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Down-Regulation of BMI-1 Cooperates With Artemisinin on Growth Inhibition of Nasopharyngeal Carcinoma Cells

Jing Wu,[1,2,3] Dong Hu,[3] Guang Yang,[1,2] Junyi Zhou,[1,2] Changfu Yang,[1,2] Yun Gao,[1,2] and Zhenyu Zhu[1,2*]

[1]*Department of Biochemistry and Molecular Biology, Zhongshan School of Medicine, Sun Yat-sen University, Guangdong, People's Republic of China*

[2]*DaAn Gene Co., Ltd, Sun Yat-sen University, Guangzhou, Guangdong, People's Republic of China*

[3]*School of Medicine, Anhui University of Science and Technology, Anhui, People's Republic of China*

ABSTRACT

Artemisinin and its derivatives are well known antimalaria drugs, particularly useful for the treatment of infection of Plasmodium falciparum malaria parasites resistant to traditional antimalarial pharmaceuticals. Artemisinin has inhibitory effects on cancer cell growth and anti-angiogenetic activity, including many drug- and radiation-resistant cancer cell lines. Moloney murine leukemia virus insertion site 1 (BMI-1) has been shown to regulate proliferation by inhibiting $p16^{ink4a}$ transcription. It is well known that BMI-1 over-expression was found in nasopharyngeal carcinoma cell lines and correlated with advanced invasive stage of the tumor progression and poor prognosis. In the present investigation, we analyzed the inhibitory effects of artemisinin on proliferation of nasopharyngeal carcinoma cell lines (CNE-1 and CNE-2, well-differentiated cells, and poorly differentiated cells). We demonstrated that artemisinin induced G1 cell cycle arrest in CNE-1 and CNE-2 cells. Artemisinin inhibited BMI-1 both in protein and transcript levels. BMI-1 knockdown made the cells more sensitive to artemisinin with an increase in G1 phase, but over-expression of BMI-1 partially reversed the artemisinin-induced G1 cell cycle arrest. Depletion of BMI-1 was able to intensifying the increment of p16 and the reduction of CDK4 induced by artemisinin. In addition, over-expression of BMI-1 was capable of attenuating the increasing p16 and decreasing CDK4 in cells treated with artemisinin. Taking together, the BMI1-p16/CDK4 axis was involved in the artemisinin-driven G1 arrest in nasopharyngeal carcinoma cells, and these results indicated that a potential treatment that the combination of artemisinin and BMI-1 downregulation could enhance the growth inhibitory affects on nasopharyngeal carcinoma cells. J. Cell. Biochem. 112: 1938–1948, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: BMI-1; ARTEMISININ; NASOPHARYNGEAL CARCINOMA; CELL CYCLE ARREST

Nasopharyngeal carcinoma (NPC) is endemic in southern china, south-east Asia and north Africa, and part of the Mediterranean basin where it is the third most common form of malignancy amongst men, with incidence rates of 80 per 100,000 [Caponigro et al., 2010]. The aetiology of NPC is complex, and includes a host of viral, genetic and environmental factors [Lo and Huang, 2002; Young and Murray, 2003; Tao and Chan, 2007]. Radiotherapy concomitant chemoradiotherapy is now acknowledged as being a standard treatment potion [Ruan et al., 2010], even though it induces a considerable incidence of acute mucosal and hematologic toxicity. The lack of therapeutics is a critical problem in the field. Therefore, a possible approach for developing clinically applicable chemotherapeutic agents is to screen traditional medicinal plants that have been used for thousands of years for their anticancer activities with few side effects [Jayaprakasam et al., 2003; Zhu et al., 2007; Jin et al., 2008; Yang et al., 2008].

Artemisinin is an endoperoxide-containing sesquiterpene isolated from the leaves of wormwood or "qinghao" [Klayman, 1985; Luo and Shen, 1987]. Artemisinin and its derivatives represent a very important new class of antimalarials [Chawira et al., 1987; Dutta et al., 1989]. Previous studies have shown that artemisinin induce growth arrest, and apoptosis. As well as, one study 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 [Chen et al., 2004; Efferth, 2006; Hou et al., 2008; He et al., 2009a; Willoughby et al., 2009]. Artemisinin also have been shown to be effective in killing cancer cells. National

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*Correspondence to: Zhenyu Zhu, Zhongshan Medical College, Sun Yat-sen University,74 zhongshan road 2, Guangzhou, 510080 Guangdong, People's Republic of China. E-mail: zhenyuzhu2008@hotmail.com
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Institutes of Health, showed that artesunate, the semisynthetic derivative of artemisinin, has anti-cancer activities against leukemia, colon, melanoma, breast, ovarian, prostate, central nervous system, and renal cancer cell lines [Efferth et al., 2001, 2004; Singh and Lai, 2004; Nakase et al., 2009; Riganti et al., 2009]. However, the effect of artemisinin on the proliferation of nasopharyngeal carcinoma cells remains unclear.

The molecular mechanism and gene expression changes that mediate the anti-proliferative activity of artemisinin are not well characterized. The BMI-1 gene was initially isolated as an oncogene which cooperates with c-Myc in retrovirus-induced B and T cell leukemia [Haupt et al., 1991]. The BMI-1 oncoprotein regulates proliferation and oncogenesis in human cells [Styczynski and Drewa, 2007; Dhawan et al., 2009; He et al., 2009b; Wang et al., 2009]. Consistent with its role in inhibiting $p16^{ink4a}$ and $p19^{arf}$ transcription, BMI-1 induces the bypassing of senescence [Molofsky et al., 2005; Song et al., 2006]. In recent years, it has been reported that BMI-1 is over-expressed in nasopharyngeal carcinoma cell lines at both mRNA and protein levels. Importantly, over-expression of BMI-1 was also observed in a significant number of nasopharyngeal carcinoma tumors, which correlated with advanced invasive stage of the tumor progression and poor prognosis [Song et al., 2006]. In this study, we examine the effect of artemisinin on the human nasopharyngeal carcinoma cell lines, well-differentiated CNE-1 cells and poorly differentiated CNE-2 cells [Teng et al., 1996]. We have discovered that artemisinin inhibited growth of human nasopharyngeal carcinoma cells by inhibiting cell proliferation and inducing G1 cell cycle arrest, to further investigate the anticancer mechanisms of artemisinin in nasopharyngeal carcinoma, we determined the effects of artemisinin on BMI-1 activity in nasopharyngeal carcinoma cells in combination with the treatment of artemisinin.

