Cordycepin, an adenosine *analog* Induces Apoptosis, ROS Generation, and Suppresses Mitochondrial potentials. Exhibits activities against Dalton's Lymphoma.

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Abstract

Cordycepin exhibits anticancer potentials against various types of cancer cells. However, the multitudes of associated aspects of its anticancer properties against T-cell Lymphoma remains obscure. Based on in vitro studies, we reported that cordycepin exhibits an antineoplastic effect, against Dalton's Lymphoma (DL) cells. This study illustrates antineoplastic effects and associated mechanisms of cordycepin against tumor cells withdrawn from murine T-cells Lymphoma, designated as Dalton's Lymphoma. Our study reveals that cordycepin demonstrates tumoricidal activities against tumor cells via activating apoptosis and necrosis, including the surplus level of Reactive oxygen species production, declined mitochondrial membrane potentials, altered cell survival, and chromatin fragmentation. Moreover, this study also reported cordycepin-induced cell cycle arrest in tumor cells which further leads to apoptosis of tumor cells. This investigation elucidates the implication of antitumor actions of cordycepin against T-Cell Lymphoma accompanied by mitochondrial-dependent apoptotic pathway and therefore, have great medicinal significance.

1. Introduction

Worldwide research efforts are focused on screening anti-cancer natural agents with minimum or no side effects. Thus, a molecule capable of targeting one or more signaling pathways that lead to cancer is being examined for its therapeutic efficacy. Cordycepin also best-known 3'-deoxyadenosine is product of cordyceps. Cordyceps is a genus of Entomopathogenic fungi that parasitizes on the larvae of arthropods. It was first entitled as Ben-Cao-Bei-Yao in 1694 and is commonly known as 'Dong-Chong-Xia-Cao' in China [1]. It contains numerous active molecules including cordycepin. Cordycepin (**fig: 1**) is a fundamental therapeutic component in the *Cordyceps militaris* and has been demonstrated to have anticancer potentials [2, 3]. Chemically it is a purine derivate analog of nucleoside adenosine that consists of two heterocyclic rings. There are approximately 400 species of cordyceps found at humid high altitude areas in the Himalayan region of which two species *Cordycepin militaris* and *Cordycepin sinensis* are most extensively studied so far [4].

The cordycepin extensively displays a broad spectrum of anticancer and therapeutic potentials [5]. Further antineoplastic action of cordycepin against numerous types of cancer has also been evaluated [6, 7, 8]. The foremost mechanisms of antineoplastic effects of cordycepin are studied to be integrated with its apoptotic and cytotoxic potentials against TdT-positive leukemic cells and its antimetastatic role accompanied with down-regulating TNF- α -influenced MMP-9 production, therefore, inhibiting invasion and migration of human bladder neoplastic cells [9]. Cordycepin has inhibited the process of transcription by binding to adjacent nucleotide as ribose moiety of cordycepin has no oxygen at 3' position. This property has been well described in vitro with purified RNA polymerases as well as poly (A) polymerases [10]. In the subsequent decades, various investigations with regards to cordycepin and cancer were reported. Cordycepin has shown apoptotic potential against Breast cancer cell lines by elevating ROS production and induction cell arrest in the G2/M phase of the cell cycle [11]. The study on human gastric

cancer SGC7901 reports cordycepin promotes apoptosis by lowering mitochondrial membrane potentials [12].

Apoptosis exerts a crucial role in anticancer drug-mediated cancer cell death. The process of apoptosis is identified by a sequence of well-defined morphological changes including membrane blebbing, chromatin condensation, and chromosomal DNA fragmentation, and disruption of mitochondrial membrane potential [13].

T-Cell malignancies are intricate for clinical management with a very high incidence rate and one of the leading causes of death globally [14]. However very little is known concerning the underlying mechanisms of anticancer effects of cordycepin, moreover no study has been carried out on apoptotic effects of cordycepin against DL thus far. Therefore there is an immediate need to identify apoptotic potentials of cordycepin and its underlying mechanism on DL cells. In this study, we explored cordycepin-induced declined mitochondrial membrane potential, ROS homeostasis, effects of cordycepin on cellular survivability, cell cycle progression, and altered nuclear morphology. To address these parameters we performed a study on murine T-cell Lymphoma cells established as DL which is considerably used for screening of anticancer drugs and exploring underlying mechanisms.

