Cordycepol C Induces Caspase-Independent Apoptosis in Human Hepatocellular Carcinoma HepG2 Cells

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Cordycepol C, a novel sesquiterpene isolated from the cultured mycelia of Cordyceps ophioglossoides, contains a hydroperoxy group and is cytotoxic to HepG2 cells. So far, no sesquiterpenes have been found in the genus Cordyceps and it would be interesting to investigate the antitumor efficacy as well as the mechanism of action of this unusual sesquiterpene. In this study, we showed that cordycepol C induced apoptosis of the HepG2 cells without affecting the normal liver cell line L-02. Cordycepol C caused poly(ADP-ribose) polymerase-1 (PARP-1) cleavage and triggered the loss of mitochondrial membrane potential (Δψm) in HepG2 cells in a time- and dose-dependent manner, resulting in the nuclear translocation of apoptosis-inducing factor (AIF) and endonuclease G (Endo G). We also found that cordycepol C induced the expression of Bax protein, followed by its translocation from the cytosol to mitochondria in both wild type and p53 knockdown HepG2 cells. However, cordycepol C could not cause cleavages of procaspase-3, -8, and -9. Caspase activities were not increased and Z-VAD-fmk, a caspase inhibitor, could not prevent the apoptosis induced by cordycepol C. These findings indicate that cordycepol C induces caspase-independent apoptosis in HepG2 cells through a p53-independent and Bax-mediated mitochondrial pathway, leading to the nuclear translocation of AIF and Endo G. Our study provides the molecular mechanism by which cordycepol C induces apoptosis in hepatocellular carcinoma cells and indicates the potential use of cordycepol C as an antitumor agent.

Key words  cordycepol C; apoptosis; bax protein; apoptosis-inducing factor; endonuclease G

Development of anti-cancer drugs from natural products is one of the most productive strategies in current drug discovery and development.1) Terpenoids, a diverse group of natural products with surprising skeletons as well as complex stereo-structures, have been widely investigated by hundreds of companies for their anti-tumor, anti-inflammatory and other therapeutic properties. Some important pharmaceutical agents, such as paclitaxel (Taxol) and terpenoid-derived indole alkaloids, belong to the family of terpenoids.2–4) Thus, research to unveil anticancer mechanism of terpenoids could further facilitate their applications in clinic as well as assist researchers to design and develop better terpene-like agents with many potential applications in basic science and industry.

Induction of apoptosis is an effective approach to eliminate cancer cells, which is mainly executed through a class of cysteine proteases called caspsases.5) However, increasing evidence has been found to support the notion of caspase-independent programmed cell death pathways.5–8) Apoptosis-inducing factor (AIF), a flavoprotein involved in complex I of the respiratory chain, was first discovered as a caspase-independent death effector.9,10) Under certain stress such as the treatment with the genotoxic agent N-methyl-N’-nitro-N-nitrosoguanidine (MNN), AIF is released from the inner mitochondrial membrane to the cytosol and then translocates into the nucleus, thereby causing nuclear condensation and caspase-independent cell death.11,12) The proapoptotic Bcl-2 family member Bax is activated by Bid and plays an indispensible role in AIF-mediated programmed necrosis.13,14) Meanwhile, endonuclease G (Endo G), a nuclease, also translocates into the nucleus and cleaves chromatin DNA into nucleosomal fragments in apoptotic cells.15–17) Endo G induces DNA degradation through a caspase-independent pathway, in a manner similar to AIF.18) Both Endo G and AIF are located in mitochondria and the mitochondria provide a majority of energy for supporting cellular functions. During apoptosis, the disruption of the mitochondrial membrane potential (Δψm) is often regarded as “the point of no return,” ensuring cell death.19,20) The loss of Δψm results not only in the interruption of ATP generation, but also leads to release of caspase-independent death effectors from the mitochondria.5,20) The integrity of the mitochondrial membrane can be regulated by Bcl-2 family proteins, which consist of anti-apoptotic and pro-apoptotic factors.21)

Cordyceps ophioglossoides is a well-known Traditional Chinese Medicine and commonly used to nourish the human body in China. Our lab has purified and identified the fungus from fruit body of C. ophioglossoides and set up an artificial fermentation system of it.22,23) Previously, we have reported the discovery of a novel spiro[4.5]decane sesquiterpene, cordycepol C, isolated from the cultured mycelia of C. ophioglossoides. Cordycepol C contains a hydroperoxy group and is cytotoxic to HepG2 cells.24) In this study, we investigated the mechanism of cell death signaling pathways induced by cordycepol C in HepG2 cells. Our results showed that cordycepol C induce caspase-independent apoptosis through a Bax-mediated mitochondrial pathway in HepG2 cells.

