

Aqueous Extract of *Huang-lian* Induces Apoptosis in Lung Cancer Cells via P53- Mediated Mitochondrial Apoptosis

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ABSTRACT

The current study was designed to evaluate the activity of the aqueous extract of *Huang-lian*, and the main apoptosis pathway induced by the extracts of *Huang-lian* was detected on lung cancer. Antiproliferative activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and TUNEL methods against human lung cancer cells (A549). *Huang-lian* regulated the Bcl-2 family protein-mediated mitochondrial pathway via p53 and was detected by western blot. It increased the activation of caspase-3 and caspase-3 cleavage increased as the time increased. These results suggested that *Huang-lian* regulated the Bcl-2 family protein-mediated mitochondrial via p53 pathway, suggesting that *Huang-lian* should be further investigation as a natural agent for treating and preventing cancer.

Key words: *Huang-lian*, aqueous extract, apoptosis

INTRODUCTION

At present, four types of cancer—prostate, breast, lung, and colorectal—exceed 100,000 new cases per year in the United States. Of these cancers, lung cancer carries the worst prognosis and has been estimated to result 159,260 deaths in 2014. Characterized risk factors include genetic susceptibility as well as environmental exposure to carcinogens such as radon, asbestos and fine-particulate matter. This multifactorial etiology for lung cancer could include long-term exposure to an inhaled carcinogen.

Apoptosis is the process of programmed cell death and is considered to be a key process for manipulation in cancer prevention (Li et al. 2012). Activation of apoptosis occurs by extrinsic and intrinsic pathways. The extrinsic pathway is characterized by caspase-8 cleavage, whereas the intrinsic pathway is characterized by cytochrome c release and caspase-9 activation (Lu et al. 2011). During apoptosis, mitochondria play a key role

and in the mitochondria-dependent intrinsic apoptosis pathway, the Bcl-2 family members are very important players. The Bcl-2 family includes pro- and anti-apoptotic proteins that maintain a dynamic balance between the cell survival and death through interactions with each other and with other proteins (Ma et al. 2008).

Huang-Lian is a famous traditional Chinese recipe that has been used to treat the toxic heat syndromes and infectious diseases. In this study, the lung cancer cell line A549 was used to investigate the activity of aqueous *Huang-Lian* extract (AHLE). In addition, the antitumor mechanisms of AHLE in lung cancer cells were investigated.

MATERIAL AND METHODS

Material

Daiichi Sangyo Corporation (Osaka, Takayama, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5diphenyl-

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2H-tetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The Mitochondrial/Cytoplasm Fractionation Kit was obtained from BioVision (San Francisco, CA, USA). All of the primary antibodies used in the study were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-linked antirabbit/mouse (Cell Signaling Technology) or Alexa-488-labeled goat anti-rabbit (Invitrogen, Carlsbad, CA, USA) IgG antibodies were used as secondary antibodies. The human lung cancer cell line A549 cell was purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China).

Preparation of AHLE

One kilogram of *Huang-lian* was crushed to powder and the powder was soaked in water at approximately 30 to 35°C for 24 h. The supernatant was collected by centrifugation at $4,000 \times g$ for 10 min and separated using zeolite dialysis membranes with apertures of $55 \times 10^4 \mu\text{m}$, $40 \times 10^4 \mu\text{m}$, $25 \times 10^4 \mu\text{m}$ and $1 \times 10^4 \mu\text{m}$. Different extract fractions were obtained using lyophilized.

Cell culture

The cancer cells were grown as a monolayer in RPMI-1640 (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum. Cells were maintained at 37°C in a humidified incubator under a 5% CO₂ atmosphere.

Cell proliferation assay

Cell proliferation was analyzed using the MTT assay. Cells were seeded in 96-well plates at densities of 1000–3000 cells/well and incubated for different time periods with or without different concentrations of AHLE. At different time points, 10 μL of MTT solution (5 mg/mL) was added to each well. The plates were incubated for an additional 4 h at 37°C. The medium was removed and 200 μL DMSO was added to each well and pipetted repeatedly to dissolve the formazan. The absorbance of each well was measured at 570 nm with a microplate reader (Tecan Group Ltd., Mannedorf, Switzerland).

TUNEL Assay

A549 were grown on chamber slides. After treatment with or without the AHLE, the slides were gently washed three times in 0.1 mol/L PBS

(pH 7.4), fixed with 4% paraformaldehyde- PBS solution (Boston Bioproducts, Worcester, MA), and immediately transferred to a freezer until use. To study the apoptosis of cultured cells, TUNEL assay was performed using *in situ* Cell Death Detection Kit, POD (number 11684 817910) according to the manufacturer's instructions (Roche, Indianapolis, IN). The terminal deoxyribonucleotidyl transferase (TDT)-mediated TUNEL was used to detect DNA fragmentation *in situ*. Fewer than 3% of the cells detached from the culture dishes and were not counted.

