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Artemisinin Represses Telomerase Subunits and Induces Apoptosis in HPV-39 Infected Human Cervical Cancer Cells

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ABSTRACT

Artemisinin, a plant-derived antimalarial drug with relatively low toxicity on normal cells in humans, has selective anticancer activities in various types of cancers, both in vitro and in vivo. In the present study, we have investigated the anticancer effects of artemisinin in human cervical cancer cells, with special emphasis on its role in inducing apoptosis and repressing cell proliferation by inhibiting the telomerase subunits, ERα which is essential for maintenance of the cervix, and downstream components like VEGF, which is known to activate angiogenesis. Effects of artemisinin on apoptosis of ME-180 cells were measured by flow cytometry, DAPI, and annexin V staining. Expression of genes and proteins related to cell proliferation and apoptosis was quantified both at the transcriptional and translational levels by semi-quantitative RT-PCR and western blot analysis, respectively. Our findings demonstrated that artemisinin significantly downregulated the expression of ERα and its downstream component, VEGF. Antiproliferative activity was also supported by decreased telomerase activity and reduced expression of hTR and hTERT subunits. Additionally, artemisinin reduced the expression of the HPV-39 viral E6 and E7 components. Artemisinin-induced apoptosis was confirmed by FACS, nuclear chromatin condensation, annexin V staining. Increased expression of p53 with concomitant decrease in expression of the p53 inhibitor Mdm2 further supported that artemisinin-induced apoptosis was p53-dependent. The results clearly indicate that artemisinin induces antiproliferative and proapoptotic effects in HPV-39-infected ME-180 cells, and warrants further trial as an effective anticancer drug. J. Cell. Biochem. 116: 1968–1981, 2015.

KEY WORDS: APOPTOSIS; ARTEMISININ; CERVICAL CANCER; E6/E7; ESTROGEN RECEPTOR; HPV; p53; TELOMERASE SUBUNITS; VEGF

Worldwide, cervical cancer kills more than 250,000 women each year despite screening programs and advent of vaccines (Arbyn et al., 2011). Till date, patients rely mostly upon ablative surgical techniques and pharmacologic treatments. Therefore, development of non-toxic, effective drugs suitable for chemotherapy would greatly support the treatment of this disease. Artemisinin, derived from the Chinese herb *Artemisia annua* (Singh and Lai, 2001; O'Neill and Posner, 2004), has been used for more than 2,000 years as an antimalarial drug (Clayman, 1985) based on an endoperoxide bond that is reduced by ferrous iron, resulting in the generation of reactive oxygen species. Recently, artemisinin and its analogs, including dihydroartemisinin, artesunate, and artemether, have been suggested to have anticancer effects (Efferth et al., 2001; Borstnik et al., 2002; Chen et al., 2003; Lai et al., 2005; Lai and Singh, 2006), mostly killed by artemisinin, apparently based upon their increased iron content (Lai et al., 2005). Consequently, artemisinin has growth inhibitory effects on various cancer cells, such as lung, melanoma, breast, renal, prostate, and also on many drug-resistant cancer cells (Wang et al., 2001; Efferth et al., 2002; Lai and Singh, 2006). Moreover, it has suppressive effects on the growth of human tumor xenografts in rats and nude mice, indicating that artemisinin has anticancer activities both in vitro and in vivo (Singh and Lai, 2004). Although the primary mechanism by which artemisinin exerts its anticancer activity is thought to be the induction of apoptosis (Singh and Lai, 2004; Nam et al., 2007), detailed mechanisms are yet to be elucidated. Interestingly, the major incentive for developing this compound as an effective anticancer agent is the fact that artemisinin is significantly less toxic to normal cells (Lai and Singh, 1995), which could be extremely beneficial for administration in humans without fear of severe side-effects of chemotherapy. However, the mechanisms by which artemisinin exerts specific anticancer activities on cervical cancer cells remains unclear, thereby limiting further development of this compound in preclinical and clinical settings.

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has been postulated that the human papillomavirus (HPV) infection alone is probably insufficient to cause cervical cancer and possible cofactors, like progesterone and estrogen, have been identified to aid promotion and progression of the disease (Webster et al., 2001). Estrogens, in particular, have been reported to increase HPV gene expression and can increase proliferation of cervical cancer cells in vivo (Bhattacharya et al., 1997; Kim et al., 2000). Consequently, the role of estrogen and its receptor, specifically estrogen receptor α (ERα), has been implicated in cervical cancer and their association with the HPV has been well elucidated (Chung et al., 2010). So, drugs that would target the ERα would be effective in prevention of cell proliferation and eventually treatment of cervical cancer. In addition, ample evidence for the role of ERs in the transcriptional regulation of the human telomerase protein hTERT, and activation of the enzyme, has also been documented (Misiti et al., 2000). Activation of telomerase is a crucial step during cellular immortalization and malignant transformation (Takakura et al., 1998). Several studies have implicated hTERT as a rate limiting determinant of telomerase activity (Wisman et al., 2001) and suggest that silencing with RNAi could inhibit proliferation and induce apoptosis in glioma and cancer cells (Wang et al., 2007).

It is well established that p53 is a powerful transcription factor and plays a central role in the regulation of cell cycle, apoptosis, DNA repair, senescence, and angiogenesis (Vousden and Lu, 2002; Fridman and Lowe, 2003; Teodoro et al., 2007). Known mainly for its prominent role as a tumor suppressor, p53 is functionally impaired by mutation or deletion in nearly 50% of human cancers (Feki and Irminger-Finger, 2004). In the remaining human cancers, p53 retains wild-type status but its function is inhibited by its primary cellular inhibitor, the murine double minute 2 (Mdm2). The Mdm2 protein inhibits p53 activity via various mechanisms: (i) by binding to the p53 transactivation domain and inhibiting its transcriptional activity; (ii) by exporting p53 out of the nucleus, promoting its degradation, and rendering it inaccessible to the target genes; and (iii) by promoting proteasome-mediated degradation of p53 by functioning as an E3 ubiquitin ligase (Momand et al., 1992; Haupt et al., 1997). In this manner, Mdm2 functions as an effective inhibitor of p53 activity.

p53 is also known to be controlled by viral oncogenes. Association between the HPV infection and cervical cancer was established after epidemiologic and molecular evidence demonstrated that infection with HPV as the first hit in cervical cancer development (Woodman et al., 2007) . In this context, expression of viral oncogenes E6 and E7 and the complex interactions between E6/E7 and cellular proteins have been shown to lead to inactivation of the tumor suppressor proteins p53. This study thereby investigated whether the plant-derived compound could inhibit cell proliferation by downregulating expression of the ERα and inhibiting telomerase activity, eventually inducing apoptosis in HPV 39-infected human cervical cancer cells.

MATERIALS AND METHODS

CHEMICALS

Human cervical cancer cell lines, ME-180 and HeLa, were procured from the cell repository of National Centre for Cell Science (Pune, India); Eagle′s minimum essential medium, fetal bovine serum, TRI reagent, FITC-conjugated Annexin V (Annexin V-FITC apoptosis detection kit), protease inhibitors, and artemisinin were purchased from Sigma; penicillin-streptomycin from Gibco; RNase inhibitor from Bioline; RevertAid^{TM} M-MuLV reverse transcriptase from Fermentas; and all antibodies were procured from Santa Cruz Biotechnology.

CELL CULTURE

ME-180 and HeLa human cervical cancer cells were cultured in Eagle′s minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in 5% CO_2 at 37 °C (Freshney, 1994). Media was changed every 48 h. Confluent cultures were detached with trypsin solution (0.05%Trypsin–0.02% EDTA) and sub-cultured every 72 h.