The authors declare no conflict of interest.

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MATERIALS AND METHODS

Cell Culture and Reagents Human hepatic carcinoma cell lines HepG2, Hep3B, Huh-7 and SMMC-7721, human liver cell line L-02, human embryonic kidney 293 (HEK293) cell line and human promyelocytic leukemia cell line HL-60 were obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai). Cells were cultured in RPMI 1640 medium (Gibco, Oklahoma, U.S.A.) containing 10% fetal bovine serum (Gibco, Oklahoma, U.S.A.), 100 units/mL penicillin and 100 units/mL streptomycin. All cells were grown in a humidified cell incubator with an atmosphere of 5% CO2 at 37°C and subcultured with 0.25% trypsin + 0.02% ethylenediaminetetraacetic acid (EDTA) (Beyotime, Jiangsu, China).

Cordycepol C (>96% (w/w) purity) was isolated from the cultured mycelia of C. ophioglossoides as described previously. A stock solution of 20 mg/mL cordycepol C was prepared in dimethyl sulfoxide (DMSO) and diluted to the appropriate concentrations before use; the final concentration of DMSO was kept below 0.1% (v/v) in all assays. Doxorubicin (Dox), paclitaxel (Taxol), staurosporine (STS), carbonylcyanide-m-chlorophenylhydrazone (CCCP), rhodamine 123 (Rhi23), 4,6-diamidino-2-phenylindole (DAPI), DMSO, ribonuclease A (RNase A), propidium iodide (PI), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 5,5′,6,6′-tetrafluoro-1′,3′,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), Annexin V-fluorescein isothiocyanate (FITC)/PI kit, Hoechst 33342, N-methyl-N-nitro-N-nitrosoguanidine (MNNNG) and the pancaspase inhibitor Z-VAD-fmk were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). An apoptotic DNA ladder kit was purchased from Roche Applied Science. The primary antibodies used were Bax, Bcl-2, Bcl-xl, AIF, cyclooxygenase (COX) IV, Caspase-3, Caspase-8, Caspase-9, Histone H3, poly(ADP-ribose)-polymerase-1 (PARP-1), and β-actin (Cell Signaling Technology, Beverly, MA, U.S.A.), and p53 and Endo G (Santa Cruz, CA, U.S.A.). The secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse immunoglobulin G (IgG) (Santa Cruz, CA, U.S.A.) or anti-rabbit IgG conjugated with Alexa6 Fluor 488 (Invitrogen, CA, U.S.A.) or anti-mouse conjugated with Dylight 549 (Jackson ImmunoResearch, West Grove, PA, U.S.A.). All other chemicals were of chemically pure grade.

Cell Viability Assay The cell viability was measured by the MTT method as previously described. Briefly, cells were seeded in 96-well microtiter plates at a density of 5×103 cells/well for 24 h. After the indicated concentrations of drug treatment for 24 h, cells were incubated with MTT (0.5 mg/ mL) for an additional 4 h at 37°C. The resulting formazan precipitate was dissolved in 150 µL DMSO and the absorbance was detected at 490 nm with a Sunrise microplate reader (Tecan Group Ltd., Seestrasse, Switzerland). Compounds were dissolved in DMSO and diluted to the proper concentrations before use. Dox was used as a positive control for all assays. Each test was repeated at least three times.

Apoptosis Assays Apoptotic rates were analyzed by flow cytometry using an Annexin V-FITC/PI kit (Sigma-Aldrich, U.S.A.) according to the manufacturer’s instruction. Briefly, HepG2 cells were treated with 0–20 µg/mL cordycepol C for 24 h, with or without 100 µmol/L Z-VAD-fmk pretreatment for 2 h. Cells were harvested, washed twice with ice-cold phosphate buffered saline (PBS), assessed for apoptosis by double staining with Annexin V-FITC and PI in binding buffer and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, U.S.A.).