2. Material And Method

Cordycepin or 3'-Deoxyadenosine (Purity >98.0%) from /of *Cordycepin militaris* purchased from TCI chemicals. RPMI-1640 and FBS were purchased from Gibco by life technologies™. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide) and DMSO were purchased from Himedia India. Thermo Fisher Scientific was the source of Acridine Orange (AO), Ethidium bromide (EB), 4', 6-diamidino-2-phenylindole Dihydrochloride (DAPI), and Rhodamine-123. DCFDA/H2DCFDA-Cellular ROS Assay Kit was obtained from Abcam US, while FITC tagged Annexin V and PI, were procured from Sigma Aldrich. All other chemicals and reagents used in experiments were of the standard purity grade.

The population of BALB/c (H²d) strain was allowed inbreeding under standard laboratory hygienic conditions. Dalton's lymphoma cells (1.0×10^6) were injected intraperitoneally(i.p.) in either male or female for the development and progression of Dalton's Lymphoma(DL) in mice peritoneum. DL was continuously maintained by in vitro passaging in culture and serial transplantation in mice. All experiments were carried out as per instructions approved by the institutional Animal Ethics Committee of the Department of Zoology, Banaras Hindu University, India.

2.1 Cell Culture-

BALB/c mice containing Dalton's lymphoma were used as a model system for growing and harvesting DL cells. Tumor cells were isolated from peritoneum DL-bearing mice via intraperitoneal injection, washed

with PBS, cultured in RPMI-1640 complete medium added with 10% FBS and placed inside 5 % CO₂ incubator.

2.2 MTT Assay for Cellular Toxicity-

MTT assay was used to determine the cytotoxicity of cordycepin against DL Cells [15]. DL cells were seeded in RPMI 1640 complete medium (10% FBS and antibiotics) in a culture plate and treated with various concentrations (25,50,100,150,200,250,300,600µg/ml) of cordycepin along with a untreated group. Culture plates were placed in 5% CO₂ incubator(shel lab model-2406-ZZMFG) for 24 h and 48 h. Thereafter MTT solution (5mg/ml) was mixed into each well and it was again incubated for additional four hrs at 37°C to enable intracellular reduction of the soluble yellow MTT to insoluble purple formazan crystal. In this sequence, Culture plates were subjected to centrifugation to remove media and cell debris. Absorbance was taken at 595 nm with the help of a microplate plate (Bio-Rad) reader after adding 100µL DMSO into each well. The cell viability percentage of the treated cells was calculated concerning untreated cells by the following equation-

$$\% \text{ Cell Viability} = [\text{Absorbance in the treated group}] / [\text{Absorbance in the untreated group}] \times 100.$$

2.3 Wright-Giemsa, DAPI staining for Cellular and Nuclear morphology

The morphological attributes of apoptotic cells include chromatin condensation, abnormal cell shape as well as disrupted nuclear symmetry [16]. These characteristics were taken as criteria to evaluate apoptosis after cordycepin treatment. DL cells were treated with cordycepin (0,125,250,500µg/ml) after 24 h of culture when cell *confluency* was maintained. After 24 hrs treatment cells were washed with PBS fixed and stained with Wright-Giemsa (5mg/ml) for 5 min at room temperature. Furthermore, DAPI staining was done to investigate alteration in nuclear structure. Treated and control cells were washed with PBS and fixed in 4%paraformaldehyde for 15 min at room temperature. DAPI Staining of cells was done after permeabilization with methanol.

The Cells of both groups (DAPI and Wright- Giemsa stained) were observed under the fluorescence microscope (evos XL digital inverted micrpscope) at magnification 20X.

