

## Ganoderic acid T from *Ganoderma lucidum* mycelia induces mitochondria mediated apoptosis in lung cancer cells

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### Abstract

*Ganoderma lucidum* is a well-known traditional Chinese medicinal herb containing many bioactive compounds. Ganoderic acid T (GA-T), which is a lanostane triterpenoid purified from methanol extract of *G. lucidum* mycelia, was found to exert cytotoxicity on various human carcinoma cell lines in a dose-dependent manner, while it was less toxic to normal human cell lines. Animal experiments in vivo also showed that GA-T suppressed the growth of human solid tumor in athymic mice. It markedly inhibited the proliferation of a highly metastatic lung cancer cell line (95-D) by apoptosis induction and cell cycle arrest at G<sub>1</sub> phase. Moreover, reduction of mitochondria membrane potential ( $\Delta\psi_m$ ) and release of cytochrome *c* were observed during the induced apoptosis. Our data further indicate that the expression of proteins p53 and Bax in 95-D cells was increased in a time-dependent manner, whereas the expression of Bcl-2 was not significantly changed; thus the ratio of Bcl-2/Bax was decreased. The results show that the apoptosis induction of GA-T was mediated by mitochondrial dysfunctions. Furthermore, stimulation of the activity of caspase-3 but not caspase-8 was observed during apoptosis. The experiments using inhibitors of caspases (Z-VAD-FMK, Z-DEVD-FMK and Z-IETD-FMK) confirmed that caspase-3 was involved in the apoptosis. All our findings demonstrate that GA-T induced apoptosis of metastatic lung tumor cells through intrinsic pathway related to mitochondrial dysfunction and p53 expression, and it may be a potentially useful chemotherapeutic agent.

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**Keywords:** *Ganoderma lucidum*; Ganoderic acid; Apoptosis; Caspases; Lung tumor cells

### Introduction

*Ganoderma lucidum* (Fr.) Karst (Polyporaceae) is an important traditional Chinese medicinal mushroom used for several thousand years in China, Japan, and other countries. Evidence has accumulated concerning the medicinal application of ganoderma in the treatment of various diseases, such as cancers and immunological disorders, and in recent years the biotechnological utilization of the mushroom has been very popular (Zhong and Tang, 2004).

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It is well documented that the natural mixtures of triterpenoids in *G. lucidum* inhibit proliferation of human and mouse carcinoma cell lines (Liu et al., 2002; Sliva, 2003, 2004). The cytotoxicity of extract of triterpene-enriched *Ganoderma tsugae* was claimed to be mediated through apoptosis and cell cycle arrest in MCF-7 human breast, prostate cancer cells and PC-3 cells (Gao and Zhou, 2003; Jiang et al., 2004). Other studies have shown that apoptosis induced by triterpene-enriched extracts of *G. lucidum* was brought about through suppressing protein kinase C, activating mitogen-activated protein kinases and G<sub>2</sub>-phase cell cycle arrest (Lin et al., 2003); through inducing a marked decrease of intracellular calcium level (Zhu et al., 2000); through inducing NAD(P)H: quinone oxidoreductase in cultured hepatic7 murine hepatoma cells (Ha et al., 2000); by activating MAP kinases in rat pheochromocytoma PC12 cells (Cheung et al., 2000) or stimulating actin polymerization in

bladder cancer cells *in vitro* (Lu et al., 2004). However, it is difficult to identify whether or not the ingredients in extracts (mixtures) have antagonistic or synergistic biological effects, and it is also unclear what compound in extracts is mainly responsible for the bioactivities, which makes the study of structure–activity relationship difficult. Therefore, the use of a purified triterpene is required to reveal the acting mechanism of responsible compounds and to further screen and rationally design structurally similar lead compounds.

Until now, there has been a lack of investigation using purified triterpenes to study bioactivity mechanism except for ganoderic acid X (GA-X) (Li et al., 2005). GA-X was shown to induce apoptosis of cancer cells, and the disruption of mitochondrial membrane, cytosolic release of cytochrome *c* and activation of caspase-3 under its treatment were also reported (Li et al., 2005). However, whether caspase-8 and p53 was involved in its induced apoptosis is unclear.

In this work, the cytotoxicity of ganoderic acid T (GA-T), a triterpenoid purified from bioreactor-cultivated mycelia of ganoderma by our lab (Fig. 1) (Tang, 2006), to various human carcinoma cell lines was investigated. Furthermore, the growth inhibitory effect of GA-T on 95-D cells and molecular events triggered by GA-T in the apoptosis of 95-D cells, including the effects on caspase-8 and p53, are elucidated. The work is considered useful to the development of interesting chemotherapeutic drugs.