EXPERIMENTAL PROCEDURES

CELL CULTURE AND TREATMENTS

The CNE-1 and CNE-2 human nasopharyngeal carcinoma cell lines were grown in Dulbecco modified Eagle's medium (DMEM) (Gibco, Invitrogen Corporation, USA) supplemented with 10% fetal bovine serum (Gibco, Invitrogen Corporation), 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine and maintained (Gibco, Invitrogen Corporation) at 37°C in humidified air containing 5%CO_2. Cells (4×10^5) were grown in six-multiwell plates (35 mm diameter) (Corning, Corning Incorporated Life Sciences, USA) with 2 ml DMEM. Artemisinin (Sigma, Sigma–Aldrich, USA) was administered to cell cultures as DMSO solution. Artemisinin was dissolved in DMSO (Sigma, Sigma–Aldrich) at concentrations that were 1,000-fold higher than the final medium concentration. For the vehicle control, 1 μl of DMSO was added per 1 ml of medium.

MTT ASSAY

Cell growth inhibition was analyzed using the MTT assay. CNE-1 and CNE-2 (3×10^3) cells were seeded in 200 μl of DMEM medium into 96-well plates, and cultured at 37°C overnight. Then the medium was replaced with fresh medium containing different concentrations of artemisinin (0–300 μM). After a further incubation for 48 h, MTT (3-(4,5-dimethylthiazol-a-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, Sigma–Aldrich) (20 μl) was added to each well followed by 4 h incubation. The medium was discarded and 150 μl of DMSO wad added into each well, and incubated for 20 min. The absorbance was recorded at 570 nm with a microplate reader. The experiments were repeated thrice.

CELL VIABILITY

Trypan blue dye exclusion assay and cell counting were used to determine viable cell numbers of CNE-1 and CNE-2 cells. Cells were seed into 96-well plates at a density of 3×10^3 cells per well, and cultured at 37°C overnight. The cells were washed with PBS (Gibco, Invitrogen Corporation) and treated with medium containing various concentrations of artemisinin dissolved in DMSO (0–300 μM). Control plates had only the DMSO dose equaling amount added to the test well. After a further incubation for 48 h, a cell suspension was prepared by brief trypsinization (Gibco, Invitrogen Corporation). An equal volume of PBS was added and the suspension was mixed with 500 μl of 0.4% trypan blue solution (Sigma, Sigma–Aldrich) and left for 5 min at room temperature. The unstained cells (viable cells) and the total cells per square of the cell chamber (four squares per suspension) were counted using Neubauer cytometer and phase contrast microscopy (Leica, Leica Microsystems, Germany). All experiments were performed in triplicate.

RNA EXTRACTION AND SYBR GREEN qRT–PCR

Total RNA was isolated by using Trizol reagent (Invitrogen, Invitrogen Life Technologies, USA) according to manufacturer's instructions, RNA was quantified using spectroscopy and the quality of RNA was confirmed using A_{260}/A_{280} and by electrophoresis on 1% agarose gels (Biowest agarose, distributed by GENE TECH Company Limited, China). Reverse transcription was performed using Oligo (dT) 20 (TAKARA, TAKARA Biotechnology Co. Ltd, Japan) and M-MLV reverse transcriptase (TAKARA, TAKARA Biotechnology Co. Ltd). Two micrograms RNA was used for the synthesis of cDNA. cDNA samples were diluted 1:1,000 and 5 μl used in each 50 μl qRT-PCR reaction, containing 25 μl SYBR Green realtime PCR master-mix (TOYOBO, TOYOBO Biotech Co. Ltd, Japan). Transcript levels were analyzed on an ABIPrism-7500 Sequence Detector System (ABI, Applied Biosystems, USA). qRT-PCR primers were designed and are listed in Table I. Normalization was performed with β-actin control gene. The PCR products were run in an agarose gel and were in all cases confined to a single band of expected size. A melting-curve analysis was also performed to ensure specificity of products. The ABI 7500 system SDS software was used for threshold (Ct) data acquisition, dissociation analyses. Relative copy number values were calculated using ddCT study. All samples were amplified in triplicate and the mean used for subsequent analyzed.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

After the indicated treatments, cells were harvested with radioimmune precipitation lysis buffer containing protease inhibitor. Debris was removed by centrifugation at 10,000*g* for 10 min at 4°C. Equal amounts of total cellular protein were mixed with loading buffer and fractionated on 12% polyacrylamide SDS resolving gels

TABLE I. Primer Sequences and Size of qRT-PCR Products

Gene	Gene description	Primers (5′–3′)	Size (bp)
GAPDH	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase	F: GAACGGGAAGCTCACTGG R: GCCTGCTTCACCACCTTCT	123
β-Actin	Homo sapiens actin, beta	F: AACTGGGACGACATGGAGAAAAT R: ATAGCACAGCCTGGATAGCAACG	190
BMI-1	Homo sapiens BMI1 polycomb ring finger oncogene	F: TAAGCATTGGGCCATAGT R: ATTCTTTCCGTTGGTTGA	140
Cdk4	Homo sapiens cyclin-dependent kinase 4	F: TTGGTGTCGGTGCCTAT R: TCCAGTCGCCTCAGTAAA	157
Cdk2	Homo sapiens cyclin-dependent kinase 2	F: AGGAGTTACTTCTATGCCTGAT R: CCTGGAAGAAAGGGTGAG	179

by electrophoresis. Rainbow marker (Fermentas, Fermentas International, Inc., Canada) was used as the molecular weight standard. Proteins were electrically transferred on to polyvinylidene difuoride membranes (Millipore, Millipore, USA). The membranes were blocked overnight in Tris-buffered saline with Tween-20 with 5% nonfat dry milk (NFDM) (Gibco, Invitrogen Corporation) at 4°C. The membranes were incubated for 2 h in primary antibody in 5% NFDM at room temperature. The antibodies used were as follows, rabbit anti-CDK2, CDK4, CDK6, cyclin D1, cyclinE, p16, and p27 (Santa Cruz, Santa Cruz Biotechnology, Inc., USA); mouse anti-beta actin (PTG, Proteintech Group, Inc., USA); mouse anti-BMI1 (R&D, R&D Systems, USA). The primary antibody diluted 1:1,000 in 5% NFDM. This was followed by incubation with a secondary antibody (PTG, Proteintech Group, Inc.) conjugated with horseradish peroxidase diluted 1:3,000 in 5% NFDM. Equal protein loading was confirmed by the level of beta-actin. Proteins were visualized using Pierce ECL Western blotting substrate (Pierce, Thermo Scientific, USA).

