Barbigerone, a Natural Isoflavone, Induces Apoptosis in Murine Lung-Cancer Cells via the Mitochondrial Apoptotic Pathway

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Key Words

Barbigerone • Lung cancer • Mitochondrial apoptotic pathway

Abstract

Barbigerone is a naturally occurring isoflavone with antioxidant activity. In present study, we investigated the antitumor activity of barbigerone against murine lung cancer cells LL/2 and the possible mechanism *in vitro*. Our results showed that barbigerone inhibited LL/2 cells proliferation in a concentration- and time-dependent manner and caused apoptotic death of LL/2 cells. Barbigerone-induced apoptosis was characterized by enhanced mitochondrial cytochrome c release, activation of caspase-3, -9, but not caspase-8. Exposure of LL/2 cells to barbigerone resulted in upregulation of Bcl-2-associated protein (Bax) and down-regulation of Bcl-2. In addition, proliferation inhibitory effect of barbigerone was associated with decreased level of phosphorylated p42/44 mitogen-activated protein kinase (p42/44 MAPK) and phosphorylated Akt. Moreover, barbigerone exhibit less toxicity to non-cancer cells than tumor cells.

In conclusion, our results indicated that barbigerone can inhibit murine lung cancer cell proliferation by inducing apoptosis via mitochondrial apoptotic pathway and by decreasing phosphorylated p42/44 MAPK and Akt. Its potential to be a candidate of anti-cancer agent is worth being further investigated.

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Introduction

Lung cancer as the most common cancer in the world represents a major public health problem [1]. One of every three cancer-related deaths is attributable to lung and it is expected to cause 10 million deaths per year worldwide by the year 2030. However, the treatment outcome of lung cancer is far from satisfactory and the dismal 5-year survival rate of approximately 14% has shown no improvement over the past 30 years [2]. Because of the poor survival figures, more effective agents are in urgent demand to better combat this malignancy.

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1015-8987/09/0242-0095\$26.00/0

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The use of botanical agents or their derivatives for medicinal purpose has been effective in treatment of cancer, such as isoflavone and its analogs which exhibited antitumor activity. Antitumor activity is an important biological activity of isoflavone. It can reduce the risk of breast and prostate cancers [3, 4], inhibit the growth and metastasis of cancer cells in vitro and in vivo [5] and enhance the sensitivity of cancer cells to chemotherapy and radiotherapy [6, 7]. Barbigerone (Figure 1), a naturally occurring isoflavone, was first found from the seeds of *Tephrosia barbiger* in 1979 [8] and could be found from other plants [9-13]. Barbigerone has exhibited profound pharmacological properties, such as antioxidant and 15-lipoxygenase inhibitory activity [12], and antiplasmodial activity [13]. Under these observations, it is possible that barbigerone might have antitumor activity and there is limited information available regarding the antitumor effects of barbigerone.

It is well known that apoptosis is a critical mechanism of anti-tumor drug-induced cell death and isoflavone can inhibit tumor cell proliferation by inducing apoptosis [14]. Moreover, barbigerone has antioxidant activity and studies showed that the compounds which showed antioxidant properties have been demonstrated to induce apoptosis in several human cell lines [15, 16], so barbigerone might exert its antitumor effect by inducing apoptosis. In general, drugs induce apoptosis in cancer cells via two pathways: intrinsic pathway and extrinsic pathway [17, 18]. On the other hand, the balance between proliferation and apoptosis influences the response of tumors to therapy [19], so inhibition of proliferation of cancer cells is another important strategy to inhibit tumor growth. Both Akt and p44/42 mitogen-activated protein kinase (MAPK) are known to play a critical role in cell growth and survival, and they are upregulated in lung cancer cells [20]. Therefore, barbigerone might be capable of inhibiting lung cancer cell proliferation by interfering with these signaling pathways. For this reason, we investigated the influences of barbigerone on apoptosis and proliferation of cancer cells to gain insights into the mechanism of antitumor effect of barbigerone.

In present study, we investigated the antitumor effect of barbigerone against lung cancer cells and its possible mechanism. Using murine Lewis lung cancer cell LL/2, we demonstrate that barbigerone exhibits antitumor activity by inducing apoptosis, as well as inhibiting proliferation. This study suggests that barbigerone induces apoptosis via the mitochondrial pathway, and down-regulation of Akt/MAPK is involved in inhibition of proliferation.

