

Cytochalasin H isolated from mangrove-derived endophytic fungus induces apoptosis and inhibits migration in lung cancer cells

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Abstract. Cytochalasin H (CyH) has been shown to exhibit promising anticancer activities against various types of cancers; however, the underlying mechanisms remain unknown. In a previous study, we isolated CyH from the mangrove-derived endophytic fungus *Phomopsis* sp. in Zhanjiang, China. In the present study, we further explored the effect of CyH on apoptosis and migration in the human lung adenocarcinoma cell line A549. Cell Counting kit-8 (CCK-8) assay was used to observe the effects of CyH on the growth of A549 cells. The cell cycle and apoptosis were determined using flow cytometry. The effect of CyH on cell migration was observed by scratch wound healing and chamber migration assays. Western blotting was used to detect the expression of apoptosis- and metastasis-associated proteins. Our results showed that CyH exhibited cytotoxicity to A549 cells. The treatment of CyH arrested A549 cells at the G₂/M phase. Furthermore, sub-G₁ peaks and fragmented DNA ladders were observed, and the mitochondrial transmembrane potential was also decreased in CyH-treated A549 cells. CyH significantly increased Bax, P53, and cleaved caspase-3 (17 kDa) protein expression and decreased Bcl-xL, Bcl-2, and full-length caspase-3 (35 kDa) protein expression, resulting in an increased ratio of the pro-apoptosis/anti-apoptosis proteins Bax/Bcl-2. Additionally, CyH treatment inhibited the migration ability of A549 cells in a dose-dependent manner. Taken

together, our results suggest that CyH may be a potential chemopreventive drug for the treatment of lung cancer.

Introduction

Cancer remains a major human health threat worldwide. The incidence of cancer has increased to 14 million new cases and 8.2 million cancer-related deaths in 2012 (1). Cancer is characterized by the uncontrolled and invasive growth of cells (2). Chemotherapy is a traditional treatment, but chemotherapeutic drugs have many side effects (3,4). Therefore, there is an urgent requirement for new anticancer drugs with advanced efficiency and fewer side-effects (5).

Mangrove ecosystems are intertidal wetlands in subtropical or tropical temperate coastal zones. These ecosystems have a great commercial and ecological value for humans (6). Endophytes are microbial entities that colonize within living tissues of living plants without causing apparent disease symptoms in its hosts and are a relatively unexplored potential source of novel and bioactive natural compounds for exploitation in medicine, agriculture and industry (7). Recently, more attention has been paid to bioactive natural products of endophytic fungi, isolated from mangrove plants. The secondary metabolites from endophytic fungi exhibit a range of biological activities, including antitumor, anti-inflammatory, antibacterial, antiviral, antioxidative and anti-angiogenic (8-13). As a result, many fungal endophytes have been isolated and subjected to extensive chemical investigations during the past two decades (14). In our previous study, cytochalasin H (CyH) was isolated from the metabolic products of endophytic fungus *Phomopsis liquidambari*, derived from a mangrove in Zhanjiang, China.

Cytochalasins are a class of structurally related fungal metabolites that are microfilament inhibitors with substantially the same biological activity as that observed in the inhibition of cell division, motility, secretion and phagocytosis (15). Different cytochalasins have their own unique functions. However, there has been a recent focus on the anti-inflammatory, anti-fungal, and antitumor pharmacological effects of

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CyH, each through different mechanisms such as the induction of apoptosis and inhibition of angiogenesis (16,17).

Apoptosis is the process of programmed cell death regulated by a complex network of proliferation and survival genes. Its disequilibrium, i.e., either through the acquisition of anti-apoptotic signals or lack of pro-apoptotic signals, can result in the failure of treatment or a variety of pathological conditions such as cancer or autoimmune and degenerative diseases (18). Therefore, chemical agents that act on molecular targets in apoptotic pathways are likely to be a promising approach for cancer therapy.