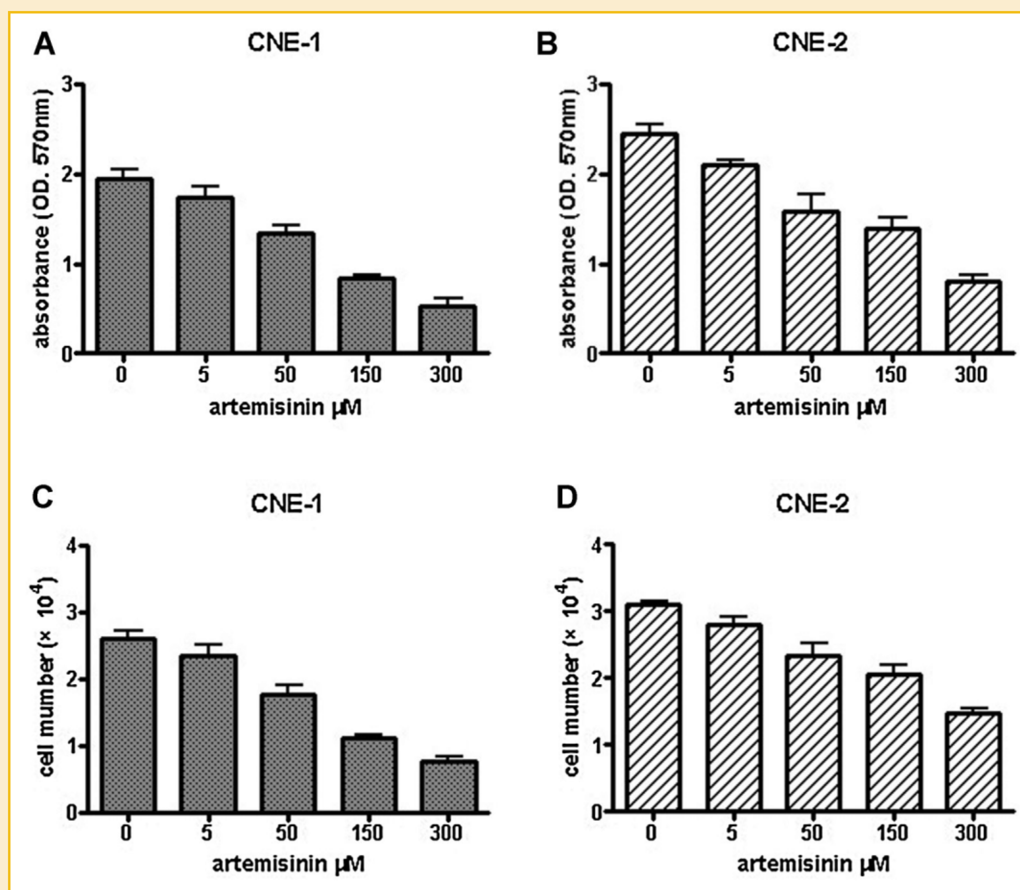

Fig. 1. Effect of artemisinin treatment on cell proliferation (MTT assay and cell counting). A,B: MTT assay. CNE-1 and CNE-2 cells were treated with various concentrations of artemisinin at 48 h. The data are presented as the mean ± SD of three independent experiments ($P < 0.05$). C,D: Cell counting and Trypan blue exclusion assay was performed to measure viable cell numbers of CNE-1 and CNE-2 cells, treated with artemisinin in various concentrations at 48 h. The data are presented as the mean ± SD of three independent experiments ($P < 0.05$).

FLOW CYTOMETRIC ANALYSIS OF DNA CONTENT

The cell cycle distribution was estimated from the flow cytometric profile of the DNA content. CNE-1 and CNE-2 cells were plated onto six-well tissue culture dishes. After treating with the indicated concentrations of artemisinin for 48 h, cells were trypsinized, washed in cold PBS, fixed in cool 75% ethanol overnight at 4°C. The fixed cells were stained with propidium iodide and analyzed by FACScalibur Flow Cytometer (BD, Becton Dickinson, USA).

GENE KNOCKDOWN

To knockdown BMI-1, CNE-1, CNE-2 cell lines were transfected with 50 nmol/L of BMI-1 targeted (siBMI1-A, 5′-AAAUGGACAUAC-CUAAUAC-3′; or siBMI1-B, 5′-CCAAUGGCUCUAAUGAAGA-3′), or negative control (siNS) small interfering RNA (siRNA) oligonucleotides (5′-ACGCATGCATGCTTGCTTT-3′) (Invitrogen, Invitrogen Life Technologies).

cDNA CLONING AND LIPOSOMAL TRANSFECTION

The total RNA isolated from LX2 human hepatic stellate cells. cDNA was synthesized by reverse transcription as described above. To obtain the insert BMI-1 DNA, PCR reaction was performed by using Pfu DNA polymerase (Fermentas, Fermentas International, Inc.). The cloning primers (sense, 5′-GAGCTCGAGATGCATCGAACAA-CGAGAATC-3′; antisense, 5′-GAAGGATCCTCAACCAGAAGAA-GTTGCTGATG-3′) were designed to carry *Xho*l I and *Bam*H1 restriction enzyme recognition sites. After *Xho*l I–*Bam*H1 (Fermentas, Fermentas International, Inc.) double digestion, BMI-1 and pIRES2-EGFP vector DNA were isolated from agarose gel containing. DNA ligation reaction was catalyzed by T4 DNA ligase (Fermentas, Fermentas International, Inc.). After heat-shock transformation. After heat-shock transformation of the ligation reaction to DH5α *Escherichia coli* competent cells, colonies were selected by means of kanamycin resistance. Four recombinant clones were obtained in a single cloning experiment. Following the isolation of plasmid, the presence of inserted BMI-1 sequence was determined by *Xho*l I–*Bam*H1 re-digestion. Recombinant hBMI1-IRES2-EGFP construct is designated as hBMI-1 whereas empty vector is designated as pIRES2. CNE-1 and CNE-2 cells were transfected with recombinant hBMI1-IRES2-EGFP construct using Lipfectamine 2000 (Invitrogen, Invitrogen Life Technologies) according to the manufacturer's protocol.