EFFECT OF ARTEMISININ ON CELL CYCLE

4×10^4 ME-180 cells were plated in 6-well tissue culture dishes and treated with different concentrations of artemisinin (0, 75, 150 and 300 μM) for 24, 48, 72, and 96 h in a complete medium. The doses of artemisinin selected were based on previous data where maximal growth inhibition occurred without adversely affecting cell viability (Sundar et al., 2008; Willoughby et al., 2009). Following treatment, cells were washed and hypotonically lysed in 1 ml of DNA staining solution containing 0.5 mg/ml propidium iodide. Cell debris was removed by filtration through 60-mm nylon mesh. Nuclear-emitted fluorescence with wavelengths of 585 nm was measured with a BD FACSCalibur (BD Biosciences). Ten thousand nuclei were analyzed from each sample at a rate of 300–500 nuclei/s. The percentages of cells distributed in the G_1, S, and G_2/M phases of the cell cycle were determined by analysis with the Cell Quest Pro 3.3 software (Chatterji et al, 2004). The optimum time and dose of artemisinin action, as determined by flow cytometry analysis, were selected for all subsequent experiments.

IN VITRO CYTOTOXICITY TEST

The MTT reduction assay was used for investigating the cytotoxicity of artemisinin in cervical cancer cells. ME-180 cells were seeded at a density of 4×10^4 cells per 100 μl per well of a 96-well plate and incubated for 24 h. Cells were subsequently treated with different doses of artemisinin and incubated for 48 and 72 h. A set of untreated control cells was also incubated for the same time. The untreated control and artemisinin-treated samples were measured in triplicates (Hu et al., 2014). Three wells of medium alone were included to provide the blanks for absorbance readings. At the end of the experiment, 10 μl of MTT reagent was added to each well and incubated for 4 h. After incubation, the cell culture medium was removed and 100 μl of the MTT solubilization solution was added to each well and swirled gently to dissolve the formazan crystals. After 2 h of incubation, the absorbance was measured at 570 nm in a Microplate Absorbance Reader (BioRad iMARK, CA).

CELL DEATH ASSAY

Following incubation with different doses on artemisinin, floating cells were collected and adherent cells were harvested by trypsinization and resuspended in a solution of 1 mg/ml propidium iodide and acquired on the FL3 channel of a flow cytometer (BD FACSCalibur). The propidium iodide positive population, recorded as sub-G_1 population, was considered dead.

RNA EXTRACTION AND SEMI-QUANTITATIVE RT-PCR

ME-180 cells were treated with artemisinin for 72 h. Total cellular RNA was isolated using TRI reagent and subjected to DNase treatment prior to reverse transcriptase-PCR. Reverse transcription reaction was performed at 42 °C with 5 μg of RNA, 100 pmol random hexamer primer, 10 mM dNTP mixture, 20 units of RNase inhibitor, and 200 units of RevertAid^{TM} M-MuLV Reverse Transcriptase. Semi-quantitative PCR amplification of the newly synthesized cDNA was performed using the primers listed in Table I. PCR was carried out for 40 cycles using an annealing temperature of 55 °C for CDK4, cyclin D1, p53, VEGF (vascular endothelial growth factor), and p21; 58 °C for ERα; 60 °C for hTR and hTERT; and 62 °C for E6 and E7 oncogenes. Samples were fractioned by 2% agarose gel electrophoresis and quantified using a BioRad Gel Documentation System (Chatterji et al, 2004). The efficiency of cDNA synthesis from each sample was estimated using GAPDH specific primers.

WESTERN BLOT ANALYSIS

ME-180 cells were treated with or without 300 μM artemisinin. After 72 h, the cells were lysed in ice-cold RIPA buffer [(150 mM NaCl, 50 mM Tris, 0.1% Triton X-100, and 0.1% SDS containing protease inhibitors, [4-(-2-aminoethyl) benzenesulphonyl fluoride)], EDTA, leupeptin, aprotinin, and bestatin]. The proteins were resolved by gel electrophoresis and electrically transferred on to PVDF (polyvinylidene difluoride) membranes (Chatterji et al, 2004). Blots were subsequently incubated with primary antibodies raised in rabbit or mouse (1:1000) for 18 h at 4 °C. Anti-β tubulin was used as a loading control. Blots were subsequently incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibodies for 1 h at 25 °C. Immunoreactive proteins were detected by staining the membranes with 3, 3′-diaminobenzidine in 50 mM Tris (pH 7) containing 0.2% H_2O_2 and bands were quantified by a BioRad Gel Documentation System.

MEASUREMENT OF NUCLEAR CHROMATIN CONDENSATION

Apoptotic cells were characterized by nuclear condensation of chromatin and/or nuclear fragmentation (Munoz et al., 2011). Briefly, ME-180 cells incubated with or without artemisinin were washed with ice-cold PBS, stained with DAPI (4′,6-diamidino-2-phenylindole; 2.5 mg/ml, 20 min), and analyzed on a high-content BD imaging system (BD Pathway^{TM} 855, BD Biosciences). A 40× objective lens was used to capture the image. A 3 × 3 montage was used to cover a larger field of focus.

ASSAY OF TELOMERASE ACTIVITY

Telomerase activity was measured using PCR-based ELISA kit (Roche Molecular Biochemicals, Germany). After treatment with artemisinin, the cells were lysed at different time points, and an aliquot containing 2 mg of protein was used for the telomeric repeat amplification protocol (TRAP) assay (Kim et al., 1994). Human embryonic Kidney 293 (HEK 293) cells and untreated ME-180 cells were used as a positive controls and the activity detected was expressed in relation to the positive control cells.

QUANTIFICATION OF APOPTOSIS

This assay combined Annexin V with the plasma membrane integrity probe, propidium iodide (Engeland van et al., 1998). Briefly, cells were treated with 300 μM artemisinin for 72 h. The cells were then harvested, washed in PBS, and incubated with 5 μl of Annexin V-FITC (fluorescein isothiocyanate) and 10 μl propidium iodide for cellular staining in binding buffer at room temperature for 10 min in the dark. The stained cells were analyzed by flow cytometry using a BD FACSCalibur (BD Biosciences) instrument equipped with Cell Quest 3.3 software. The early apoptotic cells stained with Annexin V-FITC, only were represented in the lower right quadrant of the FACS histogram, and the late apoptotic cells stained with both Annexin V-FITC and propidium iodide were represented in the upper right quadrant of the histogram.

STATISTICAL ANALYSIS

All experiments were performed in triplicates and the results expressed as mean and standard error of mean of different groups, using a statistical software package (Graphpad Sotware, Inc., La Jolla, CA). The differences between the mean values were evaluated by ANOVA and multiple Student's *t*-tests. *P*-values less than 0.05 were considered statistically significant. Densitometric analysis of the RT-PCR and Western blot results was carried out using NIH Scion Image Analysis.

RESULTS

ARTEMISININ TREATMENT CAUSES G1 CELL CYCLE ARREST OF ME-180 CELLS IN A DOSE- AND TIME-DEPENDENT MANNER

Based on preliminary assays, 75 μM, 150 μM, and 300 μM of artemisinin were used to treat ME-180 cells for different time points (24, 48, 72, and 96 h) in order to determine the optimum dose and time of artemisinin action by its effects on cell cycle progression. As shown in Figure 1A, treatment of ME-180 cells with artemisinin for

TABLE I. Sequences of the Oligonucleotides Used for Semi-Quantitative RT-PCR

Gene	Forward primer	Reverse primer	References
ERα	GGAGACATGAGAGCTGCCAAC	CCAGCAGCATGTCGAAGATC	Bodine et al., 1997
VEGF	GATCAAGTTCATGGACGTCT	GATCAAGTTCATGGACGTCT	Sugihara et al., 1998
hTR	TCTAACCCTAACTGAGAAGGGCGTAG	GTTTGCTCTAGAATGAACGGTGGAAG	Yatabe et al., 2002
hTERT	ACAGACGCCCAGGACCGCTCT	CAGCGCTGCCTGAAACTC	Ludlow et al., 2008
p53	GTTCCGAGAGCTGAATGAGG	TTATGGCGGGAGGTAGACTG	Mizuno et al., 2009
GAPDH	GACATCAAGGTGGTGAAGCAG	CACCCTGTTGCTGTAGCCATATTC	Bodine et al., 1998
Cyclin D1	CTGGCCATGAACTACCTGGA	GTCACACTTGATCACTCTGG	Kowalski et al., 2002
CDK4	TGGTGTCGGTGCCTATGGGA	GGTAGCTGTAGATTCTGGCT	Corroyer et al., 1996
$p21^{Cip1}$	ACTGTGATGCGCTAATGGC	ATGGTCTTCCTCTGCTGTCC	Braastad et al., 2003
E6	ACAAATTGCCAGACCTGTGCAC	TTTCTGCTGGACACAGCGGTTT	Akil et al., 2008
E7	AGCCCACCTTGCAGGAAATTGT	TAGTTGTCGCAGAGTATCCCGT	Tsuhako et al., 1998