2.4 Evaluation of apoptosis and necrosis

Criteria of apoptosis include initiation of apoptotic body formation, condensation of chromatin, cell shrinkage [17]. We used AO/EB dual staining to evaluate above stated apoptotic attributes. DL cells were seed in a 6- well culture plate and treated with cordycepin for 24hrs. Further Cells were washed with PBS

and resuspended in staining solution (5mg/ml of AO and 3mg/ml of EB) for 15min. The stained cell was visualized under the fluorescent microscope (Nikon ECLIPSE 90i) using band FITC and TRITC.

Moreover, we used AnnexinV/ Propidium iodide staining method to accurately enumerate apoptotic or necrotic mode of cell death. In nutshell, the DL cells (1×10^6 cells per well) were exposed to different concentrations of cordycepin for 24 h. The cells were washed with PBS and resuspended in 250 μ L of an annexin-binding buffer followed by adding of FITC-tagged Annexin-V and PI into it and kept 15 in dark. After incubation with FITC tagged annexin and PI, the cells were subjected to flow cytometry analysis (BD FACS Calibur flow cytometer). Analysis of apoptosis and necrosis of at least 10,000 cells in each apoptotic phase was performed by FlowJo software. The apoptotic and necrotic death was further confirmed by microscopic observation of the control, and Cordycepin treated DL cell.

2.5 Cell cycle analysis- Flow cytometry was employed for quantification of cellular DNA content and the analysis of the cell cycle [18]. DL cells were seeded (1×10^6 cells per well) in 6 well culture plates and treated with cordycepin for 24 hrs. Successively treated and control cells were washed with PBS and fixed with chilled methanol (70%) before treatment with RNase A. The DNA content of cells was stained with Propidium Iodide. Finally, the cells were subjected to flow cytometry analysis. The cell quest software (Becton Dickinson) was used to interpret flow cytometry data.

2.6 Estimation of Reactive Oxygen Species-

ROS estimation was done by using the standard protocol of H2DCFDA staining [19]. Briefly the control and treated DL cells (1×10^6 cells per well) were stained with 10 μ M H2DCFDA for 1 h in the dark. FL-1H channel was set in Flow cytometry (BD FACSCalibur) to capture green fluorescence per cell emitted by 2',7-dichlorofluorescein, a product formed after oxidation H2DCFDA by ROS. The control and treated DL cells stained with the dye (H2DCFDA) were also visualized under a fluorescence microscope (EVOS) at magnification 20X and photographed.

2.7 Estimation of Mitochondrial Membrane potential (MMP) – Mitochondrial mediated apoptosis is recognised by the kinetics of mitochondrial membrane potential. We used the standard protocol of Flow cytometry for analysis of MMP in Rhodamine-123 stained DL cells [20]. Treated and untreated cells were washed with PBS followed by incubation with Rh-123 (5 μ g/ml) for 10 min in the dark at room temperature. Further, the DL cells were subjected to flow cytometry (BD FACSCalibur) analysis using the FL2-H channel to collect red fluorescence emitted by Rh-123. MMP were further confirmed visualized of Rh-123 stained DL cells under a fluorescence microscope at magnification 20X and photographed.

2.8 Statistical Analysis-All results are representative of mean $\pm$ SD from experiments in triplicate. GraphPad Prism and Excel2013 were used for statistical analysis. The significance of differences between two groups and analysis of variance (two-way ANOVA) was done by an unpaired t-Test.

3. Results

3.1 Cordycepin reduced cell viability in DL cells- DL cells were exposed with various concentration of cordycepin (25, 50, 100, 150, 200, 250, 300 and 600 $\mu\text{g/ml}$) for 24 and 48 hours. The cell viability was then measured by the MTT assay. As shown in **(Fig. 2)**, the Cordycepin inhibited cell growth in a dose and time-dependent manner as the maximum percentage of cell death was observed higher dose (600 $\mu\text{g/ml}$) at 48 hrs. The IC₅₀ value (the concentration at which 50% cell death occurs) of Cordycepin in DL cells for 24 and 48 hours was approximately 150 and 250 $\mu\text{g/ml}$. Therefore, the inference of MTT results indicates that cordycepin exerted significant cytotoxic against DL cells in vitro.