## Materials and methods

### Materials

RPMI 1640 and Dulbecco's Modified Eagle Medium (DMEM), trypsin, MTT, PI were obtained from Sigma Chemical Co. (St Louis, MO). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from Huanmei Co. (Shanghai, China). Antibodies for actin and p53, Bax, Bcl-2 were purchased from BD Biosciences Pharmingen, USA. Goat anti-rabbit IgG-conjugated to horseradish peroxidase (HRP) and goat anti-mouse IgG-conjugated to HRP were purchased from Biovision (Mountain View, CA). Selective inhibitors of caspase-3 (Z-DEVE-FMK), and caspase-8 (Z-IETD-FMK) and general caspase inhibitors (Z-VAD-FMK) were purchased from Biovision (Mountain View, CA). Cytochrome *c* was purchased from Sigma (Germany).

GA-T was purified with semi-preparative liquid chromatography in our lab with its purity over 99% (Tang, 2006). Stock

solutions of GA-T were prepared in dimethyl sulfoxide (DMSO) and stored at  $-20^{\circ}\text{C}$ . Further dilutions were made with RPMI 1640 medium just before use. The final concentration of DMSO was less than 0.1%.

### Cell cultures

Human highly metastatic lung tumor cell line 95-D (lung), human liver tumor cell line SMMC7721 (liver), human epidermal cancer KB-A-1 and KB-3-1 (epidermis), human cervical cancer HeLa (cervix), and human normal cell line HLF (lung) and L-02 (liver) were purchased from the Center of Cell Culture Collection of Academia Sinica (Shanghai, China). All those cell lines were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin in an atmosphere of 5%  $\text{CO}_2$  and 95% air at  $37^{\circ}\text{C}$ . Cells ( $1 \times 10^5/\text{ml}$ ) were treated with GA-T at various indicated concentrations and for various periods. In some experiments, before addition of GA-T to the cultures, cells were pre-incubated for 60 min at  $37^{\circ}\text{C}$  with selective caspase inhibitors, i.e., caspase-3 inhibitor, Z-DEVD-FMK, 10  $\mu\text{M}$ ; caspase-8 inhibitor, Z-IETD-FMK, 10  $\mu\text{M}$ ; and pan-caspase inhibitor, Z-VAD-FMK, 10  $\mu\text{M}$ .

### Cell proliferation assay

The  $1 \times 10^5$  cells/ml were plated in 96-well tissue culture plate, and treated with different concentrations of GA-T (0, 6, 12.5, 25 and 50  $\mu\text{g}/\text{ml}$ ) after 4 h. Cells were incubated for another 24 h for cell proliferation. Viability of cells was evaluated by MTT [3-(4, 5-dimethylthiazo 1-2-yl)-2,5-diphenyltetrazolium bromide] reduction method. The cells were stained with MTT for 4 h and then incubated with lysis buffer (20% SDS in 50% *N,N*-dimethylformamide) for another 30 min. Optical density at 570 nm was detected for monitoring the cell viability. Effects of the drugs on inhibition of cell growth were calculated, and the cells treated with DMSO at same concentrations as in the drugs used as controls.

For colony formation assay, a total of 2000 cells was resuspended in RPMI 1640 medium containing 10% FBS and 0.35% agar and plated on plates with a solidified bottom layer (0.5% agar in growth medium). The plates were incubated in a humidified incubator at  $37^{\circ}\text{C}$ . Various concentrations of GA-T were added to cells, and cells were cultured for 12 days until colonies appeared. Then, cells were fixed and stained with crystal violet and counted. Dose–response curves and the concentration of GA-T inhibiting colony formation by 50% ( $\text{EC}_{50}$ ) were obtained.

### Cell cycle distribution and apoptosis evaluation

Cell cycle parameters were analyzed by flow cytometry. After induction treatment under 50  $\mu\text{g}/\text{ml}$ , cells were fixed with ethanol and then stained with propidium iodide after removing the RNA in the cells by RNase treatment, and the fluorescence of individual nuclei (about 10,000 events) was analyzed by flow cytometry (Becton Dickinson FACScan, USA). The percentage of cells in the  $G_1$ , S and  $G_2$ –M phases of the cell cycle was determined.

Apoptosis was detected by annexin V-FITC binding assay. Normal, apoptotic, and necrotic cells were distinguished by

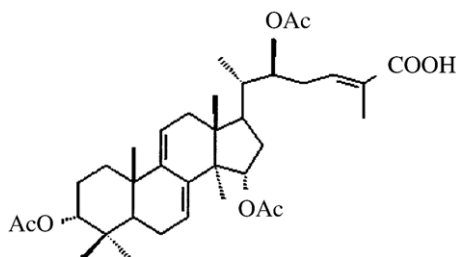


Fig. 1. Structure of ganoderic acid T (GA-T).

using the annexin V-propidium iodide (PI) kit according to the manufacturer's instructions (Roche Diagnostics, Germany). After washing in PBS buffer, cells were resuspended for 10 min in the staining solution and analyzed by flow cytometry. The percentages of viable and dead cells were determined with about 10,000 cells/sample.

