds represent a largely untapped source of potential chemotherapeutic molecules to control different types of prostate cancers with very minimal side effects. One such promising compound is artemisinin, a sesquiterpene lactone that was isolated from *Artemisia annua* (more commonly known as qinghaosu or sweet wormwood). Chinese medical practitioners have used artemisinin from plant extracts for over two thousand years to treat a variety of conditions such as fever and hemorrhoids. The compound was isolated from *A. annua* by Chinese chemists in the 1970s, and since then, artemisinin and a number of its derivatives have been used to effectively treat different forms of malaria (6). Recently, artemisinin and its derivatives have been shown to induce growth arrest and apoptosis (7–9), as well as inhibit angiogenesis by down-regulation of the vascular endothelial growth factor vascular epidermal growth factor and its cellular receptor KDR/flk-1 (10, 11). One study that analyzed 55 cell lines of the Developmental Therapeutics Program of NCI, National Institutes of Health, showed that artesunate, the semi-synthetic derivative of artemisinin, has anti-cancer activities against leukemic, colon, melanoma, breast, ovarian, prostate, central nervous system, and renal cancer cell lines (12). More-

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Artemisinin Control of CDK4 Transcription

over, the highly stable artemisinin-derived trioxane dimmers was shown to inhibit the growth of and selectively kill several human cancer cell lines without inducing cytotoxic effects on normal neighboring cells (13). The molecular mechanism and gene expression changes that mediate the anti-proliferative activity of artemisinin are not well characterized.

Eukaryotic cell growth depends on the cooperative actions of a number of cellular proteins to form a series of regulated events that drive the cell cycle from one phase to the next. The cell cycle is composed of four phases: G_1 phase, S phase, involving DNA synthesis, G_2 phase, and mitosis, or M phase where the cell divides. Critical components of the cell cycle machinery are the cyclin-dependent kinases (CDKs),² their activating binding partners called cyclins, and a variety of cyclin-dependent kinase inhibitors (CKIs). CDKs bind to specific cyclin subunits to achieve the kinase activity necessary for the phosphorylation of substrates needed for the progression of the cell cycle, such as retinoblastoma (Rb) protein (14). In the G_1 phase of the cell cycle, unphosphorylated Rb binds to the E2F family of transcription factors preventing them from activating the genes necessary for progression through S phase (15). Early in the G_1 phase, CDK4 and CDK6, interacting with D-type cyclins, phosphorylate the Rb protein in an "initiation" step. In mid to late G_1 , CDK2 can then hyperphosphorylate the Rb protein by interacting with E-type cyclins. The hyperphosphorylation of Rb causes the release the E2F transcription factor allowing the cell to enter S phase and begin DNA replication (15). The correct timing and regulation of the cell cycle is mediated through CDK activity by the control of cyclin stability, subcellular localization of the components, CDK phosphorylation events, and association of the CDKs with CKIs (16). In this study, we examine the affects of artemisinin on the LNCaP (lymph node carcinoma of the prostate) cell cycle and we have discovered that artemisinin regulates expression of key G_1 acting CDKs through the selective control of Sp1 transcription factor-promoter interactions. The results implicate artemisinin as a potential chemotherapeutic compound for controlling the proliferation of human prostate carcinoma.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, fetal bovine serum, calcium-free and magnesium-free phosphate-buffered saline (PBS), trypsin-EDTA, and the antibiotics penicillin and streptomycin were supplied by BioWhittaker (Walkersville, MD). Artemisinin and okadaic acid were obtained from Sigma. The sources of other reagents used in the study are either listed below or were of the highest purity available.

Cell Culture—The LNCaP, PC3, and DU145 human prostate carcinoma cell lines were purchased from American Type Culture Collection (Manassas, VA). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 units/ml streptomycin,

and 30 mM HEPES and maintained at subconfluency at 37 °C in humidified air containing 5% CO_2 . Artemisinin was dissolved in DMSO (99.9% high-performance liquid chromatography grade, Aldrich) at concentrations that were 1000-fold higher than the final medium concentration. Okadaic acid was diluted in sterile nano-water. In all experiments, 1 μ l of the concentrated agent was added per 1 ml of medium, and for the vehicle control, 1 μ l of DMSO was added per 1 ml of medium.

[³H]Thymidine Incorporation—LNCaP cells were plated onto 24-well Nunc tissue culture dishes (Nunc A/S, Denmark). Triplicate samples of asynchronously growing LNCaP cells were treated for the indicated times with either vehicle control (1 μ l of DMSO per 1 ml of medium) or varying concentrations of artemisinin. The cells were pulsed for 2 h with 3 μ Ci of [³H]thymidine (84 Ci/mmol), washed three times with ice-cold 10% trichloroacetic acid, and lysed with 500 μ l of 0.3 N NaOH. Lysates (250 μ l) were transferred into vials containing 4 ml of liquid scintillation mixture, and radioactivity was quantitated by scintillation counting. Triplicates were averaged and expressed as counts per minute (cpm) per well.

LNCaP Xenografts— 2×10^6 cultured LNCaP prostate cancer cells were combined with Matrigel (BD Biosciences) at a 50:50 volume ratio of cells to Matrigel and injected subcutaneously into two sites of 6- to 8-week-old male athymic nude mice (BALB/c strain, Charles River Laboratory, Wilmington, MA). 1 week later, the mice were given artemisinin (100 mg/kg/day) or DMSO (1 μ l/ml water) in their drinking water, and tumors were palpated weekly. Volumes were calculated using the equation, $V = a \times b^2/2$, where a is the width and b is the length of the tumors.

Flow Cytometric Analyses of DNA Content—LNCaP, PC3, and DU145 cells (4×10^4) were plated onto Nunc six-well tissue culture dishes (Nunc A/S, Denmark). Triplicate samples were treated with the indicated concentrations of artemisinin. The medium was changed every 24 h. Cells were incubated for the indicated times and hypotonically lysed in 1 ml of DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate, and 0.05% Triton X-100). Lysates were filtered using 60- μ m Nitex flow mesh (Sefar America, Kansas City, MO) to remove cell membranes. Propidium iodide-stained nuclei were detected using a PL-2 detector with a 575 nm band pass filter on a Beckman-Coulter (Fullerton, CA) fluorescence-activated cell sorter analyzer with laser output adjusted to deliver 15 megawatts at 488 nm. Ten thousand nuclei were analyzed from each sample at a rate of ~ 600 nuclei per second. The percentages of cells within the G_1 , S, and 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 (San Diego, CA), in the Cancer Research Laboratory Microchemical Facility at the University of California at Berkeley.

Western Blot Analysis—After the indicated treatments, cells were harvested in radioimmune precipitation assay buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% NoNidet-p40 (Nonidet P-40, Fluta Biochemitra, Switzerland), 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (50 g/ml phenylmethylsulfonyl fluoride, 10 g/ml aprotinin, 5 g/ml leupeptin, 0.1 g/ml NaF, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 0.1 mM β -glycerol phosphate). Equal amounts of total

² The abbreviations used are: CDK, cyclin-dependent kinase; LNCaP, lymph node carcinoma of the prostate; Art, artemisinin; Rb, retinoblastoma protein; pRb, phosphorylation of retinoblastoma protein; ppRb, hyperphosphorylated form of Rb; Sp1, promoter specificity factor; OA, okadaic acid; CKI, cyclin-dependent kinase inhibitor; PBS, phosphate-buffered saline; CMV, cytomegalovirus.

cellular protein were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25 ml of 14.4 M β -mercaptoethanol, 10% bromophenol blue, 3.13% 0.5 M Tris-HCl, and 0.4% SDS (pH 6.8)) and fractionated on 10% (8% for Rb and 12% for p16, p18, p21, and p27) polyacrylamide/0.1% SDS resolving gels by electrophoresis. Rainbow marker (Amersham Biosciences) was used as the molecular weight standard. Proteins were electrically transferred to nitrocellulose membranes (Micron Separations, Inc., Westboro, MA) and blocked overnight at 4 °C with Western wash buffer-5% NFD (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20, 5% nonfat dry milk).

Protein blots were subsequently incubated for 1 h at room temperature. The antibodies used were as follows, rabbit anti-CDK2, CDK4, CDK6, p16, p21, cyclin D1 and Sp1, mouse anti-alpha tubulin, cyclin E, p18, and p27 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-cyclin-D2, cyclin-D3, and Rb (NeoMarkers, Fremont, CA); and rabbit anti-phospho serine (Sigma-Aldrich). The working concentration for all antibodies was 1 μ g/ml in Western wash buffer. Immunoreactive proteins were detected after incubation with horseradish peroxidase-conjugated secondary antibody diluted to 3×10^4 in Western wash buffer (goat anti-rabbit IgG and rabbit anti-mouse IgG (Bio-Rad)). Blots were treated with enhanced chemiluminescence reagents (PerkinElmer Life Sciences), and all proteins were detected by autoradiography. Equal protein loading was confirmed by Ponceau S staining of blotted membranes.