3.2 Cordycepin induces apoptosis in DL cells- Since apoptosis plays a fundamental role in cell death induced by anticancer drugs[21] therefore we evaluated the effects of Cordycepin in the induction of apoptosis in DL cells using microscopy techniques and flow cytometry. Wright-Giemsa stained cordycepin-treated cells represented membrane blebbing, abnormal cellular morphology, condensed nuclei, apoptotic body formation as well as cell shrinkage **(Fig. 3. A)**. While normal morphology with unhampered cellular structure was seen in untreated DL cells. Cordycepin-treated DAPI stained DL cells showed deformation of nuclear structure, chromatin condensation, and aberrant cellular morphology that are hallmarks of apoptotic cell death **(Fig. 3. B)**.

Moreover, a dose-dependent enhancement of apoptotic cell number was also observed after staining with AO/EB. Dead cells have compromised cytoplasmic membrane integrity therefore allowed entry of Ethidium bromide (EB). On the other hand, AO diffuses into every cell and binds with nucleic acids. Hence, the nucleus of healthy DL cells emitted green fluorescence as AO binds double-stranded DNA and RNA. Contrarily AO produces red fluorescence when it binds with fragmented DNA as in the case of late apoptotic DL cells. Brilliant green and yellowish nucleus with fragmented chromatin were observed in early apoptotic cells. Whereas late apoptotic DL cells were distinguished by orange and red fluorescence with condensed and fragmented chromatin. Necrotic DL cells showed a dense orange nucleus **(Fig. 3 C)**.

In addition to the more precise and quantitative method of apoptotic cells detection, i.e pre apoptotic and late apoptotic dead or necrotic cells determined by AnnexinV/PI staining. In apoptotic cells, phosphatidylserine is actively flopped out to the outer leaflet of the plasma membrane. Annexin V, a fluorescent probe that can bind in a calcium-dependent manner to phosphatidylserine exposed on the outer leaflet of the plasma membrane of apoptotic cells [22] whereas propidium iodide only stained dead cells. Cordycepin treatment to T cells Lymphoma cells at various doses (125, 250, and 500 $\mu\text{g/ml}$) for 24 h resulted in a significant increase of preapoptotic cells showing 47, 58.4%, and 65.9%, respectively, as compared to control **(Fig. 3 D)**.

Cordycepin causes cycle arrest in the G2/M phase—Since apoptosis and cell cycle are closely associated [23], therefore we evaluated the effect of cordycepin on cell cycle progression. Flow cytometry analysis showed a consistent rise in the percentage of cells in the G2/M phase of the cell cycle. *Out of all concentrations cordycepin, 500 µg/mL treatment induced cell cycle arrest in the G2/M phase of most T-cell lymphoma cells. (Fig. 4).*

3.3 Cordycepin declines mitochondrial membrane potential (MMP)—To determine if the apoptotic potential of cordycepin was ascribed via the mitochondrial-mediated apoptotic pathway, DL cells were exposed to various doses of cordycepin (0, 125, 250, and 500 µg/ml) for 24 h, and MMP was analyzed using Rhodamine-123 (Rh-123) dye. Rh-123 is a fluorescent dye that binds the inner and outer aspects mitochondrial membrane. A decrease in fluorescent intensity of Rh-123 is an indicator of disrupted MMP[24]. Cordycepin significantly disrupted MMP as represented by overlaid histogram has shifted left compared to control (**Fig: 5 A**). Decrease in fluorescence intensity of Rh-123 was also confirmed by visualization of treated and control cells under the fluorescence microscope (EVOS) at magnification 20X (**Fig:5B**).

3.4 Cordycepin induces apoptosis by up-regulating ROS production-

Enhanced ROS production in the cells plays a key role in the induction of apoptosis [25]. Cordycepin treated and untreated DL stained with H2DCFDA as described in material and method. The flow cytometry analysis showed up-regulated ROS production in the DL cells dose-dependently as green fluorescence has increased corresponding to enhanced ROS production (**fig: 6A**). Cordycepin treated, H2DCFDA stained cells showed increased green fluorescence in comparison to control under a fluorescence microscope (EVOS) at magnification 20X and photographed(**Fig:6B**).