#### Mitochondrial membrane potential analysis ( $\Delta\psi_m$ )

Mitochondrial transmembrane potential was analyzed by flow cytometry. Cells under the GA-T treatment were incubated with 50 nM 3,3'-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>) (Molecular Probes, USA) for 15 min at 37 °C, and associated fluorescence alterations in 95-D cells were evaluated by FACScan flow cytometry (Becton Dickinson and Company, CA, USA). Loss in DiOC<sub>6</sub> staining indicates an association of the disruption of mitochondrial inner transmembrane potential ( $\Delta\psi_m$ ).

#### Cytochrome *c* (cyt-*c*) release analysis

The release of cytochrome *c* was detected with HPLC and ultraviolet (UV) detector. After induction treatment, cells were treated as reported (Appaix et al., 2000; Elliott et al., 2003). Cells ( $2 \times 10^6$ ) were rinsed three times with PBS, scraped from the dish and then lysed and centrifuged cells for separation of mitochondrion (4 °C, 25,000 g, 30 min). The positive control was that of cells treated with GA-T and the negative control used intact cells without any treatment. Initial spectroscopic measurements were made on a UNICO spectrophotometer (UNICO, Shanghai, China). Chromatography of cytochrome *c* was performed using a 5  $\mu$ m C<sub>18</sub> reverse-phase column (250  $\times$  4.6 mm) by Shimadzu HPLC system with UV detector (Shimadzu, Japan). The detection wavelength was 393 nm. A gradient from 20% to 60% of acetonitrile in water with trifluoroacetic acid (0.1% v/v) over 20 min with a flow rate of 1.0 ml/min was used.

#### Western blotting analysis

After GA-T treatment, cells were washed and lysed in lysis buffer (1% NP40, 20 mM Tris (base pH 7.4), 137 mM NaCl, 10% glycerol, and 1 mM phenylmethyl sulfonyl fluoride). Cell lysates cleared of debris and nuclei were resolved on 15% SDS gels and transferred to a polyvinylidene difluoride membrane (LOT 1673B22, Solon, OH, USA). After being probed with specific primary antibodies, including incubation with anti-p53, anti-Bax, anti-Bcl-2 and anti-actin, the specific protein complex formed on appropriate secondary antibody treatment (1:1000) was identified using the DAB substrate reagent (Pierce, USA). Total cellular protein was determined using the Bradford method.

#### Analysis of caspases activities

Activity of caspase-8 was measured by using caspase colorimetric assay kit (Catalog PT3356-1, BD Biosciences, USA). Briefly, cell lysates were mixed with DTT (10 mM)-rich reaction buffer containing 50 mM IETD-pNA, caspase-8 substrates, and incubated for 1 h at 37 °C. Enzyme-catalyzed release of

pNA was monitored using a microplate reader at 405 nm. The activity of caspase-3 was detected by luminometer with caspase-Glo3 assay kit (Catalog G8090, Promega, USA) according to the manufacturer's protocol. As caspase-3 and 8 was assayed by using respective specific substrate Ac-DEVD-pNA and Ac-IETD-pNA, the cross-reaction activity was avoided.

#### Animal experiments

Four-week old male BALB/c mice were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). Specific-pathogen-free (SPF) status was verified by the supplier. Mice were maintained in isolation rooms in filter top cages. The light cycle in the rooms was 12 h daily, the room temperature was at  $22 \pm 1.1$  °C and the room humidity was in the range of 40–70%. All mice were fed autoclaved mouse feed and autoclaved water.

The animals were implanted with  $1 \times 10^8$  cells/ml of 95-D in 0.1 ml at two flanks per mouse. Tumor growth was examined twice a week after implantation. The solid tumors were collected after being allowed to develop for 3 weeks and cut as cube of  $0.1 \times 0.1 \times 0.1$  cm, and then implanted into other mice at two flanks per mouse. The xenograft tumor-bearing nude mice were divided randomly into 4 groups, and each group includes 6 mice.

When the size of solid tumor in tumor-bearing nude mice reached 100 mm<sup>3</sup>, the tumor-bearing nude mice were treated with GA-T via celiac injection at the dosage of 0, 12.5 and 25 mg/kg for 12 days, and then observed for another consecutive 8 days. The control group was treated with vehicle mixture only. At the end of the experiments, animals were euthanized with carbon dioxide inhalation, followed by cervical dislocation, and then the solid tumors were picked up. Data were statistically analyzed by solid tumor weight.

The rate of inhibition (IR) was calculated according to the formula:  $IR = [(mean\ tumor\ weight\ of\ the\ experimental\ group - mean\ tumor\ weight\ of\ the\ control\ group) / mean\ tumor\ weight\ of\ the\ control\ group] \times 100\%$ .

#### Statistical analysis

All experiments were done at least three different times ( $n=3$ ) unless otherwise indicated. Data are expressed as means  $\pm$  S.D., and significance was assessed by *t* test. Differences with  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) were considered significantly different.

