

Original Research Article

Anti-proliferation effect of *Scutellaria barbata* D. Don extract on lung cancer

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Abstract

Purpose: To investigate the effect of *Scutellaria barbata* D. Don extract (SBDE) on apoptosis and proliferation in A549 human lung cancer cells.

Methods: Inverted microscope was used to observe morphological changes in A549 cells after exposure to SBDE. Trypan blue staining of living cells was applied to construct cell growth curve after treatment with varying concentrations of SBDE. The influence of SBDE on cell proliferation, apoptosis and cell cycle was determined by MTT assay while protein expressions of key apoptosis-related enzymes were evaluated by immuno-cytochemical method.

Results: SBDE inhibited the growth of A549 lung cancer cells at a concentration range of 20 - 160 µg/mL. Flow cytometry showed that SBDE induced apoptosis in the A549 cells. The proportion of cells in G0/G1-phase increased significantly ($p < 0.01$), while the proportion of cells in S-phase and G2/M-phase decreased correspondingly, indicating that the cells were in G0/G1-phase arrest. Cell cycle arrest and apoptosis-inducing effect gradually increased with increase in SBDE concentration. With increasing concentrations of SBDE, there were significant increases in the expressions of caspase-3 ($p < 0.05$), caspase-8 ($p < 0.01$) and caspase-9 ($p < 0.05$), and significant decreases in Ki-67 ($p < 0.01$) and p21 ras protein ($p < 0.01$).

Conclusion: SBDE exerts significant inhibitory effect on the proliferation of A549 lung cancer cells, which can be developed for the treatment of lung cancer patients.

Keywords: *Scutellaria barbata* D. Don, Antitumor activity, Lung cancer A549 cells, Apoptosis, Cell cycle arrest, Caspase

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INTRODUCTION

The morbidity and mortality from lung cancer account for 13 % and 19.4 % respectively, worldwide [1]. Lung cancer is classified into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The latter (NSCLC) accounts for most (about 80 %) lung

tumors. At present, early-stage NSCLC is treated by surgical intervention, while mid-late NSCLC is treated with radiotherapy and chemotherapy. Although effective for some patients in the short run, radiotherapy and chemotherapy are associated with adverse effects, toxicity, high recurrence of tumor and poor prognosis [2]. Therefore, it is necessary to reduce the side

effects of chemotherapeutic drugs by searching for more effective anticancer drugs with lower toxicity. This has been a major challenge to medical science.

Scutellaria barbata D. Don is a very valuable plant in Yunan of China. Studies have shown that *Scutellaria barbata D. Don* has anti-aging, blood pressure-lowering, hypoglycemic and immunity-enhancing properties [3]. It also has antioxidant activity [4], and exerts cytotoxic effects on various malignant tumors [5,6]. This study was carried out to investigate the effect of *Scutellaria barbata D. Don* extract on proliferation and apoptosis in A549 human lung cancer cells.

EXPERIMENTAL

Materials

Human lung cancer cell line (A549) was purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. *Scutellaria barbata D. Don* was got from Yunan, China. RPMI1640 culture medium was product of GIBCO Company, USA. Calf serum and phosphate buffered solution (PBS, American hyclone); dimethylthiozol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide (PI) and trypsin were obtained from *Sigma-Aldrich* (America). Caspase-3, caspase-8, caspase-9, Ki-67, bcl-2 p21 ras monoclonal antibodies, and SP generic kits were purchased from American NeoMarkers (San Francisco, USA) .

Samples of *Scutellaria barbata D. Don* were collected from Kunming City, Yunan Province, China in October 2017. Taxonomic identification of the plant was done by Professor He Lin of Zhejiang University in China. A voucher specimen (no. SBDE 20171006) was deposited in the herbarium of College of Pharmacy, Zhejiang University, China for future reference.

Instrumentation

The instruments used were 96-well plates (Corning Company, Wuhan, China); automatic CO₂ incubator (DuPont Company of America Napoc6100); 450 enzyme standard instrument (American BioRad); inverted microscope (Olympus CX41-32C02, Japan); 4 % hope blue mother liquor (Japanese BigWood Industrial Co., Ltd) and LKB- ultrathin sections machine (Sweden). Others were YZ-1450 laminar flow ultranet work station (Suzhou Purification Equipment Company, Suzhou, China); elite flow cytometer (Coulter, USA); blood cell counting plate; vacuum drying oven (DZF-6021 type,

Shanghai Fine Macro Experiment Equipment Limited), and ultrasonic cleaner CQ25-12 type (Shanghai Scientz Biotechnology Research Institute).

Preparation of SBDE

Dry leaves of *Scutellaria barbata D. Don* (80 g) were ground and extracted three times by refluxing with 95 % ethanol, each time for 2 h. The extracts were pooled and concentrated under reduced pressure to obtain SBDE.