Cells were seeded on 6-well culture plates and treated with cordycepol C at concentrations of 10, 15 and 20 µg/mL for 24 h, or with 100 µmol/L Z-VAD-fmk pretreatment for 2 h. DMSO was used as a negative control. Then, cells were fixed with 4% (w/v) paraformaldehyde (PFA) for 20 min, washed twice with ice-cold PBS, stained with Hoechst 33342 (10 µg/ mL in PBS) for 5 min at room temperature and observed under a fluorescence microscope (Olympus BX51, Tokyo, Japan) with an excitation wavelength of 360 nm. STS was used as a positive control.

Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate (dUTP)-biotin nick-end labeling (TUNEL) staining was conducted by a TUNEL kit (Promega, Madison, WI, U.S.A.). Briefly, cells were treated with 20 µg/mL of cordycepol C for 24 h, or with 100 µmol/L Z-VAD-fmk pretreatment for 2 h, and the apoptotic cells were stained in situ by DeadEnd colorimetric TUNEL System. Then cells were fixed in 4% PFA, washed twice with 0.2% Triton X-100 for 5 min. Cells were bound with Streptavidin HRP in the presence of terminal deoxynucleotidyltransferase (Tdt) for 1 h at 37°C and stained by 3,3′-diaminobenzidine (DAB). After washing twice with PBS, cells were directly observed under a light microscope (Olympus BX51, Tokyo, Japan).

Caspase Activity Assays Caspase activities were determined by using the Caspase-Glo 3, 8 and 9 assay kits (Promega, Madison, WI, U.S.A.), respectively. HepG2 cells (5×103/well) were seeded on a 96-well luminometer plate and treated with 0–20 µg/mL of cordycepol C for 24 h or treated with 20 µg/mL of cordycepol C for 0–24 h. Caspase-Glo 3, 8 and 9 reagents (100 µL) were added to each well, and the plate was incubated on a rotary shaker avoiding light at room temperature for 1 h. Chemiluminescence was measured using an Infinite® 200 PRO multimode reader (Tecan Group Ltd., Seestrasse, Switzerland).

Detection of Mitochondrial Membrane Potential (Δψm) Δψm was assessed in a similar fashion to the previously reported methods. Cells were treated with 0, 10, 15 or 20 µg/mL of cordycepol C for 0–24 h and then stained by the fluo-
Rescent dye JC-1 (500 nmol/L) for 20 min. The intensities of red (FL-2) represented by the J-aggregates and the intensities of green (FL-1) represented by the monomeric form of JC-1 were analyzed by using an Infinite® 200 PRO multimode reader (FL-2: excitation/emission=535 nm/595 nm; FL-1: excitation/emission=485 nm/525 nm). The value of ΔΨm was calculated by the ratio of FL-2/FL-1. The relative fluorescence intensity (F) was shown as \( F_2/F_1 \); where \( F_1 \) is the value of ΔΨm.

Fig. 2. Cordycepol C Induces Apoptosis in HepG2 Cells

(A) HepG2 and L-02 cells were treated with 10, 15 and 20 µg/mL cordycepol C, or with 100 µmol/L Z-VAD-fmk pretreatment for 2 h, and apoptosis was determined by Annexin V-FITC/PI staining. DMSO was used as a negative control. (B) Fluorescent labeling of nuclei was stained by Hoechst 33342 in 20 µg/mL cordycepol C-treated HepG2 cells for 24 h or with 100 µmol/L Z-VAD-fmk pretreatment for 2 h (magnification ×400). STS (500 nmol/L) was used as a positive control. (C) Apoptosis was determined by TUNEL assay after HepG2 cells were treated with 20 µg/mL cordycepol C or combined with 100 µmol/L Z-VAD-fmk for 24 h (magnification ×200). STS (500 nmol/L) was used as a positive control. (D) HepG2 cells were treated with 0–20 µg/mL cordycepol C for 24 h and the cleavage of PARP-1 were detected by immunoblotting. STS (500 nmol/L) was used as a positive control. (E) HEK 293, HL-60 and SMMC-7721 cells were grown in 96-well plates and then treated with 0–80 µg/mL cordycepol C for 24 h. Cell viability was detected by MTT assays as described. Dox was used as a positive control with the IC50 value <5 µg/mL to these three cell lines. All data were expressed as means±S.D. of three independent experiments (*p<0.05 versus control) or similar results from three independent experiments.
Δψ_m of control and F_2 is the value of Δψ_m of series incubation times or concentrations. The changes of the JC-Ionomeric form were observed in situ under a fluorescence microscope (Olympus BX51, Tokyo, Japan). The value of Δψ_m was also measured by Rh123 staining with the excitation/emission absorbance at 485 nm/525 nm.