In the present study, we observed the effect of CyH on apoptosis and migration in A549 cells. To the best of our knowledge, we found for the first time that CyH can induce apoptosis and inhibit migration in A549 cells. Our findings provide useful evidence for the anticancer activity of CyH in the treatment of lung cancer.

Materials and methods

Drug and reagents. CyH, isolated previously by our laboratory from mangrove endophytic fungus *Phomopsis liquidambari* in Zhanjiang, China, was identified by nuclear magnetic resonance (NMR) (Fig. 1). CyH was dissolved in 0.1% dimethyl sulfoxide (DMSO) at a concentration of 1 mM, and was then diluted in cell culture medium according to experimental requirements. Cell Counting kit-8 (CCK-8), RIPA lysis buffer, mitochondrial membrane potential (MMP) assay kit with JC-1, Hoechst staining kit, cell cycle and apoptosis analysis kit, mouse anti-human Bax antibody (1:1,000 dilution; cat. no. AB026-1), rabbit anti-human Bcl-2 antibody (1:1,000 dilution; cat. no. AB112-1), mouse anti-human β -actin antibody (1:2,000 dilution; cat. no. AA128-1), and rabbit anti-human Bcl-xL antibody (1:1,000 dilution; cat. no. AB126-1) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Phenylmethanesulfonyl fluoride (PMSF) was purchased from Biosharp Biotechnology (Hefei, China). Gibco RPMI-1640 medium was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture. The human lung adenocarcinoma cell line A549 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). A549 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μ g/ml streptomycin at 37°C and 5% CO₂ in an incubator. Cells in the logarithmic phase of growth were used in the experiment.

CCK-8 assay. The CCK-8 assay was performed to determine the effect of CyH on the growth of A549 cells. Cells in the logarithmic growth phase were plated onto 96-well plates at 5x10³ cells/well. After adhering, the cells were treated with various concentrations of CyH for 24, 48 and 72 h, respectively. CCK-8 solution (0 μ l) from the CCK-8 was added to each well and the plate was incubated at 37°C for an additional 1 h. Cell viability was calculated as the absorbance value (A) at a wavelength of 450 nm according to the manufacturer's instructions.

Analysis of cell cycle and cell apoptosis. A549 cells in the logarithmic growth phase were treated with different

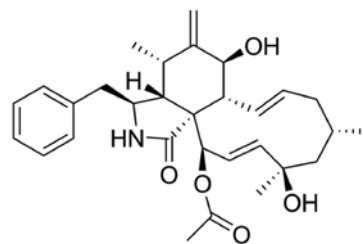


Figure 1. Chemical structure of CyH. CyH, cytochalasin H.

concentrations of CyH (0, 6.25, 12.5, 25 and 50 μ M) for 48 h. For cell cycle analysis, the cells were harvested by trypsinization and fixed in 70% ice-cold ethanol overnight. Afterwards, the cells were centrifuged at 1,000 rpm for 5 min, washed with PBS, and suspended with 0.5 ml of propidium iodide (PI) for 30 min at 37°C. Cellular DNA was stained with PI for 10 min at 4°C in the dark before analysis by flow cytometry with a MultiCycle AV DNA analysis software (Beckman Coulter, Inc., Brea, CA, USA).

Analysis of MMP. Cell apoptosis was determined using the MMP assay. MMP was detected using JC-1. Aggregate red fluorescence indicates high MMP and cells in a normal state, while green fluorescence indicates reduced MMP such as during the early events of apoptosis. The change in MMP was detected using fluorescence microscopy (Nikon Corp., Tokyo, Japan).