4. Discussion

Cancer cells have the peculiar feature of unlimited proliferation and density-independent growth, autonomous derived proliferation, capability, diminished cell adhesion, apoptotic resistance, invasion, angiogenesis, and metastasis, which makes cancer cells immortal. Treatment of Non-Hodgkin lymphoma involved radiation therapy, single-agent or combination chemotherapy, immunotherapy, or radioimmunotherapy [26]. Notwithstanding the above-stated therapies, the successful treatment of Non-Hodgkin lymphoma with no side effects has not yet been developed. Herbal molecules are evaluated as a potential antineoplastic therapeutic agent against various neoplasms because they have minimum side effects implicated with them [27]. In this consequence, Cordycepin being a natural agent has demonstrated promising antineoplastic activities against cancer. The present Investigation results indicate that Cordycepin exerted robust apoptotic actions against DL cells. Among various cell viability assays, the MTT assay is accurate and one of the simplest assays to determine cell viability. MTT is a water-soluble molecule and able to enter the inside viable cell through the plasma membrane and inside the cell MTT is subjected to reduction by the mitochondrial dehydrogenases. The reduction product formed known as formazan which reflects live cell percentage [28]. The MTT assay of Cordycepin DL

cells showed growth inhibition from 25 µg/ml whereas IC-50 is obtained at approximately 250 µg/ml, 150 µg/ml for 24 and 48 hours. Consistent with these findings, earlier studies indicated that cordycepin inhibits cell proliferation in various cancer cell lines [29]. Wright-Giemsa stained DL cell showed compromised cell membrane, membrane blebbing which are the hallmark of necrosis and apoptosis.

DAPI, a fluorescent probe that is capable of binding the A-T rich region in the minor groove of DNA. The cordycepin treated and DAPI stained DL cells showed nuclear fragmentation in DL cells under the fluorescent microscope that is characteristic of apoptosis. Furthermore, cordycepin-induced apoptosis is also demonstrated by AO/EB stained DL cells as nuclei became visible glowing green/ yellowish and orange/red nucleus by early and late apoptotic cells respectively. Besides AO/EB and DAPI staining, Apoptosis was more precisely quantified by Annexin/PI staining. FITC tagged annexin V binds to phosphatidylserine exposed by pre and post-apoptotic cells on the other hand PI (Propidium iodide) enters inside the plasma membrane of compromised necrotic and dead cells. Morphological parameters of apoptosis such as membrane blabbing, Chromatic condensation, cell shrinkage, Exposure of phosphatidylserine (PS) on the outer leaflet of plasma membrane were evident from Wright-Giemsa, DAPI, AO/EB, and Annexin V/ PI staining.

Above all Cells growth and proliferation is regulated by the surveillance strategy of cell in the form of G1, G2, and metaphase to anaphase cell cycle checkpoints. However, cell cycle check point-associated regulation is found to malfunction in cancer cells (30). Various investigations revealed antineoplastic drugs induced multi-step restraint of cell cycle, and antiproliferative effects on neoplastic cells. Moreover, the impediments in cell proliferation and enhanced apoptosis via numerous pathways cause the arrest of the cell cycle. Thereupon, a comprehension of molecular pathways underlying cell cycle arrest may indicate the road maps of cell cycle inhibition. [31].Flow cytometry was used to evaluate the events that lead to the cell cycle arrest in cordycepin-treated DL cells. The results showed an increased cell population in the G2/M phase at 125, 250, 500 µg/ml cordycepin treatment for 24 h. Hence cordycepin induced G2/M phase cycle arrest in DL cells. In concurrence with the previous report [32], the present study reports that cordycepin induced G2/M phase arrest in DL cells. This study may demonstrate that cell death accompanied by cell cycle arrest mechanism is highly correlated with cell type. Therefore, further investigations are required to decipher the molecular mechanism of cell cycle arrest in cordycepin-treated DL cells.

Mitochondria and kinetics of mitochondrial membrane potential play a crucial role in apoptosis [33].