Reverse Transcription PCR—LNCaP cells were harvested in TRIzol reagent (Sigma), and the recommended protocol was followed to extract total RNA. RNA was quantified using spectroscopy, and the quality of RNA was confirmed using A_{260}/A_{280} and by electrophoresis on 1% agarose gels. 5 μ g of total RNA was subjected to reverse transcription using murine myelogenous leukemia reverse transcriptase with 5 \times First Strand Buffer, Random Primer (hexamers), dNTPs, and RNaseOUT (Invitrogen) according to the manufacturer's instructions. 2 μ l of cDNA was then subjected to PCR using Platinum Taq, 10 \times PCR buffer, and 200 μ M each dNTP (Invitrogen) along with the following primer sets and conditions. The primer sets for CDK2 were forward 5'-TGGATGTCATTCA-CACAGAA-3' and reverse 5'-CAGGGACTCCAAAAGCT-CTG-3', and the cycling conditions were 1 min at 95 °C followed by 1 min at 58 °C for annealing and finally 1 min at 72 °C for extension for 30 cycles, giving a 267-bp product. The primer sets for CDK4 were forward (5'-CTGAGAATGGCTACCTC-TCGATATG-3') and reverse (5'-AGAGTGTAACAACCAC-GGGTGTAAG-3'), and the cycling conditions were 1 min at 95 °C followed by 1 min at 58 °C for annealing and finally 1 min at 72 °C for extension for 30 cycles, giving a 541-bp product. The primer sets for CDK6 were forward (5'-CCGAGTAGTGC-ATCGCGATCTAA-3') and reverse (5'-CTTTGCCTAGTTC-ATCGATATC-3'), and the cycling conditions were 1 min at 95 °C followed by 1 min at 58 °C for annealing and finally 1 min at 72 °C for extension for 30 cycles, giving a 406-bp product. 18S primers (Ambion, Austin TX) served as a control, and PCR was performed according to the manufacturer's instructions. The PCR products were run on 1.5% agarose gels along with a 1-kb plus DNA ladder (Invitrogen).

Generation of Luciferase Reporter Constructs—The CDK2–2400 bp promoter fragment subcloned into the PGL2-basic luciferase expression vector was a kind gift of Dr. Gary S. Stein (Department of Cell Biology, University of Massachusetts Medical School). The –2120, –867, and –404-bp fragments, containing the CDK4 promoter and subcloned into PGL3-basic luciferase expression vectors (Promega, Madison, WI), were constructed by Dr. Jaime Modiano, University of Colorado. Using the –2120-bp promoter construct as a template, 5' deletion constructs were engineered with HindIII and XhoI restriction sites engineered into the PCR primers. Constructs were generated with +43 5'-CGCGAAGCTTCATGTGACCAGC-TGCCAAAGA-3' reverse primer and the following forward primers: –1063, 5'-CGCGCTCGAGTCATTCATTGTGGT-GGTCTGGAGG-3'; –1278, 5'-CGCGCTCGAGCAAAGGG-CTGGGAGATAAAGACCT-3'; –1506, 5'-CGCGCTCGAG-TCAGGATGGAAAGAGGAGGCATCT-3'; and –1737, 5'-CGCGCTCGAGAGGTCTTATGTGGAAGGTGAGGCT-3'. For PCR amplification, 100 μ l of PCRs (1 \times Vent ThermoPol PCR buffer (New England Biolabs, Ipswich, MA), 4 mM MgSO₄, 0.2 mM dNTPs, 1 unit of Vent ThermoPol polymerase, and 0.2 μ M each primer) were amplified for 30 cycles (95 °C, 30 s/56 °C, 30 s/72 °C, 2 min) with a 72 °C, 10 min extender and a 95 °C, 5-min hot start. PCR products were gel-purified using a QIAEXII Gel Extraction Kit (Qiagen, Valencia, CA), digested with HindIII and XhoI (New England Biolabs), and subcloned into PGL3-basic (Promega, Madison, WI).

To generate the transcription factor-binding site mutant fragments, putative binding sites were altered to EcoRI restriction enzyme sites by PCR mutagenesis from the –2120-bp luciferase construct. Each primer was designed using the Stratagene QuikChange[®] Primer Design Program and was a minimum of 51 bp (contact the author to obtain the primer sequences). For each construct, 100- μ l PCRs (1 \times Vent ThermoPol PCR buffer (New England Biolabs), 4 mM MgSO₄, 0.2 mM dNTPs, 1 unit of Vent ThermoPol polymerase, and 0.2 μ M each primer) were amplified for 18 cycles (95 °C, 1 min/56 °C, 1 min/68 °C, 10 min) with a 68 °C, 10-min extender and a 95 °C, 5-min hot start. To digest the parental strands, 1 μ l of DpnI (New England Biolabs) was added to each sample for 1 h at 37 °C, and 10 μ l was used for transformation. All sequences were confirmed by DNA sequencing.

Transfection and Luciferase Assay—LNCaP cells (4×10^4) were plated onto Nunc 6-well tissue culture dishes (Nunc A/S, Denmark). For luciferase reporter assays, transfections were performed by mixing 1 μ g of reporter plasmid with 4 μ l of Lipofectamine (Invitrogen) in 1 ml of plain Dulbecco's modified Eagle's medium. For constitutive expression of human Sp1, cells were transfected with 1 μ g of pCMV-Sp1, a kind gift from Dr. Robert Tijan (Dept. of Molecular and Cell Biology, University of California at Berkeley). After 6 h, 1 ml of 2 \times Dulbecco's modified Eagle's medium without antibiotics was added to each well (20% fetal bovine serum, 60 mM HEPES). After 24 h, the transfection media was replaced with normal growth media, and the LNCaP cells were treated with artemisinin for the indicated time periods. All transfections were done in triplicate. For luciferase assays, cells were harvested by rinsing with PBS and lysed in 500 μ l of 1 \times Passive Lysis Buffer (Promega) for 15 min with gentle rocking. 20 μ l of cell lysate was added to 8 \times 75-mm

Artemisinin Control of CDK4 Transcription

cuvettes (Promega) and subsequently loaded into a TD 20/20 Luminometer (Turner Biosystems, Sunnyvale, CA) after addition of 100 μl of Luciferase Assay Reagent II (Promega). Luminescence was measured in relative light units. The luciferase specific activity was expressed as an average of relative light units produced per μg of protein present in corresponding cell lysates as measured by the Bradford Assay (Bio-Rad). LNCaP cells were also transfected with 1 μg of pCMV-CDK2 or pCMV-CDK4 (kind gift of Dr. Leonard Bjeldanes, Dept. of Nutritional Sciences and Toxicology, University of California at Berkeley). 24 h post transfection, cells were treated with artemisinin for 48 h and subjected to flow cytometric analysis as described. Expression levels were verified by immunoblotting.

Affinity Chromatography for Sp1-CDK4 Promoter Binding—Biotinylated oligonucleotides containing consensus Sp1 binding sites, wild-type endogenous transcription factor binding sites, as well as the mutated binding sites were used for the chromatographic assay. LNCaP cells treated with artemisinin or with the DMSO vehicle control were lysed using a buffer containing 10 mM HEPES, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, and 2 mM EDTA, and a mixture of protease inhibitors without dithiothreitol. Protein content of cell lysates was then normalized using the Lowry method (Bio-Rad). Streptavidin-conjugated agarose beads (Sigma) were resuspended in 400 μl of lysis buffer, and the column was divided into two Eppendorf tubes with 200 μl each. Approximately 2 mg of total protein from DMSO- or artemisinin-treated cells was added to each column and incubated at 4 °C for 1 h with constant rocking using a Nutator (Clay Adams, Sparks, MD). The column was then centrifuged at 4 °C for 5 min at 5000 rpm. The unbound lysate was stored, and the columns were washed three times using lysis buffer. The elution buffer was prepared by adding 400 mM NaCl to the lysis buffer and added to the beads. After incubation at room temperature for 5 min, the beads were centrifuged, and the supernatant was loaded and subjected to SDS-PAGE. The amount of bound Sp1 protein was assayed by immunoblotting as described above in three independent experiments.

