Grifolin, a potential antitumor natural product from the mushroom *Albatrellus confluens*, inhibits tumor cell growth by inducing apoptosis in vitro

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Abstract **Grifolin is a natural biologically active substance isolated from the fresh fruiting bodies of the mushroom *Albatrellus confluens*. Here, for the first time, we describe a novel activity of grifolin, namely its ability to inhibit the growth of tumor cells by the induction of apoptosis. Grifolin strongly inhibited the growth of tumor cell lines: CNE1, HeLa, MCF7, SW480, K562, Raji and B95-8. Analysis of acridine orange (AO)/ethidium bromide (EB) staining and flow cytometry showed that grifolin possessed apoptosis induction activity to CNE1, HeLa, MCF7 and SW480. Furthermore, the cytochrome *c* release from mitochondria was detected by confocal microscopy in CNE1 cells after a 12 h treatment with grifolin. The increase of caspase-8, 9, 3 activities revealed that caspase was a key mediator of the apoptotic pathway induced by grifolin, and the underexpression of Bcl-2 and up-regulation of Bax resulted in the increase of Bax: Bcl-2 ratio, suggesting that Bcl-2 family involved in the control of apoptosis. Owing to the combination of the significant antitumor activity by inducing apoptosis and natural abundance of the compound, grifolin holds the promise of being an interesting antitumor agent that deserves further laboratory and in vivo exploration.**

Keywords: Antitumor; Mushroom grifolin; Apoptosis

1. Introduction

About 14 000 species of mushrooms are now known in the world. They comprise a vast and yet largely untapped source of powerful new pharmaceutical products. In particular, some biologically active substances isolated from mushrooms in fruit bodies, cultured mycelium, and culture broth exhibit promising activity against tumors in vivo and in vitro. Some have been employed clinically as antitumor drugs. For example, PSK (Krestin) extracted from *Coriolus versicolor* has been developed for anticancer therapy. It is taken orally for gastric and other cancers. Irofulven derived from a family of mushroom toxins as illudins possesses significant antitumor activity [1].

Apoptosis is the process of programmed cell death through a tightly controlled program that plays important roles in many normal processes, ranging from fetal development to adult tissue homeostasis [2]. A tumor is a disease state characterized by uncontrolled proliferation and a loss of apoptosis. Compounds that block or suppress the proliferation of tumor cells by inducing apoptosis are considered to have potential as antitumor agents [3]. As a very valuable source for novel chemotherapeutic reagents, active substances isolated from the mushroom show effective antitumor activities with wide range of mechanisms. Some have been identified which induced apoptosis in the tumor cells. For example, lectin isolated from the edible mushroom *Boletopsis leucomelas* exerts the antitumor effects via apoptosis induction [4].

Grifolin is a natural biologically active substance isolated from the fresh fruiting bodies of the mushroom *Albatrellus confluens* [5], which has shown various pharmacological and microbiological effects. In 1950, grifolin was defined as an antibiotic [6]. Research has shown that grifolin added to the high cholesterol diet could significantly lower plasma cholesterol levels with limited or no toxicity [7]. Additionally, grifolin possesses antioxidative activity [8] and significantly inhibit histamine release from rat peritoneal mast cells [9]. Until now, it has never been shown that grifolin possessed antitumor activity whether in vivo or in vitro. Here, for the first time, we have discovered that grifolin inhibited the growth of some cancer cell lines and induced apoptosis. In our research, grifolin-triggered apoptosis was accompanied by the cytochrome *c* release from mitochondria and the increase of caspase-8, 9, 3 activities in nasopharyngeal carcinoma cell line CNE1. Meanwhile, the ratio between of Bax and Bcl-2, two proteins involved into the control of apoptosis in CNE1 cells, markedly increased as result of grifolin treatment. These results are significant in that they provide a mechanistic framework for further exploring the use of grifolin as a novel antitumor agent.

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Abbreviations: IC50, 50% inhibitory concentration; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; AO, acridine orange; EB, ethidium bromide; PI, propidium iodide; IETD-*p*NA, Ile–Glu–Thr–Asp conjugated to *p*-nitroanilide; LEHD-*p*NA, Leu–Glu–His–Asp conjugated to *p*-nitroanilide; DEVD-*p*NA, Asp–Glu–Val–Asp conjugated to *p*-nitroanilide

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2. Materials and methods

2.1. Cell lines and culture

The following cell lines were used: human breast cancer cell line MCF7 (ATCC HTB-22), human colon cancer cell line SW480 (ATCC CCL-228), human cervical cancer cell line HeLa (ATCC CCL-2), marmoset B lymphoblastoid cell line B95-8, human chronic myelogenous leukemia cell line K562 (ATCC CCL-243), human Burkitts Lymphoma cell line Raji (ATCC CCL-86), human nasopharyngeal carcinoma cell line CNE1, normal human bronchial epithelial cell line NHBE (Clonetics CC-2541), and mouse fibroblast cell line NIH/3T3 (ATCC CRL-1658). MCF7, SW480, HeLa, B95-8, K562, Raji and CNE1 were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, and 1% antibiotics. NHBE were maintained in culture media as suggested by the Clonetics. NIH 3T3 were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum. All cell lines were cultured at 37 °C in a humidified incubator containing 5% CO_2.