Migration assay. The effect of CyH on cell migration was evaluated by scratch wound healing and chamber migration assays. For the scratch wound healing assay, 100 μ l of A549 cell suspension was seeded onto 6-well plates at a density of 5x10⁴/well, and then cultured into monolayers. Wounds were generated in the cell monolayer by scratching with a sterile 10 μ l pipette tip. The medium was then removed and the cells were rinsed 3 times with PBS to wash away cell debris. Cells were then treated with different concentrations of CyH (0, 0.05, 0.1, 0.2, 0.4 and 0.8 μ M) and the wound migration distance was imaged at 0, 24, 48 and 72 h, respectively, under a light microscope (Nikon Corp.) at a magnification of x100. For the Transwell chamber migration assay, A549 cells (5x10⁴/ml) were pre-treated with CyH (0, 0.05, 0.10, 0.20, 0.4 and 0.8 μ M) for 48 h and then plated in the top chambers in FBS-free medium. The medium at the bottom chamber was supplemented with 10% FBS to allow migration towards the chemoattractant for 48 h in a 37°C chamber. Cells at the bottom of the membrane were fixed in 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 1 h. The number of cells was counted in at least five randomized fields under a light microscope (Nikon Corp.) at a magnification of x200.

Analysis of apoptosis-related protein expression. Proteins were extracted from A549 cells by RIPA lysis buffer containing protease inhibitors, cultured in 6-well plates and treated with CyH (0, 6.25, 12.5, 25, 50 and 100 μ M) for 48 h. Protein concentrations were detected by the BCA protein assay. Afterwards, 100 μ g of protein was separated on 10% SDS-PAGE and was then transferred to a PVDF membrane (EMD Millipore,

