Artemether attenuates the progression of non-small cell lung cancer by inducing apoptosis, cell cycle arrest and promoting cellular senescence

Jian Chen\textsuperscript{a,b,c,#}, Xiaofei Huang\textsuperscript{d,#}, Cheng Tao\textsuperscript{a,b,c,e}, Ting Xiao\textsuperscript{f}, Xinping Li\textsuperscript{c}, Qiang Zeng\textsuperscript{a,b,c}, Min Ma\textsuperscript{g,h,*}, Zhengzhi Wu\textsuperscript{a,b,c,i,*}

\textsuperscript{a} Integrated Chinese and Western Medicine Postdoctoral Research Station, Jinan University, Guangzhou 510632, China
\textsuperscript{b} The Fist Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen 518035, China
\textsuperscript{c} Shenzhen Institute of Geriatrics, Shenzhen 518020, China
\textsuperscript{d} Research Center of Integrative Medicine, School Basic Medical Sciences, Guangzhou University of Chinese Medicine, Guangzhou 510006, China
\textsuperscript{e} Dongguan Institute of Jinan University, Dongguan 523808, China
\textsuperscript{f} Shunde Hospital of GuangZhou University of Chinese Medicine, Foshan 528300, China
\textsuperscript{g} College of Traditional Chinese Medicine, Jinan University, GuangZhou 510632, China
\textsuperscript{h} The First Affiliated Hospital of Jinan University, GuangZhou 510630, China
\textsuperscript{i} The Eighth Affiliated Hospital of Sun Yat-sen University, Shenzhen 518033, China

\# Jian Chen and Xiaofei Huang contributed equally to this work

* Corresponding author. e-mail:szwzz001@email.szu.edu.cn
Summary

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.

Keywords  artemether; non-small cell lung cancer; apoptosis, cell cycle arrest; cellular senescence
Lung cancer is the most common cause of cancer death, approximately 85% of which are non-small cell lung cancer (NSCLC) 1). Currently, chemotherapy is one of the most effective treatment for NSCLC, and cisplatin-based drugs are the most efficient chemotherapeutic agents for NSCLC treatment 1). However, these chemotherapeutic agents also show highly toxic to the treated patients 2). In addition, drug resistance often appears after prolonged treatment, and the resistance toward cisplatin is multifaceted as it involves multiple cellular pathways 3-5). Therefore, there 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 6). Cellular senescence has considered as an important factor in aging and age-related disease, and it is a seductive target for therapeutic exploitation 7). Increasing studies has 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 an famous efficient antimalarial drug 11), which is revealed that also has a 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 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.

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 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 of ART (40 µM or 80 µM) for 72 h. After stimulation, whole-cell extracts were prepared in RIPA buffer, and separated by 10-15% SDS-PAGE, and transferred to nitrocellulose membranes (Millipore). The following antibodies were used in this study: rabbit monoclonal anti-cIAP1 (7065S; CST), rabbit monoclonal anti-cIAP2 (3130S; CST), rabbit monoclonal anti-Bcl2 (ab32124; Abcam), rabbit monoclonal anti-Phospho- histone H2AX (9718S; CST), mouse monoclonal anti-p16INK4a (ab54210, Abcam), mouse monoclonal anti-beta-actin (66009-1-Ig; Abcam), anti-rabbit IgG HRP-linked antibody (7074S; CST), anti-rabbit 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) 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 ± SEM. \( P < 0.05 \) 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 Fig. 1B & C, ART exhibited strong inhibitory effect on the proliferation of both A549 and NCI-1299 cells in a dose- and time-dependent manner, among which NCI-1299 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 (Fig. 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 (Fig 1D & E). Taken together, we reveal that ART not only has inhibitory effect on the proliferation of NSCLC cells but also has an 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 (Fig. 2A & B). We further detected the protein level of anti-apoptotic protein including B-cell lymphoma-2 (Bcl-2), cellular inhibitor of apoptosis protein 1 (cIAP1) and cellular inhibitor of apoptosis protein 2 (cIAP2), and found out all of these protein were down-regulated after ART (80 μM).
treatment for 72 h (Fig. 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) treatment 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 concentrations 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 (Fig. 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 of ART treatment for 72 h 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 down-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 (Fig. 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 p16INK4a (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 (Fig. 5A & B). Our results revealed that low concentration of ART promoted cellular senescence by down-regulating p16 in NSCLC cells.
DISCUSSION

In the past decades, great efforts have been made in 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 \(^1,17\). 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 \(^18,19\). 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 \(^20\). c-Myc is deregulated in more than half of all human cancers \(^21\). 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 \(^20\). Our previous study reported that artemisisitene, another derivative of artemisinin selectively kills human cancer cells but not normal cells through destabilizing c-Myc in human cancer cells. We speculate that the pathway of ART specifically suppresses cell proliferation in NSCLC cells might be similar to artemisinin, which needs to be testified in the further research.