2.2. Chemicals

Grifolin (2-*trans*, *trans*-farnesyl-5-methylresorcinol) was provided by Kunming Institute of Botany, the Chinese Academy of Sciences (purity > 99%, HPLC analysis) [5]. Dimethyl sulfoxide (DMSO, Sigma) was used to dissolve grifolin. Benzyloxy carbonyl–Val–Ala–Asp–fluoromethylketone (Z-VAD-fmk) was purchased from Promega (Madison, Wisconsin, USA).

2.3. Cell viability assay (MTT)

Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as previously described [10]. Briefly, 1 day after exponentially growing cells were seeded at 2×10^3 cells/well in a 96-well plate, the culture medium was changed to the experimental medium supplemented with grifolin at indicated concentrations. The final concentration of DMSO in the culture medium was found to have no antiproliferative effect on either cell line. Following culture with grifolin for 24, 48 and 72 h, MTT (Sigma) was added, and the plate samples were read at 570 nm on a scanning multiwell spectrophotometer. The 50% inhibitory concentration (IC50) of grifolin on cells was calculated by MTT assay.

2.4. Fluorescence morphological examination

Apoptotic morphology was studied by staining the cells with a combination of the fluorescent DNA-binding dyes acridine orange (AO) and ethidium bromide (EB). Cells were harvested and washed three times with phosphate-buffered saline (PBS) after being incubated with different concentrations of grifolin for 48 h, and were then stained with 100 μg/ml AO and EB (AO/EB; Sigma) for 2 min. Then the color and structure [11] of the different cell types were observed under a fluorescence microscope (Olympus).

2.5. Flow cytometry assay

Flow cytometry was used to quantitatively detect the apoptotic rate. Cells (1×10^6) were plated into 10-cm tissue culture dishes 1 day before the treatment, and were then treated with grifolin at various concentrations. Forty-eight hours after the treatment, floating and attached cells were harvested, washed with PBS, fixed in 70% ethanol overnight at 4 °C, and stained with propidium iodide (PI; Sigma) 50 mg/ml. The sub-G1 peak (DNA content less than 2 N) was measured with FACScan Flow Cytometry (Becton Dickinson) and analyzed by Cell Quest software.

2.6. Caspase activity assay

Caspase activation was measured using a caspase colorimetric assay kit as described by the manufacturer (BioVision). Briefly after the cell treatment with 40 μM grifolin for the indicated periods of time, cells were harvested, pelleted and frozen on dry ice. Cell lysis buffer was added to the cell pellets, and protein concentration was determined by a micro BCA kit (Pierce). Then 100 μg protein was diluted in 50 μl cell lysis buffer for each assay, and 50 μl of 2 × reaction buffer supplemented with 10 mM DTT were then added to each tube incubated at 4 °C. The substrates of Ile–Glu–Thr–Asp conjugated to *p*-nitroanilide (IETD-*p*NA), Leu–Glu–His–Asp conjugated to *p*-nitroanilide (LEHD-*p*NA), and Asp–Glu–Val–Asp conjugated to *p*-nitroanilide (DEVD-*p*NA) were added into the tubes, respectively. Formations of *p*-nitroanilide were measured by ELISA Micro-plate Reader (Bio-tek) at 405 nm after the samples were incubated for 1.5 h at 37 °C. Caspase-8-, caspase-9-, and caspase-3-like enzymatic activities were assessed in parallel.

2.7. Western blot analysis

After cells were treated for 48 h with grifolin, cells were washed with cold PBS and subjected to lysis in lysis buffer [50 mM/L Tris · Cl, 1 mM/L EDTA, 20 g/L SDS, 5 mM/L dithiothreitol, 10 mM/L

