Regular Article

Artemether Attenuates the Progression of Non-small Cell Lung Cancer by Inducing Apoptosis, Cell Cycle Arrest and Promoting Cellular Senescence

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Lung cancer is the most common cause of cancer death, approximately 85% of which are non-small cell lung cancer (NSCLC). Here we found that artemether (ART), a natural derivative of artemisinin, significantly inhibits the proliferation of NSCLC cells in a dose- and time-dependent manner. We also demonstrated that high concentration of ART induces apoptosis in NSCLC cells through down-regulating the level of anti-apoptotic protein B-cell lymphoma-2 (Bcl-2), cellular inhibitor of apoptosis protein 1 (cIAP1) and cellular inhibitor of apoptosis protein 2 (cIAP2). While low concentration of ART inhibits the mRNA level of cell cycle related genes including cyclin-dependent kinase 1 (CDK1), cyclin-dependent kinase 2 (CDK2), cyclin-dependent kinase 6 (CDK6), cyclin A2, cyclin B1 and cyclin D1, leading to cell cycle arrest in NSCLC cells. Moreover, we confirmed that low concentration of ART induces DNA double-stranded breaks (DSBs), as well as promoting cellular senescence in NSCLC cells by up-regulating the mRNA and protein level of p16. Taken together, ART represents a promising new anti-NSCLC drug candidate that could attenuate progression of NSCLC cells in a p53-independent manner through inducing apoptosis, cell cycle arrest and promoting cellular senescence.

Key words  artemether; non-small cell lung cancer; apoptosis; cell cycle arrest; cellular senescence

INTRODUCTION

Lung cancer is the most common cause of cancer death, approximately 85% of which are non-small cell lung cancer (NSCLC).1,2 Currently, chemotherapy is one of the most effective treatment for NSCLC, and cisplatin-based drugs are the most efficient chemotherapeutic agents for NSCLC treatment.3 However, these chemotherapeutic agents also show highly toxic to the treated patients.4 In addition, drug resistance often appears after prolonged treatment, and the resistance toward cisplatin is multifaceted as it involves multiple cellular pathways.5–7 Therefore, it is very necessary to seek new chemotherapeutic drugs for NSCLC treatment.

Cellular senescence is a state of irreversible cell growth arrest on cells in response to all kinds of stressors. The senescent cell is characterized by cell cycle arrest, accompanied by specific changes in morphology, along with changes in some genetic and proteomic biomarkers, especially by the presence of acidic senescence-associated-beta-galactosidase (SA-β-gal) activity.8 Cellular senescence has been considered as an important factor in aging and age-related disease, and it is a seductive target for therapeutic exploitation.9 Increasing studies have revealed that cellular senescence leads to tumor suppression,8,9 and only a few anti-tumor agents have been developed to promote cellular senescence. Therefore, developing new agents to disturb cancer cell proliferation by promoting cellular senescence is becoming a research focus.10

Over the past few decades, increasing number of natural compounds and their derivatives, acquired from traditional medicinal plants, have shown their efficacy to treat numerous human diseases, such as cancer. Artemisinin, a natural compound extracted from the plant Artemisia annua L., is a famous efficient antimalarial drug,11 which is revealed that also has an excellent anti-tumor effect.12 Our previous research also demonstrated that artemisitene, a derivative of artemisinin has strong anti-cancer activity in multiple cancers.13 Artemether (ART, Fig. 1A) is one of the derivatives of artemisinin, which can effectively resist against both Plasmodium falciparum and cerebral malaria as well as has a long history of clinical use.14 Currently, it is reported that ART has a marked antitumor effect on various types of cancer, but only few researches about the anti-lung cancer effect of ART are reported so far.14–16 Here, we identify that ART represents a promising new anti-NSCLC drug candidate that could attenuate progression of NSCLC cells in a p53-independent manner through inducing apoptosis, cell cycle arrest and promoting cellular senescence.

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MATERIALS AND METHODS

Cell Cultures  All the cells were cultured as we described previously.13)

Cell Viability Assay  The cell viability was detected by cell counting kit-8 (CCK-8) as we described previously. The cytotoxicity of ART was investigated by treating with different concentrations (20, 40 and 80 µM) of ART in detected cells for 48 and 72 h before being assessed for cell viability.