RNA extraction and qRT-PCR

Total RNA was isolated from the cells using the RNeasy kit (Qiagen, Hilden, Germany). The RNA content of samples was too low to be accurately quantified by spectrometry, and thus 6.5 μL RNA aliquots were amplified. All RNA samples were treated with RNasefree DNase I to remove any possible genomic DNA contamination. For amplification of the targets, RT and PCR were run in two separate steps (TaKaRa, Inc., Dalian, China). Primers used are shown in Table 1.

Western blotting

Cells were harvested, washed with PBS at 37°C, and lysed with a phenylmethanesulfonyl fluoride lysis buffer (Invitrogen). After centrifugation at $13,200 \times g$ for 30 min, the protein content of the supernatant was determined using the bicinchoninic acid reagent (Sigma). Total protein (50 μg) from each sample was electrophoresed on a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked with PBS containing 5% fat-free milk (Becton Dickinson, Franklin Lakes, NJ, USA) and 0.1% Tween-20 (Sigma) for 30 min at room temperature and then incubated with primary antibody for at least 1 h at room temperature or overnight at 4°C. The membranes were washed three times with PBS containing 0.1% Tween 20, incubated with peroxidase-conjugated secondary antibodies (Millipore), and developed using the ECL reagent (Pierce, Rockford, IL, USA). And the photograph was obtain by Imaging Systems (UVP, USA)

Statistical analysis

All the experiments were performed at least in triplicate. Data are presented as mean \pm standard deviation (SD). *p* values were calculated using the

Student's *t*-test accompanied by the analysis of variance (ANOVA) where appropriate.

RESULTS

A549 cell proliferation upon exposure to AHLE

To determine the activity of the AHLE, the A549 cells line were exposed to 10, 20, 40 and 60 $\mu\text{g/mL}$ of AHLE and the cell growth was examined at 12, 24, 48 and 72 h. The maximum inhibition rate appeared at an AHLE concentration of 40 $\mu\text{g/mL}$ on the 24 h of treatment (Fig. 1).

Effects of AHLE on TUNEL assay

First, the AHLE induced apoptosis, as assessed by TUNEL. However, although TUNEL was undetectable under control conditions, punctuate staining indicated that essentially all the cell nuclei exposed to the AHLE had DNA nicking

(Fig. 2) and in different time, the DNA nicking was different. At 48 h, the DNA nicking was most strong.

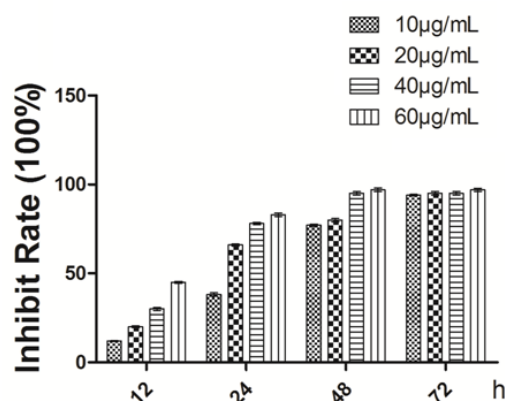


Figure 1 - Cell proliferation assay. A: Inhibit rates of A549 cells by the AHLE (10, 20, 40, and 60 $\mu\text{g/mL}$) at 12, 24, 48 and 72 h.

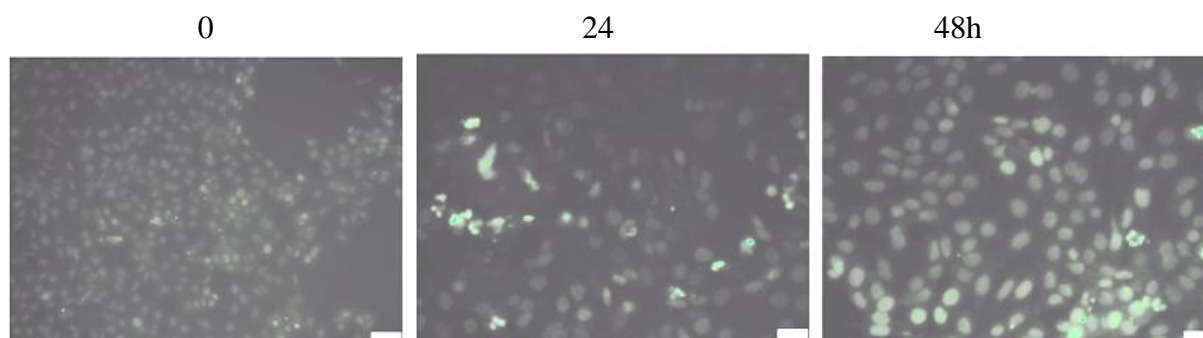


Figure 2 - Effects of AHLE on TUNEL assay at 0, 24, 48 h.