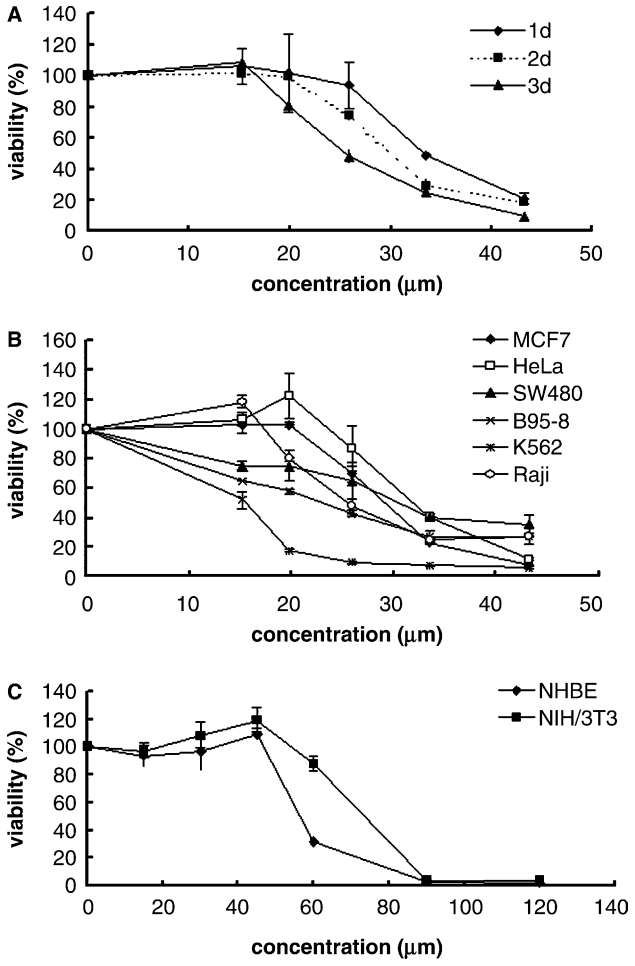

Fig. 1. Grifolin reduces the viability of CNE1 (A) at indicated time and concentrations, and MCF7, HeLa, SW480, B95-8, K562 and Raji (B), and NHBE and NIH/3T3 (C) at indicated concentrations. Cells were treated with the grifolin, and then cell viability was measured by MTT assay. In each treatment group, cells treated with medium were the control and their viability was set as 100%. Values are expressed as means ± S.D. of four experiments ($n = 4$).

Table 1
IC50 for grifolin

Cell lines	IC50 (μM)
CNE1	24
MCF7	30
HeLa	34
SW480	27
B95-8	24
K562	18
Raji	27
NHBE	49
NIH 3T3	85

phenylmethyl sulfonylfluoride]. Equal amounts of lysate (containing 50 μg protein) and rainbow molecular weight markers (Amersham Pharmacia Biotech, Amersham, United Kingdom) were separated by 12% SDS–PAGE and then electrotransferred to the nitrocellulose membrane. Membranes were blocked with buffer containing 5% fat-free milk in PBS with 0.05% Tween 20 for 2 h, and incubated with

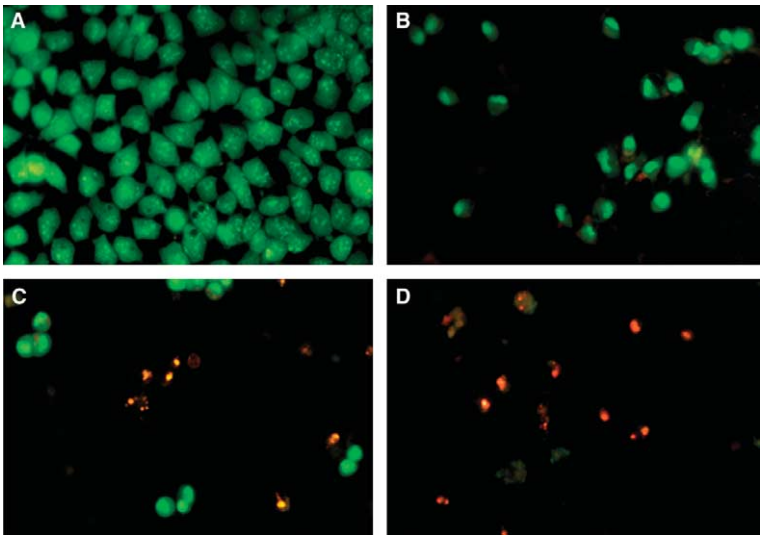

Fig. 2. Grifolin induces apoptotic morphological changes on CNE1 cells. After being treated with indicated concentrations of grifolin for 48 h. CNE1 cells were harvested, washed with PBS, and stained with AO/EB (100 μg/ml). Cell morphology was observed under fluorescence microscopy. Green live cells show normal morphology in control (A); green early apoptotic cells show nuclear margination and chromatin condensation with 30 μM (B) or 40 μM (C) grifolin treatment. Orange later apoptotic cells showed fragmented chromatin and apoptotic bodies after 40 μM (C) or 50 μM grifolin treatment (D). (×400).