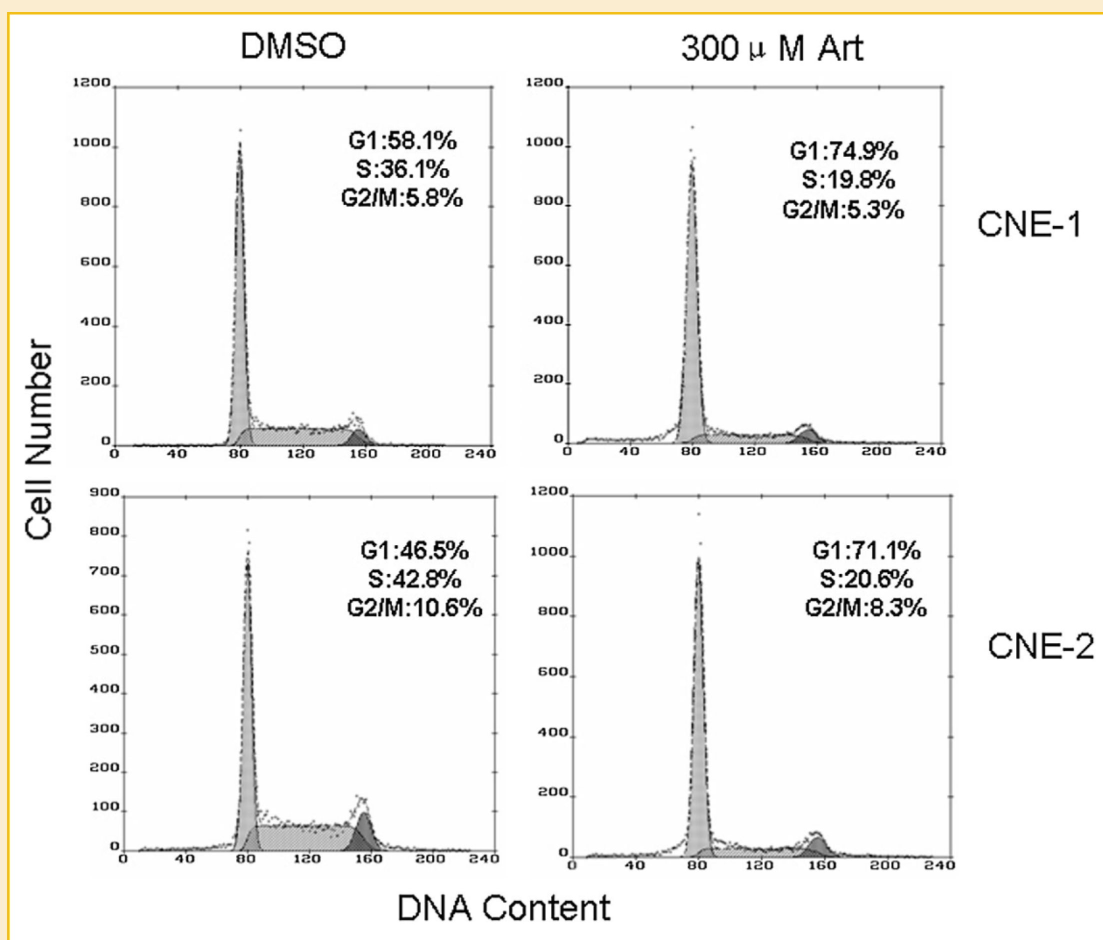

Fig. 2. Artemisinin induces a G1 cell cycle arrest in CNE-1 and CNE-2 cells. CNE-1 and CNE-2 cells were plated in six-well culture plates and treated for 48 h with vehicle control or 300 μM artemisinin. Cell were harvested and hypotonically lysed in a propidium iodide solution to stain the DNA. Nuclei were analyzed for DNA content by flow cytometry.

RESULTS

ARTEMISININ INHIBITS CELL GROWTH OF CNE-1 AND CNE-2 CELLS IN VITRO

The effect of artemisinin on the cellular proliferation of CNE-1 and CNE-2 cells was determined with MTT assay and cell counting. As shown in Figure 1, artemisinin reduced the cellular proliferation of two types of cells incubated with artemisinin for 48 h in a dose-dependent manner, compared with the DMSO vehicle control. Whereas, growth inhibition without adversely affecting cell viability occurred at 300 μM artemisinin. These reductions in cell numbers were not associated with increased cell death, as assessed by trypan blue dye exclusion. Treatment with concentration of artemisinin at 400 μM had a cytotoxic effect on these cells. As the best growth inhibition without cytotoxicity was observed when CNE-1 and CNE-2 cells were treated with 300 μM artemisinin for 48 h, we chose these experimental conditions for further investigations. The results suggest that artemisinin has a potent anti-proliferative effect on human nasopharyngeal carcinoma cells in vitro.

Artemisinin inhibits proliferation of CNE-1 and CNE-2 cells by inducing a G_1 cell cycle arrest To initially examine the cell cycle effects of artemisinin in human nasopharyngeal carcinoma cells, well-differentiated CNE-1 cells and poorly differentiated CNE-2 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 Figure 2, 48 h-treatement of CNE-1 and CNE-2 cells with artemisinin resulted in G1 distribution rates of 74.9% and 71.1%. The G1 distribution of the CNE-1 and CNE-2 cells was 58.1% and 46.5% at 48 h after treatment with DMSO vehicle control. Artemisinin-treated nasopharyngeal carcinoma cells from both cell lines exhibited increases in the percentage of cells in G1 phase of the cell cycle and decreases in the percentage of cells present in S phase. The data demonstrate that the anti-proliferative effect of artemisinin is a general property of this phytochemical in human nasopharyngeal carcinoma cells in that both poorly differentiated and well-differentiated cell lines similarly respond to this phytochemical by undergoing a G_1 cell cycle arrest.

ARTEMISININ MODULATES THE EXPRESSION OF KEY REGULATORS IN G1 CELL CYCLE PROGRESSION

Because CDK2, CDK4, CDK6, cyclin D1, cyclinE, p16, and p27 play essential roles in the regulation of G1 phase cell cycle progression, the expression levels of these components were analyzed in CNE-1 and CNE-2 cells were treated with increasing doses of artemisinin for

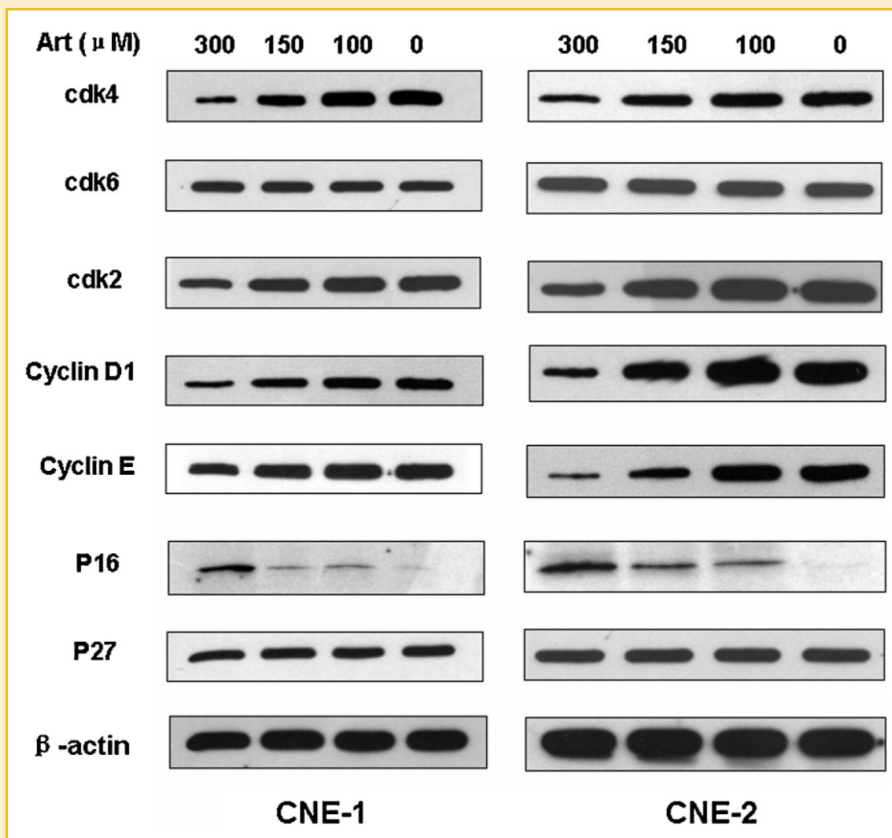

Fig. 3. Western blot analysis of artemisinin effects on expression of G1 cell cycle components. CNE-1 and CNE-2 cells were treated with the decreasing concentration of artemisinin for 48 h. Total cells lysates were electrophoretically fractionated by SDS–PAGE and expression of cell cycle proteins analyzed by Western blots. β-actin was used as loading control.