72 h resulted in a significantly higher number of cells in the G_1 phase for all the concentrations used. Compared to the untreated cells (52%), 60% cells appeared in the G_1 phase when treated with 75 μM artemisinin, 65% ($P < 0.01$) when treated with 150 μM, and 71% ($P < 0.001$) when treated with 300 μM artemisinin, indicating a G_1 cell cycle arrest. There was no further G_1 cell cycle arrest when the cells were treated for 96 h. Concomitant reduction in the number of cells in the S (11.7% in control versus 5.16% in 300 μM artemisinin-treated cells at 72 h; Fig. 1B) and G_2/M (17.2% in control versus 7.75% in artemisinin-treated cells; Fig. 1C) phases were observed. Since the percentage of growth inhibition was the highest when the cells were treated with 300 μM artemisinin for 72 h, beyond which there was no significant effect of artemisinin, all subsequent experiments were performed with the optimum effective dose of 300 μM artemisinin for 72 h.

EFFECTS OF ARTEMISININ ON ME-180 CELL VIABILITY

As revealed by the MTT assay, cell viability was unchanged by the lower concentrations of artemisinin (<150 μM), but was significantly suppressed by the higher concentrations of artemisinin (300 and 500 μM), 72 h post-exposure to artemisinin. Following exposure for 72 h, reduction was more than 60% when artemisinin was used at a concentration of 300 μM (Fig. 2). These results suggest that artemisinin suppresses cell viability in a time- and dose-dependent manner.

ARTEMISININ ALTERS EXPRESSION OF G1 PHASE CELL CYCLE PROTEINS

Since artemisinin was observed to induce a G_1 phase cell cycle arrest, we investigated the effects of artemisinin treatment on G_1 phase cell cycle regulatory proteins and their inhibitors, which are key players in the regulation of cell cycle progression. Concomitantly, we studied the expression of cyclin D1, CDK4, and $p21^{Waf1/Cip1}$, since they are chiefly involved in regulating G_1-to-S phase transition. Conforming to the flow cytometric analysis, artemisinin treatment significantly reduced cyclin D1 and CDK4 mRNA transcript levels ($P < 0.001$; Fig. 3A) and proteins ($P < 0.001$; Fig. 3B) in artemisinin-treated cells, with concomitant upregulation of the CDK-inhibitor, $p21^{Waf1/Cip1}$ mRNA and protein level as compared to the untreated cells ($P < 0.01$). The data, thus, summarize that artemisinin induces G_1 phase cell cycle arrest, possibly by downregulating the expression of G_1 phase cell cycle proteins, cyclin D1, and CDK4 and upregulating the expression of $p21^{Waf1/Cip1}$.

ARTEMISININ DOWNREGULATED EXPRESSION OF ERα IN CERVICAL CANCER CELLS

Semi-quantitative RT-PCR confirmed that the expression of ERα was significantly downregulated as a result of artemisinin treatment as compared to the control ($P < 0.01$; Fig. 4A). Western blot analysis further established that concentration of the ERα protein declined as compared to the untreated cells ($P < 0.05$; Fig. 4B). Hence, inhibition of ERα expression, both at the transcriptional and translational levels, was observed when cells were treated with artemisinin.

DOWNREGULATED EXPRESSION OF VEGF IN RESPONSE TO ARTEMISININ

VEGF is an important factor responsible for angiogenesis and a well-known downstream target of the ER signaling pathway in cervical cells. Since artemisinin downregulated ERα, we checked whether ERα downstream signaling components were also affected. Our results indicated that VEGF expression significantly decreased at the transcriptional ($P < 0.05$; Fig. 5A) and translational ($P < 0.001$; Fig. 5B) levels as a result of artemisinin treatment. Therefore, artemisinin not only downregulated ERα, it also disrupted the expression of its downstream component, indicating that angiogenesis may also be prohibited by artemisinin.

ARTEMISININ TREATMENT DECREASED TELOMERASE ACTIVITY AND DOWNREGULATED HTERT AND HTR EXPRESSION IN ME-180 CELLS

Telomerase activity is enhanced in a vast majority of immortal and cancer cells. During tumorigenesis, an important event that occurs is telomerase reactivation. Therefore, obstruction of telomerase activity could help in the control of tumor progression. In agreement with this fact, artemisinin treatment demonstrated a significant decrease in telomerase activity in ME-180 cells, which was detectable as early as 6 h and reached ~50% of control by 72 h (Fig. 6a). In addition, the expression of both the RNA (hTR) and protein (hTERT) components of the enzyme were evaluated at the transcriptional level in response to artemisinin treatment for 72 hours. Results confirmed that the levels of both hTR, which provides the template for telomere length maintenance, and hTERT, a reverse transcriptase, were significantly down regulated as a result of artemisinin treatment as compared to the control ($P < 0.001$; Fig. 6b). The fact that artemisinin suppressed expression of telomerase protein hTERT directly translate into growth inhibition of cervical cancer cells.

ARTEMISININ DOWNREGULATES VIRAL ONCOGENES E6 AND E7 IN CERVICAL CANCER CELLS

The E6 and E7 oncogenes of the high-risk HPV-39 encode oncoproteins, which bind to and degrade tumor suppressor proteins like p53 and pRb, leading to cervical carcinogenesis. Consequently, we determined whether artemisinin downregulated the HPV-39 viral oncogenes E6 and E7. Semi-quantitative RT-PCR confirmed that the levels of both E6 and E7 oncogenes were significantly downregulated as a result of artemisinin treatment as compared to the untreated cells ($P < 0.01$; Fig. 7). Thus, repression of both E6 and E7 oncogenes by artemisinin further endorses its anti-proliferation capacity.

ARTEMISININ INDUCED APOPTOSIS IN HUMAN CERVICAL CANCER CELLS

Flow cytometric analysis of artemisinin-induced dose-dependent cell death in ME-180 cells revealed that exposure of cervical cancer cells to artemisinin resulted in a dose-dependent enhancement of cells in the sub-G_1 phase of the cell cycle, indicating increase in dead cells: control (11.86%), 75 μM (28.15%), 150 μM (31.553%), and 300 μM (45.605%). The increase was most significant at 72 h, beyond which there was no further increase in sub-G_1 cells. Hence, ME-180 cells treated with 300 μM artemisinin for 72 h showed four-fold increase in the sub-G_1 phase cells compared to the untreated cells (Fig. 8A; $P < 0.01$). Chromatin condensation is a typical feature