Preparation of Subcellular Fractions and Western Blot Analysis The whole cell proteins were obtained in a buffer containing 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1% Triton X-100. Mitochondrial and cytosolic fractions were prepared using a mitochondria isolation kit (Beyotime, Jiangsu, China). Nuclear fractions were obtained with the subcellular protein fractionation kit (Beyotime, Jiangsu, China). Protein concentrations were determined by the BCA protein assay kit (Beyotime, Jiangsu, China). Cell extracts (30–100 µg protein/lane) were separated by electrophoresis on 10–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (GE Healthcare, U.S.A.). The membranes were blocked in 5% (w/v) nonfat dry milk for 1 h and incubated with indicated primary (1:1000) and secondary (1:4000) antibodies. Bands were visualized by the chemiluminescence kit (Pierce, Rockford, IL, U.S.A.).

Immunofluorescence For the AIF and Endo G redistribution analysis, HepG2 cells seeded on coverslips were washed twice in PBS and fixed in 4% PFA. Then cells were permeabilized with 0.2% Triton X-100 for 5 min and blocked with 1% bovine serum albumin (BSA), 10% goat serum and 0.1% Tween-20/PBS. Cells were incubated with appropriately primary antibodies (1:100) in 1% BSA at 4°C overnight. After washing three times with PBS, cells were incubated with Alexa® Fluor 488-labeled goat anti-rabbit IgG (1:1000) or Dylight 549-labeled goat anti-mouse IgG (1:500) in 0.1% Tween-20/PBS for 1 h. To visualize the nuclei, cells were stained with DAPI (1 µmol/L) for 5 min at room temperature in dark and then mounted with antifade mounting medium. The fluorescence was observed using a confocal laser-scanning microscopy (Carl Zeiss, Germany). Images were processed by Photoshop (Adobe, San Jose, CA, U.S.A.).

RNA Interference (RNAi) Experiments Duplex small interfering RNA (siRNA) (21-mers) was purchased from Shanghai GenePharma. The RNA sequence used for p53 was p53 siRNA-1: 5’-CUACUUCUGAA AAC AAC GTT-3’, p53 siRNA-2: 5’-UGGUUCACUGAA GAC CCA GTT-3’, p53 siRNA-3: 5’-GACUCAGUGGAUAUCUACTT-3’, control siRNA: 5’-UUCUCCGAACGUGUACGUTT-3’, as predicted by BIOPREDsi. Transfection with RNAiMAX was carried out using the reverse transfection protocol according to the manufacturer’s instructions (Invitrogen, CA, U.S.A.). Two days after transfection, cells were collected for protein expression assay by Western blot.

Statistical Analysis All data were expressed as means±S.D. Analysis was performed using a Student’s two-tailed t-test.
t-test or one-way ANOVA with $p<0.05$ versus control considered as statistically significant.

RESULTS

**Cordycepol C Induces Apoptotic Cell Death of HepG2 Cells**
Cordycepol C is a novel sesquiterpene with an unusual spiro[4.5]decane carbon skeleton (Fig. 1). When HepG2 cells were treated with 15 or 20 $\mu$g/mL of cordycepol C, the redistribution of phosphatidylserine from the inner to the outer leaflet of the plasma membrane increased from 4.5 to 24% or 47% (Annexin V-FITC positive), but their membrane retained integrity (PI negative, Fig. 2A). Nuclear condensation and apoptotic bodies were measured by Hoechst 33342 staining (Fig. 2B). Nuclear fragmentations, another morphological characteristic of apoptosis, were stained dark brown and observed by the TUNEL assay (Fig. 2C). Nuclear fragmentations, another morphological characteristic of apoptosis, were stained dark brown and observed by the TUNEL assay (Fig. 2C). The PAR polymerase-1 (PARP-1), a 113 kDa nuclear enzyme, was cleaved into an 85 kDa fragment in a dose-dependent manner after HepG2 cells were treated with cordycepol C (Fig. 2D). These findings were consistent with the central features of apoptosis.

