

ORIGINAL ARTICLE

Lycopsamine inhibits the proliferation of human lung cancer cells via induction of apoptosis and autophagy and suppression of interleukin-2

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Summary

Purpose: Lycopsamine is an active pyrrolizidine alkaloid that shows significant bioactivity. Herein, lycopsamine was evaluated for the first time for its anti-lung cancer activity. Its effects on cellular apoptosis, autophagy, cell cycle and IL-2 gene were also examined.

Methods: The human lung cancer A549 and normal MRC5 cells were used in the study. MTT assay was used to determine the cytotoxicity of lycopsamine. Transmission electron microscopy (TEM) and western blotting were implemented for analyzing autophagy. DAPI staining, Annexin V-FITC/Propidium iodide (PI) and western blotting assays were used to study cellular apoptosis. Cell cycle was examined through flow cytometry. The expression of IL-2 gene was monitored by western blotting.

Results: Lycopsamine targeted the proliferation rate and reduced it remarkably in a dose-dependent manner. On searching for underlying mechanism, the antiproliferative effect of lycopsamine was due to autophagy and the expressions of pro-autophagy proteins (LC3-I, LC3-II, Beclin-1) increased

on drug exposure. Furthermore, the antiproliferative effects were also found to be mediated via apoptosis induction and were associated with increased Bax and decreased Bcl-2 levels. Next, flow cytometry showed that lycopsamine inhibited cell cycle progression at G2/M-check point in lung cancer cells. Furthermore, the expressions of IL-2 gene decreased after lycopsamine treatment of these cells. In conclusion, on testifying the current designed hypothesis, lycopsamine showed significant antiproliferative effects in A549 lung cancer cells in a dose reliant manner. The antiproliferative effects of lycopsamine were associated with its autophagy inducing, apoptosis inducing, and inhibiting IL-2 expression, potential.

Conclusion: Taken together, lycopsamine is a potent anti-lung cancer agent and can be a lead molecule in lung cancer treatment.

Key words: pyrrolizidine alkaloids, lycopsamine, lung cancer, autophagy, apoptosis

Introduction

Plants serve as natural sources of drugs against several human diseases [1,2]. Cancer is a lethal malignancy. Cancer cells demonstrate a trend for metastasis, invasion and are extremely anaplastic. Alkaloids are an incredible class of naturally occurring phytochemicals, extracted from a variety of medicinal plants [3,4]. Alkaloids show a broad spectrum of biological activities [5]. These compounds

have been reported to play a major role as antitumor drugs through suppression of DNA replication via inhibition of the enzyme topoisomerase [6]. Alkaloids are potent p53 gene suppressors and apoptosis inducers. Even though alkaloids existed prior to human existence, few of them counterfeited neurotransmitters in human nervous system including acetylcholine, serotonin and dopamine [7,8]. The

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remarkable abilities of alkaloids on humans have been utilised to treat several human abnormalities [9]. Therefore, the medicinal importance and implications of alkaloids and the research interpreting their underlying mechanism of action in the violent proliferation of cancer cells would assist in designing novel drugs and lead molecules [9-11]. *Eupatorium maculatum* L. belonging to the family Asteraceae, commonly known as "Spotted Joe-Pye weed", is a plant growing in the wild in Canada and North America [12]. Indigenous Indians use this purple flowing plant in the treatment of diuretical problems and is believed to impact cystic and chronic renal disorders. The plant is rich in pyrrolizidine alkaloids and these compounds are reported with impressive medicinal values [13]. Lycopsamine is an active pyrrolizidine alkaloid which has been reported to induce a number of pharmacological and biological effects. Lung cancer is the prevailing dangerous malignancy among both men and women [14,15]. In 2012 alone, lung cancer represented the 14% of the total number of cancer incidence with over 1.7 million new patients [16]. In most of the lung cancer patients, the disease remains silent (asymptomatic) till advanced stages [17]. The 5-year survival rate associated with lung cancer is very poor [18]. In 2015, lung cancer was responsible for 27% of cancer related deaths in USA and in 2016, 20% of all cancer deaths in Europe were due to lung cancer. Therefore, the lethality of this malignancy is getting worse due to lack of effective treatment, and high side-effects of the current drugs. To tackle lung cancer, novel, less toxic, and more efficient drugs that can improve survival chances are the need of the hour. Herein, the current research was designed to unveil the capability of lycopsamine against human lung can-

cer. The effects of autophagy induction, apoptosis induction and inhibition of IL-2 gene expression by lycopsamine were also studied