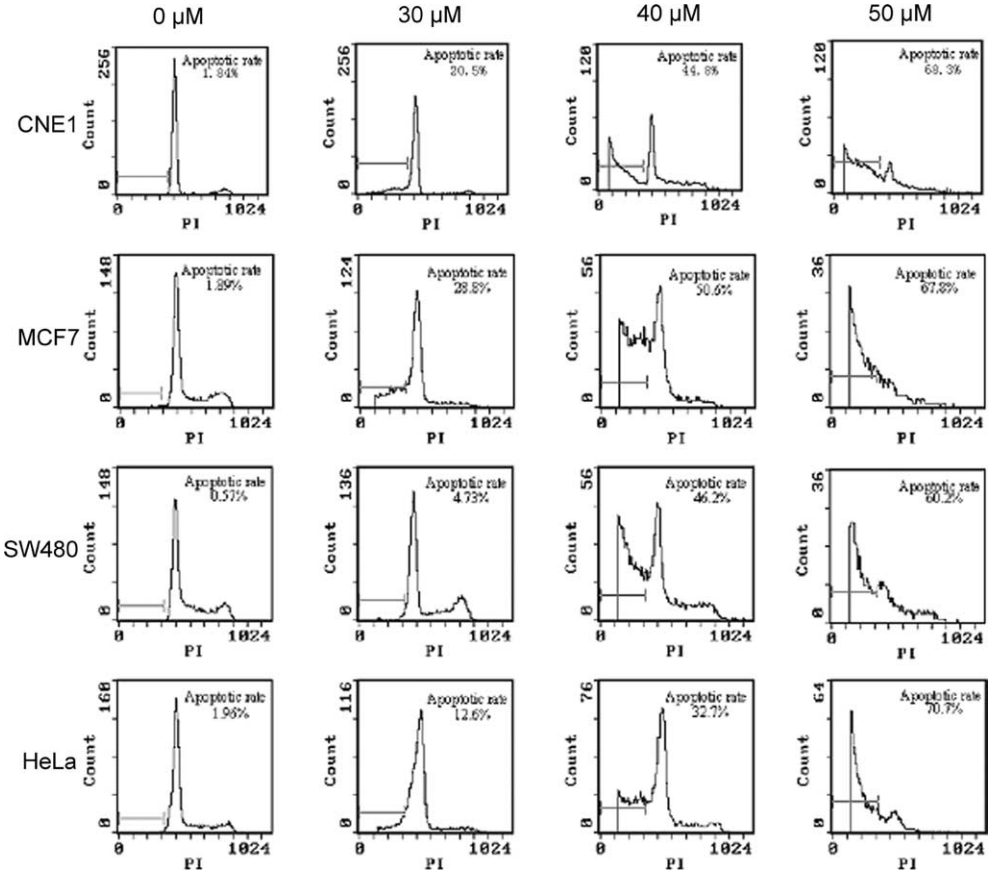

Fig. 3. Grifolin increases the apoptotic rates of cells. After CNE1, MCF7, HeLa and SW480 were grown in medium alone or treated with grifolin in indicated concentrations, cells were harvested and washed with PBS, fixed with ice-cold 70% ethanol, stained with PI, and treated with RNase A. DNA content was measured by flow cytometry. The apoptotic population was measured as the percentage of total cell populations with <G1 DNA content. Similar results were obtained in at least three other independent experiments.

anti-Bcl-2 mouse monoclonal antibody and anti-Bax mouse monoclonal antibody (Santa Cruz Biotech) overnight at 4 °C. After a second wash with PBS with 0.05% Tween 20, the membranes were incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotech) and developed with a chemiluninescence detection kit (ECL; Pierce). α-Tubulin was used as a loading control.

2.8. Confocal laser microscopy

Cells were untreated or treated with grifolin for 12 h. After fixation with 2% formaldehyde, cells were permeabilized for 10 min in 0.5% Triton X-100 in PBS. They were then incubated for 10 min in 100 mM glycine in PBS, and DNA was stained by 1 μg/ml DAPI (Vysis) in PBS and washed twice for 5 min with PBS. Incubation with the mouse anti-cytochrome *c* antibody (BioVision) was performed for 1–2 h at room temperature after the dilution in PBS and visualized with goat anti-rat IgG Cy3 antibody (Amersham Biosciences). Images were obtained by confocal laser scanning microscopy (LSM 410; Zeiss, Jena, Germany).