Inducing apoptosis and suppressing cancer cell proliferation are efficient ways for tumor treatment. DNA damaging agents can induce cell death triggered by apoptosis, which have been the mainstay of cancer chemotherapy \(^22,24\). In the current study, we demonstrated that high concentration of ART significantly induces apoptosis through down-regulating the level of anti-apoptotic protein Bcl-2, cIAP1 and cIAP2 of NSCLC cells. cIAP1 and cIAP2 are members of the inhibitor of apoptosis proteins (IAPs) family, indirectly regulate apoptosis by preventing Smac for inhibiting XIAP-caspase interaction and by preventing the formation of caspase-8-activating platform \(^25,26\). Bcl2 is one of the apoptotic protein markers, which regulated by nuclear factor κB or the cAMP-response element-binding protein (CREB) transcription factor \(^27,28\). We speculate that ART might target the protein (s) of those
pathway, which needs to be further verified. Surprisingly, we found out low concentration of ART down-regulates the mRNA level of cell cycle related genes including CDK1, CDK2, CDK6, cyclin A2, cyclin B1 and cyclin D1, leading to cell cycle arrest of NSCLC cells. However, we have not enough evidence to confirm the molecular target of ART, which will be studied in our further research. Furthermore, we also revealed that low concentration of ART induces the formation of \(\gamma\)H2AX, a known marker of DNA damage in detected NSCLC cells.

Over the decades, increasing studies have revealed that DNA damage inducers like doxorubicin, etoposide and cisplatin, are the most efficient chemotherapeutic agents inducing apoptosis in cancer cell\(^{29}\). However, these chemotherapeutic agents also show highly toxic to the treated patients. Therefore, ART can eliminate NSCLC cells with minimized cytotoxic effects on normal cells, which might be considered as 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\(^7\). 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\(^{8,9,30}\). 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\(^{\text{INK4a}}\) (p16), and affects the outcome of cancer therapy\(^{31}\). 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.

**Acknowledgements** This study were supported by Natural Science Foundation of China (Nos. 81574038 and Nos. 81673979), Shenzhen Basic Discipline Layout Project (Nos. JCYJ20170412161254416), National High Technology Research and Development Program
Conflict of interest  The authors declare no conflict of interest.

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


Fig 1. ART inhibits proliferation and colony formation of NSCLC cells  (A) Structures of Artemether (ART). (B & C) NSCLC cells (A549, NCI-H1299 cells) and normal bronch 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 days, and representative photographs of colony formation are shown, $n=3$. Error bars indicate mean ± SEM. The statistical differences were assessed using One-way ANOVA analysis. **$P < 0.01$ vs Vehicle.
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 ± SEM. The statistical differences were assessed using One-way ANOVA analysis. **P < 0.01 vs Vehicle.
Fig 3. Low concentration of ART induces cell cycle arrest and DNA damage. NSCLC cells were treated with 40 μM of ART for 72 h. (A) The cell cycle ratio of NCI-H1299 cells were determined by cell cycle staining kit, n=3. (B & C) qPCR showing the mRNA expression of cell cycle related genes including CDK1, CDK2, CDK6, cyclin A2, cyclin B1 and cyclin D1 in A549 (B) and NCI-H1299 (C) cells, n=3. (D) Western blot showing the protein level of DNA damage marker γH2AX in A549 and NCI-H1299 cells, n=3. Error bars indicate mean ± SEM. The statistical differences were assessed using One-way ANOVA analysis. *P < 0.05 and **P < 0.01 vs Vehicle.
Fig 4. Low concentration of ART promotes cellular senescence in NSCLC cells NSCLC cells were treated with 40 μM of ART for 72 h. (A & B) The senescent cells were detected by Senescence β-Galactosidase Staining Kit, and representative photographs of senescent cell are shown, n=3. Error bars indicate mean ± SEM. The statistical differences were assessed using One-way ANOVA analysis. **P < 0.01 vs Vehicle. (Color figure can be accessed in the online version.)
Fig 5. Low concentration of ART up-regulates the mRNA and protein level of p16 in NSCLC cells  NSCLC cells were treated with 40 μM of ART for 72 h. (A) qPCR showing the mRNA expression of p16 in A549 and NCI-H1299 cells, n=3. (B) Western blot showing the protein level of p16 in A549 and NCI-H1299 cells, n=3. Error bars indicate mean ± SEM. The statistical differences were assessed using One-way ANOVA analysis. **P < 0.01 vs Vehicle.
### Table 1 qPCR primers (related to Fig. 3 and Fig. 5)

<table>
<thead>
<tr>
<th>Genes</th>
<th>primer sequences (5' to 3') (forward; reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK1</td>
<td>TGAGGTAGTAACACTCTGGTA; ATGCTAGGCTTCTCTGGTT</td>
</tr>
<tr>
<td>CDK2</td>
<td>CCTGGACACTGAGACTGA; CCGATGAGAATGGCAGAA</td>
</tr>
<tr>
<td>CDK6</td>
<td>CTTCAATCACCCGAGGT; TGGACTGGAAGAACTCTTT</td>
</tr>
<tr>
<td>cyclin A2</td>
<td>ACAGCCAGACATCACTAAC; GGAAGACAGGAACCTATCAA</td>
</tr>
<tr>
<td>cyclin B1</td>
<td>TTGGTGTGTACTGCCTTCTC; TCTGACTGTGCTGCTTTC</td>
</tr>
<tr>
<td>cyclin D1</td>
<td>CCTCGGGTCTCTACTTCA; CTCTCGCACTTCTCTGTC</td>
</tr>
<tr>
<td>p16</td>
<td>ACGCACCAGAATAGTTACG; ATGGTACTGGCCTCTGGT</td>
</tr>
<tr>
<td>ACTB</td>
<td>TCGTCGTGACATTAAGG; AAGGAAGGCTGGAAAGAGT</td>
</tr>
</tbody>
</table>