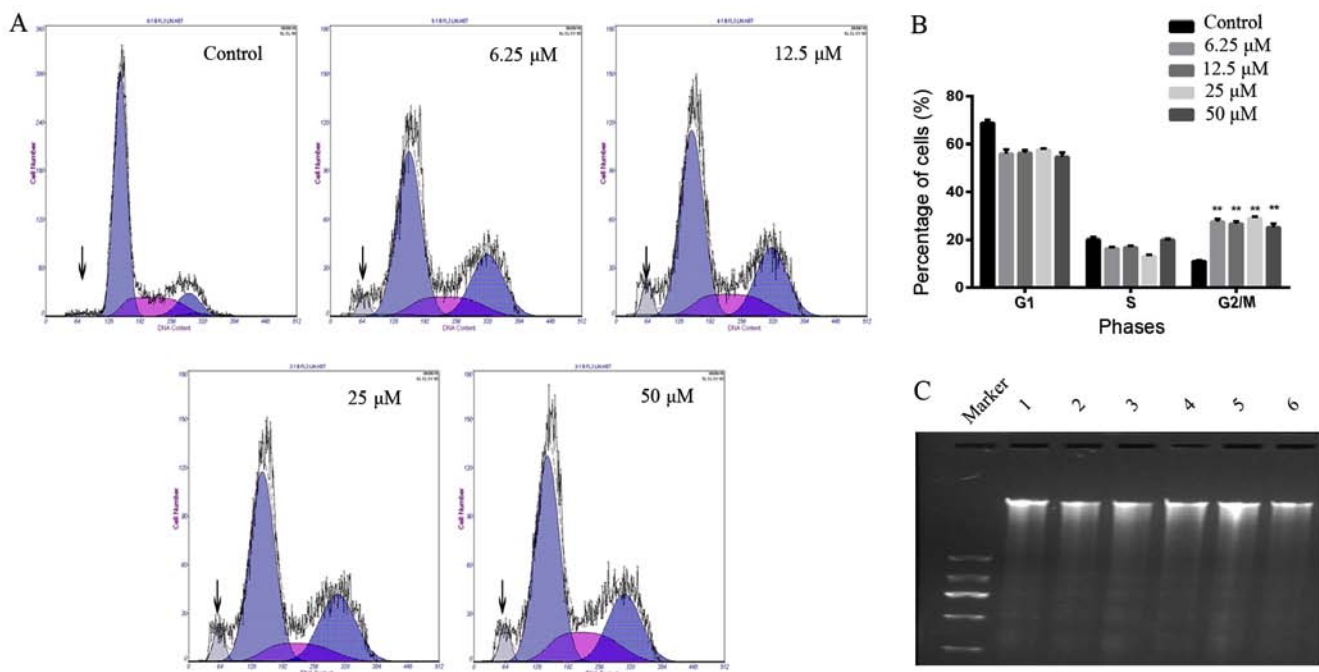


Figure 2. CyH causes cell cycle arrest at the G₂/M phase. (A) A549 cells were treated with different concentrations of CyH for 48 h and stained with PI. The arrows represent sub-G₁ peaks. The DNA content was analyzed using flow cytometry. (B) Histogram showing the flow cytometric analysis of A549 cells. Results are representative of three independent experiments. **P<0.01 compared with the control. (C) DNA was isolated from A549 cells treated with different concentrations of CyH for 48 h. Fragmented DNA was separated on 1% agarose gel for 1 h at 100 V and then stained and photographed. Lane 1, 0 μM; lane 2, 6.25 μM; lane 3, 12.5 μM; lane 4, 25 μM; lane 5, 50 μM; and lane 6, 100 μM. CyH, cytochalasin H; PI, propidium iodide.

Billerica, MA, USA). The membrane was blocked with 5% non-fat milk in TBS containing 0.1% Tween-20 (TBST) for 2 h at room temperature. The membranes were incubated overnight at 4°C with Bax, Bcl-2, Bcl-xL, caspase-3 and P53 primary antibodies, respectively and β-actin was used as an internal control. Target proteins were detected by ECL reagents and then were exposed to X-ray film (Carestream Health, Inc., Xiamen, China). The band density was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are showed as mean ± standard deviation. One-way ANOVA assay was performed for data analysis using SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA). P<0.05 was indicative of a statistically significant difference.

Results

Effect of CyH on cell viability in A549 cells. The results from the CCK-8 assay (data not shown) confirmed that CyH inhibited the proliferation of A549 cells. IC₅₀ value for CyH in the A549 cells was 159.50±1.048 μM. Subsequently, the optimal experimental concentrations of CyH (0, 6.25, 12.5, 25, 50 and 100 μM) were chosen.

Analysis of cell cycle distribution and cell apoptosis. The results from the cell cycle assay confirmed that the cell cycle was arrested at the G₂/M phase and sub-G₁ peaks were found after A549 cells were treated with different concentrations of CyH (Fig. 2A). The proportion of DNA in cells in the G₂/M phase was increased following treatment of CyH with different concentrations. Different drug concentration groups

had statistical differences when compared to the control group (P<0.01, Fig. 2B). Furthermore, fragmented DNA ladder is an important indicator of apoptosis. As expected, fragmented DNA ladders were found in the CyH-treated cells. As shown in Fig. 2C, we observed fragmented DNA ladders by agarose gel electrophoresis.

Cell apoptosis was further analyzed using MMP assay. MMP levels were examined using a JC-1 sensitive fluorescent probe by fluorescence microscopy. Moreover, the red fluorescence of JC-1 was significantly decreased and the green fluorescence was markedly increased (Fig. 3), indicating that MMP in the CyH-treated cells was decreased. Taken together, our results demonstrated that CyH induced apoptosis in the A549 cells.

Analysis of apoptosis-related protein expression. To investigate the mechanism by which CyH induces the apoptosis of A549 cells, the protein expression levels of apoptosis-related proteins were determined by western blotting. Bcl-2 and Bcl-xL, two anti-apoptotic proteins, inhibit programmed cell death; Bax and P53, two pro-apoptotic proteins, drive the cell towards apoptosis. As shown in Fig. 4A, as the concentration of CyH increased, the protein expression levels of Bcl-xL, Bcl-2 and full-length caspase-3 (35 kDa) were decreased while the protein expression levels of Bax, P53 and cleaved caspase-3 (17 kDa) were increased. The differences between the groups were statistically significant (P<0.01, Fig. 4B).