Chromatin Immunoprecipitation Assay—150-mm plates of LNCaP cells were incubated with formaldehyde at a concentration of 1% for 10 min on a shaking platform followed by glycine to a final concentration of 0.125 M for 5 min. The cells were washed twice with ice-cold PBS, collected in PBS containing in 5 mM EDTA, and then pelleted. The pellet was resuspended in 500 μl of cell lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate) containing protease inhibitors and incubated on ice for 10 min. The samples were centrifuged for 15 min at 10,000 rpm at 4 °C following sonication using a Branson Sonifier Cell Disruptor 200 (Branson Sonic Power Co., Danbury, CT). The supernatant was put in a new tube, and protein concentration was determined using the Lowry method (Bio-Rad Laboratories). 2 mg of protein was used for the immunoprecipitation and 1/10 of each sample was saved at -80 °C as "input." Samples were precleared for 1 h at 4 °C with 40 μl of a 1:1 slurry of protein G-Sepharose beads (GE Health BioSciences AB, Sweden). After a brief centrifugation to remove precleared beads, 0.5 μg of mouse anti-Sp1 (Santa Cruz Biotechnology) antibody was added to each sample and incu-

bated on a rocking platform at 4 °C overnight. Then, 40 μl of protein G-Sepharose beads were added to each sample, and the slurries were incubated on the rocking platform at 4 °C for 2 h. The samples were centrifuged briefly, and the resulting pellet was washed three times with cell lysis buffer and twice wash buffer (10 mM Tris, pH 8.0, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 1 mM EDTA). 150 μl of elution buffer (50 mM Tris, pH 8.0, 1% SDS, 10 mM EDTA) was added to the samples, as well as the "input" samples. All samples were incubated at 67 °C for 10 min. The immunoprecipitation samples were centrifuged briefly, and the elution process was repeated and the supernatants were pooled. To the pooled eluted material and the input sample, NaCl was added to a final concentration of 0.3 M, and the samples were incubated at 67 °C overnight. The DNA was purified using a PCR purification kit (Qiagen). For PCR, 1 μl of isolated chromatin was used, and the cycling conditions were 2 min at 95 °C followed by 1 min at 58 °C for annealing and finally 1 min at 72 °C for extension for 30 cycles. The Sp1 primer set used was forward (5'-TCCAGAAGGCTTACAAAGCACCCA-3') and reverse (5'-ACCCTATTTGCCACTCCTTCCACT-3') and gave a 296-bp product.

Immunoprecipitations—LNCaP cells were cultured in 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 and phosphatase inhibitors (50 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin, 0.1 $\mu\text{g}/\text{ml}$ NaF, 10 $\mu\text{g}/\text{ml}$ β -glycerophosphate, and 0.1 mM sodium orthovanadate). Samples were diluted to 1 mg of protein in 1 ml of IP buffer. Samples were precleared for 30 min at 4 °C with 40 μl of a 1:1 slurry of protein G-Sepharose beads (GE Health BioSciences AB). After a brief centrifugation to remove precleared beads, 0.5 μg of mouse anti-Sp1 (Santa Cruz Biotechnology) antibody was added to each sample and incubated on a rocking platform at 4 °C overnight. Then, 40 μl of protein G-Sepharose beads were added to each sample, and the slurries were incubated on the rocking platform at 4 °C for 45 min. 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 phospho-serine levels were examined by Western blot using the second half of the samples.

RESULTS

Artemisinin Inhibits Proliferation of LNCaP Cells in Vitro and in Vivo—The effects of artemisinin on the proliferation of human LNCaP prostate cancer cells was initially examined by treating the cells with increasing concentrations of artemisinin for 48 h, and DNA synthesis determined by pulse labeling the cells with [³H]thymidine for the last 2 h of phytochemical treatment. As shown in Fig. 1A, artemisinin strongly inhibited DNA synthesis with the half-maximal inhibition observed at a concentration of 25 μM compared with the DMSO vehicle control, whereas, maximal growth inhibition without adversely affecting cell viability occurred at 300 μM artemisinin. Treatment with concentration of artemisinin at 400 μM or greater had a

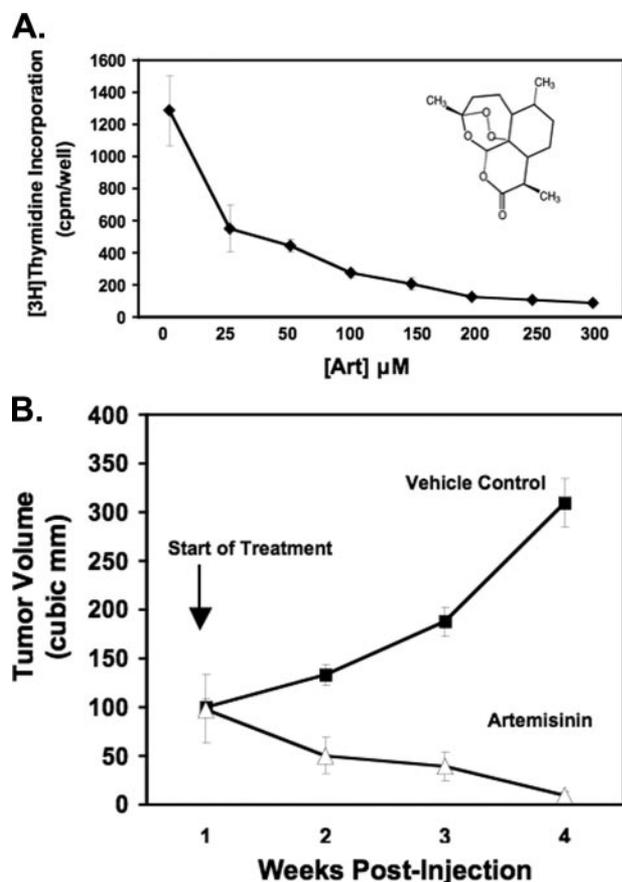


FIGURE 1. Artemisinin inhibits the growth of LNCaP cell *in vitro* and *in vivo*. *A*, LNCaP prostate cancer cells were treated with the indicated concentrations of artemisinin for 48 h and pulse labeled with [³H]thymidine for the last 2 h of treatment. [³H]thymidine incorporation into DNA was measured by acid precipitation followed by scintillation counting. Data are the mean of triplicate experiments, and error bars are standard deviation. *B*, LNCaP xenografts were transplanted into male BALB/c nude mice. One week later, mice were given DMSO vehicle control or 100 mg/kg/day artemisinin, and tumors were palpated weekly. Volume of tumors was calculated as described under "Experimental Procedures." Reported values are mean volume per tumor, and error bars are standard deviation.

cytotoxic effect on these cells (data not shown). Therefore, in the subsequent experiments artemisinin was used at a concentration of 300 μM. Time course studies showed that the maximal growth arrest was achieved within 48 h of artemisinin treatment (data not shown). To determine the effects of artemisinin on human prostate cancer cells *in vivo*, 2 × 10⁶ cultured LNCaP cells were combined with Matrigel (50:50) and injected subcutaneously into both the left and right flanks of male BALB/c athymic nude mice. After 1 week, the mice were given artemisinin (100 mg/kg/day) or DMSO (1 μl/ml water) in their drinking water, and tumors were palpated weekly. As shown in Fig. 1*B*, artemisinin not only inhibited the growth of LNCaP xenografts but also reduced the size of many of the tumors. Also, the vehicle control-treated tumors displayed significant gross vascularity (bright red in color), whereas, in contrast, in artemisinin-treated mice the xenografted tumors displayed no gross vascularity (tumors were pale yellow) that resembled avascular tissue such as optical sclera (data not shown). Throughout the study, mice treated with artemisinin did not display any signs of adverse side effects in that there were no alterations in chow consumption, water drinking rates, movement in the cage, skin

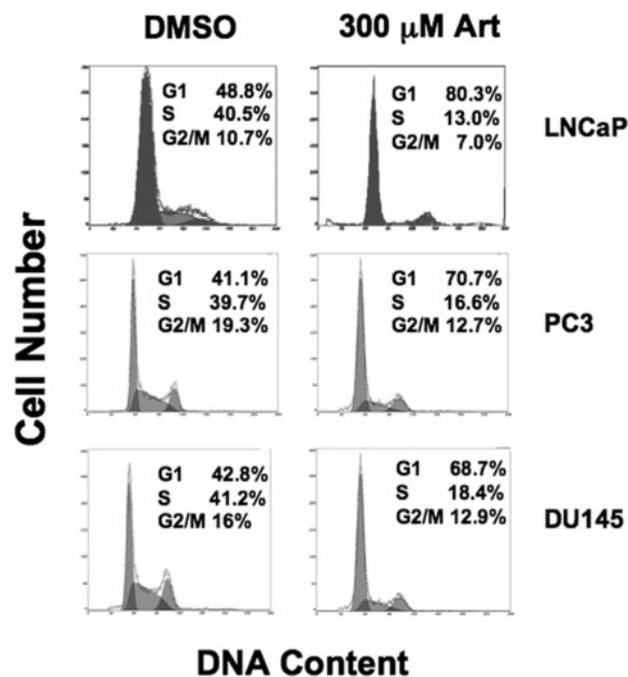


FIGURE 2. Artemisinin induces a G₁ cell cycle arrest in LNCaP, PC3, and DU145 cells. All prostate cancer cells were plated in 6-well culture plates and treated for 48 h with vehicle control or 300 μM artemisinin. Cells were harvested and hypotonically lysed in a propidium iodide solution to stain the DNA. Nuclei were analyzed for DNA content by flow cytometry.

discoloration, or weight gain compared with DMSO-treated mice. Taken together, our results suggest that artemisinin has a potent anti-proliferative effect on human prostate cancer cells both *in vitro* and *in vivo*.