Methods

Cytotoxicity assessment

Cytotoxicity of lycopsamine was assessed through MTT ((4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Sigma, St. Louis, MO, USA). Briefly, lung cancer A549 and normal MRC5 cells were placed in 96-well microplates bearing 100 μ l RMPI 1640 medium with the density of 2×10^4 cells/ml. In addition, antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) were also supplemented to each well followed by pre-culturing of both cell lines overnight at 37°C in a CO₂ (5%) humidified incubator. Thereafter, each well plate was supplied with variant lycopsamine concentrations (control, 1.6, 3.2, 6.25, 12.5, 25, 50 and 100 μ M) for 72h. Afterwards, MTT stock solution of 50 μ l with 2.5 mg MTT/ml in culture medium was added to each well and incubated for an additional one hour. During this period formazan crystals were evolved (indicated the living cells) and DMSO was used to dissolve them. Finally, the absorbance was recorded with an automatic microplate reader (LKB 5060-006 Micro PlateReader, Austria) at 540nm wavelength.

Transmission electron microscopic (TEM) analysis

Autophagy in lycopsamine treated lung A549 cancer cells was investigated via TEM. Cells were harvested at 80% growth confluence and subjected to variant lycopsamine doses (0 and 10 μ M) for 24h. Thereafter, cell assembling was performed via trypsinisation followed by washing with phosphate buffered saline (PBS) of lycopsamine treated A549 cells. Afterwards, cells were first fixed with glutaraldehyde (2%) in phosphate buffer (0.1M) followed by post fixation with osmium tetroxide (1%). Cells were then exposed to ethanol embedded

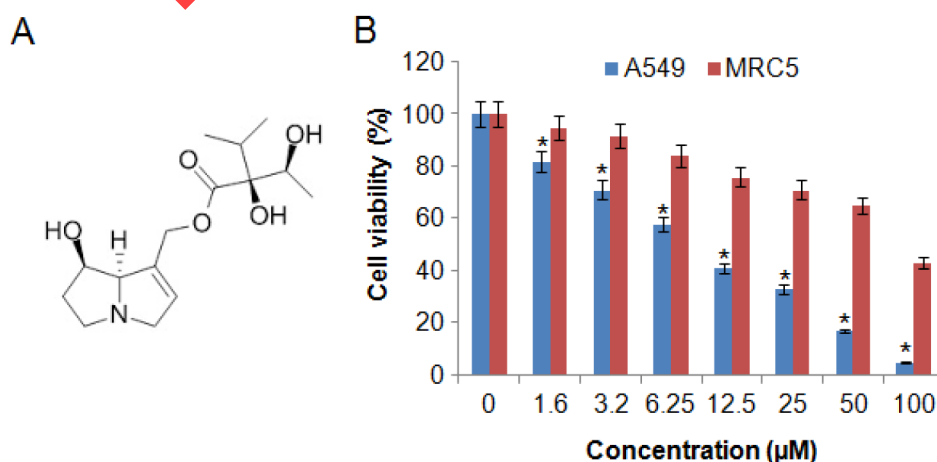


Figure 1. A: Chemical structure of lycopsamine. **B:** Graphical representation of outcomes from MTT assay. A549 and MRC5 cells were exposed to different lycopsamine doses for 72h. The results showed tremendous suppression in the proliferation of A549 cells as compared to normal MRC5 cells. Data are presented as mean \pm SD of three independent experiments (*p<0.05).

in resin followed by cutting of thin sections with an ultramicrotome. Finally, these sections were loaded on transmission electron microscope (Zeiss CEM 902, Jena, Germany) linked to a digital camera for capturing of different fields.