48 h. As shown by immunoblot analysis, artemisinin treatment resulted in downregulation of cdk4, cdk2, cyclin D1, cyclin E, and upregulation of p16, with no significant change in the expression of cdk6 and p27 (Fig. 3). The data documented the modulation effects of artemisinin on key regulators in G1 phase cell cycle progression in human nasopharyngeal carcinoma cells.

EFFECT OF ARTEMISININ ON BMI-1

The BMI-1 oncoptotein is over-expressed in nasopharyngeal carcinoma cell lines, and BMI-1 regulates proliferation and oncogenesis in human cells. To investigate the mechanism by which CNE-1 and CNE-2 G1 cell growth was inhibited, we analyzed the mRNA levels of BMI-1 by qRT-PCR and Western blot analysis. As shown in Figure 4, CNE-1 and CNE-2 cells were cultured with increasing doses of artemisinin for 48 h, immunoblotting and reverse transcription-RCR analyses revealed that artemisinin treatment down-regulated expression of BMI-1 protein and transcript by 48 h. Expression of BMI-1 was a dose dependent decrease for cultures with artemisinin.

BMI-1 KNOCKDOWN MADE THE CELLS MORE SENSITIVE TO ARTEMISININ

siRNA (siBMI1) targeting the BMI-1 gene was tested for silencing in CNE-1 and CNE-2 cells. Using qRT-PCR, we determined the levels of BMI-1 mRNA 48 h after siRNA transfection. siBMI1 was determined to be effective. It resulted in a 90% and 87% decrease of BMI-1 mRNA level in CNE-1 and CNE-2 cells (Fig. 5). Western blot analysis was used to confirm that siBMI1 was also able to reduce BMI-1 protein expression. To examine the effect of artemisinin on the survival of BMI-1 knock-down cells, cell counting and trypan blue dye exclusion assay were performed after the CNE1-siBMI1, CNE2-siBMI1, CNE1-siNS, CNE2-siNS cells were treated with or without 300 μM artemisinin for 48 h. After treatment with artemisinin, we found that both the CNE1-siBMI1 and CNE2-siBMI1 cells showed lower cell viabilities than the negative control siRNA (siNS) transfected cells. These results indicated that BMI-1 knockdown made the cells more sensitive to artemisinin.

DEPLETION OF BMI-1 ENHANCED ARTEMISININ-INDUCED G1 CELL CYCLE ARREST AND COULD REGULATE THE EXPRESSION LEVELS OF p16 AND CDK4

In order to evaluate the effect of BMI-1 knockdown on the induction of G1 cell cycle arrest, the CNE1-siBMI1, CNE2-siBMI1, CNE1-siNS, CNE2-siNS cells were treated with 300 μM artemisinin for 48 h and subjected to flow cytometry. As shown in Figure 6, depletion of BMI-1 caused increases of artemisinin-induced G1 phase of the cell cycle arrest compared with negative control siRNA transfected cells. The data showed that depletion of BMI-1 enhanced artemisinin-induced G1 cell cycle arrest. To further explore the mechanism underlying the enhancement of artemisinin-induced G1 cell cycle arrest, we examined the expression levels of p16 and CDK4 in the CNE1-siBMI1, CNE1-siNS, CNE2-siBMI1,CNE2-siNS cells. Because CDK4 activity occurs primarily in early stages of the G1 cell cycle. BMI-1 has been shown to immortalize nasopharyngeal epithelial

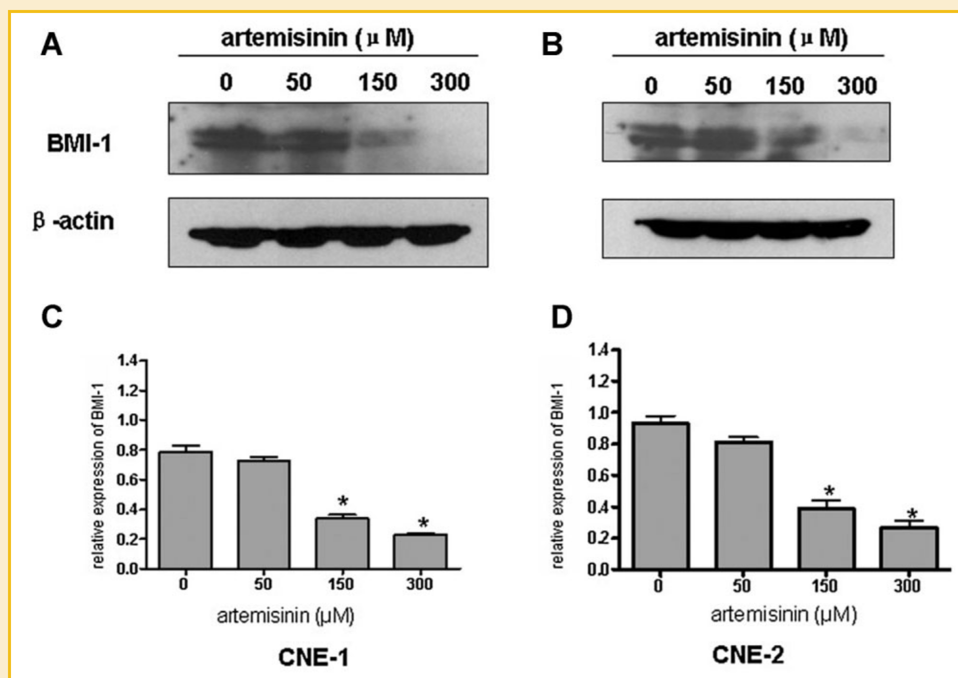

Fig. 4. Artemisinin down-regulates expression of protein and transcript of BMI-1. CNE-1 and CNE-2 cells were treated with increasing doses of artemisinin for 48 h. A,B: The treated CNE-1 and CNE-2 cells were harvested and lysed. The lysates of the cells were electrophoretically fractionated by SDS-PAGE and analyzed by Western blot. β-Actin was used as loading control. C,D: Relative expression were measured by qRT-PCR and normalized to β-actin mRNA. Data represent the mean ± SD (n = 3). $^{*}P < 0.05$ when compared with cultures with DMSO vehicle control.