Colony Formation Assay  These experiments were performed as we described previously. 13)

Apoptosis Analysis  A549 and NCI-H1299 cells were seeded on 6-well culture plates and treated with or without ART (80 µM) for 72 h. After stimulation, cells were harvested, and the apoptotic ratio was determined as we described previously. 13)

Western Blot Analysis  A549 and NCI-H1299 cells were seeded on 6-well culture plates and treated with or without ART (40 or 80 µM) for 72 h. After stimulation, whole-cell extracts were prepared in RIPA buffer, and separated by 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Millipore, U.S.A.). The following antibodies were used in this study: rabbit monoclonal anti-cellular inhibitor of apoptosis protein 1 (cIAP1) (7065S; CST, U.S.A.), rabbit monoclonal anti-cellular inhibitor of apoptosis protein 2 (cIAP2) (3130S; CST), rabbit monoclonal anti-apoptotic protein including B-cell lymphoma-2 (Bcl-2) (ab32124; Abcam, U.K.), rabbit monoclonal anti-Phospho-histone H2AX (9718S; CST), mouse monoclonal anti-p16INK4a (ab54210, Abcam), mouse monoclonal anti-beta-actin (66009-1-Ig; Abcam), and anti-mouse immunoglobulin G (IgG) HRP-linked antibody (7074S; CST), anti-mouse IgG HRP-linked antibody (7076S; CST). Quantification of band intensities by densitometry was determined by Image J software.

Cell Cycle Analysis  A549 and NCI-H1299 cells were seeded on 6-well culture plates and treated with or without ART (40 µM) for 72 h. After stimulation, cells were harvested, and the cell cycle ratio was determined by cell cycle staining kit (MultiSciences, China) and flow cytometry.

Quantitative RT-PCR (qPCR) Assay  A549 and NCI-H1299 cells were seeded on 6-well culture plates and treated with or without ART (40 µM) for 72 h. After stimulation, total RNA was isolated by Trizol reagent (TaKaRa, Fig. 1. ART Inhibits Proliferation and Colony Formation of NSCLC Cells

(A) Structure of Artemether (ART). (B, C) NSCLC cells (A549, NCI-H1299 cells) and normal branch epithelial cells (BEAS-2B) were treated with varying concentrations (20, 40 and 80 µM) of ART for 48 h (B) and 72 h (C), cell viability was determined by CCK-8 assay, n = 6. (D, E) A549 and NCI-H1299 cells were treated with 40 µM of ART for 12 d, and representative photographs of colony formation are shown, n = 3. Error bars indicate mean ± S.E.M. The statistical differences were assessed using one-way ANOVA analysis. ** p < 0.01 vs. Vehicle.

Table 1. qPCR Primers (Related to Figs. 3, 5)

| Genes    | Primer sequences (5’ to 3’) (forward; reverse) |
|----------|-----------------------------------------------|
| CDK1     | TGAGGTAGTAACACTCTGGTA; ATGCTAGGCTTCCTGGTT     |
| CDK2     | CCTGGACACTGAGACTGA; CCGATGAGAATGGCAGAA        |
| CDK6     | CTTCAATTCAACCCGAGTATG; TGGACTGAGACAGGACTT     |
| Cyclin A2| ACAGCCAGACATCACTAAC; GGAAGACAGGAACCTATCAA     |
| Cyclin B1| TTGGTTGATCTGCCTTCTC; TCTGACTGCTTGGCTT        |
| Cyclin D1| CCTCGGTTGCTCTTACCTCA; CTCTCGGACTTCTGGTC       |
| p16      | ACGACCCGATAGTTACG; ATGGTTACTGCTCTGTT         |
| ACTB     | TCGTGGTGATACATGAA; AAGGAAGGCCCTGGAAGT         |
Japan) and reversely transcribed to cDNA. The primer sequences used in qPCR were shown in Table 1 and qPCR was carried out using the two-step real-time PCR system. mRNA expression was normalized to β-Actin.

Cellular Senescence Assay A549 and NCI-H1299 cells were seeded on 12-well culture plates and treated with or without ART (40 µM) for 72 h. After stimulation, the senescent cells were tested by Senescence β-Galactosidase Staining Kit.

Statistical Analysis Statistics were calculated and analyzed by GraphPad Prism 5. All values presented are the mean ± standard error of the mean (S.E.M.). p < 0.05 is considered statistically significant.