Apoptosis analysis

DAPI staining was performed to determine the value of lycopsamine as a potential apoptosis inducer in A549 cells. Briefly, A549 cells were cultured in 6-well

plates for 12h at 37°C. Then, these cells were treated with variant lycopsamine molecule doses (control, 5, 10 and 20 μ M) for 24h. Till the lycopsamine treated A549 cells casted-off, about 20 μ l of casted cells were loaded onto glass slides for DAPI staining. Finally, the slides were cover-slipped and assessed under a fluorescent microscope for supplementary analysis. To quantify the effect of apoptosis induction by lycopsamine, Annexin V/FITC/PI assay was performed. A similar procedure was followed for Annexin V/FITC/PI as for DAPI except Annexin V/PI staining and analysis via flow cytometry.

Cell cycle check points assessment

The lung cancer A549 cells were placed in 6-well plates at a density of 2×10^5 cells/ mL well followed by overnight incubation. Afterwards, cells were exposed to lycopsamine treatment (control, 5, 10 and 20 μ M) for 72 h followed by collection of the floating and attached cells through centrifugation. Afterwards, PBS washing of attached cells was performed, followed by fixation in 70% ethanol at 20°C for 120h. Then, the cells were collected and prior to pretreatment with 50 μ g/ml RNase A, cells were again washed with PBS. Finally, Annexin V/PI staining was performed with a final concentration of 50 μ g/ml. Afterwards, FACScalibur flow cytometer (Becton Dickinson, United Kingdom) was used to perform flow cytometric analysis for analysing different cell cycle check points.

Western blotting assay

To check the protein expressions in lycopsamine treated A549 cells, western blotting assay was performed. Briefly, cells were treated with variant molecule doses (control, 5, 10 and 20 μ M) for 24h. Next, lycopsamine treated A549 cells were lysed with RIPA buffer and the lysates were analyzed through binichoninic acid (BCA) assay for protein quantification. 40 μ g of proteins were separated by SDS-PAGE and electrophoretically trans-

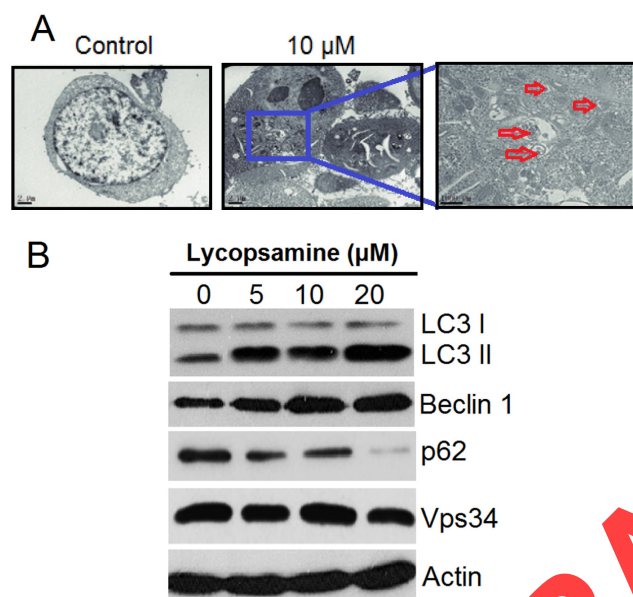


Figure 2. A: TEM analysis of A549 cells after lycopsamine exposure at represented doses (Arrows depict autophagosomes) **B:** Western blotting results specifying the levels of pro-autophagic proteins including p62 anti-autophagic protein. The experiments were performed in triplicate.