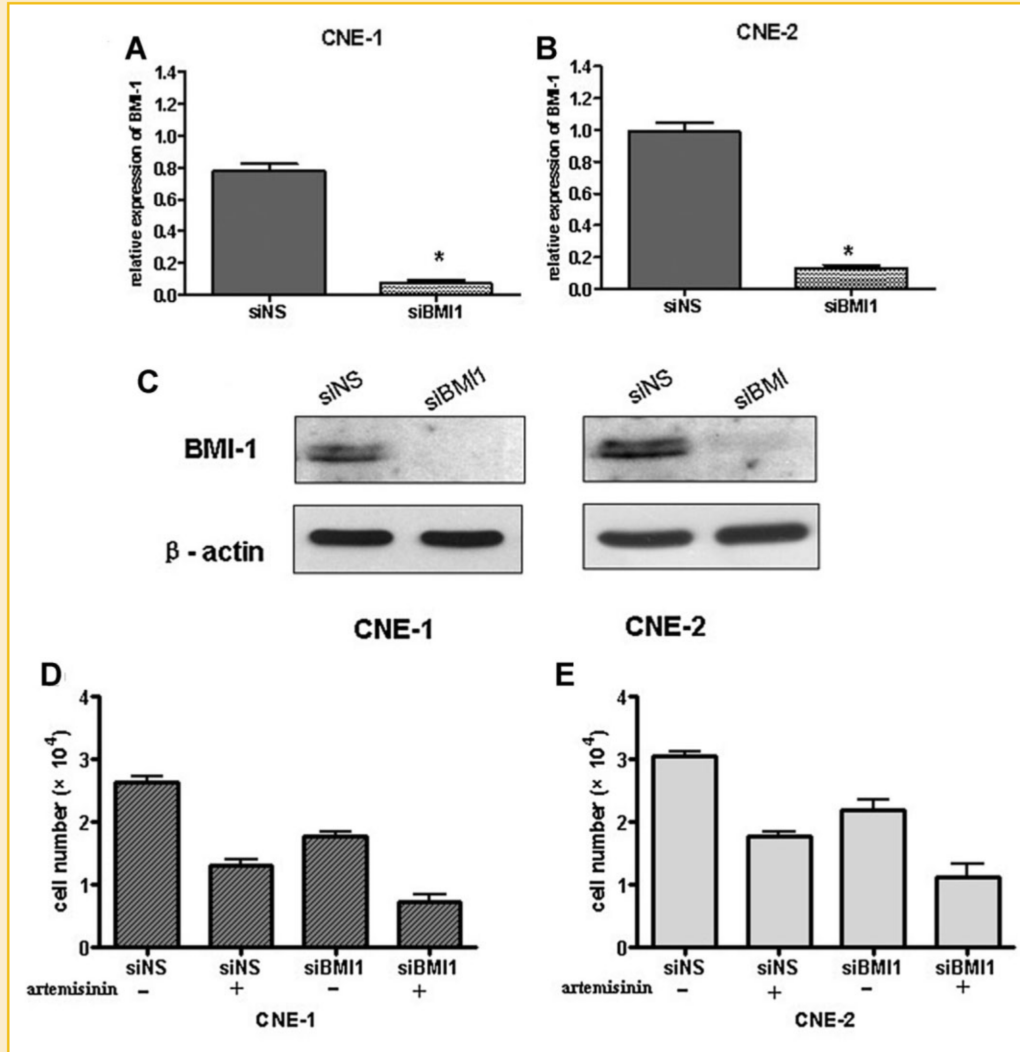

Fig. 5. Effect of BMI-1 downregulation on the chemosensitivity of CNE-1 and CNE-2 cells to artemisinin treatment. A,B: Effect of BMI-1 siRNA in CNE-1 and CNE-2 cells. Relative expression were measured by qRT-PCR and normalized to β-actin mRNA. Data represent the mean ± SD (n = 3). $^{*}P < 0.05$ when compared with cultures with the negative control siRNA. C: The protein expression of BMI-1 was detected by Western blot analysis. Triplicate experiments showed consistent results. D,E: Cell counting and Trypan blue exclusion assay was performed to measure viable cell numbers. CNE1-siNS, CNE1-siBMI1, CNE2-siNS, CNE2-siBMI1 cells were treated with or without artemisinin for 48 h. The data are presented as the mean ± SD of three independent experiments ($P < 0.05$).

cells by inhibiting $p16^{INK4A}$. And the $p16^{INK4A}$ has been demonstrated to be important target of BMI-1. Our results showed that knockdown of endogenous BMI-1 led to significantly increased expression of p16. Consistent with this increase in the p16 level, Western blot analysis showed substantial reduction in the levels of CDK4 in BMI1-knocked down cells exposed to artemisinin.

EXPRESSION LEVELS OF EXOGENOUS BMI-1 PARTIALLY REVERSE THE ARTEMISININ-INDUCED G1 CELL CYCLE ARREST

To test whether the artemisinin-mediated cell cycle arrest could be reversed by elevated levels of expressed BMI-1, CNE-1, and CNE-2 cells were transfected with the IRES2-EGFP plasmids containing cDNA of BMI-1, as well as with the empty expression vector control. Cells were treated with artemisinin for 48 h and subjected to flow cytometry. As shown in Figure 7, over-expression of BMI-1 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 S phase DNA content compared with the IRES2-EGFP control transfected cells. Western blot analysis showed over-expression of BMI-1 could inhibit p16 and increase the expression levels of CDK4. The data documented that BMI-1 over-expression partially reverses artemisinin-induced G1 cell cycle arrest. And that artemisinin-mediated increase in p16 expression and decrease in CDK4 expression is partly responsible for the observed G1 cell cycle arrest in CNE-1 and CNE-2 cells.

DISCUSSION

Nasopharyngeal carcinoma differs from other head and neck cancers in its aetiology, epidemiology, and potential therapeutic