The mitochondrial membrane potential created by proton pumps of an inner mitochondrial membrane is indispensable for the process of ATP synthesis via oxidative phosphorylation[34]. We used Rhodamine-123 for the measurement of mitochondrial membrane potential (MMP). Rh-123 is a lipophilic fluorescent dye that binds to inner and outer aspects of the mitochondrial membrane and its accumulation in mitochondrial depends on MMP [35]. Although, Rh-123 gathers within the mitochondria of normal living cells and its fluorescence is high, however disruption of MMP in the process of apoptosis results in

reduced fluorescent intensity of Rh-123 [36]. FACS analysis and fluorescence microscopic results showed that cordycepin treatment to DL cells decreases mitochondrial membrane potentials.

Apoptosis is mediated by Oxidative stress and the production of reactive oxygen species (ROS) [37]. ROS are continuously generated and removed in the cells but found elevated due to oxidative stress in neoplastic cells [38].

To investigate the events that lead reactive oxygen species-mediated apoptosis, flow cytometry, and fluorescence microscopy was used to demonstrate ROS level in cordycepin exposed DL cells. We used H2DCF-DA for the determination of ROS aggregate in DL cells. H2DCFDA can enter and be accumulated inside cell diffuses passively inside the cell until degraded by cytosolic esterases. Since ROS induces oxidation of nonfluorescent H2DCFDA into fluorescent 2'7 dichlorofluorescein [39] therefore, the result of FACS analysis and fluorescence microscopy showed an increased green fluorescence per cell in cordycepin treated DL cell corresponding to enhanced ROS production. The comprehensive anticancer action of Cordycepin has been brilliantly demonstrated against Dalton's Lymphoma (NHL) especially through the declined mitochondrial membrane potential and enhanced ROS production, apoptosis by exposing Phosphatidylserine (PS) to the outer plasma membrane, nuclear disintegration, and cell membrane blabbing with the formation of apoptotic bodies. Further research and clinically designed composition of cordycepin might improve its efficacy against lymphoma.

5. Conclusion

The outcome of this study shed light on apoptotic and antiproliferative actions of cordycepin against Dalton's Lymphoma cells concerning inhibited cell viability, dwindled mitochondrial membrane potential, enhanced ROS production, and arrest in the G2/M phase of the cell cycle. Cordycepin also induced chromatin fragmentation, membrane blabbing culminated in apoptosis of DL cells. The apoptotic potential offered by cordycepin may be mostly due to its structure. Comprehensively the present study will contribute to the design of antineoplastic drugs using cordycepin as a natural anticancer agent against malignancies of T-cell Lymphoma.

Declarations

Conflict of interest -The authors proclaim that there is no involvement of multiple interests including personal, financial, academic interest for this typescript.

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Figures

Figure 1

Chemical structure of Cordycepin.

Figure 2

The bar graph represented the cytotoxic action of cordycepin against DL cells. The results were denoted as a percentage of control $\pm$ SD and statistically analyzed via Bonferroni posttests and unpaired T-test (two-way ANOVA) for at least three independent experiments.

Figure 3

Images of Wright-Giemsa (A) and DAPI stained cells (B). AO/PI stained DL cells (C) DL Cells treated with indicated concentrations. Cordycepin for 24 h. The cells were washed with PBS, incubated with FITC tagged annexin V/PI for 15 min, and analyzed by flow cytometry using FlowJo software (D).

Figure 4

Control and cordycepin exposed DL cells were incubated with PI, and the population in different phases of the cell cycle was subjected to flow cytometry and analyzed by cell quest software (Becton Dickinson).

(A)

(B)

Figure 5

Treated and control cells analyzed by BD FACSCalibur flow cytometer. Bar graph showing the dose-dependently decrease in fluorescent intensity of Rh-123 in comparison with control (A). Decrease of the Rh-123 fluorescence intensity was also visualized under the fluorescence microscope (EVOS) representing that MMP has declined (B).

Figure 6

(A). Treated and control cells analyzed by BD FACSCalibur flow cytometer. Bar graph showing increase in fluorescent intensity of H2DCFDA dose-dependently in cordycepin treated DL cells. (B) An increase in H2DCFDA fluorescence intensity was also visualized under a fluorescence microscope (EVOS) indicating that ROS production has increased.