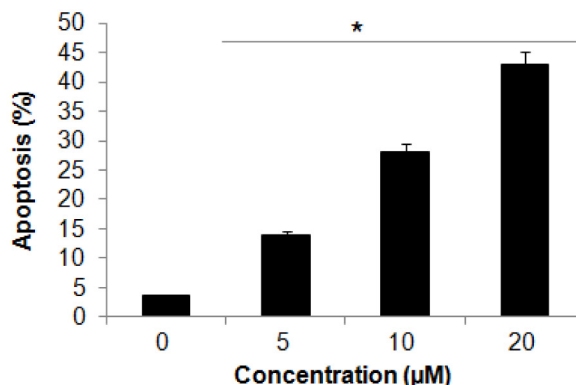
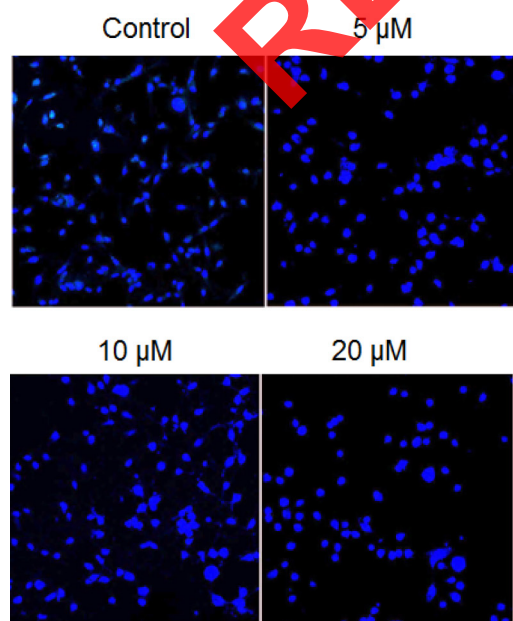


Figure 3. DAPI staining assay images presenting the apoptotic cell morphology and percentage of apoptotic A549 cells after lycopsamine exposure. The experiments were performed in triplicate and data are shown as mean \pm SD (*p<0.05).

ferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, USA). These membranes were then blotted with primary antibodies of Bax, Bcl-2, p62, LC3-I, LC3-II, Beclin-1 and Vps34 (Santa Cruz, CA, USA) having 1:1000 dilution. Thereafter, secondary antibody treatment was followed at 4°C overnight. Finally, enhanced chemiluminescence reagent (Amersham, Piscataway, NJ, USA) was utilized for the determination of protein bands.

Statistics

All the experimental data are shown as mean \pm SD of 3 independent experiments. Intergroup differences were compared by one-way analysis of variance (ANOVA: Scheffe's test for *post hoc* comparisons) using SPSS software. $P < 0.05$ was considered as statistically significant.

Results

Lycopsamine induced cytotoxicity in lung cancer A549 cells

The cytotoxicity of lycopsamine (Figure 1A) was investigated by MTT assay which showed that the molecule induced dose-dependent cytotoxicity in A549 cells. The proliferation of A549 cells decreased significantly with increased molecule doses (0-100 μ M) from 100% to 10% (Figure 1B). In normal MRC5 cells the viability remained almost unchanged. Therefore, MTT assay results showed the potential of lycopsamine as significant proliferation inhibitor in A549 cells.

Lycopsamine induced autophagic cell death in A549 cells

TEM analysis was performed to monitor autophagy in lycopsamine treated A549 cells. The results indicated formation of autophagosomes on molecule exposure (0 and 10 μ M) (Figure 2A). Autophagosomes are hallmark for autophagy, therefore TEM analysis indicated autophagic cell death in A549 cells. The fact that lycopsamine induced autophagic cell death was further reinforced by western blotting analysis. The expression of autophagy related proteins LC3-II, and Beclin-1 was increased while LC3-I, Vps34 expression remained almost unchanged upon lycopsamine exposure (Figure 2B), while the autophagy suppressor protein expression (p62) was suppressed remarkably.