Cell culture

The A549 cells were cultured in RPMI-1640 medium (Gibco, Rockville, MD) containing 10 % fetal bovine serum (v/v) (FBS; Hyclone, Logan, UT) and 1% Penicillin Streptomycin (100 U/mL and 100 µg/mL, respectively) at 37 °C in a humidified atmosphere containing 5 % CO₂, with 0.25 % trypsin digestion batches. The logarithmic phase of the cells were used for the study.

Drug sensitivity test

Cells at the logarithmic growth phase (in RPMI1640 medium) at a density of 1.0×10^5 cells/mL of cell suspension, were seeded in 48-well plates and cultured at 37 °C for 24 h, until the cells became adherent. SBDE at different final concentrations (30, 60, 120, 240 µg/mL) were added to different wells, and the wells were incubated for different periods (24, 48 and 72 h) at 37 °C. A well containing 20 µg/mL cisplatin served as positive control, while another well containing saline in place of SBDE served as negative control.

Cell morphology assay

A suspension of A549 cells at the logarithmic growth phase in RPMI 164 medium was adjusted to a density of 1.0×10^5 cells/mL in a 48-well culture plate, and cultured for 24 h at 37 °C to achieve adherence. The wells were grouped into two. One group (experimental group) received different concentrations of SBDE (62.5, 125 or 250 ug/mL) in the culture medium, while the other group (control group) received 0.04 % DMSO in place of SBDE. The cells were observed under inverted microscope after 24, 48 and 72 h for changes in cell size, cell membrane and nucleus.

Growth curve and doubling time (TD) of A549 lung cancer cells

The A549 cells at logarithmic growth phase at a density of 1×10^4 cells/mL were seeded in 24-

well plates. One milliliter was taken from each of three wells at the same time point daily, and digested. The number of cells per well was counted using a hemocytometer, and the mean number of cells was determined. Counting was done for 8 days. A growth curve was plotted with mean cell count as vertical axis, and time as horizontal axis. Doubling time (TD) for the human lung cancer A549 cells was calculated using Eq 1.

$$TD = t\{\log 2 / (\log N_t - \log N_o)\} \dots\dots\dots (1)$$

where t is time interval, while N_o , and N_t are the initial mean count and mean count after time t , respectively.

MTT assay

A suspension of the A549 cells (0.2 mL) at logarithmic phase was seeded in a 96-well plate with each well containing 5×10^3 cells, and cultured overnight for cell adhesion. In the experimental group, the original culture medium was drained, and replaced with different concentrations of freshly-prepared SBDE (one concentration in 24 wells, and 6 wells per group). The wells of the control group medium contained 0.04 % DMSO, while wells without cells served as blank control. All wells were incubated for 24, 48, 72 and 96 h at 37 °C in a humid atmosphere containing 5 % CO₂. The supernatant was discarded, and 20 µL of MTT solution (5 g/L) was added to each well and incubated at 37 °C for 4 h. Then, the supernatant was carefully removed by suction, and 150 µL DMSO was added to each well to dissolve the crystals. The absorbance of each solution was read at 490 nm against blank control, and the results were used for calculating the growth inhibition rate.

Immunocytochemical staining

The A549 cells at logarithmic phase were digested with 0.25 % trypsin, and 2×10^5 cells were seeded into a 6-well plate and cultured overnight for cell adhesion. The culture medium was removed, and 3mL of SBDE was added to

different cells to final concentrations of 30, 60, 120 and 240 µg/mL. Each SBDE concentration was replicated in six parallel wells. In the control wells, SBDE was replaced with an equivalent volume of 0.04 % DMSO. The plates were incubated for 24 and 48 h; the glass slides were taken out, dried and fixed with 4 % paraformaldehyde for 10 min at 4 °C according to instructions contained in the operation manual of S-P kit. The plates were observed for appearance of clear brown granules which is indicative of positive cells.

The presence of Bcl-2 and p21 ras in cytoplasm is indicative of positive expression; Ki67 tan is indicative of positive expression in the nucleus, caspase-9 is an index of positive expression in the cytoplasm and the nucleus; while caspase-3 and caspase-8 indicate positive expression in the cytoplasm or cytoplasm and nuclei. They are expressed less in cells without changes in color, or lightly-colored cells. The cells in the SBDE plates and control plates were observed at high magnification ($\times 400$) in 5 fields, and positive cells were counted and expressed as percentage of the total number of cells.

Statistical analysis

All data are expressed as mean \pm standard error of mean (SEM), and processed using SPSS 16.0 (SPSS Inc., Illinois, Chicago, USA). They were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of SBDE on proliferation of A549 cells

SBDE at different concentrations significantly inhibited the proliferation of A549 cells after 48 h when compared with control group ($p < 0.05$). The inhibitory effect was concentration-dependent. The results are shown in Table 1.

