Research Article

Coenzyme Q0 from *Antrodia cinnamomea* in Submerged Cultures Induces Reactive Oxygen Species-Mediated Apoptosis in A549 Human Lung Cancer Cells

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Received 10 June 2014; Revised 12 September 2014; Accepted 16 September 2014; Published 6 November 2014

Academic Editor: Yew-Min Tzeng

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We investigated the anticancer effects of *Antrodia cinnamomea*, a medicinal mushroom from Taiwan, on A549 human lung cancer cells using the ethyl acetate extract from submerged culture filtrates. Our results showed that 2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q0; CoQ0) derived from *A. cinnamomea* submerged culture filtrates has anticancer activity. CoQ0 treatment reduced the viability of A549, HepG2, and SW480 cancer cell lines. Furthermore, CoQ0 induced reactive oxygen species (ROS) generation and apoptosis in A549 cells, which was inhibited by the antioxidant ascorbic acid. To our knowledge, these data demonstrate for the first time that CoQ0 derived from *A. cinnamomea* submerged culture filtrates exerts its anticancer effect through the induction of ROS-mediated apoptosis in A549 human lung cancer cells.

1. Introduction

*Antrodia cinnamomea* is a parasitic fungus found in Taiwan on the inner cavity of the endemic species *Cinnamomum kanehirae* [1]. This fungus has been used as a traditional medicine for the prevention or treatment of various diseases, including liver disease, food intoxication, drug intoxication, hypertension, and cancer [2]. The fruiting body of *A. cinnamomea* is expensive and rarely found in nature because of its slow growth rate and the limited availability of *C. kanehirae* trees. In addition to fruiting body cultivation, *Antrodia cinnamomea* can be cultured using solid-state or submerged mycelia cultures [2]. The use of submerged cultures to produce pharmacologically active substances from cultured mycelia or broth is beneficial to overcome the slow growth rate of fruiting bodies [3–5]. Thus, isolation and characterization of bioactive compounds from submerged cultures are important for the practical use of *A. cinnamomea* in alternative medicine.

A number of studies have used *A. cinnamomea* submerged cultures to produce bioactive compounds with various pharmacological functions, including antioxidants [6], immunomodulatory [7], hepatoprotective [8], and anticancer agents [9–11]. Several reports indicate that components of the methanolic and ethanolic extracts of *A. cinnamomea* mycelia can inhibit the growth of cancer cells [9, 11]. Antroquinonol is a novel compound isolated from *A. cinnamomea* that displays anticancer activity in human hepatocellular carcinoma, pancreatic carcinoma, and non-small-cell lung carcinoma cells [12, 13]. It has received Food and Drug Administration Investigational New Drug (FDA IND) approval and is undergoing human clinical trials as a new drug in Taiwan and the United States. 4-acetyl antroquinonol B was also isolated from *A. cinnamomea* mycelia and can inhibit the proliferation of HepG2 liver cancer cells [14]. In addition to mycelia, the submerged culture broth of *A. cinnamomea* also exhibits anticancer activity. The filtrate from submerged *A. cinnamomea* cultures inhibits human ovarian carcinoma cell proliferation [15] and induces apoptosis in MCF-7 breast cancer cells *in vitro* and *in vivo* [10, 16]. Previous studies have identified new and known compounds in the submerged culture broth.

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[10, 16–18]; however, the compounds in the culture filtrate that exhibit antitumor activity are still unknown. In this study, the anticancer effects of the broth filtrate from A. cinnamomea submerged cultures were investigated using A549 human lung cancer cells. Lung cancer is the leading cause of cancer-related morbidity in many countries [19]. Thus, there is an urgent need to identify novel, effective treatments. Extracts from various medicinal mushrooms are known to have antitumor properties, making them potential sources for new anticancer compounds [20]. Studies have shown that the submerged culture filtrates from A. cinnamomea have antitumor activity [10, 21]; however, the compounds responsible for this effect remain unknown. We aimed to identify the anticancer components in submerged culture filtrates from A. cinnamomea. Our results showed that 2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q0; CoQ0) was the major anticancer compound in submerged A. cinnamomea culture filtrates and that CoQ0 induced apoptosis in A549 cells by stimulating the generation of reactive oxygen species (ROS). Furthermore, we monitored the level of CoQ0 production during the fermentation process.