Materials and Methods

Materials

3-(4, 5)-dimethylthiaziazolo(-z-y1)-3, 5-di-phenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). ³H-thymidine (³H-TdR; 74GBq/mol) was purchased from China Institute of Atomic Energy (Beijing, China). Annexin V-FITC apoptosis detection kit was purchased from Keygen Co. (Nanjing, China). All the chemicals employed in this study were of analytic pure and were of culture grade. The primary antibodies for caspase-3, caspase-8, caspase-9, Akt/p-Akt, and MAPK/p-MAPK were purchased from Cell Signaling Technology (Beverly, MA); antibodies for Bcl-2, Bax and cytochrome c were from Santa Cruz Biotechnology Co. (Santa Cruz, CA). The protein assay kit was purchased from Bio-Rad (Hercules, CA).

Barbigerone was obtained from *Suberect Spatholobus*. The extract used was a chemically pure white powder comprising 99% barbigerone and was stable when store at 4°C. It was dissolved in DMSO as a stock solution, and then was stored at 4°C. Prior to experiment the stock solution was diluted in cell-culture medium at final DMSO concentration of 0.05% (V/V).

Cell Culture

Murine lewis lung cancer cell line LL/2, mouse colorectal carcinoma cell line CT26, mouse mammary carcinoma cell line 4T1, mouse prostate cancer cell line Tramp-C1, mouse hepatoma cell line Hepa 1-6, mouse fibroblasts cell line NIH-3T3, human pancreatic island endothelial cell line MS-1, and human embryonic kidney epithelial cell line 293 were obtain from the American Type Culture Collection (ATCC, Manassas, VA). These cells were grown in RPMI 1640 (Life Technologies, Bedford, MA) or DMEM (Life Technologies) containing 10% heat-inactivated fetal bovine serum, 100units/ml penicillin, and 100units/ml streptomycin in a humid chamber at 37°C under 5% CO₂ in atmosphere.

Cell viability assay

The cell viability of barbigerone treated cancer cells was determined by the MTT assay. Briefly, Cells (4-5×10³) were seeded in 96-well plates and cultured for 24 hours, followed by barbigerone treatment for 48 hours. A volume of 10μl of 10mg/ml MTT was added per well and incubated for another 2.5 hours at 37°C, then the supernatant fluid was removed and DMSO was added 150μl/well for 15-20minutes. The light absorptions (OD) were measured at 570nm with SpectraMAX M5 microplate spectrophotometer (Molecular Devices). The effect of barbigerone on tumor cells viability was expressed by IC₅₀ of each cell lines.

Cell proliferation assay

To directly determine the proliferation inhibitory effect of barbigerone on cells, [³H]-thymidine incorporation was performed. Briefly, Cells (4-5×10³) were seeded in 96-well plates and cultured for 24 hours, followed by barbigerone treatment

Fig. 1. Structure of barbigerone.

for 24, 48, and 72 hours, respectively. The cells were pulsed with 1 μ Ci/well of [3 H]-thymidine for 5 hours and then harvested using a Wallac harvester (Wallac, Turku, Finland). The radioactivity was measured using a scintillation counter (Wallac). The cell proliferation inhibition (% of control) was calculated using the following formula: Inhibition rate = $(1 - \text{drug-treated radioactivity} / \text{control radioactivity}) \times 100\%$.

Apoptosis analysis by FCM

To investigate the apoptosis inducing effect of barbigerone, we analyzed the percentage of the early apoptotic cells by FCM with the Annexin V-FITC/PI dual-labeling technique. Briefly, cells were seeded in 6-well plates at the density of 2×10^5 /well and cultured for 24 hours, followed by barbigerone treatment for 12 hours, and then harvested and washed with PBS. Cells were stained with an annexin V-FITC apoptosis detection kit according to the manufacturer's instructions, and then immediately analyzed by flow cytometer (ESP Elite, Beckman-Coulter, Miami, FL). The experiment was repeated five times.

Morphological analysis

Apoptosis cells treated with barbigerone was further confirmed by morphological analysis. Cell culture and drug treatment were done as described above. The cells were fixed using 70% of ethanol following rinse with PBS. After examined for morphological changes by inverted microscope, the cells were stained with PI (1 μ g/ml, in PBS) and analyzed under fluorescence microscope (Zeiss, Axiovert 200, Germany) to identify the apoptotic cells.