## Results

### *GA-T inhibits proliferation of various cancer cells and affects the viability of metastatic lung carcinoma cells by inducing apoptosis and cell cycle arrest*

We first investigated the effect of GA-T on proliferation of human cancer cells and normal cells. The results in Fig. 2 indicate that GA-T caused a decrease in proliferation of some cancer cells. It had higher cytotoxicity to 95-D cell line than to normal cell lines. But the effects of GA-T on SMMC-7721 and HLF are similar. This indicates that GA-T had different cytotoxic potency

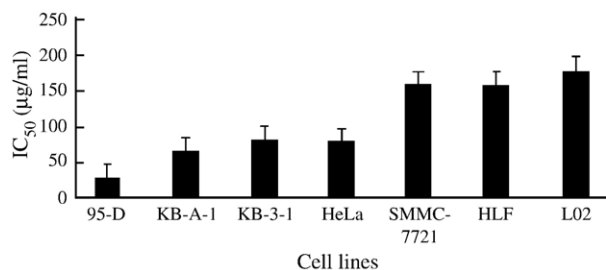


Fig. 2. Growth inhibition effects of GA-T on various cell lines. Viable cell number was detected using MTT reduction. Values are means±S.D. from triplicate cultures, and the experiments were repeated for five times with similar results.

against different tumor cells, which is also generally observed for other drugs. As shown in Fig. 3A, the viability of 95-D cells was suppressed 70% at 50 µg/ml at 24 h by GA-T. The growth inhibition was exerted in a dose-dependent manner within the indicated concentrations. Its IC<sub>50</sub> was estimated to be 27.9 µg/ml. At the same time, GA-T at a low concentration could also strongly inhibit the formation of cell colony of 95-D (Fig. 3B). The inhibitory effect also behaved in a dose-dependent manner, and the EC<sub>50</sub> was about 3.34 µg/ml.

To examine whether the decrease of viable tumor cells was caused by induced apoptosis, 95-D cells were treated with GA-T and then were analyzed for annexin V-FITC binding. The experimental results are shown in Fig. 4. The proportion of cells reactive with the annexin V-FITC conjugate indicated that target cells started to enter apoptosis after treatment. The number of apoptotic cells was about 50% at the concentration of 50 µg/ml at 8 h. This means that GA-T induced the apoptosis of tumor cells.

At the same time, the cell cycle arrest at G<sub>1</sub> phase was observed under the treatment of GA-T (Fig. 5). Compared with

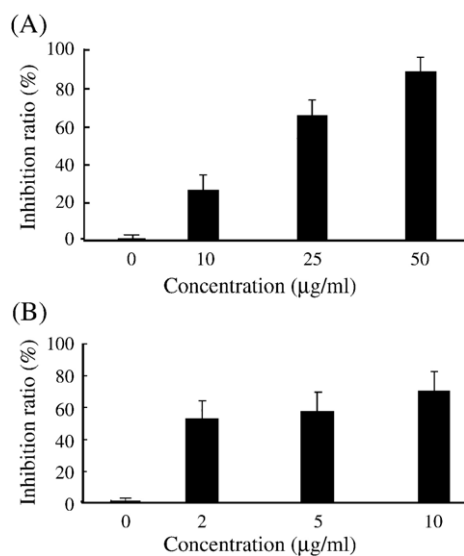


Fig. 3. Growth inhibition effects of GA-T against a highly metastatic lung cancer cell line (95-D). (A), cytotoxicity. After GA-T treatment at 0, 10, 25 and 50 µg/ml, viable cell number was detected by MTT reduction. (B), colony formation. After GA-T treatment at 0, 2, 5, 10 mg/ml, cells were fixed and stained with crystal violet and counted. Values are means±S.D. from triplicate cultures, and the experiments were repeated for five times with similar results.

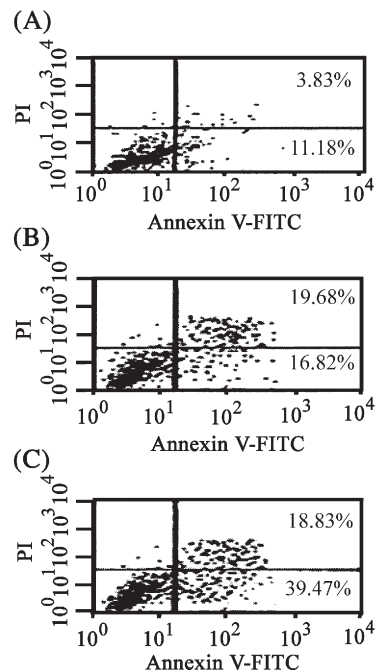


Fig. 4. GA-T induced apoptosis in 95-D cells. The cells were treated at the indicated time at 50 µg/ml of GA-T and apoptosis was assayed by annexin V-FITC binding with flow cytometry. *Top right quadrant*, dead cells in late stage of apoptosis; *Bottom right quadrant*, cells undergoing apoptosis; *Bottom left quadrant*, viable cells. (A), 0 h; (B), 4 h; (C), 8 h. Shown are typical data from one of three independent experiments with similar results. For the apoptosis percentage, it takes the total percentage of viable cells, cells undergoing apoptosis, apoptotic cells and necrosis cells as 100%.

control, the percentage of cells in G<sub>1</sub> phase increased from 48% to 76% (at 24 h) with an addition of 50 µg/ml of GA-T, whereas the percentage of cells in S phase decreased to 13% from 37% at the same condition.