2. Materials and Methods

2.1. Reagents and Chemicals. CoQ0 standard and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Malt extract and peptone were purchased from BD Bacto (Sparks, MD, USA). All other chemicals were purchased from commercial sources at the highest purity available.

2.2. Organism and Inoculums. A. cinnamomea (BCRC 35716) was obtained from the Bioresource Collection and Research Center (BCRC) at the Food Industry Research and Development Institute, Hsinchu, Taiwan. The mycelium culture was maintained on petri dishes (medium components: 20 g/L glucose, 20 g/L malt extract, 1 g/L peptone, and 20 g/L agar).

2.3. Submerged Fermentation. A 4-week mycelium culture on agar was inoculated into 100 mL of preculture medium (20 g/L glucose, 20 g/L sucrose, 2 g/L peptone, pH 5.0) in a 500-mL flask and incubated for 7 days at 25°C on a rotary shaker (100 rpm). The mycelia were then inoculated in a 5-L fermenter (B. Braun Biotech International, Melsungen, Germany) with 3 L of medium. The medium consisted of 20 g/L glucose, 20 g/L malt extract, 2 g/L peptone, 0.3 g/L MgSO4, 0.3 g/L KH2PO4, and 0.3 g/L K2HPO4. The pH of the medium was adjusted to 5. After inoculation with 1% inoculants, fermentation was carried out at 28°C, 1vvm, and 100 rpm for 28 days.

2.4. Preparation of Broth Extracts. The fermentation product was harvested every week and was separated using filter papers (ADVANTEC, Tokyo, Japan) into culture filtrates and mycelia. The culture filtrate was extracted with an equal volume of ethyl acetate and then vacuum dried. For the cell assay, the extract was dissolved in dimethyl sulfoxide (Sigma-Aldrich) and passed through a 0.2 mm filter. The A. cinnamomea submerged culture filtrate extract (ACFE) was stored at −20°C until use in experiments.

2.5. Cell Culture. Human A549 lung cancer cells, HepG2 hepatoma cells, and SW480 colon cancer cells were purchased from the Bioresource Collection & Research Centre (Food Industry Research and Development Institute, Hsinchu, Taiwan). The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS (v/v), penicillin (100 units/mL), and streptomycin (100 mg/mL). Cells were cultured in a humidified incubator at 37°C with 5% CO2 in air.

2.6. Activity Guided Purification of Anticancer Compounds. Thin layer chromatography (TLC) plates were prepared by making slurry of 30 mg of silica gel G (Merck, Darmstadt, Germany) with 60 mL of distilled water. Spreading was done manually over glass plates (20 × 20 cm) and air dried. The plates were activated in an oven for 3 h at 110°C. ACFE was fractionated by petroleum ether (Merck) into two fractions, namely, petroleum ether soluble (PES) and petroleum ether insoluble (PEI). PES (50 mg sample dissolved in 500 µL of ethyl acetate) was used for TLC spotting. Separation of the TLC spots was done using chloroform:ethyl acetate (8:2, v/v) as mobile phase. Spots developed on TLC plates were observed under visible light. Spots were eluted by ethyl acetate separately and isolated fractions were tested for their inhibition against A549 cancer cells. The bioactive fraction was then analyzed by the GC-MS and 1H NMR.

2.7. Cell Viability Analysis. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability. Cells were seeded in 96-well plates (1 × 104 cells/well) overnight in DMEM medium and then

![Figure 1: The IC50 of ACFE was prepared at different culture times on SW480, A549, and HepG2. The IC50 values were measured by the MTT assay as described in the Section 2.](image-url)
treated with different concentrations of ACFE. The effect of CoQ0 on cell growth was examined with the MTT assay. Briefly, 100 µL of MTT solution (2 mg/mL in PBS; Sigma-Aldrich) was added to each well and incubated for 2 h at 37°C. The supernatant was aspirated, and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 100 µL of DMSO. The absorbance was measured by a microplate reader at a wavelength of 570 nm.