Migration assay. To analyze the effect of CyH on the metastasis of A549 cells, scratch wound healing and Transwell migration assays were used to analyze cell migration. The results from the scratch wound healing assay showed that the migration

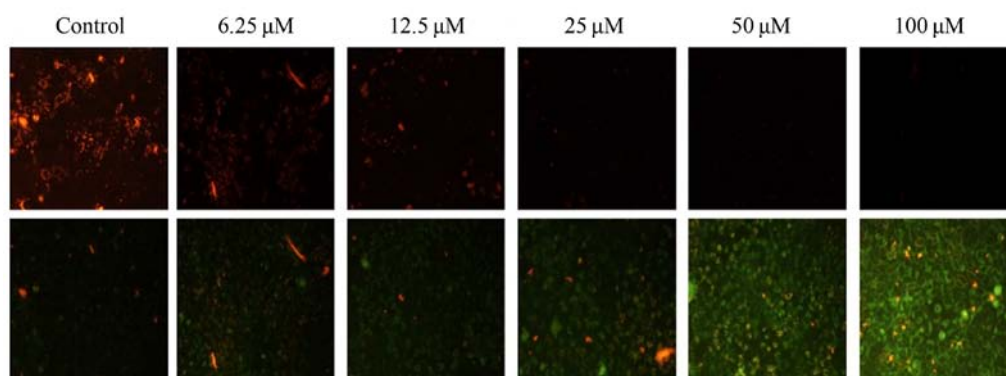


Figure 3. MMP was measured using JC-1 dye staining under fluorescence microscopy. A549 cells were treated with different concentrations (0, 6.25, 12.5, 25, 50 and 100 μ M) of CyH for 48 h. In upper row, red fluorescence represents mitochondria with intact membrane potential. After treated, the red fluorescence was decreased. In lower row, green fluorescence represents de-energized mitochondria. In treated cells, green fluorescence was noted in A549 cells indicating the dissipation of the MMP. MMP, mitochondrial membrane potential; CyH, cytochalasin H.