*GA-T influences the integrity of mitochondria by decreasing the mitochondrial transmembrane potential ( $\Delta\psi_m$ )*

The disruption of mitochondrial integrity is one of the early events leading to apoptosis. To assess whether the GA-T affects the function of mitochondria, potential changes in mitochondrial membrane were analyzed by employing a mitochondria

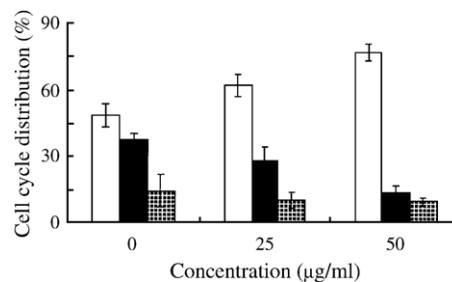


Fig. 5. Cell cycle progression was blocked under GA-T treatment in 95-D cells at the indicated time at 50 µg/ml. Symbols: blank bar, G<sub>0</sub>-G<sub>1</sub> phase; dark bar, S phase; gray bar, G<sub>2</sub>/M phase. After GA-T treatment, cells were stained with PI and analyzed with flow cytometry. The results are the average of triplicates. For the percentage of cell cycle distribution, it takes the total percentage of cells at G<sub>1</sub>-G<sub>0</sub> phase, S phase, and G<sub>2</sub>/M phase as 100%.

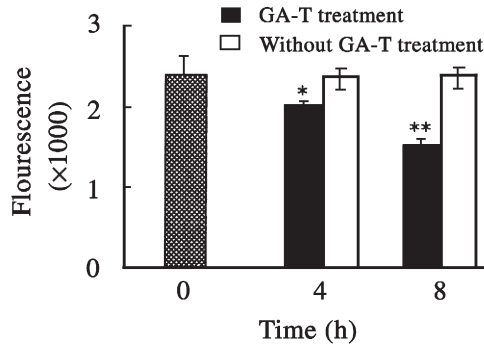


Fig. 6. GA-T influences the mitochondrial membrane potential. The cells were treated at the indicated time at 50  $\mu\text{g/ml}$  of GA-T. Cells were analyzed as described in “Materials and methods”. The results are the average of triplicates. Samples without drug treatment were taken as control.

fluorescent dye, DiOC<sub>6</sub>. As shown in Fig. 6, a drop in the mitochondrial membrane potential was observed. The data show that changes in the membrane potential were induced in treated 95-D cells during 4 and 8 h treatments. A significant decrease of  $\Delta\psi_m$  was detected in cells treated with 50  $\mu\text{g/ml}$  GA-T for 4 and 8 h. Compared to control, the decrease in the mean fluorescence density was about 10% and 16.7%, respectively. The results illuminate that GA-T could induce  $\Delta\psi_m$  dissipation in a time-dependent manner. A drop in the mitochondrial membrane potential is usually accompanied by release of cyt-*c* into the cytosol. As shown in Fig. 7, cyt-*c* levels in the cytosol of GA-T treated cells showed a rapid increase. A one-fold increase was seen within 4 h. At 8 h, about 3-fold increase in the cyt-*c* level was observed. The data indicate that GA-T increased the release of cyt-*c* to cytosol in treated tumor cells.

*GA-T induces activation of caspase-3 but not caspase-8*

In general, induced apoptosis is often associated with activity of a series of caspases, such as caspase-3 and caspase-8. In this work, the activity of caspase-3 was detected in 95-D cells treated by GA-T. The data are shown in Fig. 8A. GA-T noticeably stimulated the activity of caspase-3 in tumor cells in a dose-dependent manner. The activity of caspase-3 increases 2- and 3-fold respectively at 4 and 8 h under the drug concentration of

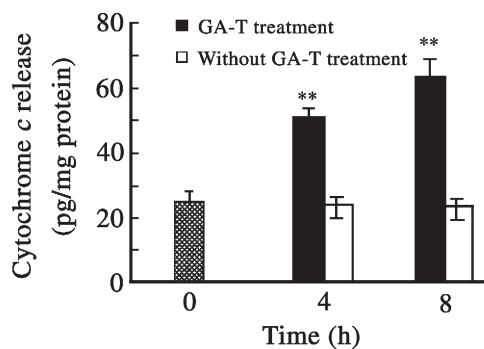


Fig. 7. Release of cytochrome *c* from mitochondria after GA-T treatment at the indicated time at 50  $\mu\text{g/ml}$ . Cells were analyzed as described in “Materials and methods”. The results are the average of triplicates.