Lycopsamine induced apoptotic cell death in A549 cells

The results showed cell morphology of apoptotic cells in lycopsamine treated A549 cells. Morphological features like membrane blebbing, condensed nucleus and DNA-fragmentation were observed, indicating apoptosis in A549 cells (Figure 3). The apoptotic cell percentage increased up to 45% after molecule exposure. Furthermore, the

number of apoptotic cells was analysed by Annexin V/FITC/PI assay which showed increased early and late apoptotic cell population and significant necrotic cell population on higher molecule doses (Figure 4). The apoptotic cell death by lycopsamine was further supported by western blotting assay which depicted that the levels of Bax were increased and the levels of Bcl-2 were decreased by lycopsamine (Figure 5). Therefore, lycopsamine induced apoptotic cell death in A549 cells.

Lycopsamine possesses cell cycle inhibitory effects in A549 cells

Cell cycle check points were monitored by flow cytometric analysis. Herein, lycopsamine resulted

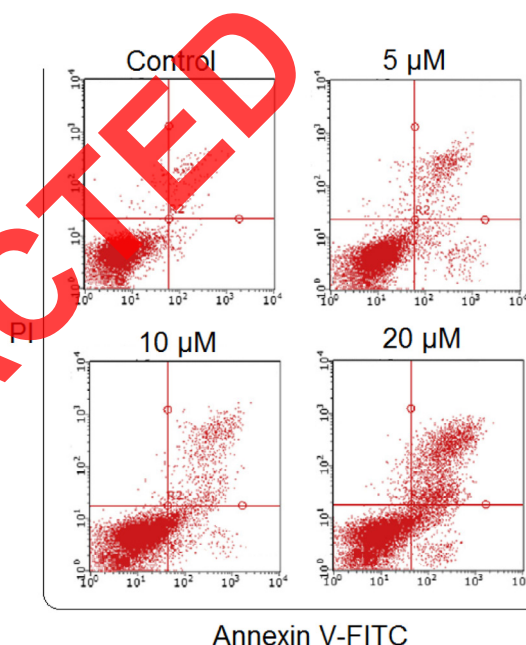


Figure 4. Annexin V/FITC/PI assay results developed via flow cytometry indicating enhanced early and late apoptotic cells. As the lycopsamine concentration increased the number of necrotic and apoptotic cells increased. The experiments were performed in triplicate and data are shown as mean \pm SD ($p < 0.05$).

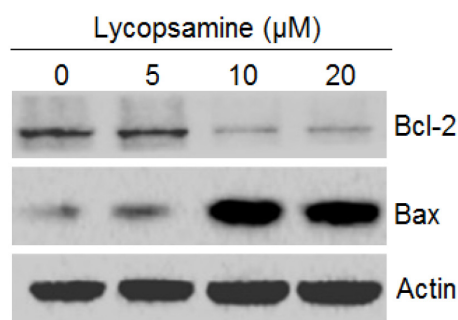


Figure 5. Western blotting indicating the levels of Bax and Bcl-2 proteins after 24h of lycopsamine exposure. The experiments were performed in triplicate.

to increase in the percentage of G2/M-phase cells as compared to controls (Figure 6). Therefore, it is evidenced that cell cycle was blocked at this check point after lycopsamine exposure. Cells in other phases of the cell cycle decreased with higher molecule doses.

Inhibition of IL-2 proteins lycopsamine

There are several molecular pathways that operate to maintain the integrity of cancer cells. The IL-2 protein is among one of the important proteins that help in cancer cell survival. After lycopsamine treatment for 24h, cell lysates were examined with Western blotting and the results indicated significant suppression of IL-2 expression after lycopsamine treatment of A549 cells (Figure 7). The effect of IL-2 suppression was observed to be dose-dependent.