However, under the same conditions, no apparent apoptosis was observed in the human liver cell line, L-02 (Fig. 2A). Then, the cytotoxic activity of cordycepol C towards an additional hepatoma cell line SMMC-7721, a human promyelocytic leukemia cell line HL-60 and a human embryonic kidney 293 cell line were measured by MTT assays. Cordycepol C could inhibit SMMC-7721 cells proliferation with an IC$_{50}$ value (50% inhibitory concentrations, 24h) of 42.1 $\mu$g/mL and HL-60 cells with the IC$_{50}$ value of 57.3 $\mu$g/mL, but this compound was less harmful to HEK293 cells with the IC$_{50}$ value above 80 $\mu$g/mL (Fig. 2E). Therefore, cordycepol C was cytotoxic towards cancer cells and induced apoptosis in the human hepatic carcinoma cell line HepG2, showing less cytotoxicity to normal cell lines.

**Cordycepol C Induces Apoptosis in a Caspase-Independent Manner**
Caspase activation plays a central role in the execution of apoptosis. In this study, caspase-3, -8 and -9 activities were not increased significantly after incubated with different concentrations for different exposure times of cordycepol C (Figs. 3A, B). Nearly no cleavage of procaspase-3, -8 and -9 in HepG2 Cells were measured by Western blot (Fig. 3C). Furthermore, there was little effect of the pancaspase inhibitor Z-VAD-fmk on cordycepol C-mediated cell death using MTT assays (Fig. 3D). The apoptotic-like characteristics such as chromatin condensation, oligonucleosomal DNA fragmentation and the exposure of phosphatidylserine still persisted in the presence of 100 $\mu$mol/L Z-VAD-fmk (Figs. 2A–C). These results indicated that cordycepol C-induced apoptosis was independent of caspase activation in HepG2 cells.

Fig. 4. Loss of Mitochondrial Membrane Potential ($\Delta$Ψ$_{m}$) and the Mitochondrial Translocation of Bax in HepG2 Cells
(A) Cells were incubated with 0–20 $\mu$g/mL cordycepol C for 24h and analyzed by JC-1 and Rh123 staining. The relative fluorescence intensity ($F$) was shown as $F/F_0$. $F_0$ is the value of $\Delta$Ψ$_{m}$ of control and $F_2$ is value of $\Delta$Ψ$_{m}$ of indicated incubation times or concentrations. (B) Cells were incubated with 20 $\mu$g/mL cordycepol C for 0–24h and the fluorescence intensity was measured. (C) HepG2 cells were treated with 0 or 20 $\mu$g/mL of cordycepol C for 24h and the subcellular fractions were extracted as described. The expression level of Bax was analyzed by Western blot. $\beta$-Actin and COX IV were used as loading controls for the cytoplasm and mitochondria, respectively. (D) Cells were treated with 0–20 $\mu$g/mL of cordycepol C for 24h and the expression levels of Bcl-2 and Bcl-xL were analyzed by Western blot. (E) The expression level of p53 was analyzed by Western blot. STS (500 nmol/L) was used as a positive control. All data were presented as means±S.D. of three independent experiments ($^*p<0.05$ versus control) or similar results from three independent experiments.
Cordycepol C Induces the Loss of $\Delta \psi_m$ and Mitochondrial Translocation of Bax in a p53-Independent Way in HepG2 Cells