Western blot analysis

To identify the mechanism of apoptosis induced by barbigerone, immunoblot analysis was performed. Briefly, 5×10^6 cells were lysed in 1 ml lysis buffer, and the protein concentration was determined by the Bio-Rad protein assay reagent. The samples were denatured in sample buffer, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were electroblotted onto a polyvinylidene difluoride membrane. The membrane blots were rinsed with TBS/T (20 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 7.6) and blocked by 5% nonfat dry milk in blocking buffer. The membrane was incubated with the desired primary antibody overnight at

Tumor cell line	Cell type	IC50(μ M)
CT26	Mouse colorectal cancer cell line	5.03 $\pm$ 0.70
Hepa 1-6	Mouse hepatoma cell line	2.07 $\pm$ 0.33
4T1	Mouse breast cancer cell line	6.5 $\pm$ 0.49
LL/2	Mouse lung cancer cell line	0.92 $\pm$ 0.05
Tramp-C1	Mouse prostate cancer cell line	5.8 $\pm$ 0.34

Table 1. The cytotoxicity effect of barbigerone on tumor cells. Each cell line was treated with various concentrations (0-16 μ M) of barbigerone for 48 hours, respectively. Cell viability was detected by MTT assay. Data are expressed as the mean $\pm$ SD from three experiments.

4°C. The membrane was then incubated with appropriate peroxidase-conjugated secondary antibody and the immunoreactive bands were visualized using the enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ) method.

Analysis of cytochrome c in cytosolic and mitochondrial extracts

For detection of cytochrome c, the cytosolic and mitochondria fractions were prepared as described previously [21]. Briefly, LL/2 cells were treated with barbigerone at various concentrations for 48 hours and then harvested by centrifugation at 800g at 4°C for 15 min. After washing three times with ice-cold PBS, cell pellets were resuspended in HEPES-buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, pH 7.5) containing 250 mM sucrose, homogenized with a homogenizer, and centrifuged at 800g at 4°C for 15 min. The supernatants were centrifuged at 10000g for 15 min at 4°C, and the mitochondrial pellets were dissolved in SDS sample buffer (25 μ l), subjected to 15% SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblotting with monoclonal antibodies against cytochrome c. Aliquots (20 μ l) of the supernatant (i.e., cytosolic fraction) were also analyzed for cytochrome c expression by western blotting.

Statistical Analysis

Data were expressed as the means $\pm$ SD. Statistical comparisons were made by ANOVA test. All data were analyzed using SPSS software (SPSS for Windows, ver. 13.0; SPSS, Chicago, IL). In all statistical analysis, $P < 0.05$ was taken as significant.

Results

The cytotoxicity effect of barbigerone on tumor cells

A small panel of five cancer cell lines (LL/2, CT26, 4T1, Hepa 1-6 and Tramp-C1) was used to investigate the cytotoxicity of barbigerone. After 48 hours treatment,

barbigerone (0.125 - 16 μ M) inhibited viability of all cell lines. The IC₅₀ for LL/2, CT26, 4T1, Hepa 1-6 and Tramp-C1 were 0.92 μ M, 5.03 μ M, 6.5 μ M, 2.07 μ M and 5.8 μ M, respectively (Table 1). A comparison between the five cell lines examined showed that LL/2 cells were more sensitive to barbigerone treatment.

The proliferation inhibitory effect of barbigerone on LL/2 cells

We tested the antiproliferative effect of barbigerone on LL/2 cells by the [³H]-thymidine incorporation assay which is reliable to detect proliferation of cells. Exposure of cells to 0.125-16 μ M barbigerone for 24, 48, and 72 hours, respectively, resulted in decrease of the cell proliferation ($P < 0.05$) (Figure 2). When the cells were treated with barbigerone for 24 hours, the IC₅₀ was 2.67 μ M, whereas the IC₅₀ decreased to 0.90 μ M and 0.46 μ M when cells were treated for 48 hours and 72 hours, respectively. The results directly suggested that barbigerone inhibited the proliferation of LL/2 cells in a time- and concentration-dependent manner.

Induction of apoptosis by barbigerone

Because a decrease in cell proliferation may result from the induction of apoptosis, we investigated whether barbigerone induced LL/2 cells apoptosis. LL/2 cells were treated with barbigerone and annexin V – FITC and PI fluorescence was determined by flow cytometry (Figure 3). The percentage of early apoptotic cells as measured by Annexin-V-positive and PI-negative (the forth quadrant) were 3.5%, 10.7% and 19.8%, respectively, after treated with 0.25 μ M, 0.5 μ M and 1 μ M barbigerone for 12 hours. The results suggested that barbigerone inhibited proliferation of the LL/2 cells by inducing apoptosis in concentration-dependent.

Furthermore, fluorescence microscopic examination of PI-stained cell was performed to confirm the apoptosis induction effect of barbigerone. Treatment with barbigerone resulted in morphological changes characteristic for apoptosis: a brightly red-fluorescent condensed nuclei (intact or fragmented) by fluorescence microscopy of PI-stained nuclei, blebbing, reduction of cell volume, condensation of nuclear chromatin, nuclear fragmentation, and apoptotic bodies, and the change was concentration-dependent (Figure 4).