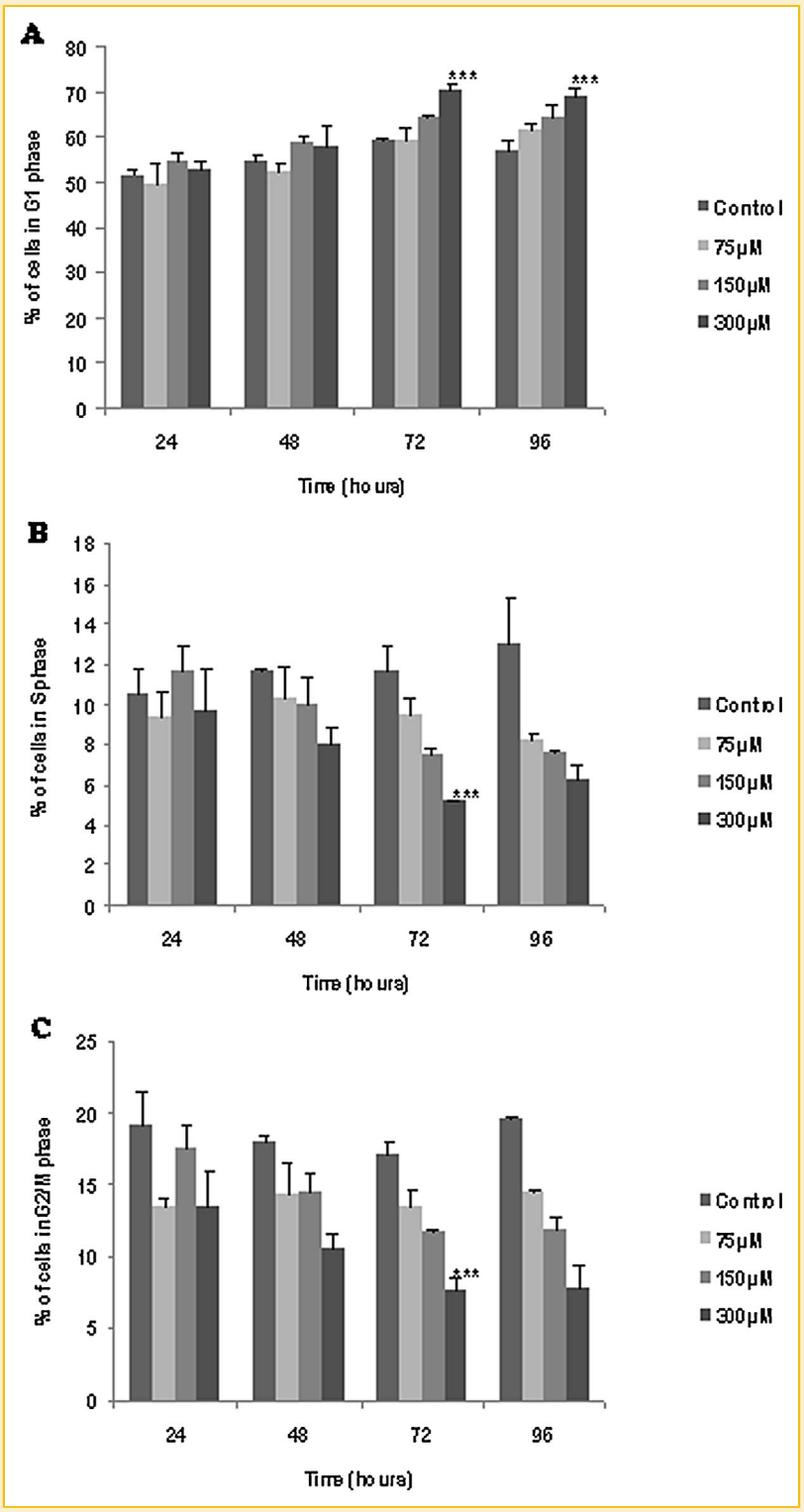

Fig. 1. Effects of artemisinin on the DNA content of ME-180 cervical cancer cells. ME-180 cells were treated with DMSO and various concentrations of artemisinin (75 μM, 150 μM, 300 μM) for 24, 48, 72, 96 h. Cells were then lysed and stained with propidium iodide, and the nuclei were analyzed for DNA content by flow cytometry. (A) Cells distributed in the G_1 phase indicated G_1 cell cycle arrest (71% cells compared to 52% in the control) at 72 h when cells were treated with 300 μM artemisinin. (B) Effect of artemisinin on S-phase cell cycle indicated progressive reduction of artemisinin-treated cells in the proliferation phase as compared to artemisinin-untreated cells. (C) Treatment of ME-180 cells with artemisinin led to significant downregulation of cells in the G_2/M phase, indicating a prominent G_1 phase arrest. All assays were done in triplicate. ***$P < 0.001$.

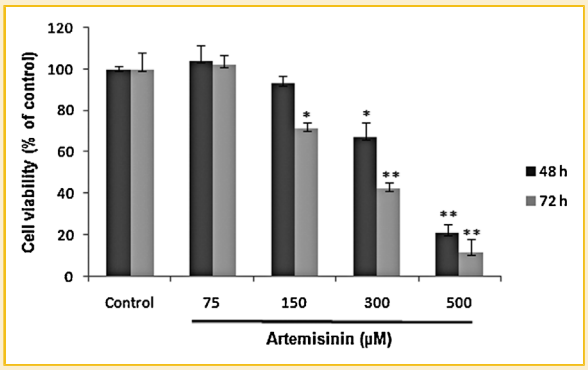

Fig. 2. Effect of artemisinin on ME-180 cell viability. After ME-180 cells were treated with designated concentrations of artemisinin for 48 and 72 h, respectively, cell viability was determined by MTT assay. *$P < 0.05$, **$P < 0.01$ compared to the control group. Data are expressed as mean ± SD; n = 5.

of apoptotic cells. Artemisinin-induced nuclear chromatin condensation was observed using DAPI, a nucleic acid binding-dye, in ME-180 cells (Fig. 8B and C), as additional evidence of its apoptotic potential.

To further validate that artemisinin-induced cell death was associated with the induction of apoptosis, ME-180 cells were treated with artemisinin and assessed using FITC-conjugated Annexin V. This assay was combined with analysis of exclusion of the plasma membrane integrity probe, propidium iodide. Apoptotic cells were recorded as early or late apoptotic cells, which are shown in the lower right and upper right quadrants of the histograms, respectively (Fig. 8D). There was a significant decrease in the percentage of viable cells in the treated cells (68.54%) as compared to the control (83.26%) cells. The lower right quadrant (PI^{-ve}, Annexin V^{+ve}) indicated 1.8-fold increase in cells undergoing early apoptosis (26.43%) as compared to the untreated cells (14.49%). The percentage of cells undergoing late apoptosis (PI^{+ve}, Annexin V^{+ve}) increased by 4.8-fold in the artemisinin-treated group (3.41%) when compared to the control cells (0.71%). The cells in the upper left quadrant, which indicates the percentage of necrotic cells, did not show significant difference between the treated and untreated cells, confirming that artemisinin induced apoptotic and not necrotic cell death in ME-180 cells.

ARTEMISININ-INDUCED APOPTOSIS IS P53-DEPENDENT

p53, which serves as a tumor suppressor by inducing cell cycle arrest, apoptosis, senescence and DNA repair, was studied for its involvement in artemisinin-induced apoptosis. Our results indicated a significant increase in both the p53 transcript and protein levels ($P < 0.01$) in the artemisinin-treated cells (Fig. 9A). On the contrary,