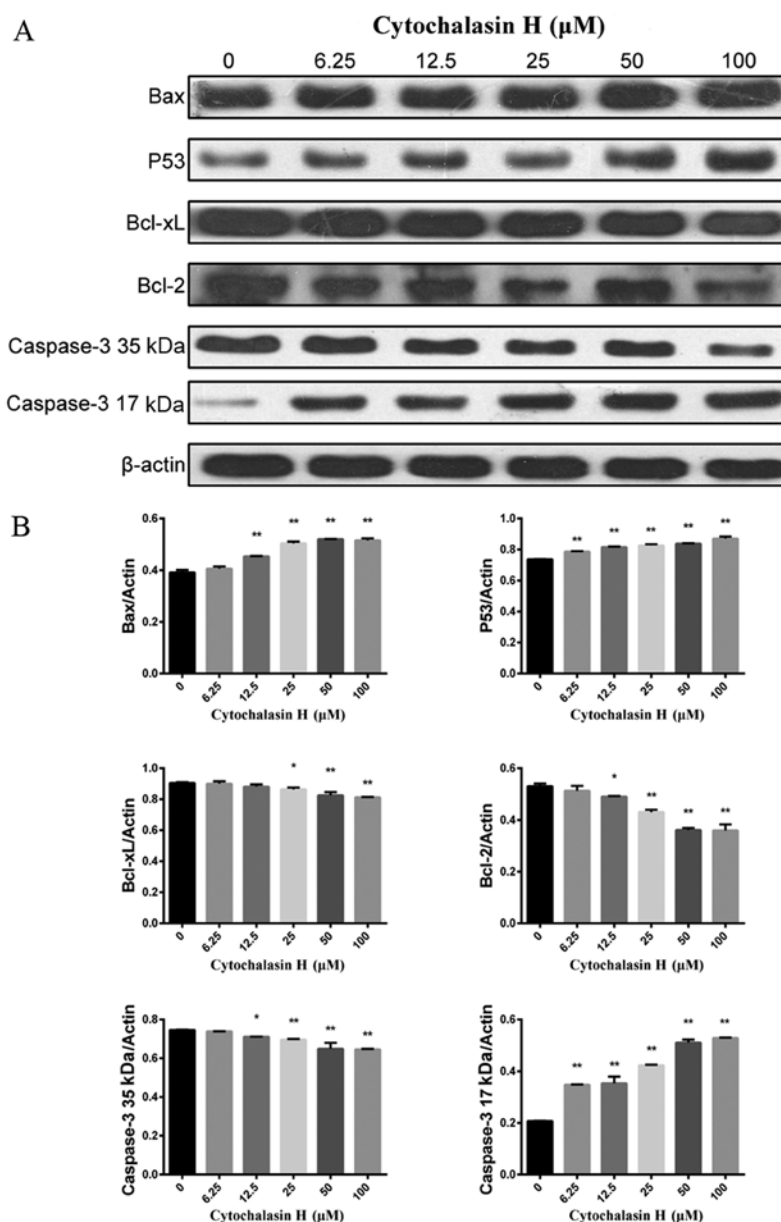


Figure 4. Effect of CyH on the expression of apoptosis-related proteins in A549 cells. Cell extracts were prepared and separated on 10% polyacrylamide-SDS gels, and the expression of P53, Bax, Bcl-xL, Bcl-2, cleaved caspase-3 (17 kDa) and full-length caspase-3 (35 kDa) proteins were analyzed by western blotting. (A) A549 cells were treated with different concentrations of CyH for 48 h. (B) The differences between the groups were statistically significant, * $P < 0.05$ and ** $P < 0.01$. Relative expression level: the value was evaluated by the ratio of target protein expression to reference protein expression. CyH, cytochalasin H.

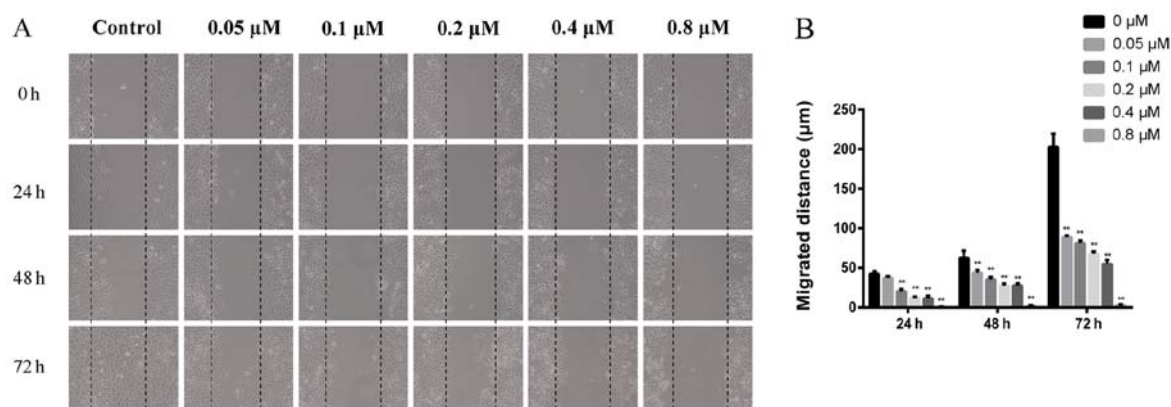


Figure 5. Effect of CyH on the migration ability of A549 cells by a scratch wound healing assay. (A) Wounds were made by scraping a plastic tip across the cell monolayer and then A549 cells were treated with different concentrations of CyH (0, 0.05, 0.10, 0.20, 0.4 and 0.8 μ M) and wound distances were imaged at 0, 24, 48 and 72 h. (B) The relative cell migration ability was analyzed in the A549 cells. Results are representative of three independent experiments. ** $P < 0.01$, compared with the control at the same time-point. CyH, cytochalasin H.