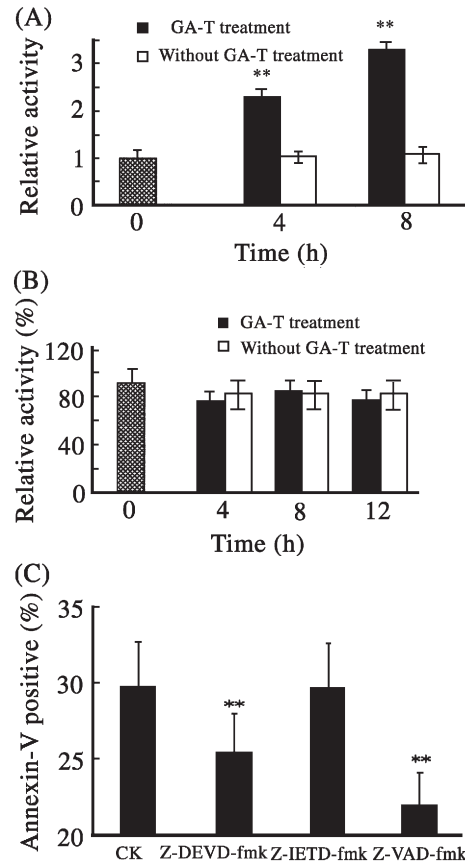


Fig. 8. Effect of GA-T on activities of caspases. The cells were treated at the indicated time at 50  $\mu\text{g/ml}$  of GA-T. (A), caspase-3, which activity was detected by luminometer. (B), caspase-8, which activity was monitored using a microplate reader at 405 nm. (C), percentage of apoptosis was quantified by annexin-V staining in the presence or absence (CK, control) of selected caspase-8 and -3 inhibitors. Results are the average of triplicates from one of the three independent experiments.

50  $\mu\text{g/ml}$  compared with the control. In contrast, the activation of caspase-8 was not detected during the 4 h and 8 h incubations (Fig. 8B). To identify which caspases are functionally important for GA-T induced apoptosis, selective caspase inhibitors were used in our investigation. As shown in Fig. 8C, the caspase-3 inhibitor or pan-caspase inhibitor alone but not caspase-8 inhibitor can reduce the apoptosis mediated by GA-T.

*GA-T modulates the p53 and Bax expression, but does not affect bcl-2 protein expression*

The expression of proteins including p53, Bcl-2 and Bax may be involved in an intrinsic apoptosis pathway. In our case, under GA-T treatment, the expression of p53 and Bax was found up-regulated in a time-dependent manner, whereas the expression of Bcl-2 was not changed (Fig. 9).

*GA-T inhibits the growth of solid tumor implanted in athymic mice*

When solid tumors in athymic mice were treated with the GA-T, the suppression of tumor growth was observed. Fig. 10



Fig. 9. Time-dependent expression of apoptosis regulating proteins under GA-T treatment as observed. (A), p53; (B), Bcl-2; (C), Bax. Following 4 h and 8 h exposure to 50  $\mu\text{g/ml}$  GA-T, the total cellular protein was extracted, resolved using SDS-PAGE, and western blotting analysis was done with indicated antibodies. Data represent means from two independent experiments.

shows the inhibition ratio of tumor growth for the GA-T treatment in nude mice. The results demonstrated that GA-T could suppress tumor growth *in vivo*.

## Discussion

In this work, the treatment of a tumor cell line (95-D cells) with GA-T resulted in the inhibition of the cell growth in a dose-dependent manner. Our further experiments revealed that apoptosis induction and cell cycle progression block were simultaneously responsible for the inhibition of the tumor cell growth. The percentage of cells in  $G_1$  cell cycle phase was increased in 95-D cells under the GA-T treatment. For the effect of ganoderma extracts, the cell cycle blocked at the transition from  $G_1$  to S phase (Zhu et al., 2000) and  $G_2/M$  phase (Lin et al., 2003) were observed. The different effect may be caused by the variety of triterpenes in extracts, because the activities of triterpenes are dependent on their structures (Gan et al., 1998).

Caspase cascade plays a key role in apoptosis procedure (Oubrahim et al., 2001). Caspase-3 typically functions at the downstream of other caspases and directly activates enzymes that are responsible for DNA fragmentation in intrinsic apoptosis pathway. Caspase-8 is considered as a signaling and key caspase in extrinsic pathway. Our data suggest that activation of caspase-3 but not caspase-8 was involved in the tumor cells' apoptosis induced by GA-T. The use of caspase inhibitors proved that caspase-3 was associated with the induced apoptosis. Consistent with this conclusion is the finding that caspase-8 inhibitor had no additive effect in preventing GA-T-mediated apoptosis. At the same time, stress-mediated apoptosis is often triggered by mitochondrial function loss and subsequent cyt-*c* release from mitochondria to cytosol. The role of mitochondria in GA-T-mediated apoptosis was also explored in this work. GA-T induced a loss of mitochondrial potential and the release of cyt-*c* to cytosol. Taken together, these results indicate that the apoptosis induced by GA-T was through intrinsic pathway related to mitochondrial dysfunction.