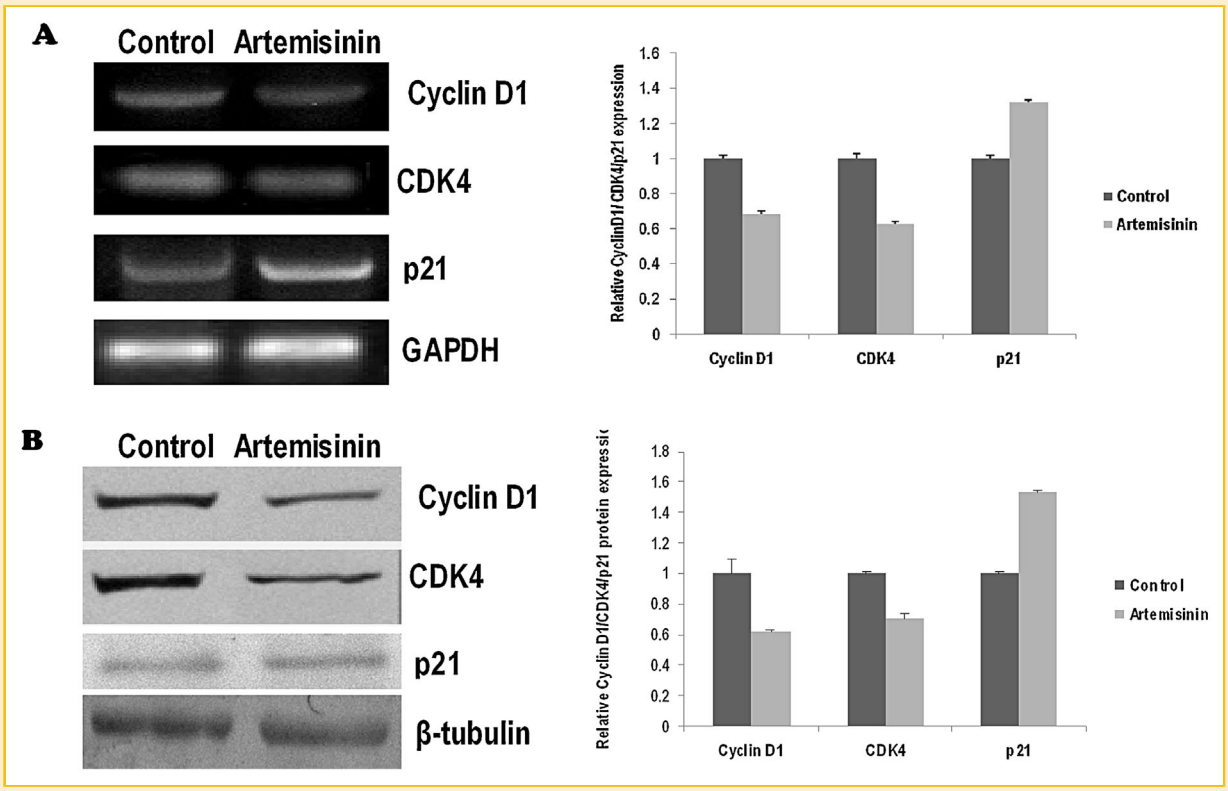

Fig. 3. Effect of artemisinin on the expression of G_1 cell cycle regulatory components in ME-180 cells. (A) RT-PCR analysis of total mRNA transcript levels of cyclin D1, CDK4 and p21 indicated that 300 μM artemisinin treatment for 72 h led to significant downregulation of the early G_1 phase cell cycle components cyclin D1 and CDK4, ***$P < 0.001$, along with concomitant increased expression of the CDK inhibitor, $p21^{Waf1/Cip1}$, **$P < 0.01$. (B) Western blot analysis indicated that artemisinin led to downregulation of the early G_1 cell cycle components at the protein level, and upregulation of the CDK inhibitor, $p21^{Waf1/Cip1}$. Densitometric analysis of expression levels, represented by the histograms, was carried out with respect to GAPDH and β-tubulin, respectively. The data shown are a representative of three independent experiments.

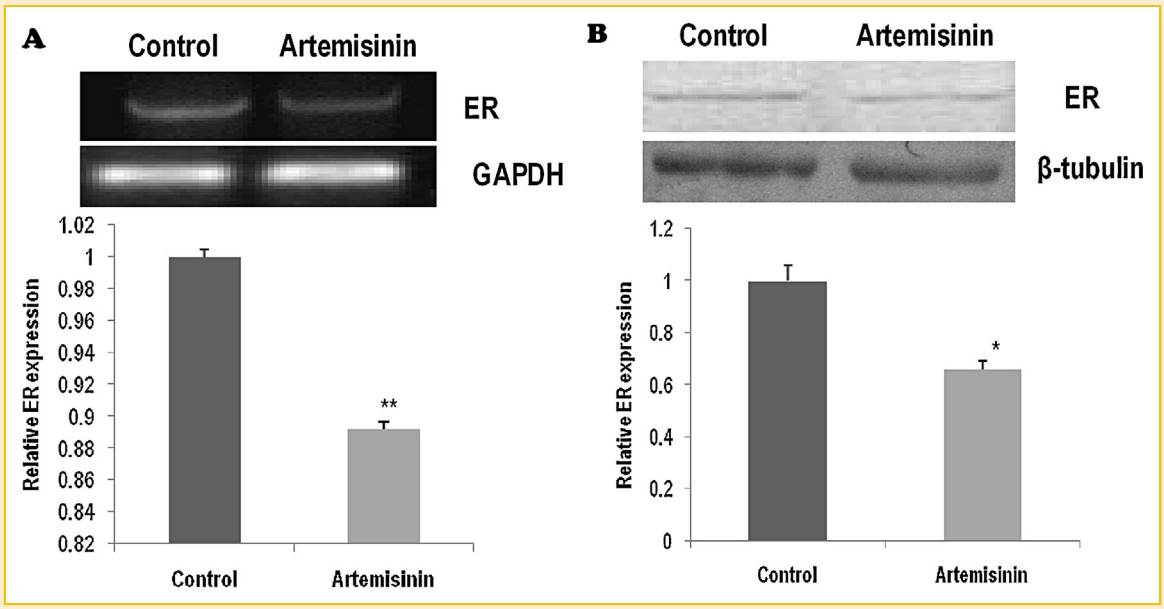

Fig. 4. Effect of artemisinin on the expression of ERα in ME-180 human cervical cancer cells. Cells were treated with or without 300 μM artemisinin for 72 h. Total RNA and protein were extracted from the cells after treatment and processed for analysis. (A) RT-PCR analysis of mRNA transcript levels of ERα indicates that ART significantly (**$P < 0.01$) downregulated the expression of ERα transcripts in cervical cancer cells. (B) Western blot analysis further supports the downregulated protein expression of ERα. The data shown are a representative of three independent experiments. *$P < 0.05$.

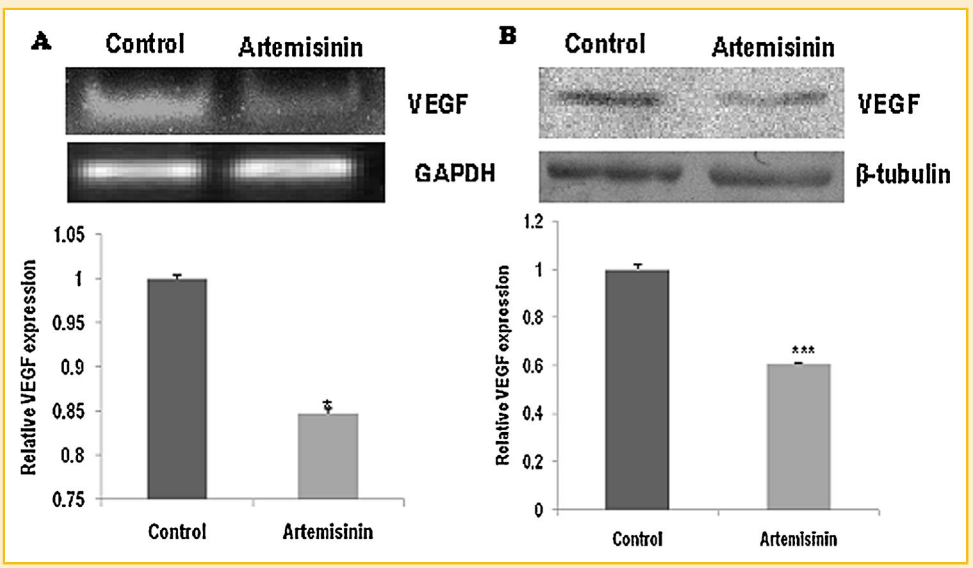

Fig. 5. Effect of artemisinin on the expression of VEGF in ME-180 human cervical cancer cells. Treatment of ME-180 cells with or without 300 μM artemisinin reveal that artemisinin reduced the expression of VEGF at the transcriptional level (A), as indicated by RT-PCR analysis, *$P < 0.01$, and at the translational level (B), as indicated by Western blot analysis, ***$P < 0.001$. GAPDH and β-tubulin were used as loading controls, respectively. The histograms are a representation of the differences of expression levels in relation to the controls in three independent experiments.