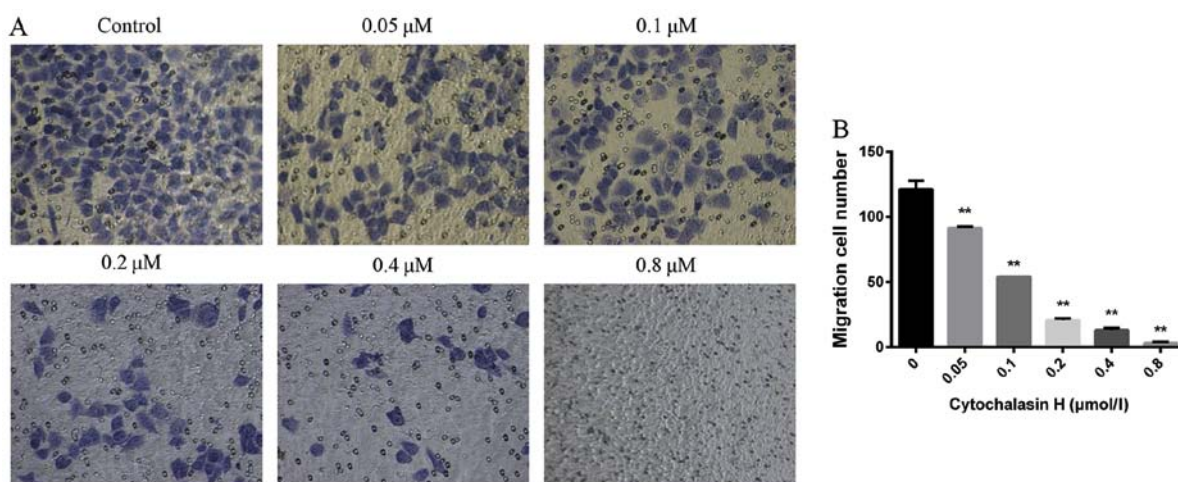


Figure 6. Effect of CyH on the migration ability of A549 cells by a Transwell migration assay. The cells were plated in the top chambers and treated with different concentrations of CyH (0, 0.05, 0.10, 0.20, 0.4 and 0.8 μ M). (A) The number of cells was counted under a microscope after 48 h (magnification, $\times 200$). (B) The results are representative of three independent experiments. The migration cell number, $n=3$; ** $P < 0.01$ compared with the control. CyH, cytochalasin H.

ability in CyH-treated A549 cells was significantly inhibited compared with that of the control group (Fig. 5A). Furthermore, it revealed that the migration distances of CyH-treated A549 cells were significantly decreased compared with that of the control cells ($P < 0.01$, Fig. 5B). A Transwell migration assay was carried out to further confirm the effect of CyH on the migration of A549 cells. Our results showed that the number of A549 cells moving into the chamber was reduced gradually as the concentration of CyH increased compared with the control group (Fig. 6A). These results demonstrated that the difference between the CyH-treated cells and the control cells was statistically significant ($P < 0.01$, Fig. 6B), indicating that CyH suppressed the migration ability of A549 cells.

Discussion

High rates of morbidity and mortality have made cancers a major public health concern (19). The treatment of cancers mainly involves radiotherapy combined with chemotherapy and other comprehensive treatments (20). However, conventional chemotherapy drugs have serious adverse reactions and

are prone to induce drug resistance, and the long-term effects are not ideal (21). The secondary metabolites from mangrove endophytic fungi have the advantages of high biological activity and low toxicity.

In our previous study, we isolated CyH from the endophytic fungus *Phomopsis liquidambari*. In the present study, we further analyzed the effects of CyH on proliferation in A549 cells. We found that CyH inhibited the proliferation of A549 cells. The cell cycle is important for the proliferation of cancer cells (22). The goal of targeting cell proliferation is to arrest the cell cycle or induce cancer cell death using cytotoxic compounds. Cells treated with Taxol extracted from yews are unable to proceed normally through the cell cycle and are arrested in the G_1 and G_2/M phases (23-25). As shown in Fig. 2, our findings suggest that CyH inhibits the proliferation of A549 cells attributed to G_2/M phase arrest. However, G_2/M arrest may stop cell cycle progression, which will also affect DNA synthesis, thus further investigation is needed.