3. Results

3.1. Growth inhibitory effect of grifolin on tumor cell lines

In a primary screening test, we found that grifolin was able to decrease nasopharyngeal carcinoma cell line CNE1 viability in a dose- and time-dependent manner (Fig. 1A), whereas most of the chemicals isolated from higher fungi had no effect on it (Data not shown). Thus, the cell killing effects of this compound were further evaluated in vitro in six tumor cell lines of different origins. Cells were treated with various concentrations of grifolin for 72 h. The survival rates of cells were decreased in a dose-dependent manner (Fig. 1B). The concentrations required to inhibit cell growth by 50% (IC50) for CNE1, MCF7, HeLa, SW480, K562, Raji, and B95-8 were 24, 30, 33, 27, 18, 27 and 24 μM, respectively (Table 1). In addition, we examined the effect of grifolin on the survival rate of normal, non-tumor cell lines, such as NHBE (normal human bronchial epithelial cell line) and NIH/3T3 (mouse fibroblast cell line) (Fig. 1C). As compared with the panel of tumor cell lines analyzed, we found grifolin had some selective antitumor effect. The comparison of the respective IC50 (Table 1) showed that normal cells were less sensitive to grifolin in vitro.

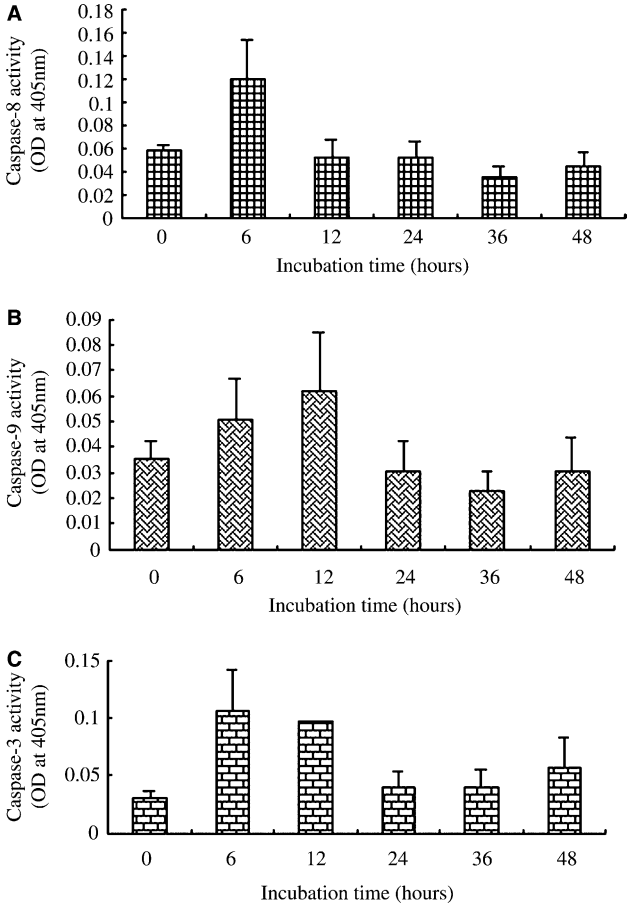

Fig. 4. Activation of caspase-8, caspase-9, caspase-3 by grifolin. CNE1 cells were incubated with grifolin (40 μM) for indicated time before the caspase-8 substrate IETD-*p*NA, or caspase-9 substrate LEHD-*p*NA, or caspase-3 substrate DEVD-*p*NA (50 mM) was added. Assay mixtures were incubated for 1.5 h at 37 °C. Activation of caspase-8 (A), caspase-9 (B), caspase-3 (C) was measured by ELISA Micro-plate Reader (Bio-tek) at 405 nm. Results are presented as means ± S.D. (three independent experiments).

3.2. Induction of apoptosis by grifolin

To determine whether the growth inhibitory activity of grifolin was related to the induction of apoptosis, morphological assay of cell death was investigated using the AO/EB staining for fluorescence microscopy. After CNE1 cells were exposed to various concentrations of grifolin for 48 h, different morphological features were analyzed. Uniformly green live cells with normal morphology were seen in the control group (Fig. 2A), whereas green early apoptotic cells with nuclear margination and chromatin condensation occurred in the experimental group with 30 and 40 μM grifolin (Fig. 2B and C), orange later apoptotic cells with fragmented chromatin and apoptotic bodies were seen when grifolin was at the concentration of 40 and 50 μM (Fig. 2C and D). The results suggested that grifolin was able to induce marked apoptotic morphology in CNE1 cells. Meanwhile, apoptotic morphology was also found in MCF7, HeLa, SW480, Raji, K562 and B95-8 (Data not shown). Further, we quantified the apoptotic cells after grifolin treatment by flow cytometry analysis using PI staining. By this technique, apoptotic nuclei are identified in the subdiploid re-