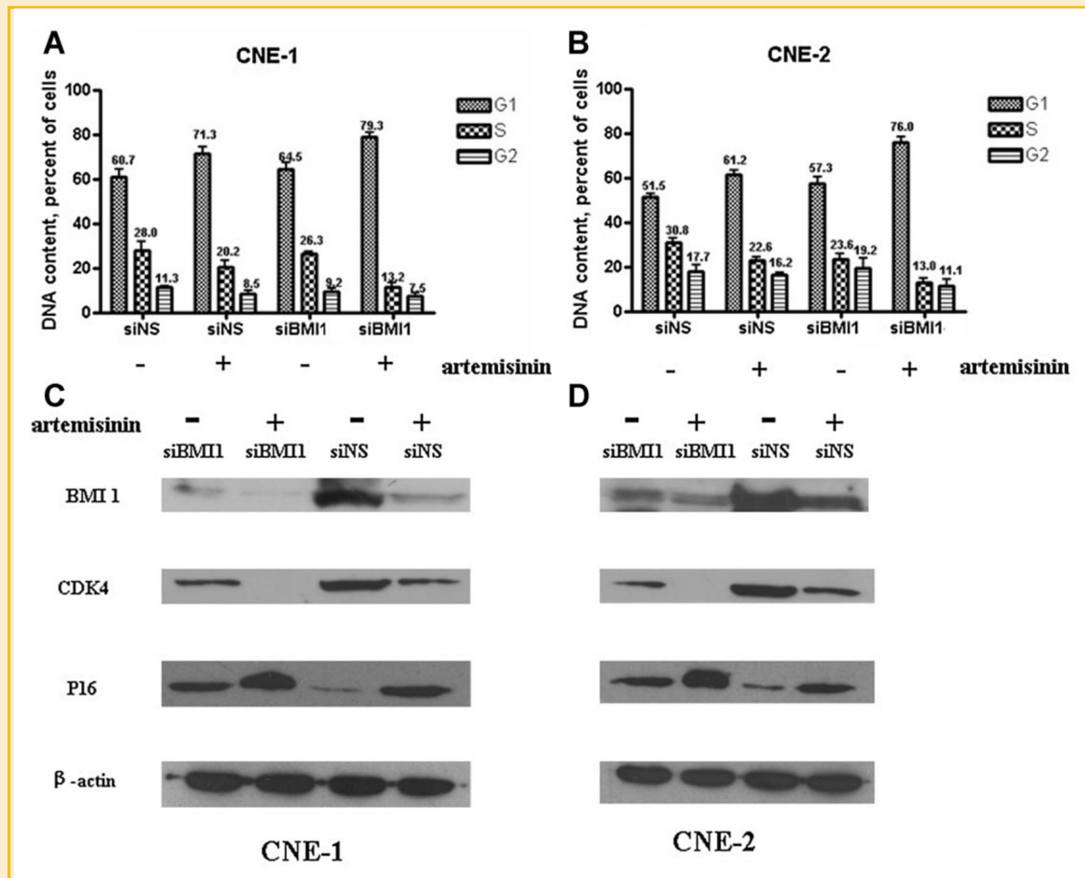

Fig. 6. Depletion of BMI-1 caused increases of artemisinin-induced G1 cell cycle arrest and regulated the expression levels of p16 and CDK4. A,B: CNE-1 and CNE-2 cells were transfected with siBMI1 as well as with the negative control siRNA. Transfected cells were treated with DMSO vehicle control or 300 μM artemisinin 48 h, subjected to subsequent flow cytometric analysis. C,D: Transfected cells were treated with DMSO vehicle control or 300 μM artemisinin 48 h. The protein expression of BMI-1, CDK4, and p16 was detected by Western blot analysis. Experiments were performed in triplicate per treatment. The bar graphs with SE results form this analysis.

options. Concomitant chemoradiotherapy is now acknowledged as being a standard treatment potion, even though it induces a considerable incidence of acute mucosal and hematologic toxicity. The lack of treatment of patients with refractory nasopharyngeal carcinoma and the management of long-term toxicities highlights the necessity to identify new potent anti-cancer compounds. Artemisinin is an effective novel anti-malarial drug with low toxicity. Although it has been reported that artemisinin inhibits cell proliferation in many types of cancers, it is still not known whether artemisinin work as growth inhibitors in nasopharyngeal carcinoma cells. Here we demonstrate that artemisinin may induce cell growth arrest in nasopharyngeal carcinoma cell lines, CNE-1 and CNE-2. Artemisinin can induce dose-dependent growth arrest. And that this anti-proliferative response is due to G1 cell cycle arrest as reported in other cancer cells [Firestone and Sundar, 2009; Chen et al., 2010; Gravett et al., 2010; Morrissey et al., 2010]. Therefore, the anti-proliferative effects of artemisinin represent a general property in distinct types of human nasopharyngeal carcinoma cells, well-differentiated CNE-1 cells and poorly differentiated CNE-2 cells, and not a cell line-specific effect.

It has been well documented that cell cycle is primarily regulated by complexes containing CDKs and cyclins, which are critical for progression of cell cycle and their inactivation leads to cell cycle arrest [Hirai et al., 1995; Baghdassarian and Ffrench, 1996; Ferguson et al., 2000; Yokota et al., 2007]. The complexes responsible for the progression of cells through the G1 cell cycle include cyclin D-CDK4/CDK6 and cyclin E-CDK2 complexes [Reed et al., 1994; Mayol et al., 1996]. Most anti-cancer agents that induce a G1 arrest in cancer cells typically do so through decreased enzymatic activity of CDKs through the increased expression of cyclin-dependent kinase inhibitors, p27 and $p16^{INK4a}$ [Ye et al., 2007; Miao et al., 2009]. The inhibitory effects of artemisinin on CDK2, CDK4, cyclin D1, cyclinE were observed, with its upregulating effects on p16. Artemisinin treatment had no effect on expression of p27 and CDK6. Our data demonstrated that artemisinin arrests nasopharyngeal carcinoma cells in G1 phase of cell cycle via modulating cell cycle regulatory molecules suggesting yet another important molecular mechanism through which artemisinin inhibits the growth of nasopharyngeal carcinoma cells.

The BMI-1 oncoprotein plays an important role in the development and progression of nasopharyngeal carcinoma, and that BMI-1 is a valuable marker for assessing the prognosis of nasopharyngeal carcinoma patients [Song et al., 2006]. The $p16^{INK4a}$ has been demonstrated to be important target of BMI-1 [Silva et al.,