Artemisinin Inhibits Proliferation of LNCaP, PC3, and DU145 Prostate Cancer Cells by Inducing a G₁ Cell Cycle Arrest— To initially examine the cell cycle effects of artemisinin in human prostate cancer cells, androgen-responsive LNCaP cells, as well as androgen-unresponsive PC3 and DU145 cells, were treated with or without 300 μM artemisinin for 48 h and nuclear DNA stained with propidium iodide and quantified by flow cytometry. As shown in Fig. 2, the vehicle control cells grow as an asynchronous population represented by cells in all stages of the cell cycle. In all three cell lines, artemisinin induced a marked increase in the percentage of cells in the G₁ phase with a corresponding decrease in S phase cells. These data demonstrate that the anti-proliferative effect of artemisinin is a general property of this phytochemical in human prostate cancer cells in that both androgen-responsive and non-responsive cell lines similarly respond to this phytochemical by undergoing a G₁ cell cycle arrest. The differences in the proportion of artemisinin treated cells in G₁ phase is due to intrinsic differences between the three cell lines employed. Furthermore, the loss in the percentage of cells in S phase is consistent with the [³H]thymidine incorporation results described in Fig. 1 for LNCaP cells. The percentage of artemisinin-treated cells in the G₂ phase of the cell cycle remained relatively equal to DMSO vehicle control-treated cells. Given the similarities in efficiency of the overall G₁ cell cycle arrest of the three tested prostate cancer cell lines, the following experimental strategies employ exclusively the LNCaP cells to allow a more in depth analysis of the anti-proliferative mechanism of artemisinin.

Artemisinin Control of CDK4 Transcription

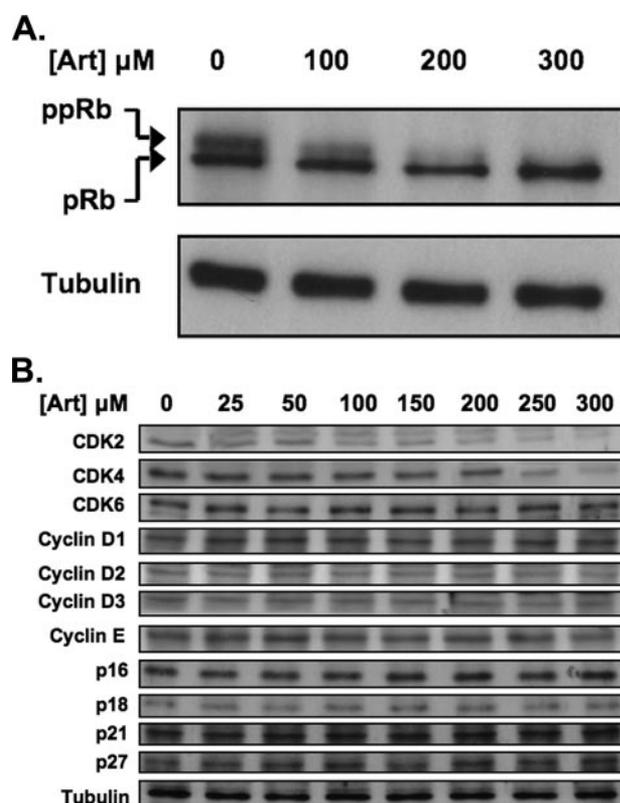


FIGURE 3. Western blot analysis of artemisinin effects on hyperphosphorylation of Rb and on expression of G_1 cell cycle components. *A*, LNCaP prostate cancer cells were treated with the indicated concentrations of artemisinin for 48 h. Total cell lysates were electrophoretically fractionated by SDS-PAGE and Rb was analyzed by Western blot using an antibody that recognizes both hypo- and hyperphosphorylated Rb. *B*, LNCaP prostate cancer cells were treated with the indicated concentrations of artemisinin for 48 h. Total cell lysates were electrophoretically fractionated by SDS-PAGE and expression of cell cycle proteins analyzed by Western blots. α -Tubulin was used as loading control as well as Ponceau S staining.

Effects of Artemisinin on Production of Phosphorylated Rb and on Expression of G_1 -acting Cell Cycle Components—The G_1 phase of the cell cycle is largely controlled by the phosphorylation status of retinoblastoma protein (pRb), which is complexed with E2F family of transcription factors. Hyperphosphorylation of Rb causes a release of E2F transcription factors allowing them to induce expression of genes necessary for the progression of the cells through G_1 and into S phase (15). To determine whether the artemisinin-induced G_1 cell cycle arrest is associated with a change in phosphorylation status of Rb, LNCaP cells were treated with increasing doses of artemisinin for 48 h, and cell extracts were analyzed by immunoblotting for total Rb and for phosphorylated Rb. As shown in Fig. 3*A*, artemisinin treatment had no effect on the expression levels of Rb, however it caused the loss of the hyperphosphorylated form of Rb (ppRb) to a state in which the predominant form is the hypophosphorylated Rb (pRb). This result further establishes that artemisinin mediates its growth arrest of LNCaP prostate cancer cells by inducing G_1 block in cell cycle progression.

Because Rb phosphorylation is tightly regulated by the activities of the major G_1 -acting cell cycle components, the expression levels of these components were analyzed in LNCaP cells were treated with the indicated concentrations of artemisinin up to 300 μ M for 48 h. Immunoblotting of electrophoretically

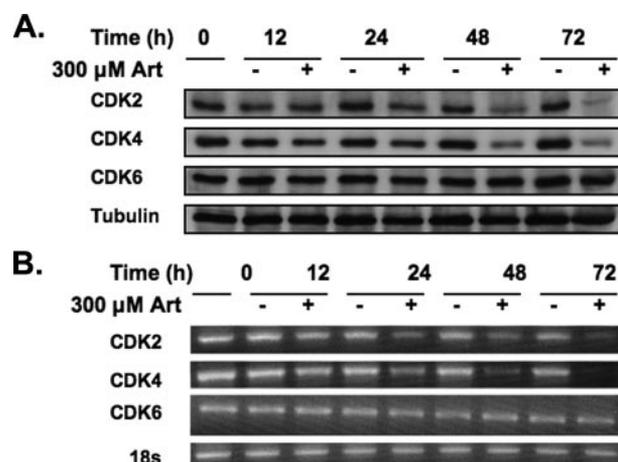


FIGURE 4. Artemisinin down-regulates expression of protein and transcript of both CDK2 and CDK4. LNCaP prostate cancer cells were treated with either DMSO vehicle control or 300 μ M artemisinin for the indicated times. Cells were harvested in two equal portions. *A*, total cell lysates of the first half of the cells were electrophoretically fractionated by SDS-PAGE and analyzed by Western blot. Blot of α -tubulin was used as loading control as well as Ponceau S staining. *B*, RNA was isolated from the second half of the cells and subjected to reverse transcription-PCR as described under "Experimental Procedures." 18s was used as loading control as well as agarose gel electrophoresis of isolated RNA.

fractionated cell extracts revealed that artemisinin treatment strongly down-regulated production of both cyclin-dependent kinase-2 (CDK2) and cyclin-dependent kinase-4 (CDK4), with no significant change in the expression of cyclin-dependent kinase-6 (CDK6) (Fig. 3*B*). The G_1 -acting CDKs function in distinct protein complexes that include the activating cyclins and CDK inhibitors (16). Artemisinin treatment had no effect on expression of cyclin D1, D2, D3, and E, or on the CDK inhibitors p16, p18, p21, or p27 (Fig. 3*B*). During cell cycle progression, the Rb protein is phosphorylated early in G_1 by CDK4 and CDK6 to induce an allosteric change that allows access of CDK2 to Rb for formation of the hyperphosphorylated Rb and subsequent release of the E2F transcription factors (15). Thus, the selective artemisinin-induced loss of CDK4 and CDK2 production can account for the G_1 cell cycle arrest mediated by this phytochemical.