Apoptosis, the process of programmed cell death, has been recognized as one of the major processes that mediate the inhibition of cell proliferation, which may be targeted by anticancer

agents (26). Cells undergoing apoptosis exhibit morphological and biochemical modifications including chromatin segregation, nuclear condensation, DNA fragmentation, partition of the membrane, and vesicle formation (27,28). The late-stage of apoptosis can be visualized by standard agarose gel electrophoresis as a ladder pattern because of DNA cleavage (29). In the present study, we found fragmented DNA ladders in the CyH-treated A549 cells (Fig. 2C). To further confirm the effects of CyH on apoptosis, MMP assay, PI staining, and flow cytometry were performed. Our results showed that CyH induced apoptosis in the A549 cells. p53, a tumor-suppressor transcription factor, plays a vital role in cell cycle arrest and apoptosis in response to cellular stress (30). The functional p53 encodes a nuclear phosphoprotein that regulates the synthesis of gene products involved in growth arrest, DNA repair, apoptosis and the inhibition of angiogenesis (31). According to the present results, the expression level of p53 was significantly upregulated following CyH treatment (Fig. 4). It could be concluded that CyH induces apoptosis in a p53-dependent pathway. The potent anticancer activity of p53 has been linked to its ability to induce apoptosis through the intrinsic mitochondrial-mediated apoptotic pathway (32). The mitochondrial apoptotic pathway is mainly regulated by Bcl-2 family proteins. Any imbalances in the expression level of pro-apoptotic Bax and anti-apoptotic Bcl-2 members lead to the disruption of the outer mitochondrial membrane (33,34). Upon apoptotic stimulation, the expression of Bax is increased, leading to a lower level of Bcl-2 (35). Bax forms oligomers and is transported from the cytoplasm to the mitochondrial membrane, resulting in mitochondrial membrane depolarization (36). Subsequently, cytochrome *c* is released from mitochondria to the cytosol, triggering caspase pathway activation (37). Caspases, playing a key role in apoptotic events, adjust cell death, and the appearance and function of caspase apoptotic features are closely associated (38). Caspase-3, a crucial downstream effector of the caspase family, is thought to be involved in both the mitochondrial apoptotic pathway and the death receptor pathway (39). Full-length caspase-3 (35 kDa), an inactive protein, is cleaved between Asp28 and Ser29 as well as between Asp175 and Ser176 to produce cleaved caspase-3 (17 kDa), an active peptide. Apoptosis will occur in the cells when full-length caspase-3 (35 kDa) protein levels are decreased and cleaved caspase-3 (17 kDa) protein levels are increased. In this study, we demonstrated that Bcl-2, full-length caspase-3 (35 kDa) and Bcl-xL protein levels were significantly decreased. Meanwhile, Bax and cleaved caspase-3 (17 kDa) protein levels were significantly increased (Fig. 4). As a result, the ratios of anti-apoptotic proteins and pro-apoptotic proteins were significantly reduced during apoptosis. This imbalance led to the loss of MMP after CyH treatment (Fig. 3).

Migration is a critical step in the initial progression of cancer that facilitates metastasis. The scratch wound healing assay is a classic and common method used for the discovery and validation of molecules that affect cell migration and metastasis (40-42). The results from this study indicated that CyH showed strong anti-migratory activities (Figs. 5 and 6). The underlying mechanisms of how CyH inhibits migration ability in A549 cells require further investigation.

In summary, we demonstrated for the first time and to the best of our knowledge that CyH significantly induced cell apoptosis and inhibited migration in A549 cells. Furthermore,

we found that CyH induced apoptosis in A549 cells by the downregulation of Bcl-xL, Bcl-2 and full-length caspase-3 (35 kDa) protein levels and the upregulation of Bax, P53 and cleaved caspase-3 (17 kDa) protein levels. These findings suggest that CyH may be developed into a potential chemotherapeutic drug for the treatment of lung cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XT, YM and XW conceived and designed the study. YM, ZX, XL, BH, LH, JL and ZZ performed the experiments. YM and XT wrote the report. XT and XW reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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