Effects of AHLE on expression of p53, Bcl-2 proteins (Bcl-2, Bcl-xL, and Bax)

To further investigate the effect of AHLE on A549 cells, the protein expression levels of Bcl-2 proteins (Bcl-2, Bcl-xL, and Bax) and p53 were examined upon exposure of the to 40 $\mu\text{g/mL}$ AHLE at 0, 20, 40 and 60 min. As shown in Figure 3, the protein expression levels of p53, Bcl-2 and Bcl-xL decreased as the time increased. However, Bax expression increased with increasing time. Then, the p53 was inhibited by PFT- α (p53 inhibitor) and the protein expression levels of Bcl-2 proteins (Bcl-2, Bcl-xL, and Bax) and p53 were examined upon exposure of cells to 40 $\mu\text{g/mL}$ AHLE at 0, 20, 40 and 60 min. The p53, Bcl-2 and Bcl-xL increased as the time increased. But, the Bax was decreased (Fig. 4).

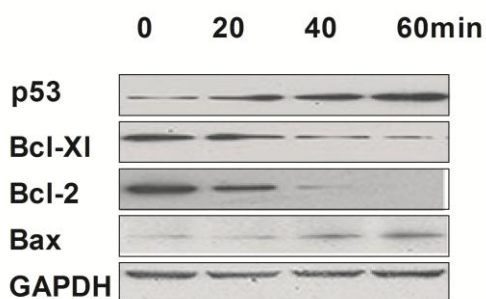


Figure 3 - Protein expression of proteins of the p53 and Bcl-2 family (Bcl-2, Bcl-xl, and Bax) after treatment of A549 cells with AHLE at 0, 20, 40 and 60 min.

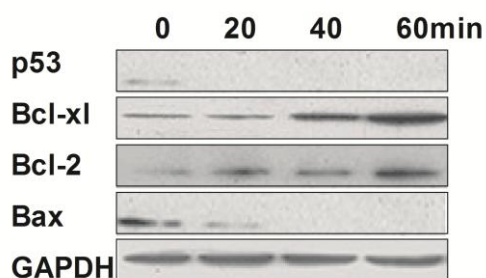


Figure 4 - Protein expression of proteins of the p53 and Bcl-2 family (Bcl-2, Bcl-xl, and Bax) after treatment of A549 cells with AHLE at 0, 20, 40 and 60 min when p53 was inhibited.

Effects of AHLE on caspase-3 expression

To determine whether caspase-3 and PARP was involved in apoptosis mediated by AHLE, their expression levels were analyzed by western blotting. As shown in Figure 5, caspase-3 cleavage increased as the time increased.

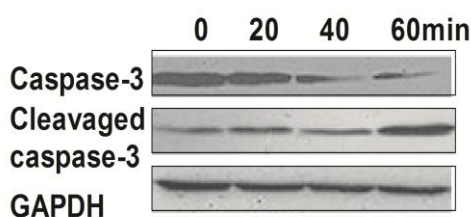


Figure 5 - Effects of AHLE on caspase-3 expression, caspase-3 cleavage at 0, 20, 40 and 60 min according to western blot analysis.

DISCUSSION

Huang-lian is used widely in TCM to treat different ailments for a long time. Recently, many biological activities of *Huang-lian* such as anticancer, antidiabetic, antimutagenic, antibacterial, antifungal, and antiviral effects have been reported (Zhang et al. 2007; Zeng et al. 2009). The product of *Huang-lian* decoction has been shown to inhibit the bacterial growth as well as cancer (Kim et al. 2012). Therefore, *Huang-lian* seemed very useful in TCM. The present results showed that AHLE could inhibit the growth lung cancer cell line and caused cell morphology changes as well (data not shown). The MTT assay results showed that the inhibition of cancer cell viability was time and dose dependent.

Apoptosis induced by some anticancer agents constitutes one aspect of their treatment effect. Two major pathways involved in the process have been investigated in great depth (Gustafsson and Gottlieb 2007). As p53 responds to both DNA damage and oxidative stress to trigger downstream apoptotic signalling, the mechanism for apoptosis seems to originate both from the pharmacological consequences of DNA injury and from oxidative stress (Yeh et al. 2009; Zhou et al. 2012). In this study, AHLE induced reduced expression of Bcl-2, Bcl-xl and greater expression of Bax. Therefore, AHLE could promote A549 cell apoptosis via p53. The study on the mechanism of apoptosis induced by AHLE showed anti-proliferative activity in A549 cells. Apoptosis is initiated through the mitochondrial pathway under physiological conditions such as oxidative stress, mitochondrial disturbance, and DNA damage (Gotoh et al. 2012) (another important key player in the mitochondrial pathway of apoptosis). The typical executioners of apoptosis are proteolytic enzymes called caspases (Floros et al. 2006). The present results clearly demonstrated that AHLE increased the activation of caspase-3. Given that aqueous AHLE could play a novel role as a complementary medicine in lung cancer treatment, further studies on its anticancer mechanisms should be done. However, aqueous AHLE extract did not show this effect on normal mouse lung cells.

CONCLUSION

In summary, results showed that aqueous HUANG-LIAN inhibited cell proliferations by inducing apoptosis and cell-cycle arrest in A549 breast cancer cells. These results contributed to the understanding of the anticancer activity of *Huang-lian*.

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