Artemisinin Down-regulates CDK2 and CDK4 Transcript Levels and Inhibits Promoter Activity—To uncover the cellular processes regulated by artemisinin that leads to the down-regulation of CDK2 and CDK4 production, LNCaP cells were cultured in the presence or absence of 300 μ M artemisinin, and the levels of CDK protein and transcripts were compared over a 72-h time course. Immunoblotting (Fig. 4*A*) and reverse transcription-PCR (Fig. 4*B*) analyses revealed that artemisinin treatment rapidly down-regulated expression of CDK2 and CDK4 protein and transcripts by 24 h. Maximal down-regulation was observed by 48-h exposure to artemisinin and maintained through 72 h. The down-regulation of CDK2 and CDK4 transcripts accounts for the loss of the corresponding protein levels. Artemisinin did not alter the stability of the CDK2 or CDK4 transcripts (data not shown), suggesting that artemisinin regulates promoter activity of CDK2 and CDK4. This possibility was directly tested in LNCaP cells transfected with either a 2400-bp CDK2-luciferase reporter construct or a 2120-bp CDK4-luciferase reporter construct. The cells were treated

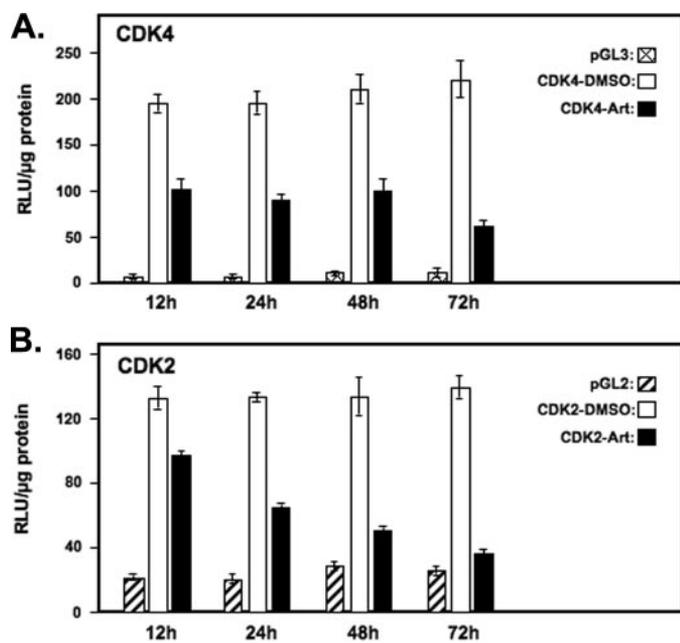


FIGURE 5. Artemisinin abrogates the activities of both CDK2 and CDK4 promoters. LNCaP prostate cancer cells were transfected with either *A*, –2120-bp CDK4 promoter cloned in PGL3-basic luciferase reporter vector or *B*, –2400-bp CDK2 promoter cloned in PGL2-basic luciferase reporter vector. Cells were treated with DMSO vehicle control or 300 μM artemisinin for the indicated times. Cells were harvested and relative light units (RLU) were measured as described under “Experimental Procedures” and normalized to protein concentration of the same sample. Data are the mean of triplicate experiments, and error bars are standard deviation.

with or without 300 μM artemisinin, and over a 48-h time course luciferase reporter activity was assayed. Treatment with 300 μM artemisinin significantly down-regulated the promoter activities of both CDK4 and CDK2 by 12 h (Fig. 5, *A* and *B*, respectively). These results suggest that artemisinin alters the function of specific transcriptional regulators that directly or indirectly regulate expression of these critical CDK genes. The following experiments will focus on artemisinin regulation of CDK4 expression, because CDK4 activity occurs primarily in early stages of the G_1 phase of the cell cycle and CDK4 is expressed at a higher level than CDK2 in LNCaP cells.

Identification of an Artemisinin-responsive Region in the CDK4 Promoter—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 reporter plasmid containing CDK4 promoter constructs was transiently transfected into LNCaP cells, the cells were treated with or without artemisinin for 24 h, and cellular luciferase activity was assayed to quantify CDK4 promoter activity. As shown in Fig. 6, in addition to the 2120-bp CDK4 promoter fragment shown in the previous figure, artemisinin strongly down-regulated the activity of the –1737-bp CDK4 promoter deletion construct. In contrast, artemisinin treatment has no effect on level of reporter plasmid activity driven by the –404, –867, –1063, –1278, and –1506-bp CDK4 promoter deletion constructs. These results suggest there is a 231-bp region of the CDK4 promoter between –1506 bp and –1737 bp that confers artemisinin responsiveness.

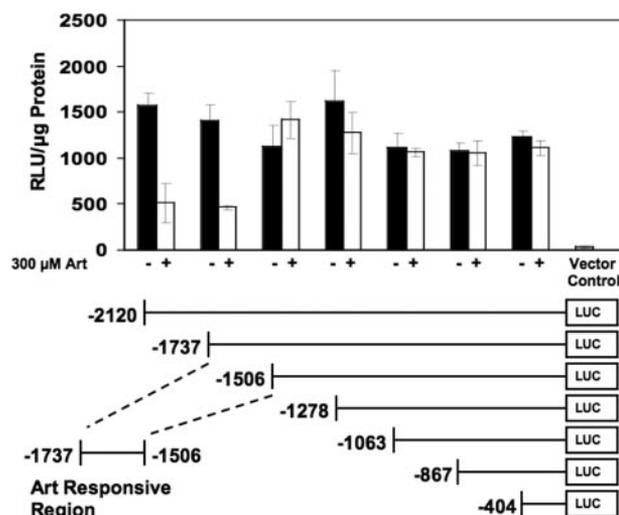


FIGURE 6. Identification of the artemisinin-responsive region of the CDK4 promoter. LNCaP prostate cancer cells were transfected with a series of CDK4-luciferase reporter plasmids that contain the indicated 5' deletions of the CDK4 promoter. Cells were treated with DMSO vehicle control or 300 μM artemisinin for 48 h. Cells were harvested and relative light units (RLU) were measured according to “Experimental Procedures” and normalized to protein concentration of the same sample. Data are the mean of triplicate experiments, and error bars are standard deviation.

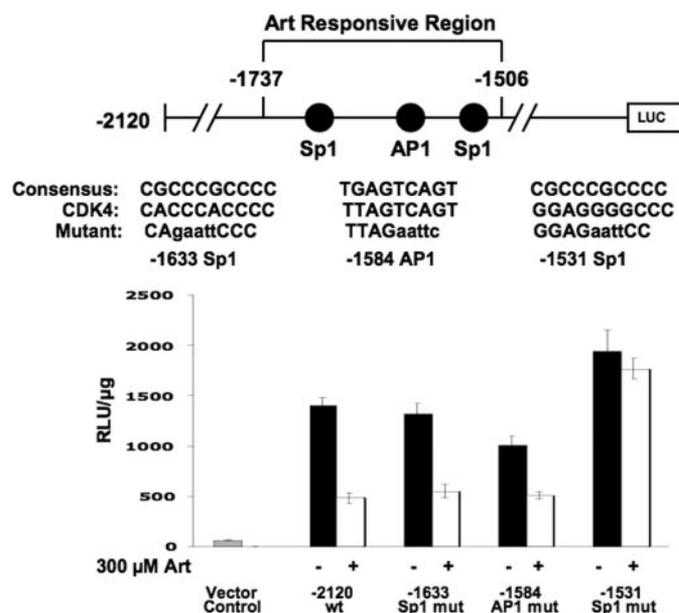


FIGURE 7. Site-directed mutagenesis of AP-1 and Sp1 DNA-binding sites within the artemisinin-responsive region of the CDK4 promoter. LNCaP prostate cancer cells were transfected a series of CDK4-luciferase reporter plasmids that contain the indicated transcription factor binding site mutations. In these –2120-bp CDK4 promoter-luciferase reporter plasmids, AP-1- and Sp1-like DNA-binding sites were mutated by PCR to EcoRI restriction endonuclease sites. Cells were treated with or without 300 μM artemisinin for 48 h. Cells were harvested, and relative light units (RLU) were measured as described under “Experimental Procedures” and normalized to protein concentration of the same sample. Data are the mean of triplicate experiments, and error bars are standard deviation.