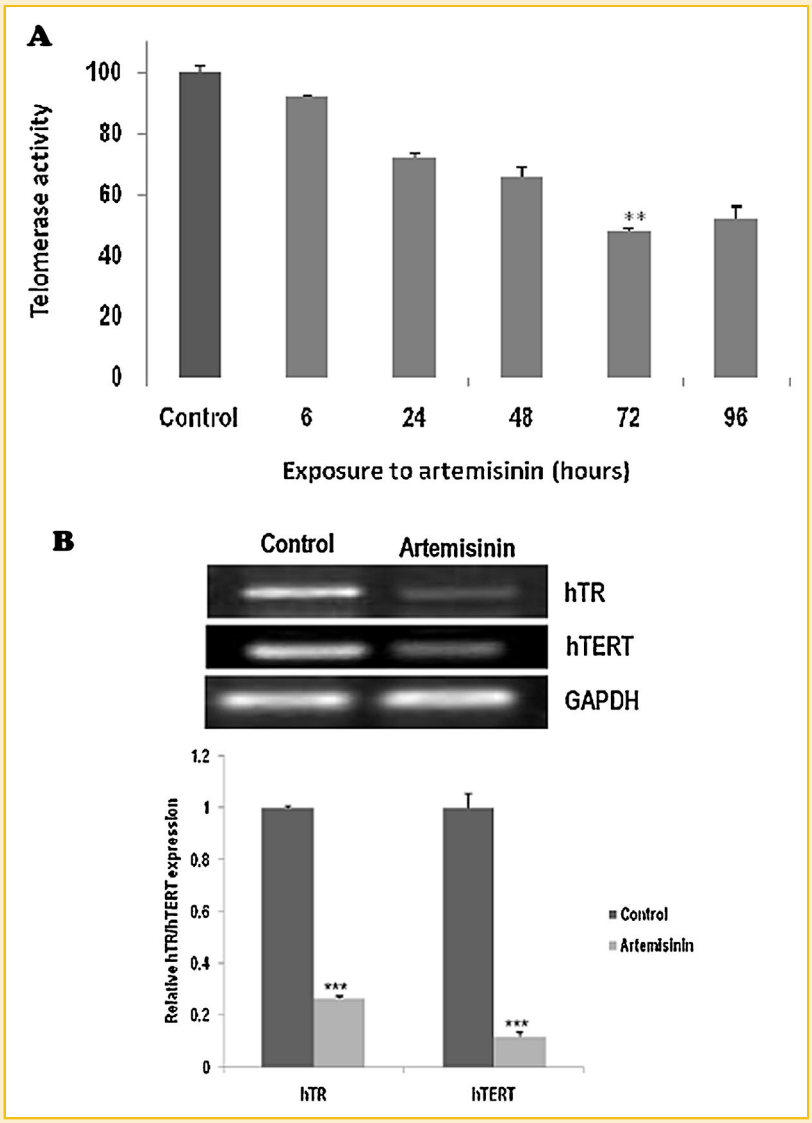

Fig. 6. Effect of artemisinin on activity and expression of telomerase components in ME-180 cells. (A) Analysis of telomerase activity indicated that treatment with 300 μM artemisinin for 72 h led to 50% decrease in telomerase activity. The results shown are mean of three different experiments. $^{**}P < 0.01$. (B) RT-PCR analysis of total mRNA transcript levels of hTR and hTERT indicated that 300 μM artemisinin treatment for 72 h led to significant downregulation of both the telomerase components. $^{***}P < 0.001$.

expression of Mdm2, the negative regulator of p53, was seen to be reduced in artemisinin-treated cells as compared to the untreated cells (Fig. 9B), indicating artemisinin-induced p53 stability and further endorsing its role in assisting apoptosis of the ME-180 cells.

DISCUSSION

In recent times, several anticancer phytochemicals, like artemisinin, indole-3-carbinol, resveratrol, curcumin, and epigallocatechin gallate, have been tested for their efficacy in cancer cells. These phytochemicals have shown to inhibit growth and induce apoptosis in a wide range of human cancer cell lines and their respective tumor xenografts in athymic mice, and hence predict eventual development as efficient anticancer drugs (Firestone and Sundar, 2009). Consequently, we have focused our attention on the anticancer properties of artemisinin, a FDA-approved anti-malarial drug. In our study, we have identified for the first time the effects of artemisinin on telomerase subunits, which are critical markers for cell proliferation, and artemisinin-induced p53-dependent apoptosis in human cervical cancer cells. We have also shown that artemisinin disrupts the estrogen signaling pathway, by downregulating the expression of ERα and its downstream component, VEGF.

Nearly all cervical cancers are etiologically attributable to HPV infection, and pharmaceutical treatments targeting HPV-infected cells would be of great medical benefit. ME-180 cells are known to be

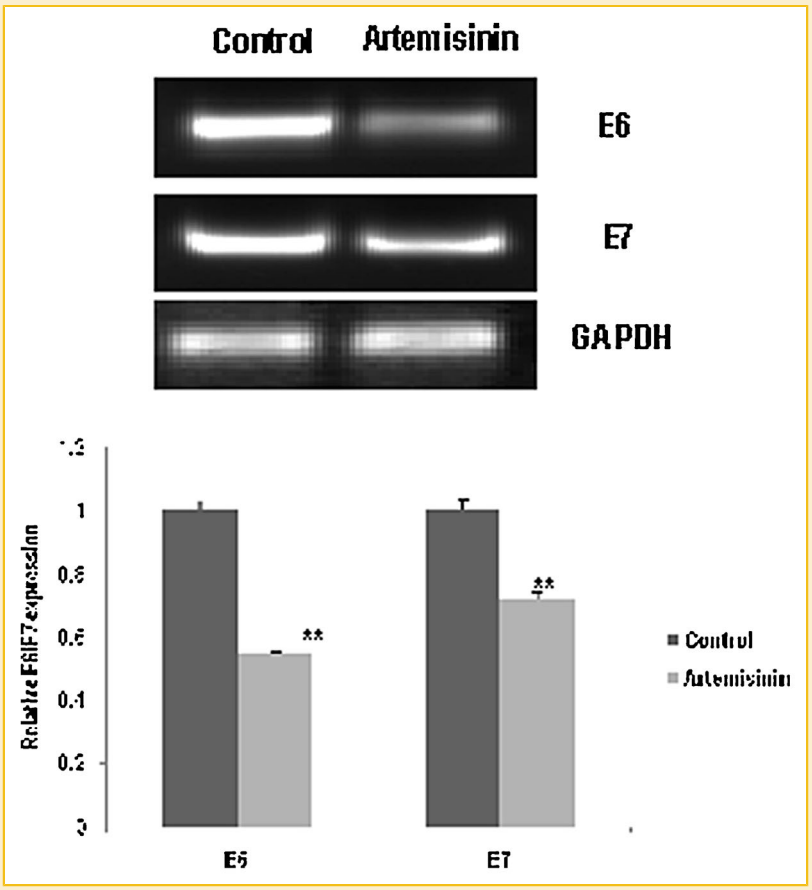

Fig. 7. Effect of artemisinin on the expression of E6 and E7 oncogenes in ME-180 cells. RT-PCR analysis of total mRNA transcript levels of E6 and E7 indicated that 300 μM artemisinin treatment for 72 h led to significant downregulation of both the viral oncogenes. $^{**}P < 0.01$.

infected with HPV-39 (Reuter et al., 1991). HPV-39 contributes to being one of the most prevalent high oncogenic risk genotypes (Vet et al., 2008; Şahiner et al., 2014), but there is no specific vaccine or drugs targeting cancers caused by this virus. A study involving the Indian population revealed that in women, the six most common types were HPV-16, HPV-89, HPV-39, HPV-52, HPV-62, and HPV-18 (Bhatla et al., 2008), thus emphasizing the relevance of using ME-180 cells for our study, in addition to studies which have already shown effectiveness of artemisinin in HPV-16-infected HeLa cells (Disbrow et al., 2005).