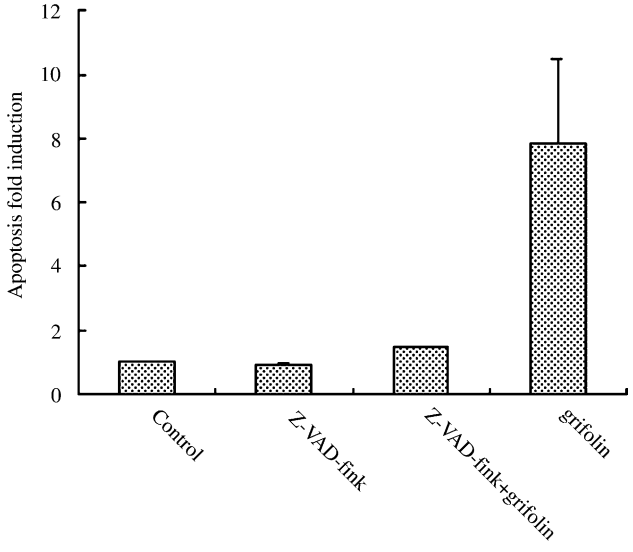

Fig. 5. Inhibition of grifolin-induced apoptosis by the caspase inhibitor Z-VAD-fmk. CNE1 cells were treated with either 20 μM Z-VAD-fmk alone or 20 μM Z-VAD-FMK in combination with 40 μM for 48 h and analyzed for apoptosis by flow cytometry. Results are presented as means ± S.D. (three independent experiments).

gion (sub-G_1) of the histograms. The result showed that treatment at different concentrations of grifolin resulted in a dramatic increase of apoptosis. Apoptotic rate in CNE1, MCF7, HeLa and SW480 cells increased from 1.84% to 68.3%, 1.89% to 67.8%, 1.96% to 70.7% and 0.57% to 60.2%, respectively, in a dose-dependent manner (Fig. 3).

3.3. Activation of caspase induced by grifolin

The caspase family is at the heart of apoptotic machinery, where these enzymes play key roles in the execution of apoptosis [12]. Morphological assay of apoptosis led us to hypothesize that grifolin might activate the caspase-dependent cell death pathway. Thus, the activation of caspase induced by grifolin was further evaluated in CNE1 cells. To identify whether caspase was involved in the mechanism, we measured the catalytic activity of caspase-8, caspase-9 and caspase-3. The result showed that caspase-3 and caspase-8 activities were rapidly elevated and peaked at 6 h of grifolin treatment (Fig. 4A and C), whereas caspase-9 activity peaked at 12 h (Fig. 4B). However, caspase-9 activity levels were lower than those of caspase-8. In addition, we found that caspase-3 activity remained at a high level at 12 h (Fig. 4C), which may be associated with the increased level of caspase-9 activity.

To further demonstrate the involvement of caspase activation in the apoptotic effect, we analyzed whether the pan-caspase inhibitor Z-VAD-fmk prevented apoptosis. When CNE1 cells were incubated with 40 μM grifolin in the presence of the 20 μM caspase inhibitor Z-VAD-fmk, we observed that the apoptotic response was reversed by more than 80% (Fig. 5). These findings allow us to conclude the involvement of caspase-dependent pathway(s) in grifolin-induced apoptotic death of CNE1 cells.

3.4. Release of cytochrome C induced by grifolin

Cytochrome *c* is localized in the intermembrane space and loosely attached to the surface of the inner mitochondrial membrane. In response to a variety of apoptosis-inducing agents, cytochrome *c* is released from mitochondria to the cytosol [13]. To examine this step in the apoptotic cell death pathway initiated by grifolin, the subcellular localization of cytochrome *c* was determined by confocal laser microscopy after immunolabeling cytochrome *c* in CNE1 cells. After cells were treated for 12 h at 40 μM, the staining pattern became diffuse in most cells (Fig. 6B), consistent with a translocation of cytochrome *c* into the cytosol, whereas cytochrome *c* displayed a dotted pattern in untreated cells, consistent with its location within the mitochondria (Fig. 6A). Meanwhile, the condensation and margination of the chromatin of the nucleus was also observed by staining DNA with DAPI (Fig. 5) in these cells with diffuse cytochrome *c* staining. Therefore, we conclude that cytochrome *c* release is part of the apoptosis pathway induced by grifolin.

3.5. Up-regulation of Bax and down-regulation of Bcl-2 by grifolin

Although the caspase proteolytic cascade is a central point in apoptotic response, its activity was tightly regulated by a