Mutation of the –1531 Sp1 Binding Site within the Artemisinin-responsive Region of the CDK4 Promoter Ablates Artemisinin Down-regulation of Promoter Activity—As shown in Fig. 7, sequence analysis of the 231-bp artemisinin responsive region by the Alibaba 2.1 transcription factor-binding site computer program revealed two putative Sp1 transcription factor binding

Artemisinin Control of CDK4 Transcription

sites (at -1633 and -1531) as well as an AP1 transcription factor binding site (at -1584 bp). To determine if one or more of these sites plays a role in conferring artemisinin responsiveness to the CDK4 promoter, each of the three DNA elements was mutated to an EcoR1 restriction enzyme site (GAATTC) within the -2120 -bp CDK4 promoter-luciferase reporter vector (see *diagram* in Fig. 7). Based on known DNA binding specificities (17), substitution of the wild-type DNA binding sites with the GAATTC sequence will disrupt transcription factor interactions with their corresponding sites. The three mutant and wild-type -2120 -bp promoter luciferase reporter vectors were transfected into LNCaP cells and assayed for artemisinin responsiveness. As shown in Fig. 7, mutation of the -1531 -bp Sp1 DNA element completely prevented the artemisinin down-regulation of CDK4 promoter activity. In contrast, mutation of either the -1611 -bp Sp1 site or the -1584 bp AP1 had no effect on artemisinin responsiveness. These results demonstrate that the -1531 -bp Sp1 binding site plays a functional role in the artemisinin signaling pathway that leads to the down-regulation of CDK4 transcript levels in LNCaP prostate cancer cells.

Artemisinin Disrupts Sp1 Binding to the CDK4 Promoter—An *in vitro* DNA binding assay was employed to determine whether artemisinin regulates Sp1 transcription factor interactions with the CDK4 promoter. Three sets of biotinylated oligonucleotides were designed that correspond to the wild-type -1531 -bp Sp1 binding site from the CDK4 promoter, a mutated -1531 -bp Sp1 site containing the EcoR1 restriction site, and a consensus Sp1 binding site. These oligonucleotides were conjugated to streptavidin-agarose beads and incubated with cell lysates isolated from artemisinin-treated or vehicle control-treated LNCaP cells. The bound proteins were eluted with a high salt buffer, electrophoretically fractionated and analyzed by immunoblot using Sp1 specific antibodies. As shown in Fig. 8A, artemisinin treatment significantly reduced the level of Sp1 that binds to the wild-type oligonucleotides corresponding to the -1531 -bp Sp1 site in the CDK4 promoter, but had no effect on Sp1 binding to the consensus Sp1 DNA site. No binding to the oligonucleotide with the mutant Sp1 site was observed. These results suggest that artemisinin down-regulates the binding of Sp1 to the CDK4 promoter in a context-specific manner, given that artemisinin had no effect on the binding of Sp1 to the consensus Sp1 sequence (Fig. 8A). Chromatin immunoprecipitation was used to confirm the *in vitro* DNA binding results to determine the endogenous interactions of Sp1 with the CDK4 promoter. LNCaP cells were treated for 48 h with or without artemisinin and formaldehyde cross-linked. After sonication to shear the chromatin and immunoprecipitation of Sp1, the cross-link was reversed, and DNA was isolated and analyzed by PCR using primers specific to the artemisinin-responsive -1531 -bp Sp1 site in the CDK4 promoter. As shown in Fig. 8B, artemisinin significantly reduced Sp1 interactions with the endogenous CDK4 promoter. This results show that the artemisinin down-regulation of CDK4 gene expression can be accounted for by the selective loss of Sp1 transcription factor interactions with the -1531 -bp Sp1 site within the CDK4 promoter.

Artemisinin Down-regulation of Sp1 Phosphorylation—Several studies have shown that activity and DNA binding of the

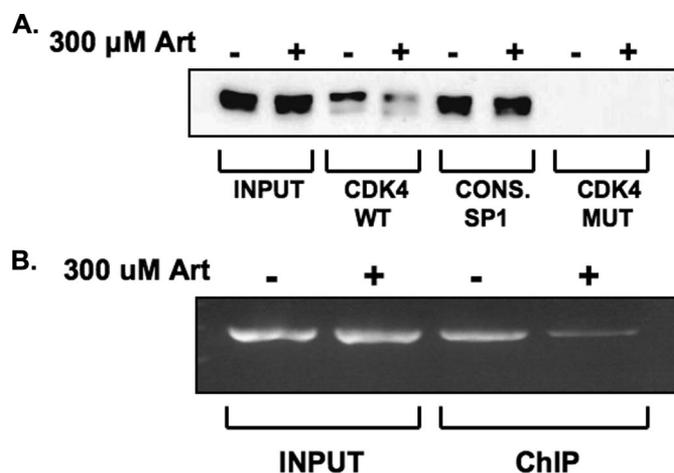


FIGURE 8. Artemisinin inhibits SP1 binding to the artemisinin-responsive region of the CDK4 promoter. LNCaP prostate cancer cells were treated with vehicle control DMSO or $300 \mu\text{M}$ artemisinin. **A**, whole cell extracts were subjected to affinity column chromatography using biotinylated primers conjugated to streptavidin-agarose beads. Primers contained either consensus (CONS) Sp1 sequences, the wild type (WT) artemisinin-responsive region, or the mutant (MUT) artemisinin-responsive region. After elution, input sample (as loading control) and eluates were subjected to immunoblot analysis for Sp1. **B**, before harvesting, LNCaP cells were formaldehyde cross-linked. Following sonication, samples were normalized and subjected to immunoprecipitation (IP) with anti-Sp1. Cross-linking was reversed, and DNA was isolated from the samples and subjected to PCR as described under "Experimental Procedures." Using input as loading control, samples were resolved on 1% agarose gels.

Sp1 transcription factor can be regulated by phosphorylation of certain serine residues (18). To determine if artemisinin has an effect on the serine phosphorylation of Sp1, this transcription factor was immunoprecipitated from artemisinin-treated and untreated LNCaP cells, and the immunoprecipitating protein was electrophoretically fractionated and analyzed by immunoblot using phospho-serine specific antibodies. As shown in Fig. 9A, artemisinin significantly reduced the level of phosphorylated Sp1 serine residues. This reduction was reversed by the addition of okadaic acid (Fig. 9A), a compound known to inhibit serine/threonine-specific protein phosphatase types 1 and 2A enhancing the Sp1 phosphorylation state in other systems (19). The ratio of phosphorylated Sp1 to total Sp1 protein was quantified after densitometric analysis of the Western blot protein bands, and, as also shown in Fig. 9A (*lower panel*), artemisinin reduced the level of serine-phosphorylated Sp1 compared with total Sp1 protein, and this response was reversed in the presence of okadaic acid. These results suggest that the artemisinin inhibition of phosphorylated Sp1 results in reduced protein-DNA interactions that lead to the selective loss of CDK4 gene expression.

One prediction of the observed artemisinin down-regulation of Sp1 phosphorylation is that the reversal of this effect should prevent or attenuate the ability of artemisinin to down-regulate CDK4 gene expression and promoter activity. As a functional test of this notion, LNCaP cells were transfected with the -2120 CDK4 promoter luciferase reporter plasmid and then treated for 48 h with or without combinations of $300 \mu\text{M}$ artemisinin and/or 10 nM okadaic acid. As shown in Fig. 9B (*top panel*), okadaic acid treatment abolished the artemisinin down-regulation of CDK4 promoter activity.

