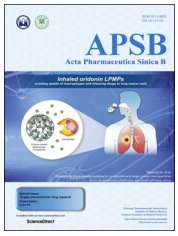
Acta Pharmaceutica Sinica B 2017;7(1):65–72

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

Asiatic acid inhibits lung cancer cell growth *in vitro* and *in vivo* by destroying mitochondria

Tiancong Wu[a], Ji Geng[b], Wenjie Guo[c], Jing Gao[b,*], Xixu Zhu[a,*]

[a]*Department of Radiation Oncology, Jinling Hospital, Medical School of Nanjing University, Nanjing 210002, China*
[b]*School of Pharmacy, Jiangsu University, Zhenjiang 212013, China*
[c]*School of Life Science, Nanjing University, Nanjing 210093, China*

Received 12 January 2016; received in revised form 18 February 2016; accepted 9 March 2016

KEY WORDS

Asiatic acid;
Lung cancer;
Apoptosis;
Mitochondria;
Reactive oxygen species

Abstract Asiatic acid (AA), a pentacyclic triterpene found in *Centella asiatica*, displays significant anti-proliferative effects on cancer cells *in vitro* although the underlying mechanism of this effect remains unknown. This study investigated the efficacy and mechanism of action of AA against lung cancer both *in vivo* and *in vitro*. Using the MTT assay, AA was found to induce apoptosis in a dose- and time-dependent manner, an effect enhanced by pretreatment with an autophagy inhibitor. It also elevated expression of microtubule-associated protein 1 light chain 3 (LC3) and decreased the expression of p62. Furthermore, exposure to AA resulted in collapse of the mitochondrial membrane potential and generation of reactive oxygen species (ROS), suggesting mitochondria are the target of AA. In the mouse lung cancer xenograft model, oral administration of AA significantly inhibited tumor volume and weight accompanied by significant apoptosis of lung cancer cells. In addition, it led to a significant decrease in the expression of proliferating cell nuclear antigen (PCNA). In summary, the results show that AA significantly reduces lung cancer cell growth both *in vitro* and *in vivo* and that the associated apoptosis is mediated through mitochondrial damage.

*Corresponding author.
E-mail addresses: guowj@nju.edu.cn (Wenjie Guo), jinggao@ujs.edu.cn (Jing Gao), zhuxixu@hotmail.com (Xixu Zhu).

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

http://dx.doi.org/10.1016/j.apsb.2016.04.003

1. Introduction

Lung cancer is associated with high morbidity and mortality to such an extent that in 2014 it was the leading cause of death among all cancer patients in the United States[1]. In fact, in that year, an estimated 221,200 cases of lung cancer were diagnosed and 158,040 people died from the disease[1]. Although there have been advances in our knowledge of the biology of the disease and treatment options for lung cancer have increased, nevertheless the 5-year relative survival remains low at currently only 18%[1]. In the case of non-small cell lung cancer (NSCLC), drugs targeting epidermal growth factor receptor (EGFR), such as gefitinib and erlotinib[2–6], have been approved but their efficacy is limited and more than 50% of NSCLC patients are not suitable to receive them[5,7]. Therefore, lung cancer remains a public health challenge and novel anticancer drugs are needed.

Asiatic acid (AA) is a triterpene extracted from *Centella asiatica* (L.) Urban (Umbelliferae) that has a long history of successful use in both traditional Chinese and Indian Ayurvedic medicine. Previous studies have demonstrated that AA exhibits a variety of pharmacological effects not only as an antioxidant, antiinflammatory and neuroprotective agent[8–11], but also against cancer. For example, AA induces apoptosis in human SK-MEL-2 melanoma cells by triggering generation of reactive oxygen species (ROS)[12] and does the same thing in HepG2 human hepatoma cells by releasing intracellular Ca^{2+} and enhancing expression of p53[13]. In addition, AA activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways and induces human breast cancer apoptosis and cell cycle arrest[14]. While these *in vitro* data testify to the anti-cancer efficacy of AA, evidence from *in vivo* studies is limited. How ever, it has been shown to prevent 9,10-dimethylbenz[*a*]anthracene (DMBA)-initiated and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-promoted mouse skin tumorigenesis by inhibiting NO and COX-2 signaling[15].

In the present study, we evaluated the effects of AA on lung cancer cells *in vitro* and in the mouse Lewis lung cancer xenograft model *in vivo*. As part of the work, we attempted to clarify the mechanism of AA-induced apoptosis and its efficacy against lung cancer

2. Materials and methods

2.1. Materials

Materials (suppliers) were as follows: AA, *N*-acetylcysteine (NAC), chloroquine (CQ) and 3-methyladenine (3-MA, Sigma–Aldrich); 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Amresco); JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technology, Waltham, MA, USA); annexin V/propidium iodide (PI) kits for apoptosis, transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay kit and DCFH-DA kit (Beyotime Institute of Biotechnology, Nantong, China); antibodies against peroxisome proliferator-activated receptor (PARP), cytochrome c, caspase-3, caspase-9, cytochrome oxidase subunit IV (COXIV), LC3 and p62 (Cell Signaling Technology, Boston, MA, USA); β-actin and proliferating cell nuclear antigen (PCNA, Santa Cruz Biotechnology, Santa Cruz, CA, USA). All other chemicals were of high purity and purchased from commercial sources.

2.2. Cell culture

Human A549 and H1299 lung cancer cell lines and mouse Lewis lung cancer (LLC) cells were purchased from the Shanghai Institute of Cell Biology, Shanghai, China. Cells were maintained in DMEM medium supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2 (Thermo Fisher Scientific, MA, USA).

2.3. Animals

C57BL/6J mice (6–8 weeks old) were purchased from the Shanghai Laboratory Animal Center, Shanghai, China. Briefly, mice were housed in plastic cages at 21 ± 2 °C on a 12 h light–dark cycle with free access to pellet food and water. Animal welfare and experimental procedures were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and related ethical regulations of Nanjing University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.4. MTT assay

Cells were plated at a density of approximately 4×10^3 cells/well in 96-well plates and treated with various concentrations of AA in triplicate. After incubation for various times, the MTT assay was applied to determine cell viability using a 96-well plate reader (Spectra MAX 190, Molecular Devices Corporation, CA, USA).

2.5. Cell apoptosis and cell cycle assay

Into 6-well plates 1×10^5 A549 cells were seeded and subsequently treated with AA (20, 40 and 80 mol/L) for up to 24 h. Cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI) and the nuclei photographed. In another experiment, cells were collected, washed with PBS, stained with annexin V/PI in binding buffer at room temperature for 15 min in the dark and then analyzed by fluorescence activated cell sorting (FACS) using a Calibur flow cytometer (Becton Dickinson, NJ, USA). annexin V^+/PI^- and annexin V^+/PI^+ cells were considered to be in the early and late phases of apoptosis. To examine the cell cycle, cells were collected, washed with PBS and then suspended in 1 mL DNA staining solution (20 mg/mL of PI and 100 mg/mL of RnaseA in PBS) for 30 min on ice. DNA content was analyzed by FACS (Becton Dickinson, USA) and the resulting DNA histograms quantified using Cell Quest Pro software.

2.6. Western blot analysis

Proteins were extracted in lysis buffer (30 mmol/L Tris pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 10% glycerol and phosphatase and protease inhibitors), separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4 °C and then incubated with a horse radish peroxidase-coupled secondary antibody. Detection was performed using the LumiGLO chemiluminescent substrate system (KPL, Gaithersburg, MD, USA).