In induced apoptosis, interactions between Bax and Bcl-2 proteins on mitochondria have been postulated to associate with apoptotic pathways (Zong et al., 2003). Many chemotherapeutic drugs activate apoptosis as a function of their anticancer activity. Bcl-2 and Bax have also been implicated as major players in the control of apoptosis pathway. Bcl-2 and Bcl-xL

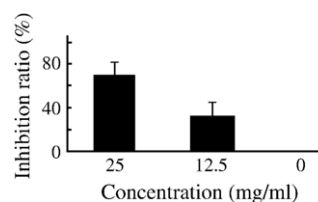


Fig. 10. The inhibition ratio of solid tumors in athymic mice by GA-T.

promote cell survival, whereas Bax promotes cell death (Yashita et al., 1994). It was suggested that the ratio of Bcl-2 to Bax determines survival or death following apoptotic stimulus. Triterpene extract was found to modulate the expression of Bcl-2 and Bax in some tumor cell lines (Choudhuri et al., 2002). In this work, the increase of Bax expression was observed whereas the expression of Bcl-2 was not changed. This result was consistent with the reported data (Sang et al., 2001). The work suggests that the apoptosis under the GA-T treatment was by regulating the ratio of Bax/Bcl-2 groups. The conclusion was similar to the effect of other triterpenes such as asiatic acid (Park et al., 2005) on tumor cells but it has not been known what effects ganoderma triterpenes have on tumor cell lines until now.

In many cases, the p53 protein has been identified as the effector of apoptosis signals (Levin, 1997). It is a regulator of cell cycle progression and mediator of apoptosis in various cases. The key role of p53 in the  $G_1/S$  checkpoint was its response to DNA damage. Now, there is some evidence that ganoderic acids can inhibit the topoisomerases and damage cellular DNA in our research (data not shown) and other work (Li et al., 2005). In this study, the expression of p53 was up-regulated. This means that the biochemical events induced by GA-T were possibly associated with p53 protein. Activation of p53 may be stimulated by DNA damage under GA-T treatment, and activated p53 might either trigger the onset of cell cycle arrest or induce the apoptosis in 95-D cells. On the other hand, we admit that this drug may not work in tumors with p53 mutation. At this stage, the acting mechanism of GA-T against other tumor cell lines is not clear, and whether GA-T has another anti-cancer acting target or not is also unknown to us, and will require further studies.

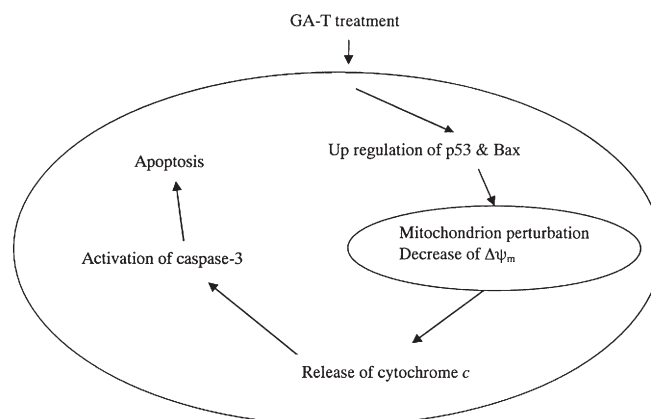


Fig. 11. Proposed pathway involved in apoptosis induced by GA-T.

Taken together, based on the results obtained above and the current paradigms of apoptosis reported in the literature, a molecular pathway of apoptosis induced by GA-T was proposed as in Fig. 11. As it is not yet completely verified, further work can be done to investigate related interesting issues such as the confirmation of apoptosis blockage by blocking p53 or Bax induction. This work also suggests that GA-T may be a natural potential apoptosis-inducing agent for highly metastatic lung tumor and it may be also applied to treat other tumor cell lines. Further investigation is on the way in our lab.

