

The inhibitory effect of cyclopamine on human non-small cell lung cancer

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Lung cancer is the leading cause of cancer-related death worldwide nowadays. Non-small cell lung cancer (NSCLC) is relatively resistant to both chemotherapy and radiotherapy.¹ Sonic hedgehog (SHH) signaling pathway plays an important role in developmental biology, and recently, the activation of SHH signaling pathway has been reported to have a critical role in a subset of lung cancers.² Cyclopamine is a specific inhibitor of the SHH pathway. The objective of this study was to investigate the antiproliferative effects, in vitro and in vivo, by cyclopamine in human non small cell lung cancer cell A549.

This study was conducted between April 2009 and July 2009 in the Department of Oncology, Renmin Hospital of Wuhan University, Wuhan, China. All animal experimental protocols were approved by the Animal Care and Use Committee of Wuhan University, and conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, Chinese Version, 1996). All statistical analyses were performed by the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) 11.0, software package for Windows. A two-tailed *p*-value less than 0.05 was considered statistically significant.

Effect of cyclopamine on A549 cell proliferation. Human lung cancer cell line A549 was obtained from American Type Culture Collection. The A549 cell was grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, USA), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to investigate the cell viability of A549 after cyclopamine treatment. The A549 cells were seeded into a 96-well plate at a density of 1×10^4 cells/well. Cyclopamine (Toronto Research Chemicals, Toronto, Canada) of different concentrations were added to the culture medium. After treatment for 24 hours, 20 μ L MTT (5mg/ml) was added to each well and incubated for an additional 4 hours, and then culture media was discarded, followed by addition of 0.15 mL Dimethyl sulfoxide and vibrated for 10 minutes. A model 550 microplate reader was used to measure the absorbance at 490 nm. The inhibitory rates (IR) were calculated as follows: $IR(\%) = (1 - \text{Absorbance of the treated wells}) / (\text{Absorbance of the control wells}) \times 100\%$.

The results show that cyclopamine could inhibit the growth of A549 lung cancer cells in a dose-dependent manner. Even at a minimum dose, cyclopamine

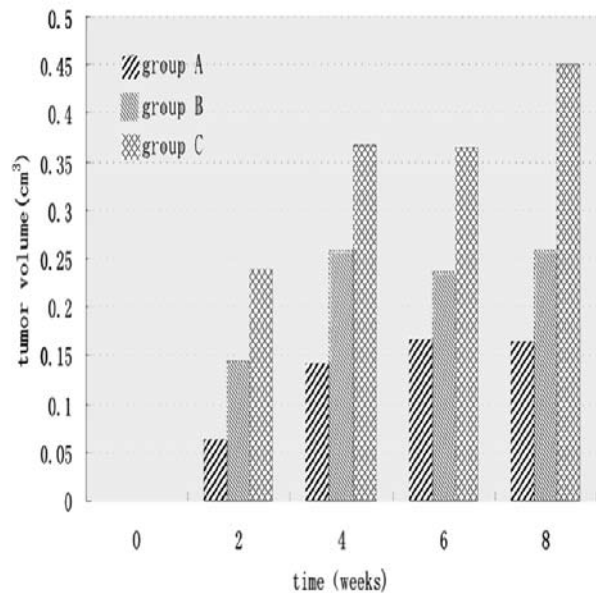


Figure 1 - The growth inhibitory effect of cyclopamine in A549 xenografts.

showed an antiproliferative effect on A549 cells. When the concentration of cyclopamine reached 10mmol/L, the inhibitory rate amounted to nearly 90% at 24 hour.

Effects of cyclopamine on A549 cell cycle progression and apoptosis. The A549 cell with the density of 1.0×10^7 cells/ml was serum-starved for 24 hours and then treated with different concentrations of cyclopamine. The percentage of cells in each phase of the cell cycle, G0/G1, S, and G2/M was determined by flow cytometry after cells were treated. The cells were pelleted by centrifugation and washed twice with phosphate buffer solution (PBS). Then, the cell pellets were resuspended in 0.5 ml PBS and fixed in 5 ml ice-cold 70% ethanol. After resuspension with 1 ml PBS, the cells were incubated with RNase A (20 mg/L, Sigma) and proliferative index (PI) (50 mg/L, Sigma). Then the cells were assayed using a FACScan flow cytometer in combination with BD FACSDiva software (BD Biosciences, San Jose, USA). The apoptosis index (AI) and PI were calculated: $AI = \text{Hypodiploid peak cell} / \text{total cell number} \times 100\%$; $PI = (\text{S phase} + \text{G2/M phase}) / (\text{G0/G1 phase} + \text{S phase} + \text{G2/M phase}) \times 100\%$.