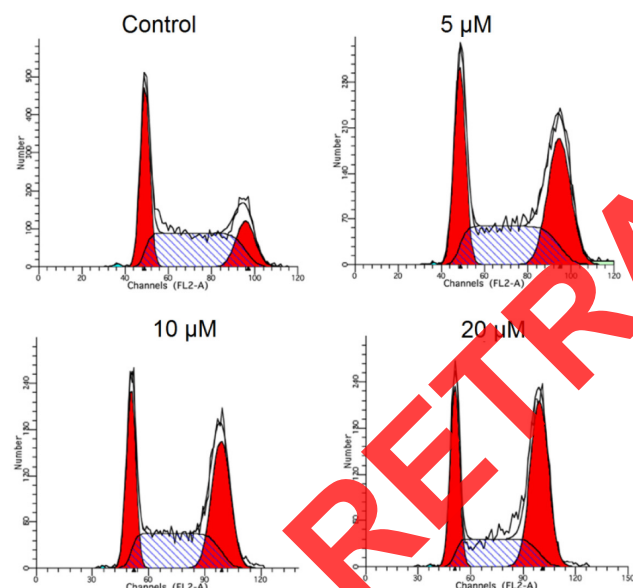


Figure 6. Flow cytometric examination of different cell cycle check points. The results presented significant dose-dependent accumulation of G2/M-phase cells indicating cell cycle arrest. The experiments were performed in triplicate and data are shown as mean \pm SD ($p < 0.05$).

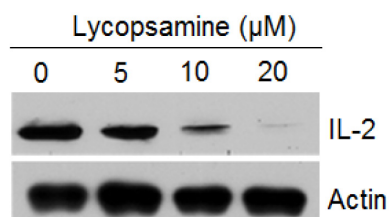


Figure 7. Western blotting assay indicating the levels of IL-2 protein after 24h of lycopsamine exposure. The experiments were performed in triplicate and show that lycopsamine treatment suppresses the IL-2 expression in a dose-dependent manner.

Discussion

Lung cancer is a global health issue that needs to be addressed as it accounts for very high mortality. The 5-year survival rate in lung cancer patients is very poor. The major hurdles present in lung cancer management include late diagnosis, severe side effects of the present used drugs, low survival rates and poor prognosis. Therefore, an urgent situation for novel therapeutic agents arises to curb the present lung cancer situation. Autophagy is a pivotal mechanism of living cells to dispose-off cytoplasmic materials including organelles, protein aggregates and macromolecules [21,22]. These disposables are transported to lysosomes where they are degraded. Autophagy bears three distinct types i.e. microautophagy, chaperone-mediated autophagy and macroautophagy. Macroautophagy simply stands for autophagy and it is induced after energy stress or nutrient starvation via suppression of mTOR (rapamycin) [23,24]. Suppression of mTOR initiates a number of molecular interactions in a stressed cell and ultimately results to formation of "omegasome". Omegasome is then surrounded by isolation membranes and evolves as an autophagosome [25]. Another mechanism that is used by mammals for elimination of damaged and malfunctioning cells is termed as apoptosis. Apoptosis is often related with enhanced Bax protein expressions and marks significant morphological changes in a target cell [26,27]. Herein, lycopsamine - a pyrrolizidine alkaloid- was evaluated for anti-lung cancer activity along with examining its effects on cellular apoptosis, autophagy, cell cycle and IL-2 gene. The results portrayed that lycopsamine is a potential antiproliferative representative against lung cancer as depicted from MTT assay. Furthermore, on investigating the underlying antiproliferative molecular mechanism of lycopsamine autophagic analysis it was revealed that the autophagosomes were associated with increased levels of LC3-I, LC3-II, and Beclin-1. Next, the results also depicted that for the antiproliferative effects of lycopsamine in A549 cells, apoptosis plays an important part. Western blotting indicated increased Bax expression and decreased Bcl-2 expression, indicating induction of apoptotic cell death. Cell cycle was also blocked by the exposure of lycopsamine in A549 cells at G2/M-check point of the cell cycle. Finally, the expression of IL-5 gene (important growth and survival regulatory gene) was observed to diminish after the test molecule exposure.

Conclusion

In conclusion, all the performed investigations

in the current study indicated that lycopsamine acts as an anti-lung cancer candidate. It was observed that lycopsamine induced antiproliferative effects via stimulation of apoptosis, autophagy, cell cycle arrest and inhibition of IL-2. Therefore, this molecule can be considered for lung cancer treat-

ment provided further clinical investigations are carried out.

Conflict of interests

The authors declare no conflict of interests.

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