Apoptotic effect of cordycepin on A549 human lung cancer cell line

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Abstract: Cordycepin, an active ingredient in the insect fungus Cordyceps militaris, is in a category of compounds that exhibit significant therapeutic activity. The aim of the present study was to investigate the effect of cordycepin on cell proliferation, apoptosis, and cell cycle in A549 human lung cancer cells. MTT assay was used to evaluate the cytotoxic effect of cordycepin on A549 cell growth. Apoptotic effect was observed using cell morphology, DAPI staining, and DNA fragmentation studies. Flow cytometry (FCM) analysis was used to analyze cell cycle status after cordycepin treatment. Furthermore, apoptosis was assayed using annexin V-Alexa Fluor 488. Results of MTT assay showed that cordycepin significantly inhibited cell proliferation with an IC50 value of 64 µg/mL. The number of rounding-up cells increased with cordycepin treatment and changes in cellular morphology were seen. In DNA fragmentation studies, a typical ladder pattern was observed on agarose gel and formation of apoptotic bodies was further confirmed using DAPI staining. The FCM analysis of cordycepin-treated cells showed that apoptosis rate increased with the increase in dosage. In conclusion, cordycepin induces apoptosis in A549 human lung cancer cell line and could be a potential therapeutic candidate for lung cancer treatment.

Key words: Cordyceps militaris, cordycepin, lung cancer, flow cytometry, cell cycle, apoptosis

1. Introduction

In the Western world, lung cancer is still a leading cause of mortality, responsible for 170,000 deaths per year, more than deaths due to breast, colon, and prostate cancer combined (Danesi et al., 2003). Approximately 80%–85% of all lung cancers are classified as nonsmall-cell lung cancer (NSCLC). It is very difficult to cure NSCLC, even in the early stage, as it develops resistance against chemotherapeutic agents. Therefore, treatment of NSCLC remains elusive despite the availability of a variety of chemotherapeutic agents that exhibit sophisticated mechanisms of action (Kim et al., 2003; Jemal et al., 2010; Vejselova and Kutlu, 2015). In the last few decades, a large number of natural bioactive metabolites, including paclitaxel, vinblastine, and camptothecin, have been isolated and used for cancer treatment (Kosty et al., 1994; Douillard et al., 2001; Koshkina et al., 2003). The inadequate availability of cancer treatment invites scientific communities to propose the development of novel therapeutic strategies for lung cancer treatment. Apoptosis, also referred to as programmed cell death, is an important mechanism of many anticancer drugs, including cordycepin (Tuli et al., 2013).

The biometabolite cordycepin was first isolated from the fermented broth of the medicinal mushroom Cordyceps militaris (Cunningham et al., 1950), which is an entomopathogenic fungus that grows parasitically over lepidopteran larvae and insect pupae. The genus Cordyceps is well known in traditional Chinese medicine and exhibits a variety of clinical health effects including immunomodulatory, anticancer, antioxidant, antiinflammatory, and antimicrobial activities. Cordycepin is a type of nucleoside analogue that is structurally similar to adenosine, except that it lacks a 3’ hydroxyl group. It is known to interfere with a number of biochemical and molecular processes, such as purine biosynthesis, DNA/RNA synthesis, and mammalian target of rapamycin signal transduction pathway (Tuli et al., 2014b). In ethnopharmacological studies, Cordyceps extract has been shown to induce apoptosis (Park et al., 2009; Thakur et al., 2011) in human lung carcinoma cells. To the best of our knowledge there is no scientific report available in support of the apoptotic effect of cordycepin on A549 human lung cancer cells. The aim of the present study was to extract cordycepin from fermented broth of Cordyceps militaris and to evaluate its apoptotic effect on the A549 lung cancer cell line.
2. Material and methods

2.1. Chemicals and microbial strain
Chemicals and nutrient medium ingredients of analytical grade were purchased from Sigma Chemical Corporation (USA), E-Merck India, and HiMedia Ltd. (India). *Cordyceps militaris* 3936 was procured from the microbial type culture collection at IMTECH, Chandigarh, India, and it was regularly maintained on potato dextrose agar (PDA) slants and stored at 4 °C.