To study whether the mitochondrial events were involved in the caspase-independent apoptosis induced by cordycepol C, $\Delta \psi_m$ was measured by the fluorescent dye JC-1 and Rh123. $\Delta \psi_m$ (shown as JC-1 or Rh123) decreased in a dose- and time-dependent manner (Figs. 4A, B). The relative mean fluorescence intensity of JC-1 dropped to 33.6±5.1% of the control after treatment with 20 $\mu$g/mL cordycepol C for 24h. Similar results were obtained by Rh123 staining. The expression levels of several Bcl-2 family proteins (Bcl-2, Bcl-xL or Bax) were measured to see whether they were involved in the permeability change of mitochondrial membrane. Cordycepol C could induce the pro-apoptotic protein Bax expression without affecting the expression of anti-apoptotic members such as Bcl-2 and Bcl-xL (Figs. 4C, D). The translocation of Bax to mitochondria was also observed (Fig. 4C). Both of these results indicated that cordycepol C could induce the loss of $\Delta \psi_m$ and mitochondrial translocation of Bax in HepG2 cells.

Tumor suppressor protein p53 was the upstream regulator of Bax.27 Interestingly, the expression level of p53 protein was found unchanged in cordycepol C-treated HepG2 cells (Fig. 4E). HepG2 cells expressed wild-type p53. However, cordycepol C was harmful to HL-60 cells (p53-deficient) with the IC$_{50}$ value of 57.3 $\mu$g/mL (Fig. 2F). Cordycepol C could also inhibit Hep3B cells (p53-deficient) proliferation with an IC$_{50}$ value (24h) of 42.4 $\mu$g/mL and Huh-7 cells (p53 mutant) with the IC$_{50}$ value of 43.4 $\mu$g/mL by MTT assays (Fig. 5A), while these data were less variant compared with the IC$_{50}$ value in HepG2 cells (37.9 $\mu$g/mL) or SMMC-7721 (42.1 $\mu$g/mL)24 (Fig. 2F). Then, expression of endogenous p53 in HepG2 cells was knocked down by siRNA. Among the three siRNA oligos, p53 siRNA-3 was most efficient in knocking down the expression of p53 and it was used for the following experiments (Fig. 5B). The $\Delta \psi_m$ of p53-knockdown HepG2 cells was measured by JC-1 staining and it reduced to 35.1±6.1%, showing no significant difference with the wild type HepG2 cells. Translocation of Bax was also found in p53-knockdown HepG2 cells (Figs. 5C, D). These data implied that cordycepol C induced the loss of $\Delta \psi_m$ and mitochondrial translocation of Bax in a p53-independent manner.

Nuclear Translocation of AIF and Endo G in Cordycepol C-Treated HepG2 Cells

Both AIF and Endo G are mitochondrial factors, which can be released to the nucleus during the process of caspase-independent cell death.5,26 To assess whether AIF and Endo G were involved in the cordycepol C-induced apoptosis, the subcellular fractions were isolated and analyzed by Western blot. We found that AIF and Endo G were released from the mitochondria into nuclear fraction after cells were treated with 20 $\mu$g/mL cordycepol C for 24h (Fig. 6A). We further confirmed the time-dependent translocation of AIF (green) and Endo G (red) to the nucleus (blue) by immunofluorescence analysis (Figs. 6B, C).
of AIF and Endo G began at 6 h after exposure to 20 µg/mL cordycepol C. When the incubation time extended to 24 h, more granular staining of AIF and Endo G spread into the nucleus with the distribution of combined color turning cyan and purple. All these results suggested that AIF and Endo G were involved in cordycepol C-induced apoptosis.