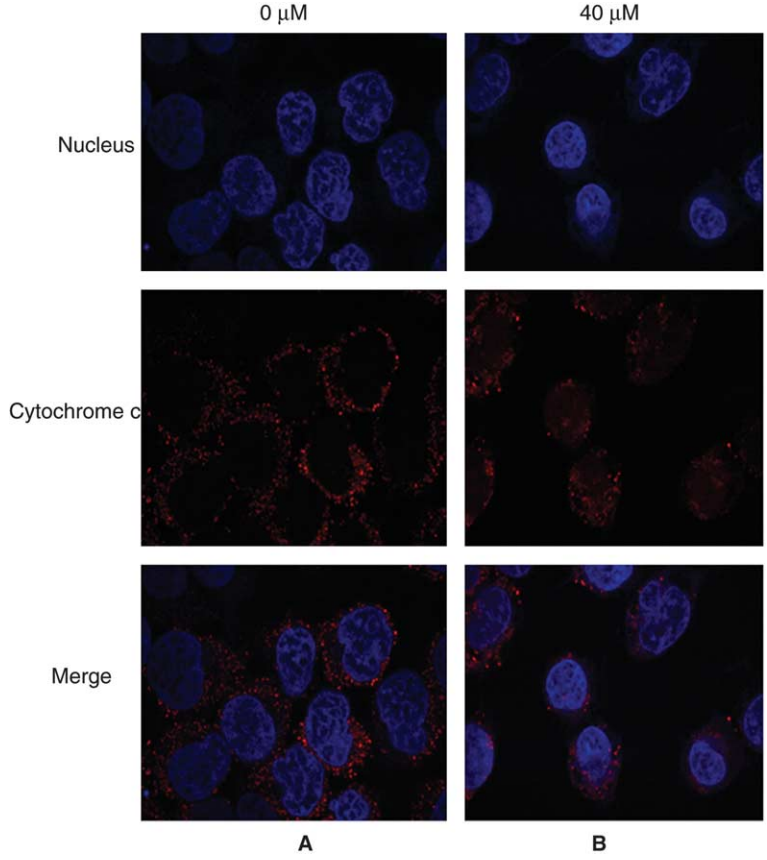

Fig. 6. Effect of grifolin on cytochrome *c* release. Microscopic analysis of cytochrome *c* and DNA-stained CNE1 cells. CNE1 cells with control medium (A) and cells treated for 12 h with 40 μM grifolin (B) were fixed and labeled for cytochrome *c* (red) and DNA (blue). Images were obtained with confocal laser scanning microscopy.

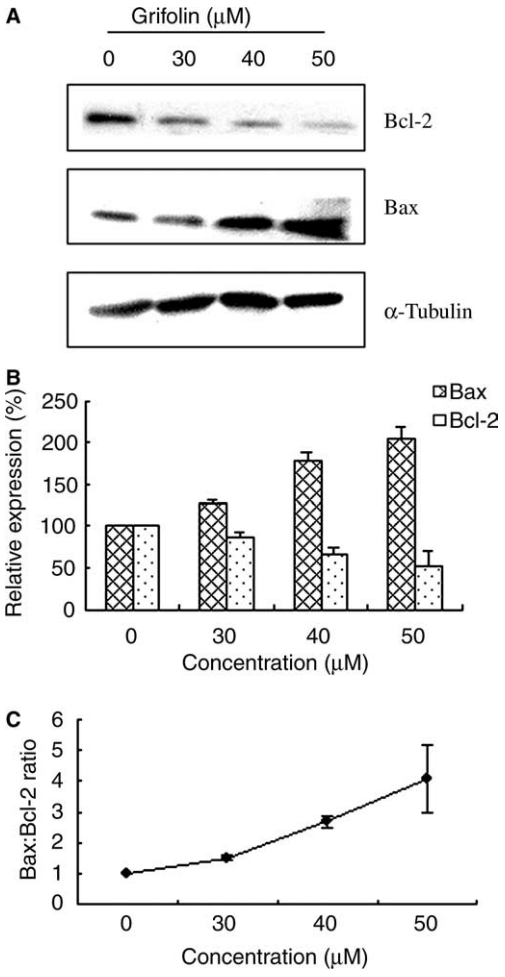

Fig. 7. Effect of grifolin on the expression of Bcl-2 and Bax in CNE1 cells. Exponentially growing CNE1 cells were treated with indicated concentrations of grifolin for 48 h, cell lysates were prepared and protein level of Bcl-2 and Bax was determined by Western blotting analysis. The expression of Bcl-2 and Bax after grifolin induction at indicated concentrations (A). α-Tubulin was used for normalization and verification of protein loading. Quantitative Bcl-2 and Bax expression after normalization to α-tubulin ($n = 3$ at each concentration point) (B). The Bax:Bcl-2 ratio corresponding to indicated concentrations of grifolin (C). Values are means ± S.D.

variety of factors. Among these, Bcl-2 family proteins, including anti-apoptotic members (such as Bcl-2) and pro-apoptotic members (such as Bax), play a pivotal role [14]. To further analyze the possible mechanism underlying the grifolin-induced apoptosis, we tested the expression of Bcl-2 and Bax in CNE1 cells after grifolin treatment. After being normalized to α-tubulin, the expression of Bcl-2 was decreased significantly, while Bax protein level was increased markedly in a dose-dependent manner (Fig. 7A and B). The ratio of Bax: Bcl-2 was dramatically increased in a dose-dependent manner (Fig. 7C).