2.2. Production, extraction, and purification of cordycepin
*Cordyceps militaris* 3936 was initially grown on PDA medium in a petri dish and then transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a sterilized cork borer. The seed culture was grown in a 250-mL flask containing 50 mL of basal medium (glucose 15 g L–1, peptone 5 g L–1, KH2PO4 3 g L–1, K2HPO4 1 g L–1) at 25 °C on a rotary shaker incubator at 150 rpm for 4 days. The batch-mode fermentation experiments were performed in a 1000-mL flask containing 500 mL of the medium with 4% (v/v) inocula of the seed culture. The inoculated bottles were initially incubated at 25 °C for 7 days in stationary phase and then transferred to shaking mode for 10 days followed by stationary condition again for 7 days. Cordycepin was extracted and purified from fermented culture medium according to the procedure we described previously (Tuli et al., 2014a, 2014c).

2.3. Cell culture
A549 human lung cancer cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were maintained at 37 °C in a humidified 5% CO2 atmosphere.

2.4. Cytotoxicity assay
MTT assay was performed on A549 human lung cancer cells to determine cell viability (Lu et al., 2014). Briefly, 4 × 10⁴ cells were seeded in 96-well plates. After incubation for 24 h at 37 °C under 5% CO2, the cells were exposed to different concentrations of cordycepin ranging from 0 to 100 µg/mL for 48 h. MTT solution (10 µL, 5 mg/mL) was added to each well and further incubated for 4 h at 37 °C. The medium was removed and formazan crystals were dissolved by adding 100 µL of DMSO into each well and then shaking for another 20 min. Optical density (OD) was measured at 570 nm with a microplate reader (Bio-Rad) and percentage of viability was calculated as follows:

Percent (%) viability = 100 × mean of test OD / mean of control OD,

Percent (%) cytotoxicity = 100 – (percent viability).

2.5. 4’,6-Diamidino-2-phenylindole (DAPI) staining
Cells were grown on cover slips for 24 h followed by treatment with cordycepin (40 and 80 µg/mL) for 48 h. Then medium was removed and cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Fixed cells were washed with PBS and stained with 2.5 µg/mL 4,6-diamidino-2-phenylindole solution for 10 min at room temperature. The cells were again washed twice with PBS and analyzed under fluorescence microscope.

2.6. DNA fragmentation assay
In order to further investigate if cordycepin could induce cell apoptosis, fragmentation assay was performed according to manufacturer’s instructions (Apoptotic DNA Ladder Kit, Roche, Germany). Cells were grown in the presence of various concentrations (0, 40, and 80 µg/mL) of cordycepin for 48 h. After cordycepin treatment, A549 cells were harvested and subjected to DNA extraction and fragmentation assay. Approximately 10 µg of DNA was loaded onto 1% agarose gel using gel loading dye followed by electrophoresis and run at 50 V for 90 min. Visualization was done under UV transilluminator and photographed in the gel doc system (Vilber Lourmat, Germany).

2.7. Cell cycle analysis
A549 human lung cancer cells (50 × 10⁴) were seeded in 24-well plates with complete DMEM medium. After 70%–80% confluence, cells were treated with and without cordycepin-containing medium for 24 h. Cordycepin-treated and untreated cells were harvested, washed with PBS, and placed in 70% ethanol overnight at –20 °C. After fixation, cells were resuspended in 400 µL of PBS and added to 50 µL of propidium iodide (PI) solution (400 µg/mL) and 50 µL of RNase solution (1 mg/mL) followed by 30 min of incubation at 37 °C under dark conditions. Cell cycle analysis was done on a BD FACS Calibur flow cytometer using BD CellQuest, after gating out doublets in the PI area vs. width plot. Cell fractions in different phases of the cell cycle were determined using Cylogic (Cyflo Ltd., Finland) analysis software.