DISCUSSION

Fungicolous fungi, which colonize other fungal species, are a rich resource of new secondary metabolites due to their special niche.28–30) *Cordyceps ophioglossoides*, one of these fungi, is parasitized on certain types of *Elaphomyces*.23) The extracts from the genus *Cordyceps* showed various biological activities and a lot of anti-tumor compounds have been isolated and identified, such as ergosta-4,6,8(14),22-tetraen-3-one, H1-A, cordyheptapeptide A and cordycedipeptide A.31–34) Cordycepin, 3'-deoxyadenosine, a famous compound isolated from *C. militaris* 60 years ago could induce apoptosis in various cancer cell lines with an IC₅₀ value above 100 µmol/L.35–37) However, to the best of our knowledge, no sesquiterpenes were found in the genus *Cordyceps*. In our previous study, we identified four novel sesquiterpenes (including cordycepol C) from cultured mycelia of *C. ophioglossoides* and the IC₅₀ value of cordycepol C against HepG2 cells was 37.9 µg/mL.24) The cell morphology was significantly changed after cordycepol C (20 µg/mL) treatment for 24 h (Supplementary Fig. 1). Cordycepol C was also cytotoxicity towards other cancer cells such as SMMC-7721 and HL-60 cells. Here, we reported cordycepol C (20 µg/mL) could induce HepG2 cell apoptosis through a caspase-independent way, but do minimal harm to normal cell lines, L-02 and HEK293. The hydroperoxy group of cordycepol C is unstable and can form free radicals from RO•.38) Many studies have shown that more cancer cells tend to get damaged by additional oxidative stress than normal cells.25,39) The hydroperoxy group might be the reason for selective cytotoxicity of cordycepol C to cancer cells. Considering its unique structure and the ability to selectively kill tumor cells, cordycepol C may be a potential lead for future drug development.
Mitochondria contain several potentially apoptogenic factors, including cytochrome c, procaspases 2, 3 and 9, AIF and Endo G, which have crucial roles in caspase-independent or caspase-dependent apoptosis. \(\Delta \psi_m\) is often regarded as an indicator of cell viability. The disruption of \(\Delta \psi_m\) leads to leakage of these aforementioned proapoptotic proteins into the cytosol and triggers apoptosis. Some pro-apoptotic members of Bcl-2 family like Bax and Bak can be oligomerized to form membrane channels or pores, resulting in the loss of \(\Delta \psi_m\). Bax was also found to be indispensable for AIF-mediated cell death. On the contrary, the anti-apoptotic molecules such as Bcl-2 and Bcl-xL can be activated to prevent the conformational change and oligomerization of Bax and Bak. In this study, we found cordycepol C could induce the disruption of \(\Delta \psi_m\) and the translocation of Bax from cytoplasm to mitochondria. These data suggested that Bax was oligomerized to anchor onto outer mitochondrial membrane, thus forming mitochondrial permeability transition pore to disrupt the \(\Delta \psi_m\). Bax upregulation could also induce the mitochondrial permeability transition. However, the expressions of Bcl-2 and Bcl-xL were not changed, and the opening of the permeability transition pore was not inhibited. The tumor suppressor p53 can directly activate Bax to permeabilize mitochondria and trigger apoptosis. Meanwhile, Bax can also mediate apoptosis in a p53-independent pathway. We found cordycepol C inhibited cell proliferation in SMMC-7721 (wild-type p53), HepG2 cells (wild-type p53), Hep3B cells (p53-deficient) and Huh-7 cells (p53 mutant) with no significantly difference in IC\(_{50}\) values. To further verify the role of p53 in cordycepol C-induced apoptosis, we used RNAi technology to knock down the endogenous expression of p53 in HepG2 (p53+/+) cells. Both the loss of \(\Delta \psi_m\) and mitochondrial translocation of Bax were detected in p53 knockdown HepG2 cells. These results suggested that p53 didn’t activate Bax in cordycepol C-treated HepG2 cells.

AIF and Endo G, proapoptotic proteins located in the mitochondria, play key roles in caspase-independent cell death. AIF can induce chromatin condensation and cleavage of DNA into high molecular weight fragments (50–300 kb), while endonuclease G can produce oligonucleosomal DNA fragments. Our results revealed that cordycepol C induced the release of AIF and Endo G from the mitochondria and their translocation to the nucleus in a time-dependent manner. The \(\Delta \psi_m\) was lost in a time-dependent manner consistent with the translocation of AIF and Endo G, however, the collapse of \(\Delta \psi_m\) was prior to that. This suggested that the dysfunction of the mitochondria caused the release of AIF and Endo G, leading to caspase-independent apoptosis.

In conclusion, our study suggests that cordycepol C from C. ophioglossoides could induce caspase-independent apoptosis through a p53-independent mitochondrial pathway in HepG2 cells. Bax was activated and targeted to the outer mitochondrial membrane, leading the collapse of \(\Delta \psi_m\) and the translocation of AIF and Endo G (summarized in Fig. 7). Understanding the anticancer pharmacology of this novel sesquiterpene will help scientists to design better drugs.

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