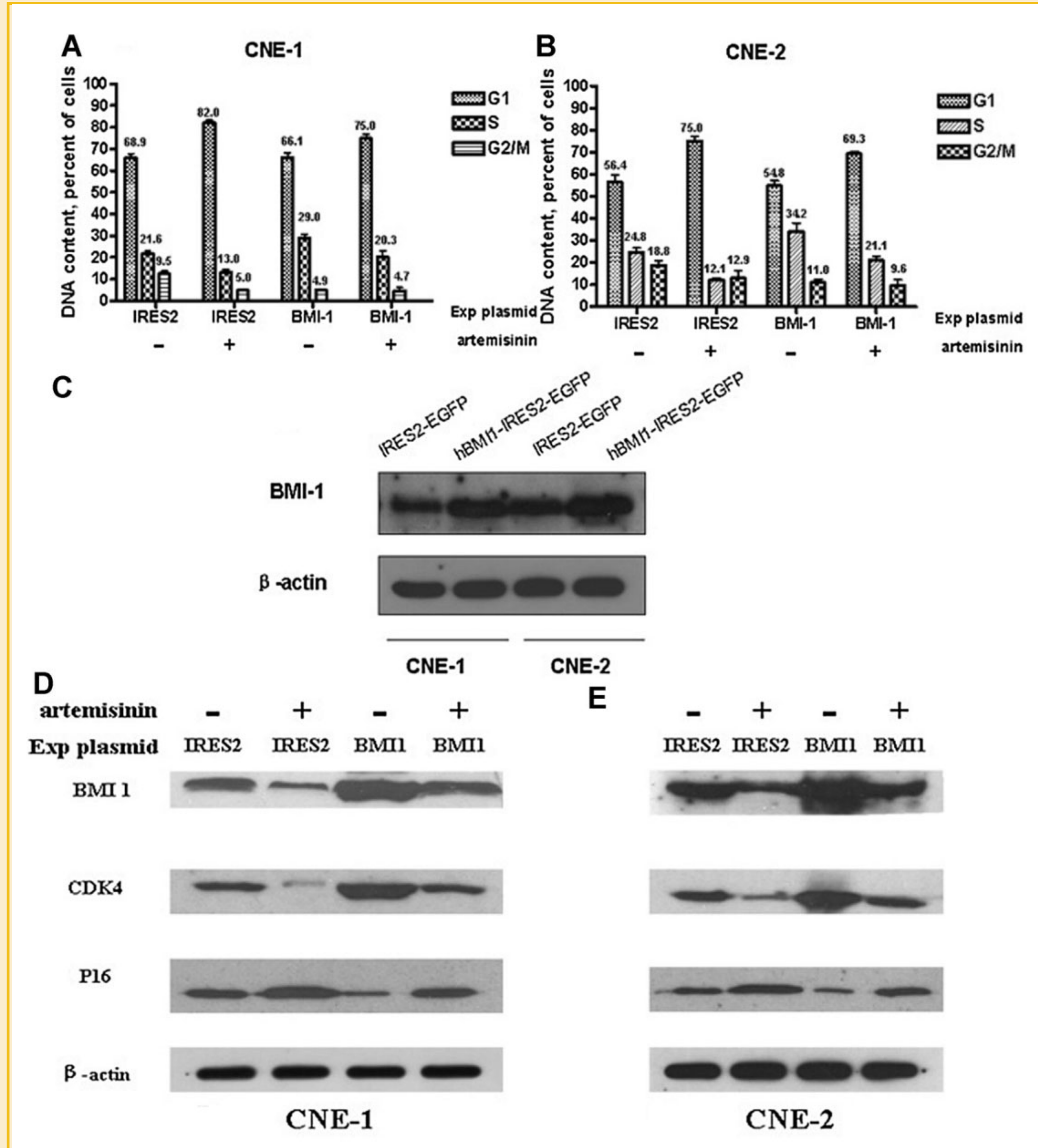

Fig. 7. BMI-1 over-expression partially reverses artemisinin-induced G1 cell cycle arrest and regulated the expression levels of p16 and CDK4. A,B: CNE-1 and CNE-2 cells were transfected with IRES2-EGFP constitutive expression vectors for BMI-1 as well as with the IRES2-EGFP empty vector control. Transfected cells were treated with DMSO vehicle control or 300 μM artemisinin 48 h, subjected to subsequent flow cytometric analysis. C: Western blots demonstrated the over-expression of BMI-1 in the appropriate cells. D,E: Transfected cells were treated with DMSO vehicle control or 300 μM artemisinin 48 h. The protein expression of BMI-1, CDK4, and p16 was detected by Western blot analysis. Experiments were performed in triplicate per treatment. The bar graphs (A,B) with SE results form this analysis.

2006]. Therefore, BMI-1 could promote cell proliferation by suppressing p16/Rb (retinoblastoma protein) tumor suppressor pathways [Jacobs et al., 1999; Guo et al., 2007; Dhawan et al., 2009]. In most normal and tumorigenic mammalian systems, cell proliferation is highly regulated in the G1 phase of the cell cycle. CDK4 activity occurs primarily in early stages of the G1 cell cycle. $p16^{INK4a}$ protein could inactivate CDK by directly binding to CDK4 and CDK6, and lead to the cell cycle arrest in the G1/S phase [Bartkova et al., 1996].

In the present study, CNE-1 and CNE-2 cells, in which BMI-1 is highly expressed, were used in our study. We observed that artemisinin down-regulated expression of BMI-1 protein and transcript, and the depletion of BMI-1 in these cells resulted in an increased sensitivity of these cells to artemisinin. Further FACS revealed that silencing BMI-1 expression could enhance artemisinin-induced cell cycle arrest, and the inhibitory ability of artemisinin could be attenuated by BMI-1 over-expression. But we did not find a more significant decrease in cell number in cells

treated with artemisinin and siBMI-1. Obviously BMI-1 was already decreased by artemisinin, so the additive effect of siBMI-1 was limited. CNE-1 and CNE-2 cell lines are both cancer cell lines, the growth of cancer cells is fast, so over-expression of BMI-1 could not induce to a more significant decrease in G1 phase in cells. These results indicated that artemisinin signaling pathways inhibit nasopharyngeal carcinoma cell growth in part by targeting BMI-1 thereby induced a G1 block in cell cycle progression. Which pathway was responsible for the artemisinin-induced G1 cell cycle arrest? The following experiments were focused on artemisinin regulation of p16 and CDK4 expression because the artemisinin treatment had no effect on expression of p27 and CDK6. We examined the expression levels of p16 and CDK4 in the CNE1-siBMI1, CNE1-siNS, CNE2-siBMI1, CNE2-siNS cells, that knock-down of endogenous BMI-1 led to significantly increased expression of p16. Consistent with this increase in the p16 level, Western blot analysis showed substantial reduction in the levels of CDK4 in BMI1-knocked down cells exposed to artemisinin. Furthermore, over-expression of BMI-1 could inhibit p16 and increase the expression levels of CDK4. Together, these results indicated that the BMI1-p16/CDK4 axis was involved in the artemisinin-driven G1 arrest in nasopharyngeal carcinoma cells. This evidence implicates the role of BMI-1 and suggests that block BMI-1 activation could be an effective method to combat the growth of nasopharyngeal carcinoma.

In conclusion, we report for the first time the anticancer potential of the combination of artemisinin treatment and BMI-1 depletion. The present study demonstrated the effect of artemisinin on cell cycle arrest in human nasopharyngeal carcinoma cells and down-regulated BMI-1. The knockdown of BMI-1 makes cancer cells more sensitive to artemisinin-induced cell cycle arrest and BMI-1 over-expression partially reverses artemisinin-induced G1 cell cycle arrest. Furthermore, we demonstrated that the knockdown and over-expression of BMI-1 regulates the expression level of p16 and CDK4. As such, artemisinin has the potential to be developed as a potent anti-nasopharyngeal carcinoma therapeutic. Further examination of BMI-1 will be necessary to characterize possible artemisinin-mediated changes, the combination of artemisinin and BMI-1 depletion might be a potent clinical strategy for caner therapeutic.

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