2.8. GC-MS Analysis. GC-MS analysis was performed using an Agilent GC-MS system (6890N GC system and 5973N mass selective detector, CA, USA) equipped with a HP-5 capillary column (5% diphenyl and 95% dimethyl polysiloxane phase; 0.25 µm film thickness and 30 m x 0.25 mm i.d.). The electron ionization (EI) source was operated at 230°C. Helium was used as the carrier gas at a constant flow rate of 0.8 mL/min. The injection volume was 1 µL, and the injection port was maintained at 250°C. The column temperature protocol was 70°C for 2 min with a ramp rate of 3°C/min to 160°C and then 20°C/min to 280°C, where the temperature was maintained for 20 min. The total run time was 58 min. Data were acquired in a full scan mode with
Figure 3: Identification of CoQ0 in PEY by GC-MS. (a) PEY and the CoQ0 standard were analyzed by GC-MS. (b) Mass spectra (MS-EI) of PEY and the CoQ0 standard.
2.9. \(^1\)H NMR Measurements at 400 MHz. NMR measurements were performed on a Bruker Avance 400 Ultra-shield spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5-mm PABBO BB-probe head, using a Bruker Automatic Sample Changer (B-ACS 120). \(^1\)H NMR spectra were acquired at 300.0 K, and number of scans was 64. The sample was dissolved in CDCl\(_3\), and tetramethylsilane was used as the internal standard. Chemical shifts were expressed in ppm.

2.10. Apoptosis Analysis. Early apoptotic cells were detected using the Alexa Fluor 488 Annexin-V/propidium iodide (PI) kit (Invitrogen, Carlsbad, CA). The apoptosis of A549 cells (1 \( \times 10^6 \) cells/well) was measured 24 h after, and cells treated with PBS containing 0.1% DMSO served as negative control. The experimental groups were treated with various concentrations of CoQ0. The quantification of PI and FITC signals was analyzed by a FACSCanto II flow cytometer (BD Bioscience, San Diego, CA, USA). The percentage of stained cells in each quadrant was analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA). Cells positive for Annexin-V FITC and negative for PI (Q4 quadrant) were in early apoptosis, and cells positive for both Annexin-V FITC and PI (Q2 quadrant) were in late apoptosis or necrosis.

2.11. Determination of the Intracellular ROS Level. To evaluate the intracellular ROS level in A549 cells, the cells were incubated with CoQ0 (25 \( \mu \)g/mL) for the indicated periods. Cells were then incubated with 10 \( \mu \)M 2′,7′-dichlorofluorescin diacetate (DCF-DA) for 30 min prior to harvesting. The presence of ROS causes DCF-DA to be oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF). The fluorescence intensity of the cells was analyzed by flow cytometry.

2.12. Statistical Analysis. The results are presented as the mean ± standard deviation (mean ± SD). All study data were analyzed using analysis of variance followed by Dunnett’s test for pair-wise comparison. An asterisk indicates that the experimental values were significantly different from those of the controls (*) \( P < 0.05 \).

3. Results

3.1. Ethyl Acetate Extract from A. cinnamomea Submerged Culture Filtrates Inhibits Cancer Cell Survival. To investigate the anticancer activity of the ACFE and identify the bioactive compound, we first examined the effect of ACFE on the survival of three cancer cell lines, HepG2, A549, and SW480. Cells were treated with ACFE at different culture times, and cell viability was determined by the MTT assay. The results showed that ACFE inhibited the viability of all three cancer cell lines, and the inhibitory effect was stronger when ACFE was prepared at later culture times (Figure 1). In the following experiments, we used the ACFE from 4-week culture filtrates to identify the compound(s) with anticancer activities.

3.2. Isolation and Identification of CoQ0 as the Major Anticancer Compound in A. cinnamomea Submerged Culture Extract. To purify the anticancer compound(s) in ACFE, we first fractionated ACFE by petrol ester into two fractions, namely, the soluble PES and the insoluble PEI fractions, which were then evaluated for their anticancer effects using A549 cancer cells. We found that the PES fraction was more potent than the PEI fraction in suppressing A549 cell proliferation (Figure 2(a)). Components in the PES fraction were further separated by TLC, and the chromatographic profile showed two major bands on the TLC plate under visible light: a purple band (PEP) and a yellow band (PEY) (Figure 2(b)). The PEP and PEY fractions were tested for their anticancer activity by the MTT assay (Figure 2(c)), and the results indicated that the major anticancer activity resided in the PEY fraction. The identity of PEY was first analyzed by GC-MS analysis. The GC-MS spectrum of PEY was identical to that of authentic compound. The purified PEY standard also had the same spectra in the GC-MS total ion chromatograms (TICs) (Figure 3(b)).