Estrogens regulate a plethora of physiological functions in the maintenance of the human cervix and act predominantly via the activation of the ERs, particularly the ERα, and not ERβ, in the cervix (Chen et al., 2008). In addition, estrogen and ERα synergize with the HPV oncogenes to develop cervical cancer (Chung et al., 2010). Although the mechanism by which estrogens acts synergistically with E6 and/or E7 to induce cervical cancer has not been identified, there is a possibility that estrogens might act as survival factors, enabling enhanced proliferation of cells expressing E6 and E7, leading to spontaneous development of tumor cells (Webster et al., 2001). On the other hand, targeting neo-angiogenesis is considered a promising therapeutic approach for cancer. Angiogenesis plays a vital role in tumor growth, invasion, and metastasis, and inhibition of angiogenesis provides an efficient strategy for preventing development of malignancy. VEGF is an important factor responsible for angiogenesis and a well-known target of the ER signaling pathway in cervical cells (Shifren et al., 1996; Hyder et al., 2000; Tangjitgamol et al., 2005). Interestingly, there are evidences to support that artemisinin and its derivatives may have a potential role on anti-angiogenesis (Wartenberg et al., 2003; Zhou et al., 2007; Wang et al., 2008). Thus, the above conjectures strongly suggest that repression of ERα and VEGF could be one mechanism to prevent cervical cancer. Our results have indicated that artemisinin treatment of ME-180 cells decreased the expression of ERα, both at the transcriptional and translational levels, and thus endorsed its capacity to prevent the proliferative role of estrogens in cervical cancer. As a critical angiogenic factor, VEGF exhibited abundant expression in cervical cancer cells. Consistent with decrease in ERα, expression of VEGF was significantly lowered in artemisinin-treated cells, further endorsing possible anti-angiogenic effects of artemisinin in cervical cancer cells.

Functional telomerase is known to establish and/or maintain the transformed phenotype of cells (Kim et al., 1994), and the level of telomerase activity acts as a useful prognostic marker for cancer

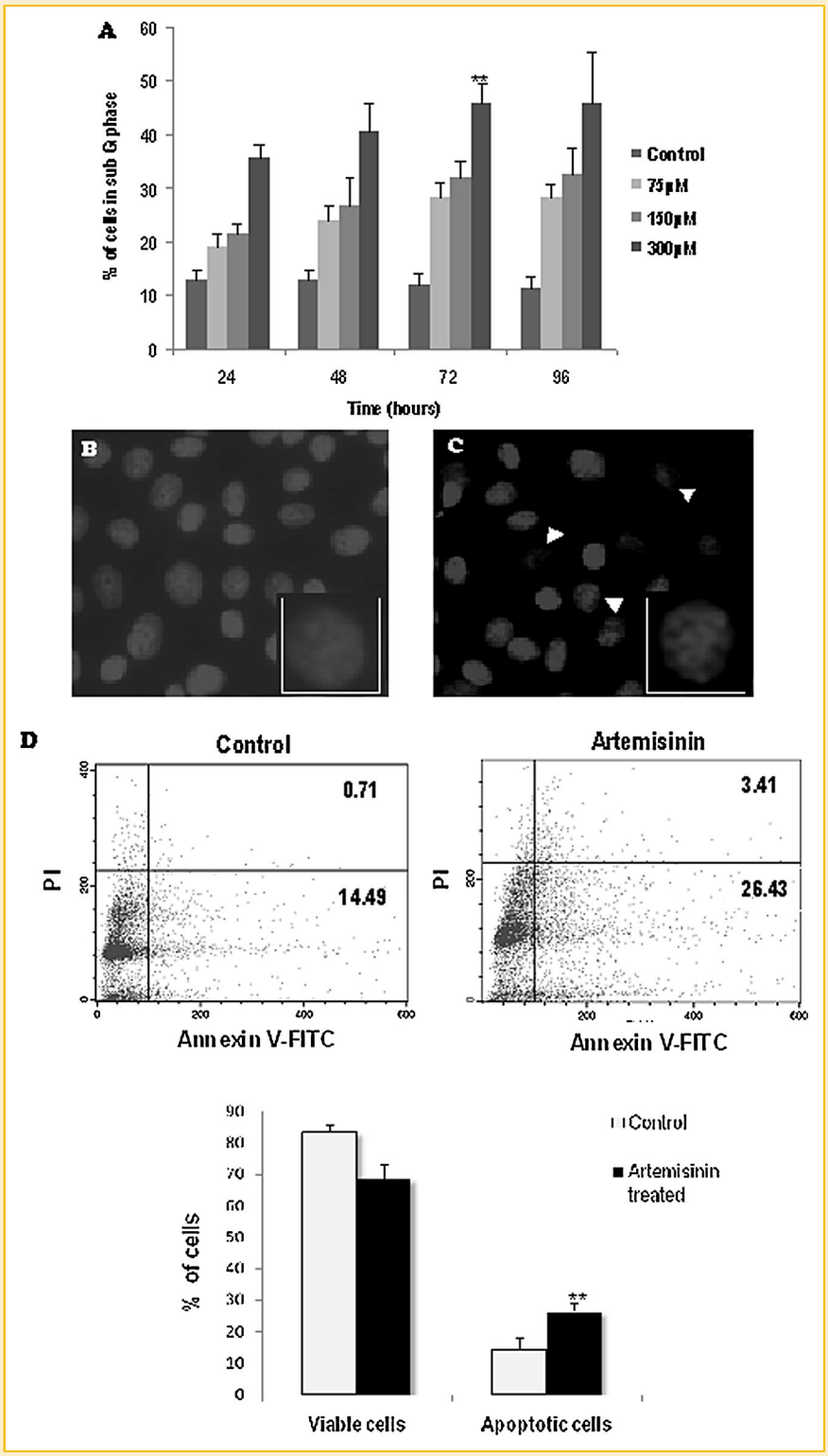

Fig. 8. Artemisinin induced apoptosis in human cervical cancer cells. (A) Induction of apoptosis in human cervical cancer cells in a dose–time dependent manner. Cells were exposed to various concentrations of artemisinin (75 μM, 150 μM, 300 μM) for 24, 48, 72, 96 h. All assays were done in triplicate. The optimum dose and time were 300 μM artemisinin and 72 h, respectively within which 45.605% of cells underwent apoptosis in artemisinin-treated cells as compared to 11.86% in control cells. $^{**}P < 0.01$. (B and C) Apoptosis-related nuclear events induced by artemisinin. Nuclear chromatin condensation of ME-180 cells treated without (B) and with (C) 300 μM artemisinin for 72 h. Cells were labeled with DAPI, and fluorescence was analyzed by the BD Pathway analyzer as described in Materials and Methods section. The figure is a representative profile of three experiments. Magnification: 400×. (D) Externalization of phosphatidylserine in artemisinin-treated cervical cancer cells. ME-180 cells were incubated with 300 μM artemisinin for 72 h, co stained with PI and Annexin V-FITC, and analyzed by flow cytometry. Annexin^{+ve}, PI^{-ve} and Annexin^{+ve}, PI^{+ve} staining indicated cells undergoing early and late apoptosis, respectively. Histogram showing apoptotic cells in response to artemisinin treatment. $^{**}P < 0.01$.