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### References

- Appaix, F., Minatchy, M.N., Riva, L.C., Olivares, J., Antonsson, B., Saks, V.A., 2000. Rapid spectrophotometric method for quantization of cytochrome *c* release from isolated mitochondria or permeabilized cells revisited. *Biochimica et Biophysica Acta* 1457, 175–181.
- Cheung, W.M., Hui, W.S., Chu, P.W., Chiu, S.W., Ip, N.Y., 2000. *Ganoderma* extract activates MAP kinases and induces the neuronal differentiation of rat pheochromocytoma PC12 cells. *FEBS Letters* 486, 291–296.
- Choudhuri, T., Pal, S., Agwarwal, M.L., Das, T., Sa, G., 2002. Curcumin induces apoptosis in human breast cancer cells through p53-dependent Bax induction. *FEBS Letters* 512, 334–340.
- Elliott, D., Crouser, M.E., Gadd, M.W., Julian, J.E., Huff, K.M., Robbins, K.A., Douglas, R.P., 2003. Quantitation of cytochrome *c* release from rat liver mitochondria. *Annual Review of Biochemistry* 317, 67–75.
- Gan, K.H., Fann, Y.F., Hsu, S.H., Kuo, K.W., Lin, C.N., 1998. Mediation of the cytotoxicity of lanostanoids and steroids of *Ganoderma tsugae* through apoptosis and cell cycle. *Journal of Natural Products* 61, 485–487.
- Gao, Y.H., Zhou, S.F., 2003. Cancer prevention and treatment by *Ganoderma*, a mushroom with medicinal properties. *Food Review International* 19, 275–325.
- Ha, T.B., Gerhauser, C., Zhang, W.D., Ho, N., Fouraste, I., 2000. New lanostanoids from *Ganoderma lucidum* that induce NAD(P)H:quinone oxidoreductase in cultured hepalc1c7 murine hepatoma cells. *Planta Medica* 66, 681–684.
- Jiang, J., Slivova, V., Valachovicova, T., Harvey, K., Sliva, D., 2004. *Ganoderma lucidum* inhibits proliferation and induces apoptosis in human prostate cancer cells PC-3. *International Journal of Oncology* 24, 1093–1099.
- Levin, X., 1997. p53, the cellular gatekeeper for growth and division. *Cell* 88, 323–331.
- Li, C.H., Chen, P.Y., Chang, U.M., Kan, L.S., Fang, W.H., Tsai, K.S., Lin, S.B., 2005. Ganoderic acid X, a lanostanoid triterpene, inhibits topoisomerases and induces apoptosis of cancer cells. *Life Sciences* 77, 252–265.
- Lin, S.B., Li, C.H., Lee, S.S., Kan, L.S., 2003. Triterpene-enriched extracts from *Ganoderma lucidum* inhibit growth of hepatoma cells via suppressing protein kinase C, activating mitogen-activated protein kinases and G2-phase cell cycle arrest. *Life Sciences* 72, 2381–2390.
- Liu, X., Yuan, J.P., Chung, C.K., Chen, X.J., 2002. Antitumor activity of the sporoderm-broken germinating spores of *Ganoderma lucidum*. *Cancer Letters* 182, 155–161.
- Lu, Q.Y., Jin, Y.S., Zhang, Q.F., Zhang, Z.F., Heber, D., Go, V.L., Li, F.P., Rao, J.Y., 2004. *Ganoderma lucidum* extracts inhibit growth and induce actin polymerization in bladder cancer cells in vitro. *Cancer Letters* 216, 9–20.
- Oubrahim, H., Stadtman, E.R., Chock, P.B., 2001. Mitochondria play no roles in Mn(II)-induced apoptosis in HeLa cells. *Proceedings of the National Academy of Sciences of the United States of America* 98, 9505–9510.
- Park, B.C., Bosire, K.O., Lee, E.S., Lee, Y.S., Kim, J.A., 2005. Asiatic acid induces apoptosis in SK-MEL-2 human melanoma cells. *Cancer Letters* 218, 81–90.
- Sang, S.M., Pan, M., Cheng, X., Bai, N., Stark, R., Rosen, R.T., Lin-Shiau, S.Y., Lin, J.K., Ho, C.T., 2001. Chemical studies on antioxidant mechanism of garcinol: analysis of radical reaction products of garcinol and their antitumor activities. *Tetrahedron* 57, 9931–9938.
- Sliva, D., 2003. *Ganoderma lucidum* (Reishi) in cancer treatment. *Integrative Cancer Therapies* 2, 358–364.
- Sliva, D., 2004. Cellular and physiological effects of *Ganoderma lucidum* (Reishi). *Mini-Reviews in Medical Chemistry* 4, 873–879.
- Tang W., 2006. Purification and antitumor activity of ganoderic acids from *Ganoderma lucidum*. Ph.D. Dissertation, East China University of Science and Technology, Shanghai, China.
- Yashita, T., Krajewski, S., Krajewski, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B., Reed, J.C., 1994. Tumor suppressor p53 is a regulator of Bcl-2 and Bax gene expression in vitro and in vivo. *Oncogene* 9, 799–805.
- Zhong, J.J., Tang, Y.J., 2004. Submerged cultivation of medicinal mushrooms for production of valuable bioactive metabolites. *Advances in Biochemical Engineering, Biotechnology* 87, 25–59.
- Zhu, H.S., Yang, X.L., Wang, L.B., Zhao, D.X., Chen, L., 2000. Effects of extracts from sporoderm-broken spores of *Ganoderma lucidum* on HeLa cells. *Cell Biology and Toxicology* 16, 201–206.
- Zong, W.X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q.C., Yuan, J., Thompson, C.B., 2003. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. *Journal of Cell Biology* 162, 59–69.