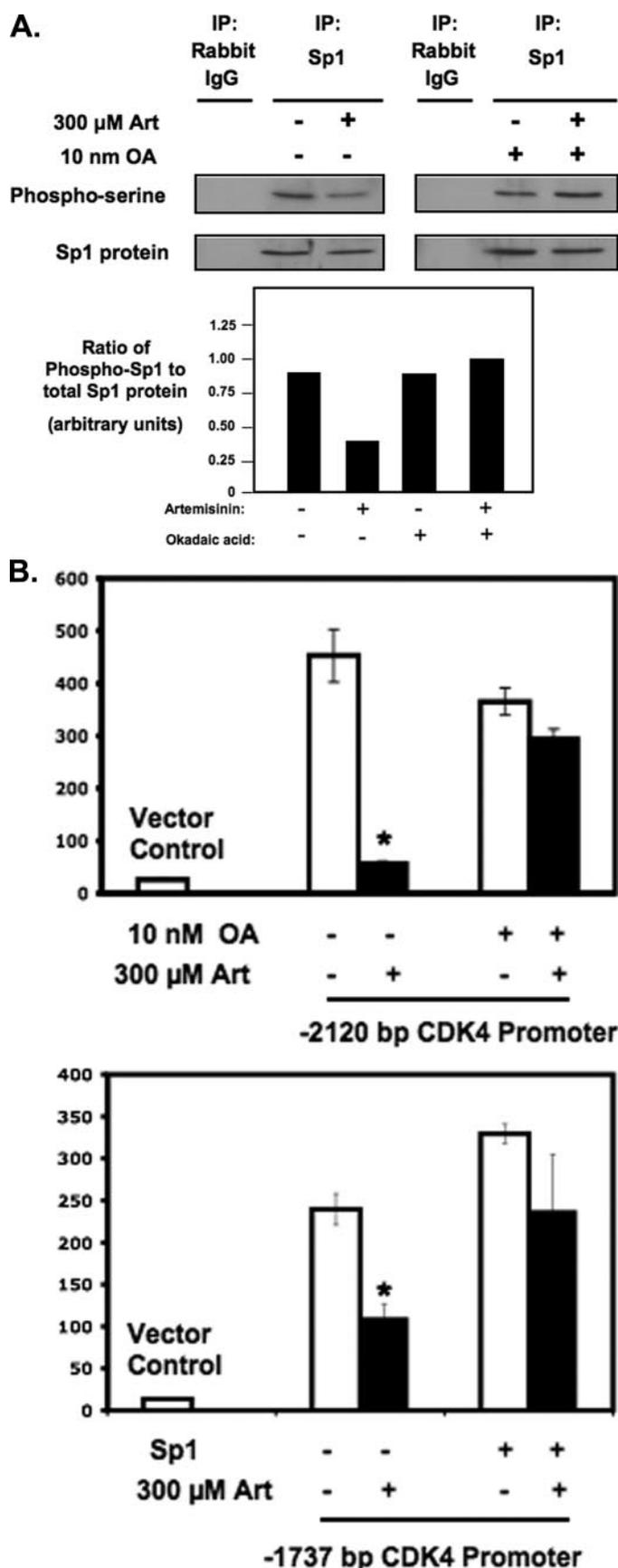


FIGURE 9. Effects of artemisinin on Sp1 phosphorylation and CDK4 promoter activity. A, LNCaP prostate cancer cells were treated with DMSO or 300 μ M artemisinin in the presence or absence of 10 nM okadaic acid (OA) for 48 h.

The role of Sp1 in mediating the artemisinin down-regulation of CDK4 expression was further characterized by overexpression of wild-type Sp1, which at high enough levels should overcome the reduced activity of the nonphosphorylated transcription factor. LNCaP cells were co-transfected with the -1737 CDK4 promoter luciferase reporter plasmid in the presence of either the pCMV-Sp1 constitutive expression vector or the pCMV-neo control vector. The -1737 CDK4 promoter fragment was used for this experiment, because it contains the artemisinin-sensitive Sp1 site, but eliminates seven other Sp1 sites that exist between in the region spanning -2120 to -1737 . Luciferase activity was monitored in LNCaP cells treated for 24 h with or without 300 μ M artemisinin. As also shown in Fig. 9B (bottom panel), artemisinin down-regulation of CDK4 promoter activity was reversed by elevated expression of the Sp1 transcription. Taken together, these results demonstrate that the artemisinin down-regulation of Sp1 phosphorylation and activity mediates the subsequent effects on CDK4 gene expression.

Expression Levels of Exogenous Sp1 Partially Reverses the Artemisinin-mediated G₁ Cell Cycle Arrest of LNCaP Cells—To functionally test whether the artemisinin-mediated cell cycle arrest could be reversed by elevated levels of expressed CDK2, CDK4, or Sp1, LNCaP cells were transfected with the constitutive cytomegalovirus promoter (pCMV)-driven expression plasmids containing cDNA of CDK2, CDK4, or Sp1, as well as with the empty expression vector control (neo). Cells were treated with artemisinin for 48 h and subjected to flow cytometry. As shown in Fig. 10, expression of CDK2 and CDK4 did not alter the artemisinin-induced G₁ cell cycle arrest. However, ectopic expression of Sp1 caused a partial reversal of artemisinin-induced cell cycle arrest in that the resulting artemisinin-treated cell population had a lower level of cells with a G₁ DNA content and a higher level of cells with a S1 DNA content compared with the pGL3 control transfected cells (neo). Taken together, these data confirm that artemisinin-mediated decrease in Sp1 function is partly responsible for the observed cell cycle arrest in LNCaP cells.

DISCUSSION

The lack of effective long term treatments for prostate cancer highlights the necessity to identify new potent anti-cancer compounds. Naturally occurring plant compounds represent a possible source of molecules that may have anti-proliferative effects on a variety of cancers. Previous work has suggested that

Cells were harvested and Sp1 was immunoprecipitated from whole cell lysates and phospho-serine was detected by Western blot according to "Experimental Procedures." B, LNCaP cells were transfected with the -2120 -bp CDK4 promoter-luciferase reporter plasmid, then treated with DMSO or 300 μ M artemisinin in the presence or absence of 10 nM okadaic acid (OA) for 48 h. Cells were harvested and relative light units (RLU) were measured as described under "Experimental Procedures" and normalized to protein concentration of the same sample. LNCaP cells were also co-transfected with the artemisinin-responsive -1737 -bp CDK4 promoter luciferase reporter plasmid with either the pCMV-Sp1 constitutive expression plasmid for human Sp1 or with the pCMV-neo control vector. Luciferase activity was assayed in cells treated with or without 300 μ M artemisinin for 24 h. Data are the mean of triplicate experiments, and error bars are standard deviation ($p < 0.01$), the asterisk denotes a significant difference between artemisinin-treated and untreated samples.

Artemisinin Control of CDK4 Transcription

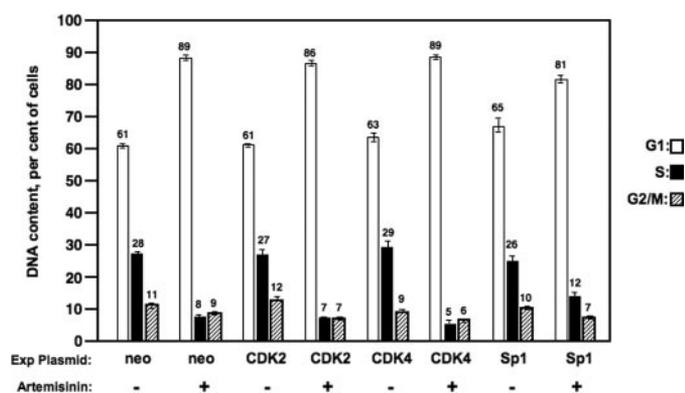


FIGURE 10. Sp1 overexpression partially reverses artemisinin-induced G₁ cell cycle arrest. LNCaP prostate cancer cells were transfected with pCMV constitutive expression vectors for CDK2, CDK4, and Sp1 as well as with the pCMV-neo empty vector control (*neo*). Western blots demonstrated the overexpression of these genes in the appropriate cells (data not shown). Transfected cells were treated with or without 300 μ M artemisinin 48 h, subjected to propidium iodide staining and subsequent flow cytometric analysis as described under "Experimental Procedures." Experiments were performed in triplicate per treatment. The bar graph with S.E. represents results from this analysis.

artemisinin and several of its derivatives inhibit the growth of various cancer cell types (12), although the mechanism of this effect was never investigated. Here we demonstrate that artemisinin inhibits the growth of LNCaP cells both *in vitro* and *in vivo*, and that this anti-proliferative response is due to a G₁ cell cycle arrest. It is interesting to note that artemisinin caused a dose- and time-dependent G₁ cell cycle arrest in androgen-responsive LNCaP as well as in androgen-unresponsive PC3 and DU145 cells in culture. Therefore, the anti-proliferative effects of artemisinin represent a general property in distinct types of human prostate cancer cells, and not a cell line-specific effect.

In most normal and tumorigenic mammalian systems, cell proliferation is highly regulated in the G₁ phase of the cell cycle, because cells that progress out of G₁ into S phase automatically progress through subsequent stages of the cell cycle (20). The G₁ phase of the cell cycle can be regulated by the interactions of cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors. Most anti-cancer agents that induce a G₁ arrest in cancer cells typically do so through decreased enzymatic activity of the G₁-acting CDKs through the increased expression of cyclin-dependent kinase inhibitors, decreased expression of cyclins and expression of different isoforms of cyclins that alter CDK activity (21–23). In contrast, we observed that artemisinin down-regulates CDK2 and CDK4 protein and transcript expression in LNCaP cells by inhibiting their respective promoter activities. More detailed transcriptional analysis of the CDK4 promoter revealed that this transcriptional response was mediated by a selective loss in Sp1 transcription factor interactions with the promoter.