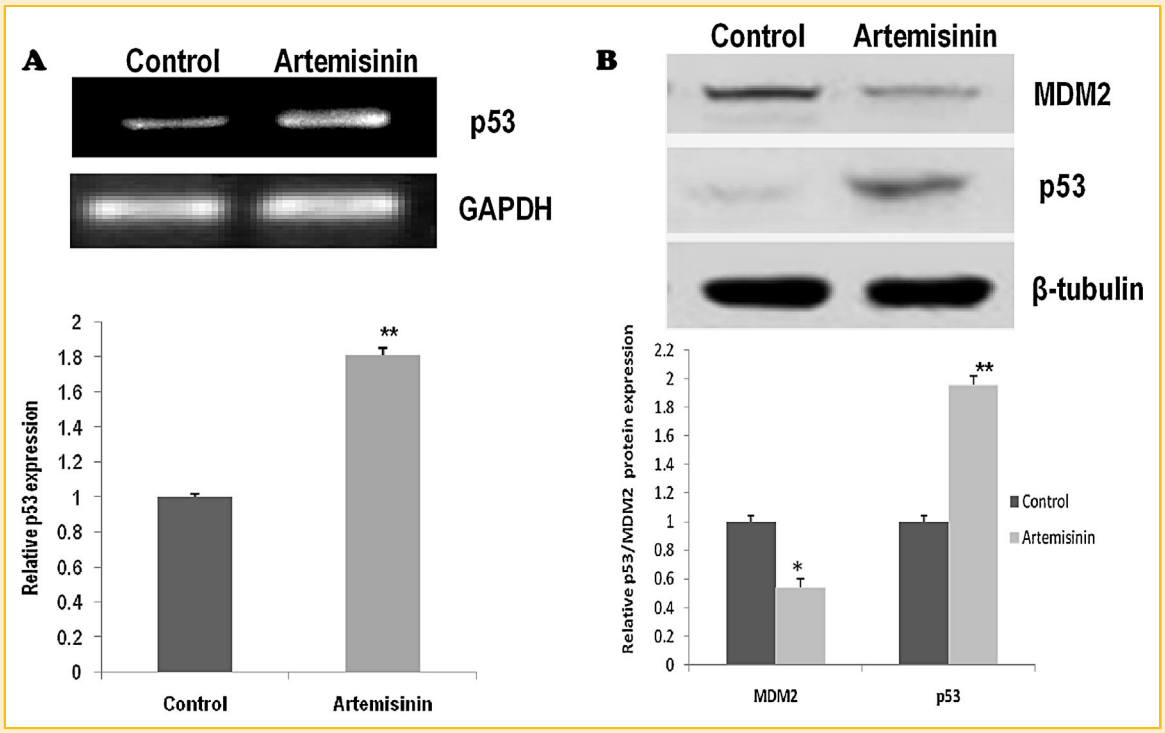

Fig. 9. Artemisinin-induced apoptosis is p53 dependent. p53-mediated apoptosis in artemisinin treated cells. Cells were treated with or without 300 μM artemisinin for 72 h. (A) RT-PCR analysis of mRNA transcript levels of p53 indicated that artemisinin significantly (**$P < 0.01$) upregulated the expression of p53 transcripts in cervical cancer cells. (B) Western blot analysis indicated increased p53 expression with concomitant decrease in protein levels of the p53 inhibitor Mdm2 (**$P < 0.01$). The data shown are a representative of three independent experiments.

patients (Piatyszek et al., 1995). On treatment of cervical cancer cells with artemisinin, both the telomerase subunits hTR and hTERT were significantly downregulated, indicating inactivation of the enzyme. These observations suggest that since telomerase activation is a rate-limiting step in cellular immortality and carcinogenesis, repression of telomerase subunits by artemisinin can act as an effective tumor-suppressive mechanism. In addition, we have shown a 50% decrease in telomerase activity in ME-180 cells following artemisinin treatment. The observed decrease in telomerase activity can also be explained by the alteration of E6 and p53 levels, both of which can modulate telomerase activity. It is well established that E6 along with E7 oncoprotein can activate telomerase and confer immortality to a cell (Jeong Seo et al., 2004). On the other hand, p53 represses telomerase activity through downregulation of hTERT transcription (Kanaya et al., 2000). Although studies using HPV-16 E6 mutants showed that there was no correlation between the ability of the mutants to activate telomerase and their ability to target p53 for degradation, suggesting that telomerase activation by HPV-16 E6 is p53 independent (Klingelhutz et al., 1996), it appears from our studies that telomerase activity may be closely linked to the levels of E6 and p53 as the decrease in telomerase activity in response to artemisinin treatment coincided with a significant decrease in E6 expression and prominent increase in p53 protein expression.

Since several neoplastic cells overexpress the transferrin receptor to increase their iron uptake, we hypothesized that iron-dependent antimalarial drugs, such as artemisinin, may be selectively taken up by cancer cells at higher concentrations as compared to the normal cells, and may lead the cell toward apoptosis. The data indicated that artemisinin induced G_1 phase cell cycle arrest, possibly by down-regulating the expression of G_1 phase cell cycle proteins, cyclin D1 and CDK4 and upregulating the expression of $p21^{Waf1/Cip1}$. Our results also confirmed that artemisinin induced apoptotic cell death in HPV 39-immortalized cervical cells in vitro, as observed by increased sub-G_1 population, elevated Annexin-V staining, and increased nuclear chromatin condensation. Next, we determined whether artemisinin-induced cell death was dependent on p53, which is known to act as a tumor suppressor by inducing cell cycle arrest, apoptosis, senescence, and DNA repair (Bañuelos et al., 2003). It was observed that expression of p53 in ME-180 cells increased after artemisinin treatment both at the mRNA and protein levels. It is a well-established fact that p53-dependent apoptosis is a consequence of drug-induced reduction in viral oncogene expression, since the viral E6 oncogene interacts with p53 and leads it to proteasomal degradation in cancer cells (Disbrow et al., 2005). We have confirmed downregulation of the E6 transcripts by artemisinin. In addition, levels of p21 were enhanced following artemisinin treatment. These findings further established that increased p21 expression and reduced interaction of E6 with p53, due to decreased expression of the viral E6 component in response to artemisinin, eventually inhibited p53 degradation and led to apoptosis in ME-180 cells.

In order to further understand the regulation of p53 by factors other than the cell cycle inhibitor and viral components in artemisinin-induced cells, we determined the expression of Mdm2, which also dynamically controls p53 deactivation. Contrary to conventional genotoxic anticancer agents and radiation, which induce the accumulation and activation of p53 by posttranslational modifications of p53, such as phosphorylation, several Mdm2 inhibitors (for example Nutlin and MI-219) are known to block the intracellular Mdm2-p53 interaction and induce the accumulation of p53 (Shangary and Wang, 2008). According to our data, artemisinin treatment of cervical cancer cells led to decreased expression of Mdm2, which further corroborated that reduced Mdm2 expression led to decreased Mdm2-p53 interaction, culminating in reactivation of p53 function. Thus, artemisinin may act as an Mdm2 inhibitor, though further analyses are required to confirm its specific action. In addition, there is evidence that activation of p53 effectively inhibits angiogenesis (Teodoro et al., 2007), and Mdm2 inhibitors are known to reduce angiogenesis through the activation of p53 (Binder, 2007). Subsequently, it may be proposed that artemisinin targets VEGF and inhibits angiogenesis through two mechanisms: (i) as a downstream event of ER inhibition and (ii) by lowering the expression of Mdm2 and leading to activation of p53. The inhibitory effect of artemisinin on telomerase subunits in cervical cancer cells also conforms to the previous reports that p53 gene transduction directly inhibits telomerase activity (Kusumoto et al., 1999). Interestingly, specific types of the HPV, which express the E6 oncoprotein, are causative agents of at least 90% of cervical cancers (zur Hausen, 1996). Oncogenic E6 proteins interact with p53 and target it for proteasome-mediated degradation (Scheffner et al., 1993). In other words, in some cases, E6 substitutes for Mdm2 (Hengstermann et al., 2001), and like Mdm2-mediated degradation of p53, E6-mediated degradation can be abrogated by inhibitors of transcription (Thomas et al., 1999). Since artemisinin inhibits both transcription of the E6 subunit of telomerase and expression of Mdm2, it stabilizes p53 and leads to p53-mediated cell death in HPV-39-induced cervical cancer cells. Further analysis of artemisinin-induced cell death and in vivo studies may help develop this compound as an effective chemotherapeutic intervention for cervical cancer patients.

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