4. Discussion

The ability to induce tumor cell apoptosis is an important property of a candidate anticancer drug, which discriminates between anticancer drugs and toxic compounds [3]. Here, we showed grifolin isolated from the mushroom *A. confluens* was able to inhibit the growth of 7 tumor cell lines in a dose-dependent manner. Further, we evaluated that the growth inhibitory activity of grifolin was associated with the induction of apoptosis in CNE1 cells. Treatment of CNE1 cells with grifolin resulted in a dose-dependent generation of apoptosis-specific morphological changes and apoptotic rates. In addition, we found grifolin was also able to induce apoptosis in other cancer cell lines, including MCF7, HeLa, SW480, Raji, K562 and B95-8.

Caspases are a ubiquitous family of cysteine proteases that include both upstream (initiator) and downstream (effector) caspase [15]. The initiator caspases, typically caspase-8 and caspase-9, are activated by two alternative pathways. The first involves cell death receptor-mediated apoptosis through caspase-8 [16]. It is characterized by binding cell death ligands and cell death receptors, and subsequently activates caspase-8 and caspase-3 [17,18]. The second involves mitochondria-mediated apoptosis through caspase-9. The key element in the pathway is the efflux of the cytochrome *c* from mitochondria to cytosol. Once cytochrome *c* is in the cytosol, cytochrome *c* together with Apaf-1 activates caspase-9, and the latter then activates caspase-3 [19]. In both pathways the initiator caspase cleaves and activates downstream, effector caspases, such as caspase-3 [20]. This caspase cascade ultimately leads to proteolytic cleavage and induces the broad range of the morphological changes of apoptosis. Our results indicate that caspase-8, caspase-9 and caspase-3 activities were rapidly elevated after the grifolin treatment, and the kinetic activation of caspase-3 was associated with the activation of caspase-8 and caspase-9. An important role for caspases in grifolin-induced apoptosis was also suggested by the observation that Z-VAD-fmk reversed the apoptotic effect exerted by grifolin in CNE1 cells. Because Z-VAD-fmk is a general inhibitor of caspases [21], this finding demonstrates the involvement of caspase-dependent mechanisms in grifolin-mediated apoptosis of CNE1 cells. But, we also found grifolin was able to induce apoptosis in caspase-3-deficient MCF-7 cells by flow cytometry analysis. It deserved further investigation on about whether grifolin may induce other death pathway. On the other hand, we found that grifolin was able to induce cytochrome *c* release from the mitochondria in CNE1 cells by confocal laser microscopy after immunolabeling cytochrome *c*. Therefore, our study indicated that mitochondrial pathway and death receptor signaling pathway were both involved in apoptosis induced by grifolin in CNE1 cells.

Apoptosis is a tightly regulated process, which involves changes in the expression of distinct genes [22]. Bcl-2 family proteins that consist of anti-apoptotic and pro-apoptotic members are important regulators of apoptosis [23]. Overexpression of pro-apoptotic Bax induces the release of cytochrome *c* from mitochondria. Released cytochrome *c* contributes to apoptosis through binding Apaf-1 and caspase-9, whereas, anti-apoptotic Bcl-2 binds to Bax and can form heterodimers, which inhibit Bax activity and block apoptosis in cells. The ratio of Bcl-2 to Bax protein has been reported to be associated with apoptosis [23–26], and used as a predictive marker for therapeutic response to radiotherapy [27]. Our data clearly demonstrated that grifolin treatment to CNE1 cells resulted in a dose-dependent increase in the level of Bax with a concomitant decrease in Bcl-2 levels and increase in Bax/Bcl-2 ration. Studies have shown P53 is very important regulatory protein targeting

many other apoptosis regulatory genes such as Bcl-2 and Bax. Wild-type p53 can down-regulate the expression of Bcl-2 and up-regulate that of Bax, altering the balance of the couple genes in favor of apoptosis [28]. Meanwhile, several studies have demonstrated that Stat3 can up-regulate the expression of Bcl-2 [29]. EGCG can decrease the level of the Bcl-2 protein by inhibiting Stat3 [30]. However, the precise mechanism by which grifolin decreased Bcl-2 and increased Bax expression in CNE1 cell remains to be determined.

In conclusion, grifolin significantly inhibited the growth of some cancer cell lines in vitro. The cancer-suppressing mechanism involved the induction of apoptosis. We further proved that apoptosis of CNE1 cells was induced by the activation of caspase-8, caspase-9, and caspase-3, release of cytochrome *c* from mitochondria, decrease of the Bcl-2 level, and increase of the Bax level. Determining the detailed mechanism of grifolin action and exploring its pharmacokinetics may lead to the development of a novel antitumor drug.

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