Sp1 is a ubiquitously expressed transcription factor that regulates expression of a large number of mammalian genes by binding to specific GC-rich promoter elements. Sp1 is one member of a multigene family, including Sp1, Sp2, Sp3, and Sp4. Here we show that the artemisinin-induced changes in expression of CDK4 in LNCaP cells are due to inhibition of Sp1 interactions with a specific Sp1 DNA site in the CDK4 pro-

motor by reduced serine phosphorylation of Sp1. Addition of okadaic acid, which inhibits phosphatase activity, prevented the artemisinin-mediated down-regulation of Sp1 phosphorylation and reversed the artemisinin inhibition of CDK4 promoter activity. Okadaic acid had no effect on the artemisinin-induced G₁ arrest of LNCaP cells, which further suggests that the growth inhibitory effects of artemisinin on LNCaP cells may be due to the effect of artemisinin on multiple pathways. Our results implicate Sp1 being involved in controlling prostate cancer cell growth, because ectopic expression of Sp1 partially reversed the artemisinin-mediated cell cycle arrest. Sp1 activation can be influenced by several distinct cell signaling pathways (24), including epidermal growth factor receptor and CDK2 signaling. We are currently attempting to characterize the cellular proliferative pathways that involve Sp1 and are under artemisinin control.

We propose that Sp1 may be one of the key targets of phytochemical regulation of growth of human cancers. 3,3'-Diindolylmethane and indole-3-carbinol both induce a G₁ arrest in MCF-7 breast cancer cells due in part to Sp1-mediated increase in p21^{Waf1/Cip1} expression and Sp1-mediated decrease in CDK6 expression, respectively (21, 25). Interestingly, the CDK2 promoter contains a number of Sp1 sites, and we are currently examining whether the artemisinin down-regulation of CDK2 in LNCaP cells is due to reduced interactions of Sp1 with the CDK2 promoter analogous to the effects of artemisinin on CDK4 promoter activity.

Artemisinin signaling pathways inhibit prostate cancer cell growth in part by targeting the transcription of CDK4 and CDK2 thereby induced a G₁ block in cell cycle progression. Angiogenesis inhibitors have been shown to retard tumor growth and vascularization of prostate cancer cells *in vivo*, and artemisinin and its derivatives show similar anti-angiogenic effects (10, 11, 26). Our data corroborate these findings in that LNCaP xenografts excised from BALB/c mice treated with artemisinin showed little to no vascularization and appeared bloodless, whereas xenografts taken from the control mice were much larger and highly vascularized and sanguinary (data not shown). This result suggests that artemisinin may affect multiple pathways associated with tumor proliferation *in vivo* in addition to the control of cell cycle progression.

The anti-malarial effects of artemisinin are achieved by a two-step mechanism. First, intraparasitic heme-iron catalyzes cleavage of the endoperoxide bridge. Breakage of the endoperoxide bridge then leads to formation of free hydroxyl radicals that cause extensive damage eliminating the parasites (27). Iron is an essential micronutrient for cell growth that plays an important role in energy metabolism and DNA synthesis, and iron levels are much higher in cancer cells compared with normal cells (28). This suggests that artemisinin may be more selective in its toxicity and in that it is more effective at killing cancer cells while normal cells are relatively unaffected. Although this effect may play a role in the anti-proliferative effects of artemisinin on LNCaP cells, it does not appear to be the primary mode of action. LNCaP cells treated with artemisinin in the presence of anti-oxidants ascorbic acid or dithiothreitol still displayed a strong G₁ cell cycle arrest and down-regulation of CDK2 and CDK4 expression mirroring LNCaP cells

treated with artemisinin alone (data not shown). This result suggests that anti-proliferative effects of artemisinin on prostate cancer cells, and likely other cancer cell types, are independent of its oxidative potential. In this regard, we observe a similar effect of artemisinin on other types of human reproductive cancer cells.³

Taken together, our results suggest that artemisinin is a very potent anti-cancer compound that exhibits unique effects on the cell cycle regulation of human prostate cancer cells. As such, artemisinin has the potential to be developed as a potent anti-prostate cancer therapeutic. Further examination of the Sp1 interactions with the CDK4 promoter will be necessary to characterize possible artemisinin-mediated changes in protein-protein interactions of Sp1 and other transcription factors and/or co-regulators. Furthermore, as part of understanding the precise artemisinin mechanism of action, we plan to identify the direct cellular targets of artemisinin that act upstream of Sp1 and mediate the transcriptional effects of this phytochemical.

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REFERENCES

- Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., and Thun, M. J. (2007) *CA Cancer J. Clin.* **57**, 43–66
- Lynch, J. H., Batuello, J. T., Crawford, E. D., Gomella, L. G., Kaufman, J., Petrylak, D. P., and Joel, A. B. (2001) *Rev. Urol.* **3**, Suppl. 2, S39–S48
- Labrie, F., Dupont, A., Belanger, A., Cusan, L., Brochu, M., Turina, E., Pinault, S., Lacourciere, Y., and Emond, J. (1989) *Br. J. Urol.* **63**, 634–638
- Smaletz, O., Scher, H. I., Small, E. J., Verbel, D. A., McMillan, A., Regan, K., Kelly, W. K., and Kattan, M. W. (2002) *J. Clin. Oncol.* **20**, 3972–3982
- Halabi, S., Small, E. J., Kantoff, P. W., Kattan, M. W., Kaplan, E. B., Dawson, N. A., Levine, E. G., Blumenstein, B. A., and Vogelzang, N. J. (2003) *J. Clin. Oncol.* **21**, 1232–1237
- Duffy, P. E., and Mutabingwa, T. K. (2004) *The Lancet* **363**, 3–4
- Beekman, A. C., Woerdenbag, H. J., Van Uden, W., Pras, N., Konings, A. W., and Wikstrom, H. V. (1997) *J. Pharm. Pharmacol.* **49**, 1254–1258
- Sadava, D., Phillips, T., Lin, C., and Kane, S. E. (2002) *Cancer Lett.* **179**, 151–156
- Yamachika, E., Habte, T., and Oda, D. (2004) *Anticancer Res.* **24**, 2153–2160
- Chen, H. H., Zhou, H. J., and Fang, X. (2003) *Pharmacol. Res.* **48**, 231–236
- Chen, H. H., Zhou, H. J., Wu, G. D., and Lou, X. E. (2004) *Pharmacology* **71**, 1–9
- Efferth, T., Dunstan, H., Sauerbrey, A., Miyachi, H., and Chitambar, C. R. (2001) *Int. J. Oncol.* **18**, 767–773
- Paik, I. H., Xie, S., Shapiro, T. A., Labonte, T., Narducci-Sarjeant, A. A., Baega, A. C., and Posner, G. H. (2006) *J. Med. Chem.* **49**, 2731–2734
- Weinberg, R. A. (1995) *Cell* **81**, 323–330
- Helin, K., Lees, J. A., Vidal, M., Dyson, N., Harlow, E., and Fattaey, A. (1992) *Cell* **70**, 337–350
- Sherr, C. J. (1996) *Science* **274**, 1672–1677
- Emami, K. H., Navarre, W. W., and Smale, S. T. (1995) *Mol. Cell. Biol.* **15**, 5906–5916
- Samson, S. L., and Wong, N. C. (2002) *J. Mol. Endocrinol.* **29**, 265–279
- MacKintosh, C., and MacKintosh, R. W. (1994) *Trends Biochem. Sci.* **19**, 444–448
- Pardee, A. B. (1989) *Science* **246**, 603–608
- Hong, C., Kim, H. A., Firestone, G. L., and Bjeldanes, L. F. (2002) *Carcinogenesis* **23**, 1297–1305
- Yokota, T., Matsuzaki, Y., Koyama, M., Hitomi, T., Kawanaka, M., Enoki-Konishi, M., Okuyama, Y., Takayasu, J., Nishino, H., Nishikawa, A., Osawa, T., and Sakai, T. (2007) *Cancer Sci.* **98**, 1447–1453
- Garcia, H. H., Brar, G. A., Nguyen, D. H., Bjeldanes, L. F., and Firestone, G. L. (2005) *J. Biol. Chem.* **280**, 8756–8764
- Chu, W., and Ferro, T. J. (2005) *Gene (Amst.)* **348**, 1–11
- Cram, E. J., Liu, B. D., Bjeldanes, L. F., and Firestone, G. L. (2001) *J. Biol. Chem.* **276**, 22332–22340
- Dalrymple, S. L., Becker, R. E., and Isaacs, J. T. (2007) *Prostate* **67**, 790–797
- Meshnick, S. R. (1994) *Trans. R. Soc. Trop. Med. Hyg.* **88**, Suppl 1, S31–S31
- Reizenstein, P. (1991) *Med. Oncol. Tumor Pharmacother.* **8**, 229–233

³ J. A. Willoughby Sr., S. N. Sundar, M. Cheung, A. S. Tin, J. Modiano, and G. L. Firestone, manuscript in preparation.