The percentage of cell population of the G0/G1 phase was significantly increased, while that in the S and G2/M phase decreased. Cyclopamine caused cell cycle arrest in the G0/G1 phase in a dose-dependent manner. The AI of A549 increased quickly along with cyclopamine dose escalation, whereas the PI decreased. Cyclopamine induced apoptosis of A549 cells in a dose-dependent manner.

The growth inhibitory effect of cyclopamine in A549 xenografts. We used the A549 cells in vivo model to

investigate the inhibitory effects of cyclopamine on the growth of subcutaneously (SC) implanted lung cancer. The concentration of cyclopamine used in this step was 5 mmol/L. The A549 cells were harvested from subconfluent cultures. Suspensions consisting of single cells with >90% viability were used for the injections. Four- to five-week old Balb/C nude mice were obtained from Wuhan University, and 1×10^6 A549 cells in 200 μ L PBS were inoculated SC into the left flank. Fifteen mice were divided into 3 groups (group A, B, C), each group consisting of 5 mice. The mice in group A were given 1 ml/100g of cyclopamine intraperitoneally (IP), concurrent with the tumor inoculation. The mice in group B were given 1 ml/100g of cyclopamine IP. One week after the tumor inoculation; the mice in group C were given physiologic saline as control. The frequency of IP injection of cyclopamine was once a week. The tumor volume was estimated by measuring tumor size and using the following formula: tumor volume = $0.5 \times L \times W^2$, where L and W represent the largest diameter and the smallest diameter. As shown in Figure 1, the tumor volume was increasing quickly in group C. The tumor volume increased quickly at first but slowed down latterly in group B. The growth speed was significantly slower in group A compared with the other 2 groups.

Lung cancer has the highest cancer mortality rate today. Non-small cell lung cancer accounts for approximately 80% in these patients, and the other 20% are small cell lung cancer. Since NSCLC was not sensitive to chemotherapy and radiotherapy, there is limited treatment for these patients, especially for those with advanced stage disease. New therapeutic strategies are needed to deal with these patients. This study was designed to investigate the antiproliferative effects, in vitro and in vivo, through targeting SHH signal pathway in human NSCLC cell line A549. This may provide some clues to develop new drugs for NSCLC.

The SHH signaling pathway is important for the development of the mammalian embryo. Recently, some studies have reported abnormal activation of the SHH pathway in several types of tumors, including lung cancer.³ Cyclopamine is a special inhibitor of the SHH signaling pathway. The inhibitory role of cyclopamine has been tested for some kinds of cancers, such as prostate cancer.⁴ We have also reported that cyclopamine could induce inhibition in pancreatic cancer cells with activated SHH signaling.⁵ In this study, we tested the inhibitory effect of cyclopamine in none small cell lung cancer cell line A549 in vitro and in vivo.

Our results showed that the cyclopamine could inhibit the proliferation of A549 lung cancer cells in a dose-dependent manner. Even at a minimum dose, cyclopamine starts to show an antiproliferative effect on A549 cells. At the dose of 5mmol/L, almost half of the

A549 cells were inhibited at 24 hours. We then tested the inhibitory effect of cyclopamine on the growth of a SC implanted lung cancer model. We found that the growth speed of the tumor was significantly slowed down when animals were treated with cyclopamine concurrently with tumor inoculation in group A. This may due to the features of cyclopamine; it can inhibit the proliferation of tumor cell.

Moreover, we found that the A549 cells were arrested in the G0/G1 phase after cyclopamine treatment, concomitant with a decrease of the cell number in the S and G2/M phases. As SHH could activate the cell cycle regulators, such as the cyclins, cyclopamine could down-regulate cyclins through the inhibition of SHH signaling. This may cause the cell cycle arrest in lung cancer cell. The apoptotic effect induced by cyclopamine in A549 cells was also tested in this study. The AI of A549 increased quickly along with cyclopamine dose escalation, whereas the PI decreased. It seems that cyclopamine induced apoptosis of A549 cells in a dose-dependent manner. Cyclopamine could induce apoptosis in cancer cells without harmful effects on normal tissues. Therefore, targeted inhibition of SHH signaling pathways should be possible without disturbing normal cell function, and also minimizing toxicity from such therapy.

The aberrant SHH in NSCLC makes it a promising target for therapy. Further understanding of the molecular basis of the cyclopamine effect will help us plan better ways to treat NSCLC in the future.

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