Table 1: Inhibitory effect of SBDE on proliferation of A549 cells

Group	Dose (µg/mL)	Absorbance (250 nm)	Inhibition (%)
Negative control	—	0.331 \pm 0.005	—
Positive control	10	0.064 \pm 0.002 ²	98.35 \pm 0.14
SBDE	20	0.185 \pm 0.004 ¹	43.29 \pm 0.17
	40	0.194 \pm 0.003 ¹	53.08 \pm 0.24
	80	0.181 \pm 0.003 ²	56.21 \pm 0.28
	160	0.142 \pm 0.004 ²	63.34 \pm 0.17

Values are mean \pm SEM (n = 4); ¹ $p < 0.05$, ² $p < 0.01$ relative to control

Inhibitory activity of SBDE

Results from MTT assay showed that SBDE exerted a concentration and time-dependent inhibitory effects on the growth of human lung cancer A549 cells (Table 2). The inhibitory effect was highest at a concentration of 160 $\mu\text{g/mL}$. In addition, for each SBDE concentration, the inhibitory effect increased with time and became maximum at 96 h.

Effect of SBDE on A549 cell apoptosis and cell cycle

SBDE at a concentration of 40 $\mu\text{g/mL}$ brought about a cell cycle arrest at G0 / G1 phase in 24 and 48 h, and blocked the transformation from G1 phase to S phase (Table 3). With increase in G0 - G1 phase transition time, there was reduction in the number of cells in the S phase. The increase in the percentage of cells in the G0/G1 phase, and decrease in the cells in S phase, were concentration-dependent and time-dependent ($p < 0.01$).

DISCUSSION

The results obtained in this study show that SBDE has significant time- and concentration-dependent inhibitory effects on the proliferation of A549 cells. The extract also exerted strong apoptotic effects on the A549 cells, as seen from the morphological changes in the cells. The dynamics of tumor cell proliferation are due to loss of control of cell cycle, resulting in uncontrolled G1/S and G2/M transformations. The cells were mainly in the active DNA synthesis S phase, which led to abnormal cell proliferation [5]. Thus blockage of the G1/S phase and G2/M phase can effectively control

the tumor cell cycle and inhibit tumor proliferation.

Ki-67 antigen is a more positive marker of nuclear proliferation, and Ki-67 may be used as a biomarker for determining high risk individuals in pre-cancer population [7-9]. Results from Ki-67 antigen assay showed that SBDE significantly decreased the expression of Ki-67 in A549 cells. This implies that a large number of the A549 cancer cells were in the stationary phase of cell cycle. In effect, SBDE may contain active principles that regulate the gene which inhibits cancer cell mitosis, thereby inhibiting the growth of tumor cells. P21 ras cancer gene is involved in transmission of intracellular information which regulates the cell cycle, and it is the "initiation factor" in the occurrence of tumors [10-13]. The expression of p21ras antigen was also significantly decreased in the SBDE-treated A549 cells, in a time- and concentration-dependent manner, when compared with untreated controls. Again, this is an indication that most of the cancer cells were in the S phase of the cell proliferation cycle. This supports the presence of anti-proliferative and mitosis-inhibiting agents in SBDE.

Caspases are proteolytic enzymes which constitute key components of the apoptosis system. They are a family of cysteine proteases involved in regulation of apoptosis, which is considered to be a key to cancer cell death [14-17]. In this study, SBDE treatment led to significantly lowered expression of Bcl-2, and significant increase in the expression of caspase-9. These results demonstrate that the anti-proliferative effects SBDE also involve the mitochondrial apoptotic pathway.

Table 2: Inhibitory effects of SBDE on A549 lung cancer cell growth

Group	Dose ($\mu\text{g/mL}$)	24h	48h (%)	72h	96h
Control	—	—	—	—	—
SBDE	5	1.24	2.08	2.18	2.14
SBDE	10	5.37	12.35	19.41	26.38
SBDE	20	23.24	23.14	38.22	41.09
SBDE	40	32.11	38.19	49.15	56.17
SBDE	80	46.24	52.73	64.34	70.27
SBDE	160	44.29	54.35	67.21	75.36

Table 3: Effect of SBDE on cell cycle distribution of A549 cells at 24 and 48 h

Group	G0/G1 (%)	S (%)	G2/M (%)
Control	47.28 \pm 3.04	45.31 \pm 3.08	7.75 \pm 1.25
SBDE 24 h	59.37 \pm 3.10	38.43 \pm 3.28	4.96 \pm 1.15
SBDE 48 h	69.18 \pm 4.07	23.27 \pm 4.54	3.37 \pm 1.09

Values are mean \pm SEM (n = 8); $p < 0.01$ compared to control

Caspase-3 is a pro-apoptosis caspase. Its expression in the SBDE-treated A549 human lung cancer cells was significantly higher than in the untreated control cells, indicating that SBDE promotes apoptosis in these cells. Thus, SBDE induced apoptosis by inhibiting the signal transduction pathway enzymes to achieve elevated expressions of the pro-apoptosis caspases 3, 8 and 9; as well as the apoptosis-related gene Bcl-2.

CONCLUSION

The findings of this study reveal that SBDE has significant effect on the inhibition of proliferation and induction of apoptosis in human lung cancer A549 cells. These findings suggest that SBDE might be a potent source of drugs for the treatment of lung cancer patients.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Duan Jing designed all the experiment and revised the paper. Zhang Hu performed the experiment and wrote the manuscript.

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