RESULTS

ART Inhibits Proliferation and Colony Formation of NSCLC Cells To evaluate the anti-NSCLC activity of ART, we investigated the effect of ART on the viability of human NSCLC cells (A549 and NCI-H1299). As shown in Figs. 1B and C, ART exhibited strong inhibitory effect on the proliferation of both A549 and NCI-H1299 cells in a dose- and time-dependent manner, among which NCI-H1299 cells were more sensitive to ART. P53 gene is null in NCI-H1299 cells, indicating that the inhibitory effect of ART in NSCLC cells was not dependent on p53 status. In addition, we also tested the cytotoxicity of ART at the same conditions in normal bronchial epithelial cell line BEAS-2B, and observed that normal cells were not sensitive to ART (Figs. 1B, C). These results indicated that ART exhibits selective sensitivity on NSCLC cells. Furthermore, according to the colony forming assay, we found that low concentration of ART significantly inhibited the colony formation of detected NSCLC cells (Figs. 1D, E). Taken together, we reveal that ART not only has inhibitory effect on the proliferation of NSCLC cells but also has a excellent selectivity against NSCLC cells over the normal cells.

High Concentration of ART Significantly Induces Apoptosis in NSCLC Cells Since the changes of cell viability can be caused by cell apoptosis, we speculated that ART may have these effect on NSCLC cells. Apoptosis analysis was used to assess the effect of ART on inducing apoptosis of NSCLC cells. Our results showed that high concentration of ART (80 µM) treatment for 72 h led to apoptosis in detected NSCLC cells (Figs. 2A, B). We further detected the protein level of anti-apoptotic protein including Bcl-2, cIAP1 and cIAP2, and found out all of these protein were down-regulated after ART (80 µM) treatment for 72 h (Figs. 2C, D). These findings suggested that high concentration of ART induced apoptosis through down-regulating the level of anti-apoptotic protein Bcl-2, cIAP1 and cIAP2 in NSCLC cells.

Low Concentration of ART Induces Cell Cycle Arrest and DNA Damage in NSCLC Cells Next, we further detected the effect of ART on cell cycle of NSCLC cells. The results showed that treated with low concentration of ART (40 µM) for 72 h led to increasing cell populations at the S phase in A549 (data not shown) and NCI-H1299 cells (Fig. 3A), which suggested that low concentration of ART induced cell cycle arrest in detected NSCLC cells. To understand the mechanism how ART induces cell cycle arrest, we detected the mRNA level of cell cycle related genes including cyclin-dependent kinase 1 (CDK1), cyclin-dependent kinase 2 (CDK2), cyclin-dependent kinase 6 (CDK6), cyclin A2, cyclin B1 and cyclin D1 in NSCLC cells after a 72 h-treatment of ART (40 µM). Coincidently, all the checked genes were down-regulated (Figs. 3B, C), which suggested that low concentration of ART inhibited the mRNA expression of CDK1, CDK2, CDK6, cyclin A2, cyclin B1 and cyclin D1, leading to cell cycle arrest in NSCLC cells. In addition, we found out 40 µM

Fig. 2. High Concentration of ART Significantly Induces Apoptosis in NSCLC Cells

NSCLC cells were treated with 80 µM of ART for 72 h. (A, B) Apoptotic cells of A549 (A) and NCI-H1299 (B) were determined by flow cytometry, n = 3; (C, D) Western blot showing the protein level of Bcl-2, cIAP1 and cIAP2 in A549 (C) and NCI-H1299 (D) cells, n = 3. Error bars indicate mean ± S.E.M. The statistical differences were assessed using one-way ANOVA analysis. ** p < 0.01 vs. Vehicle.
of ART treatment for 72h induced the formation of γH2AX, a known marker of DNA damage in detected NSCLC cells (Fig. 3D). These findings suggest that low concentration of ART induces cell cycle arrest and DNA damage in NSCLC cells.

**Low Concentration of ART Promotes Cellular Senescence by Up-Regulating p16 in NSCLC Cells**

It is reported that cellular senescence is defined as a signal transduction program result in irreversible arrest of cell proliferation, which brought our attention that ART might promote cellular senescence in NSCLC cells. So we detected the effect of ART on cellular senescence in NSCLC cells. Consistent with our hypothesis, increased SA-β-gal was observed after ART treatment in detected NSCLC cells (Figs. 4A, B), indicating that ART treatment led to cellular senescence in NSCLC cells. Cellular senescence is reported to be controlled by cyclin-dependent kinase (CDK) inhibitor, such as p16<sup>INK4a</sup> (p16). To understand how ART promotes cellular senescence in NSCLC cells, we further detected the expression level of p16. Our results showed that a 72h-treatment of ART (40 µM) led to increase of mRNA and protein level of p16 in detected NSCLC cells (Figs. 5A, B). Our results revealed that low concentration of ART promoted cellular senescence by up-regulating p16 in NSCLC cells.
In the past decades, great efforts have been made to defeat NSCLC. However, most NSCLC cases finally relapsed and progressed to intractable metastatic disease. Though immunotherapy targeting the checkpoint PD1 or PDL1 has been used in the treatment of NSCLC, cisplatin-based drugs are the most efficient chemotherapeutic agents for NSCLC treatment.\(^\text{1,17}\) In the treatment of NSCLC, cisplatin-based drugs are the most efficient chemotherapeutic agents for NSCLC treatment.\(^\text{1,17}\) In other hand, the critical tumor suppressor p53 is mutated in over half of tumors. Tumors harboring p53 mutations are often refractory to current cancer therapy.\(^\text{2,3}\) Therefore, it is important to identify novel drug candidates that can induce the apoptosis of tumors in a p53-independent manner.