2.8. Detection of apoptosis using annexin V-Alexa Fluor 488
A total of 5 × 10⁴ cells were cultured in 24-well plates overnight followed by treatment of cordycepin at concentrations of 40, 60, 80, and 100 µg/mL. After 24 h of treatment, cells were harvested and resuspended in annexin V binding buffer. Cells were stained with 5 µL of antiannexin V-Alexa Fluor 488 (Molecular Probes, USA) and PI according to the manufacturer’s instructions followed by analysis on the BD FACS Calibur equipped with Cylogic (Cyflo Ltd.) software.

3. Results
In this study, the fermented broth of *Cordyceps militaris* was extracted with various solvents such as hexane,
chloroform, and butanol. Cordycepin was detected in butanolic fractions using various identification tools such as TLC, spectrophotometer, and HPLC. The butanolic fraction was further eluted on a silica gel column with a stepwise gradient of methanol:chloroform (5:95, 15:85, 25:75, 35:65, 45:55, and 55:45) and 6 fractions were obtained (F1–F6). Presence of cordycepin was detected in fraction F2 by TLC, which was subfractionated into 4 fractions (S1–S4) using elution gradient methanol:chloroform (15:85). Subfraction S3 was found to carry cordycepin, which was further vacuum-dried and recrystallized in methanol, giving a white/creamy powdery product. Spectral analysis of purified cordycepin was performed using UV, HPLC, and NMR spectroscopy and the data were consistent with our earlier published values (Tuli et al., 2014a, 2014c). We performed anticancer assays to examine the apoptotic effect of cordycepin on A549 human lung cancer cell line.

3.1. Cordycepin inhibits the proliferation of A549 lung cancer cells
The effect of cordycepin on the growth and viability of A549 cell lines was observed using MTT assay. After 48 h of treatment with different concentrations of cordycepin, a significant dose-dependent decrease in the number of metabolically active viable cells was observed (Figure 1). The results showed that cordycepin has potential to inhibit the growth of A549 with an IC50 value of 64 µg/mL.

3.2. Cordycepin-induced morphological change
The morphological changes were observed in cordycepin-treated cells under phase contrast microscope. Untreated cells were polygonal in shape, whereas the treated cells appeared with blebbing of plasma membrane and loss of cell structure and cell adhesion property. The number of floating cells in the cordycepin-treated group was greater than in the control group. These observations suggested that cordycepin is cytotoxic to A549 cells in a concentration-dependent manner.

3.3. DAPI and DNA fragmentation assay
The DAPI staining of A549 cells treated with cordycepin indicated that cells had undergone morphological changes including cell shrinkage, along with the formation of preapoptotic bodies, which are indicative of apoptosis. DNA fragmentation, another hallmark known for induction of apoptosis, was also analyzed in cordycepin-treated cells. The typical ladder pattern of DNA fragmentation was noticed on agarose gel (1%), pointing towards the apoptotic effect of cordycepin on A549 lung cancer cells (Figure 2).

3.4. Cell cycle analysis
Flow cytometric analysis was carried out to analyze cell cycle distribution via apoptosis induction. Apoptosis is an important mechanism in many anticancer drugs, including cordycepin (Tuli et al., 2013). In the present study, the A549 cells were treated with various concentrations of cordycepin for 24 h and stained with PI. Results showed that a significant proportion of cells were in the sub-G1 phase and apoptosis rates increased with the increase in

![Figure 1. The inhibitory effect with different concentrations of cordycepin on A549 cell growth. The data are represented as the mean ± SD of 3 independent experiments.](image1)

![Figure 2. Gel electrophoresis patterns of isolated DNA from A549 cells: lane 1, genomic DNA of cordycepin-untreated cells; lanes 2 and 3, genomic DNA of cordycepin-treated cells at concentrations of 40 and 80 µg/mL, respectively.](image2)
cordycepin dosages (Figure 3a). The histograms in Figure 3b represent cells from the untreated cultures (control) and from the cultures treated with the cordycepin, which affects the cell cycle distribution and induces apoptosis.