The \(^1\)H NMR data were similar to CoQ0 reported in literature [22] and the authentic compound. The purification method yielded 65 mg of PE and 16 mg of PEY from 300 mg of ACFE, and the purity of CoQ0 in PEY was 95.5%. The results identified that the major anticancer compound isolated from the submerged culture filtrate of A. cinnamomea was CoQ0.
3.3. CoQ0 Induced Apoptosis of A549 Lung Cancer Cells. To confirm that CoQ0 is a cytotoxic agent for the A549 lung cancer cell, we examined the effect of CoQ0 treatment on cell viability by the MTT assay. CoQ0 showed dose-dependent inhibition on A549 cell viability, with an IC₅₀ value of approximately 15 μg/mL (Figure 4). We then examined the induction of apoptosis in A549 cells using the Annexin-V/PI assay. The results showed that CoQ0 treatment resulted in a significant increase in both early apoptotic (Annexin-V⁻PI⁻) and late apoptotic (Annexin-V⁺PI⁺) cells (Figure 5). These results demonstrate that CoQ0 exerts its cytotoxic effect on A549 cells via the induction of apoptotic cell death.

3.4. CoQ0 Triggered A549 Cell Apoptosis via Inducing ROS Generation. ROS play an important role in the induction of apoptosis [23]. To determine if CoQ0 induced apoptosis of A549 cells through ROS accumulation, we used the DCF-DA fluorescent dye to detect the levels of superoxide radicals and hydrogen peroxide in the cell. The results showed that CoQ0 induced a rapid production of intracellular ROS at 0.5 h after treatment (Figure 6(a)). To study if ROS generation was directly associated with CoQ0-induced apoptosis, we assessed apoptotic events in A549 cells pretreated with the antioxidant ascorbic acid (ASC) for 1 h before CoQ0 treatment. ASC is a thiol-free antioxidant and should not interact directly with CoQ0 [24]. Pretreatment with ASC effectively blocked CoQ0-induced apoptosis (Figure 6(b)) and loss of cell viability (Figure 6(c)) of A549 cells. These data clearly demonstrate that CoQ0-induced production of ROS plays an essential role in trigging apoptosis of A549 cells.

3.5. Production of CoQ0 by A. cinnamomea Submerged Culture in a 5-L Fermenter. After identifying CoQ0 as the key anticancer compound in A. cinnamomea culture filtrates and characterizing its anticancer mechanism, we next performed a scaled-up mycelial culture of A. cinnamomea in a 5-L fermenter and monitored the kinetics of CoQ0 production. As shown in Figure 7, the mycelial biomass reached the highest level of 3.51 g/L on day 21, and CoQ0 appeared after 2 weeks of culture. The concentration of CoQ0 in culture filtrates increased from weeks 2 to 4, and the maximum CoQ0 content of 43 mg/L was obtained after 4 weeks of culture. These results indicate that accumulation of CoQ0 occurs later than biomass formation in A. cinnamomea submerged culture.

4. Discussion

Fruiting body of A. cinnamomea in the wild is well-known as an effective and expensive folk remedy for cancer, in Taiwan, and most studies on the anticancer function of A. cinnamomea have examined extracts from the fruiting bodies or mycelia. In this study, we demonstrate that the ethyl acetate extract of the culture broth of A. cinnamomea has anticancer activity and identify that CoQ0 is the major cytotoxic compound produced by A. cinnamomea during the submerged mycelial culture. Using human A549 lung cancer cell line as a model to characterize the mechanism of CoQ0-induced cancer cell death, our results showed that CoQ0 treatment results in the generation of ROS, which leads to the induction of apoptosis of A549 cells. Batch culture of A. cinnamomea in a 5-L fermenter indicated that the maximum level of CoQ0 was produced at a later stage of fermentation.
Figure 6: CoQ0 provokes ROS-mediated apoptosis. (a) A549 cells were treated with 15 μg/mL of CoQ0 for 0.5 and 1 h, and the ROS levels were measured by DCF-DA staining and flow cytometry. Numbers in parentheses indicate mean fluorescence intensities. (b) A549 cells were pretreated without or with ASC (1 mM) for 1 h and then incubated with CoQ0 (15 μg/mL) for 6 h. Apoptotic cells were measured by the Annexin V/PI assay. (c) A549 cells were pretreated without or with ASC (1 mM) for 1 h and then incubated with different concentrations of CoQ0 for 24 h. Cell viability was measured by the MTT assay. Data shown are representative of three independent experiments with similar results. * P < 0.05.