In this study, we evaluated the anti-NSCLC activity of ART and found out ART exhibited potent cytotoxicity in detected NSCLC cells but not normal cells, suggesting that ART is a promising anticancer agent that causes fewer adverse effects. P53 gene is null in NCI-H1299 cells, indicating that the inhibitory effect of ART in NSCLC cells was not dependent on p53 status. The c-Myc oncoprotein is an essential transcriptional factor that regulates many genes involved in multiple biological processes including cell growth, proliferation and apoptosis.\(^\text{20}\) c-Myc is deregulated in more than half of all human cancers.\(^\text{21}\) Accumulating studies have provided convincing evidence that normal cells have adapted several ways to control c-Myc levels, but these mechanisms can be disrupted in cancer cells.\(^\text{20,22}\) Our previous study reported that artemisinin, another derivative of artemisinin selectively kills human cancer cells.\(^\text{20}\) In the current study, we found that low concentration of ART promotes cellular senescence in NSCLC cells with minimized cytotoxic effects on normal cells, suggesting that ART is a promising new anti-NSCLC drug candidate.

Cellular senescence has considered as an important factor in aging and age-related disease, and it is a seductive target for therapeutic exploitation.\(^\text{7}\) Recent studies have shown that cellular senescence contributes to the anticancer effects of chemotherapeutic drugs, and cellular senescence-inducing agents might be a feasible strategy.\(^\text{8,9,30}\) In the current study, we found that low concentration of ART promotes cellular senescence in NSCLC cells. As cellular senescence can be controlled by CDK inhibitor p16\(^\text{Nk4}\) (p16), and affects the outcome of cancer therapy.\(^\text{31}\) To further reveal the mechanism how ART promotes cellular senescence, we tested the expression level of p16 in NSCLC cells, and found out ART up-regulates the mRNA and protein expression of p16, which provides a mechanism how ART promotes cellular senescence.