Barbigerone induces apoptosis through intrinsic pathway

Caspase-3 is an effector caspase that plays a central role in cell apoptosis. Therefore, we investigated the

Fig. 2. Proliferation inhibitory effect of barbigerone on LL/2 cells. Concentration- and time-dependent inhibition of LL/2 cells proliferation by barbigerone shown by ³H-TdR incorporation assay. Cells were seeded in 96-well-plate and treated with various concentrations of barbigerone for 24 hours, 48 hours and 72 hours respectively. Data were expressed as mean $\pm$ SD. for at least 5 independent experiments. The asterisk indicates a significant difference between control group and barbigerone-treated groups (* $P < 0.05$ vs. control group, ** $P < 0.01$ vs. control group, *** $P < 0.001$ vs. control group)

Fig. 3. Barbigerone concentration-dependently induced apoptosis in LL/2 cells. The cells harvested were processed for apoptosis assessment by using the Annexin V-FITC/PI dual-labeling technique. LL/2 cells were untreated (A) or treated with barbigerone at 0.25 μ M (B), 0.5 μ M (C), and 1 μ M (D) for 12 hours.

Fig. 4. Morphological changes induced by barbigerone. Bright-field microscope images (E-H) and Fluorescence microscopic appearance of PI-stained nuclei (A-D) of LL/2 cells after incubation with barbigerone for 48 hours at varying concentrations: 0 μ M (A, E); 0.25 μ M (B, F); 0.5 μ M (C, G); 1 μ M (D, H) (original magnification 400 $\times$). Apoptotic cells containing condensed and fragmented fluorescent nuclei are visible in barbigerone-treated cells, but not in untreated cells.

effect of barbigerone on the activation of caspase-3. Treatment LL/2 cells with barbigerone for 48 hours resulted in a concentration-dependent increase of cleavage caspase-3 (Figure 5).

To further characterize barbigerone-induced apoptosis and determine which apoptotic pathway barbigerone activates, we examined the patterns of proteolytic processing of caspase-8 and -9, the apical proteases in the extrinsic and intrinsic pathways respectively. Our results showed that the levels of procaspase-9 decreased, and levels of the cleaved caspase-9 accumulated in a concentration-dependent way after 48 hours of barbigerone treatment. However, levels of caspase-8 did not change upon barbigerone treatment (Figure 5). These data suggest that in the apoptosis induced by barbigerone caspase-9 is activated but caspase-8 is not involved. According to the results, we propose that barbigerone triggers apoptosis not through extrinsic pathway but intrinsic pathway.

Barbigerone induces cytochrome c release

A key step in the intrinsic apoptotic pathway is damage of mitochondria and the release of cytochrome c to activate Apaf-1, which turns on the caspase cascade [22]. After treatment of LL/2 cells with barbigerone at various concentrations for 48h, cytochrome c was detectable in the cytosolic fraction and increased in a concentration-dependent manner, whereas a decrease

Fig. 5. Effect of barbigerone on caspase activation. (A) LL/2 cells were treated with barbigerone at various concentrations (0-1 μ M) for 48 hours and then we analyzed the expression levels of caspase-8, 3, 9, and their activated forms by Western blotting. Tubulin was used as a loading control. (B) Densitometric analysis using image analyzing software. Barbigerone dose-dependently induced caspase-9 and -3 activation, but caspase-8 was not influenced. Data were expressed as the mean $\pm$ SD (n=3).

Fig. 6. Barbigerone induced cytochrome c release from mitochondria. (A) LL/2 cells were treated with barbigerone at various concentrations (0–1 μM) for 48 hours and then we analyzed the levels of cytochrome c in the cytosol and mitochondrial fraction. (B) Densitometric analysis of cytochrome c using image analyzing software. Barbigerone dose-dependently increased cytochrome c level in cytosol fraction and decreased in mitochondrial fraction. Data are expressed as the mean ± SD (n=3).

Fig. 7. Effects of barbigerone on Bcl-2 and Bax expression. (A) LL/2 cells were treated with barbigerone at various concentrations (0–1 μM) for 48 hours and then we analyzed the expression levels of Bcl-2 and Bax by Western blotting. Total proteins extracts were subjected to 15% SDS-PAGE for western blot analysis. Tubulin was used as a loading control. (B) Densitometric analysis of Bcl-2 and Bax using image analyzing software. Data were expressed as the mean ± SD (n=3). Barbigerone concentration-dependently inhibited Bcl-2 expression and increased Bax expression simultaneously.

level of cytochrome c in the mitochondria fraction (Figure 6). This result indicated that barbigerone induces the release of cytochrome c from the mitochondria to the cytosol in LL/2 cells.