To our knowledge, this is the first report to show that CoQ0 is an anticancer compound produced by the submerged culture of *A. cinnamomea*. Our data showed that ACFE is cytotoxic to multiple cancer cell lines, including HepG2 hepatoma cells, A549 lung cancer cells, and SW480 colon cancer cells. Through a series of purification steps, we have identified that CoQ0 is the major cytotoxic compound in ACFE. CoQ0 was also shown to modulate cell cycle and induce apoptosis in estrogen receptor-negative human breast cancer cells [25]. These data together suggest that CoQ0 has a general cytotoxicity against various cancer cells and can potentially be developed into an anticancer drug. Similar to our finding, CoQ0 and other quinones have been isolated from the culture broth of *A. cinnamomea* [26] and the mycelium of *Antrodia salmonea* [27]. Many compounds with anticancer
ROS was also shown to mediate apoptosis of HER-2/neu-overexpressing breast cancer cells induced by the culture broth of *A. cinnamomea* [34]. We found that CoQ0 exerts a higher cytotoxicity in A549 cancer cells (IC_{50} ≈ 15 µg/mL) than in normal human fibroblast Detroit 551 cells (IC_{50} ≈ 42 µg/mL, data not shown). In a previous study, CoQ0 was shown to inhibit PTP opening in primary rat hepatocytes and cultured rat liver Clone-9 cells, whereas it induced PTP opening in cancerous rat liver MHICl cells [33]. These data suggest that CoQ0 may induce stronger ROS production and cell death in cancer cells than in normal cells. However, since CoQ0 was shown to be toxic to neuron [35], and insulin-producing cells [33, 36], the dose of CoQ0 that gives selective toxicity to cancer cells should be examined in more detail before it can be considered for use in cancer chemotherapy.

In the current study, we investigated the kinetics of mycelial growth and CoQ0 production of *A. cinnamomea* in a 5-L fermenter. The production of CoQ0 was evident after 2 weeks of submerged culture, when the mycelial growth had reached the stationary phase (Figure 7). The level of CoQ0 in the culture filtrate continued to rise till week 4, the endpoint of culture in this study. The time course of CoQ0 accumulation correlated well with the cytotoxicity of ACFE prepared at different culture times (Figure 1), consistent with our finding that CoQ0 is the major anticancer compound in the culture broth of *A. cinnamomea*. We predict that the amount of CoQ0 might accumulate further when the culture time is extended. Our data suggest that submerged culture of *A. cinnamomea* can be a new way of CoQ0 production besides chemical synthesis [22]. One advantage of producing CoQ0 using the submerged culture of *A. cinnamomea* is that additional anticancer compounds can also be isolated from the mycelia [9, 11].

### 5. Conclusions

*A. cinnamomea* is a medicinal mushroom found in Taiwan and has been used for the prevention or treatment of various diseases [2]. Our results provide evidence that CoQ0 in the filtrate from submerged *A. cinnamomea* cultures induces ROS-mediated apoptosis in A549 human lung cancer cells. Future studies will focus on the anticancer activity and safety profile of CoQ0 *in vivo*, which will determine whether CoQ0 has a potential to be developed into a novel chemotherapeutic agent for cancer treatment.

### Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of the paper.

### Authors’ Contribution

Cheng-Han Chung, Szu-Chien Yeh, Chun-Jen Chen, and Kung-Ta Lee designed research. Cheng-Han Chung and Szu-Chien Yeh performed research. Cheng-Han Chung, Chun-Jen Chen, and Kung-Ta Lee wrote the paper. All authors read and approved the final version of the paper.
Acknowledgments

We would like to acknowledge the excellent technical assistance provided by the Flow Cytometry Core Facility of Technology Commons, College of Life Science, NTU. We are grateful to the Council of Agricultural Development (Grant no. 103AS-14.3.4-ST-a2) for financial support.

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