Taken together, the findings in this study demonstrate that ART represents a promising new anti-NSCLC drug candidate that could attenuate progression of NSCLC cells in a p53-independent manner through inducing apoptosis, cell cycle arrest and promoting cellular senescence. It provides the experimental foundation for the future development of ART.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) Wakelee H, Kelly K, Edelman MJ. 50 Years of progress in the systemic therapy of non-small cell lung cancer. Am. Soc. Clin. Oncol. Educ. Book, 34, 177–189 (2014).
2) Arriagada R, Bergman B, Dunant A, Le Chevalier T, Pignon JP, Vansteenkiste J. Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. N. Engl. J. Med., 350, 351–360 (2004).
3) Amable L. Cisplatin resistance and opportunities for precision medicine. Pharmacoil. Res., 106, 27–36 (2016).
4) Feng X, Liu H, Zhang Z, Gu Y, Qiu H, He Z. Annexin A2 contributes to cisplatin resistance by activation of JNK-p53 pathway in non-small cell lung cancer cells. J. Exp. Clin. Cancer Res., 36, 123 (2017).
5) Xiao L, Lan X, Shi X, Zhao K, Wang D, Wang X, Li F, Huang H, Liu J. Cytoplasmic RAP1 mediates cisplatin resistance of non-small cell lung cancer. Cell Death Dis., 8, e2803 (2017).
6) Grimes A, Chandra SB. Significance of cellular senescence in aging and cancer. Cancer research and treatment. Official Journal of Korean Cancer Association, 41, 187–195 (2009).
7) Childs BQ, Durik M, Baker DJ, van Deursen JM. Cellular senescence in aging and age-related disease: from mechanisms to therapy. Nat. Med., 21, 1424–1435 (2015).
8) Larsson LG. Oncogene- and tumor suppressor gene-mediated suppression of cellular senescence. Semin. Cancer Biol., 21, 367–376 (2011).
9) Galluzzi L, Bravo-San Pedro JM, Kroemer G. Autophagy mediates tumor suppression via cellular senescence. Trends Cell Biol., 26, 1–3 (2016).
10) Shang D, Wu Y, Ding Y, Lu Z, Shen Y, Zhu F, Liu H, Zhu C, Tu Z. Identification of a pyridine derivative inducing senescence in ovarian cancer cell lines via p21 activation. Clin. Exp. Pharmacol. Physiol., 45, 452–460 (2018).
11) Hsu S. The history of qing hao in the Chinese materia medica. Trans. R. Soc. Trop. Med. Hyg., 100, 505–508 (2006).
12) Ooko E, Saeed ME, Kadioglu O, Sarvi S, Colak M, Elmasaoudi K, Janah R, Greten HJ, Effertth T. Artemisinin derivatives induce iron-dependent cell death (ferropotosis) in tumor cells. Phytomedicine, 22, 1045–1054 (2015).
13) Chen J, Li W, Cui K, Ji K, Xu S, Xu Y. Artemisitene suppresses tumorigenesis by inducing DNA damage through deregulating c-Myc-topoisomerase pathway. Oncogene, 37, 5079–5087 (2018).
14) Wu ZP, Gao CW, Wu YJ, Zhu QS, Chen Y, Liu X, Liu C. Inhibitory effect of artemether on tumor growth and angiogenesis in the rat C6 orthotopic brain gliomas model. Integr. Cancer Ther., 8, 88–92 (2009).
15) Tan WQ, Chen G, Ye M, Jia B. Artemether regulates chemosensitivity to doxorubicin via regulation of B7-H3 in human neuroblastoma cells. Med. Sci. Monit., 23, 4252–4259 (2017).
16) Tan L, Liu J, Jia Q, Ying Y, Yang Z, Huang G. Preparation and evaluation of artemether liposomes for enhanced anti-tumor therapy. AAPS PharmSciTech, 19, 512–521 (2018).
17) Nagasaka M, Zaki M, Kim H, Raza SN, Yoo G, Lin HS, Sukari A, PD1/PD-L1 inhibition as a potential radiosensitizer in head and neck squamous cell carcinoma: a case report. Journal for Immunotherapy of Cancer, 4, 83 (2016).
18) Gurley KE, Moser R, Gu Y, Hasty P, Kemp CJ. DNA-PK suppresses a p53-independent apoptotic response to DNA damage. EMBO Rep., 10, 87–93 (2009).
19) Bohman S, Manfredt JJ. p53-independent effects of Mdm2. Sub. cell. Biochem., 85, 235–246 (2014).
20) Farrell AS, Sears RG. MYC degradation. Cold Spring. Harb. Perspect. Med., 4, 2014M63 (2014).
21) Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nat. Rev. Cancer, 8, 976–990 (2008).
22) Zhang P, Wang H, Rowe PSN, Hu B, Wang Y, MEPE/OF45 as a new target for sensitizing human tumour cells to DNA damage inducers. Br. J. Cancer, 102, 862–866 (2010).
23) Roos WP, Kaina B. DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. Cancer Lett., 332, 237–248 (2013).
24) Roos WP, Kaina B. DNA damage-induced cell death by apoptosis. Trends Mol. Med., 12, 440–450 (2006).
25) Vandenabeele P, Bertrand MJ. The role of the IAP E3 ubiquitin ligases in regulating pattern-recognition receptor signalling. Nat. Rev. Immunol., 12, 833–844 (2012).
26) Yang C, Wang H, Zhang B, Chen Y, Zhang Y, Sun X, Xiao G, Nan K, Ren Q, Qin S. LCL161 increases paclitaxel-induced apoptosis by degrading cIAP1 and cIAP2 in NSCLC. J. Exp. Clin. Cancer Res., 35, 158 (2016).
27) Catz SD, Johnson JL. Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. Oncogene, 20, 734–7357 (2001).
28) Kwon OS, Hong SK, Kwon SJ, Go YH, Oh E, Cha HJ. BCL2 induced by LAMTOR3-MAPK is a druggable target of chemoradiotherapy resistance in mesenchymal lung cancer. Cancer Lett., 403, 48–58 (2017).
29) Nittis JL. Targeting DNA topoisomerase II in cancer chemotherapy. Nat. Rev. Cancer, 9, 338–350 (2009).
30) Yang H, Wang H, Ren J, Chen Q, Chen ZJ. cEGF is essential for cellular senescence. Proc. Natl. Acad. Sci. U.S.A., 114, 4612–4620 (2017).
31) Schmitz CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM, Lowe SW. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. Cell, 109, 335–346 (2002).