3.5. Annexin V/PI staining

To further validate the apoptotic effect of cordycepin, cells were stained with annexin V/PI and analyzed by flow cytometry (FCM). Annexin V/PI staining revealed that cordycepin (40–100 µg/mL) significantly induced apoptotic cell death in a dose-dependent manner. In Figure 4, the plot between annexin V and PI from the gated cells shows the percentage of viable and nonapoptotic (annexin V–PI–), early (annexin V+PI–), and late (annexin V+PI+) apoptotic cells at various concentrations of cordycepin. We found that cordycepin was able to induce apoptosis even at 40 µg/mL concentration in A549 human lung cancer cells. Percentage of live cells was higher in untreated cells and it was decreased with concomitant increase in apoptotic cells as a result of treatment with cordycepin.

4. Discussion

*Cordyceps militaris* has been used as a traditional Chinese medicinal mushroom for the treatment of many diseases, including cancer. More recently, many studies have shown that the extracts of *C. militaris* or the bioactive metabolite cordycepin may induce apoptosis in various cell lines such as human colorectal cancer cell lines (SW480 and SW620), breast cancer cell line MDA-MB-231, leukemia (U937 and THP-1), neuroblastoma SK-N-EB(2)-C (CRL-2268), and melanoma SK-Mel-2 (HTB-68) cell lines (Park et al., 2005; Lee et al., 2006; Lui et al., 2007; Wu et al., 2007; Paterson, 2008; Tuli et al., 2013). Apoptosis is characterized mainly by a series of distinct changes in cell morphology, such as blebbing, loss of cell attachment, cytoplasmic contraction, DNA fragmentation, and other biochemical changes including the activation of caspases through extrinsic and/or intrinsic mitochondrial pathways (Tuli et al., 2013). However, the apoptotic effect of the active ingredient of *Cordyceps*, i.e. cordycepin, on A549 cells has not yet been studied in detail. In this study we extracted cordycepin from fermented broth of *Cordyceps militaris* and observed its inhibitory effect on A549 cell growth in a dose-dependent manner. Cellular morphology and DAPI staining of drug-treated cells showed characteristics typical of apoptosis.

Furthermore, the cell cycle analysis by flow cytometer of cordycepin-treated A549 cells revealed apoptosis in a concentration-dependent manner. FCM is one of the best methods to detect and quantify apoptosis after drug treatment (Wlodkowic et al., 2009). Significant accumulations of cells were found in the sub-G1 phase.

**Figure 3.** Apoptotic effect of cordycepin on A549 lung cancer cells determined by flow cytometric assay. (a) Representation of flow cytometric plots at various concentrations (0, 40, 60, 80, and 100 µg/mL) of cordycepin after 24 h of treatments. (b) The histograms represent the redistribution of cells in the cordycepin-treated group in comparison to untreated cells.
Furthermore, the percentage of annexin V+ cells also increased with the increase in cordycepin dosage. In the untreated/control sample, the majority of cells (86%) were viable and nonapoptotic (annexin V–PI–). In contrast, when cells were treated with 40–100 µg/mL of cordycepin for 24 h, the percentages of annexin V–PI– cells were significantly decreased up to 41.99%. Similarly the percentage of early and late apoptotic cells increased in comparison to untreated cells. These results demonstrated that the growth inhibitory effect of cordycepin in A549 human lung cancer cell line might be associated with the induction of apoptotic cell death.

In conclusion, our study showed that there is a concentration-dependent cytotoxic effect of cordycepin on the human lung cancer A549 cell line. Cordycepin inhibits the cell cycle progression and leads to accumulation of cells in the sub-G1 phase, which results in apoptosis induction. However, further studies are required to elucidate the exact mechanism at the molecular level underlying the apoptotic property of cordycepin and to propose a novel therapeutic strategy for cancer treatment.

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