Barbigerone induces apoptosis via Bax activation and Bcl-2 inhibition

Mitochondrial integrity is regulated by pro-apoptotic and anti-apoptotic members of the Bcl-2 group of proteins such as Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic). Bcl-2 protects cells from the induction of apoptosis by interacting with pro-apoptotic members of the Bcl-2 group and thus blocking the release of cytochrome c from the mitochondria.

To determine the effects of barbigerone on the expression of the Bcl-2 protein group, we examined Bcl-2 and Bax expression in the LL/2 cells after treatment with barbigerone at various concentrations for 48 hours. As shown in Figure 7, inhibition in expression of Bcl-2 and increase in Bax were confirmed in response to

treatment with barbigerone. The increase in the Bax/Bcl-2 ratio has been described in association with activation of the mitochondrial apoptotic route. Both Bax activation and Bcl-2 inhibition are required for the release of cytochrome c from mitochondria [23]. The result of inhibition of Bcl-2 and concomitant increase in Bax expression was coincident with the result of cytochrome c release.

Effect of barbigerone on MAPK and Akt signaling pathways

We investigated whether p44/42 MAPK and Akt, which are known to be very important in cell growth and survival, were involved in barbigerone-mediated proliferation inhibitory effect. Our result showed barbigerone clearly reduced phosphorylated Akt and phosphorylated p44/42 MAPK in a concentration-dependent manner (Figure 8), indicating that barbigerone can inhibit Akt and p44/42 MAPK activities. The levels of total Akt and MAPK were not visibly changed,

Table 2. The cytotoxicity effect of barbigerone on normal cells. Each cell was treated with various concentrations (0-32 μ M) of barbigerone for 48 hours, respectively. Cell viability was detected by MTT assay.

Cell line	Cell type	IC50(μ M)
NIH-3T3	Mouse embryonic fibroblast cell line	32
MS-1	Mouse endothelial cell line	32
HUVEC	Human umbilical vein endothelial cells	32
293	Human embryonic kidney epithelial cell line	32

however, phosphorylated form, as the main part of this protein system, was inhibited much more significantly.

Effects of barbigerone on non-tumor cell lines and primary cells

Compared with tumor cell lines, the sensitivity of nontumor cell lines to the cytotoxic effects of barbigerone was much lower. Barbigerone did not exhibit a significant effect on the viability of these cells at the higher doses (32 μ M) (Table 2). Thus, these data suggest that nontumor cells are more resistant to the barbigerone-mediated cytotoxicity than tumor cells.

Discussion

In the present study, we investigated the antiproliferation effect of barbigerone which obtained from *Suberect Spatholobu* on murine Lewis lung cancer cell line LL/2 and the possible mechanism. Our study demonstrated that barbigerone inhibited LL/2 proliferation by inducing apoptosis and the mitochondrial pathway played a predominant role in this process. Moreover, cell growth inhibitory effect was associated with a decrease in the phosphorylated levels of Akt and p44/42 MAPK. To our knowledge, this study is first to show the barbigerone, a naturally occurring isoflavone, inhibited lung cancer cell proliferation and induced apoptosis in lung cancer cell.

We demonstrated that barbigerone has significant anti-cancer activities against a panel of murine cancer cells and murine Lewis lung cancer cell LL/2 is most sensitive to barbigerone treatment. In addition, barbigerone exhibited proliferation inhibitory effect of human tumor cell lines, such as human lung cancer cell line A549 (data not shown). Therefore, we focused on the effect of barbigerone on murine lung cancer cells LL/2.

The mechanism of action by which isoflavones inhibit proliferation in a variety of cancer cells has yet to be clearly determined. Apoptosis is an important mechanism of isoflavone-induced cell death, and susceptibility to apoptosis of tumor cells is an important determinant of

Fig. 8. Barbigerone inhibited Akt and p 44/42 MAPK phosphorylation. (A) LL/2 cells were treated with barbigerone at various concentrations (0-1 μ M) for 48 hours and then we analyzed the expression levels of Akt, p 44/42 MAPK and their phosphorylation status by Western blotting. (B) Densitometric analysis of Akt, p 44/42 MAPK and their phosphorylation using image analyzing software. Barbigerone inhibited Akt and p 44/42 MAPK phosphorylation, but not influenced the levels of total Akt or p 44/42 MAPK. Data are expressed as the mean $\pm$ SD (n=3).

chemotherapy efficacy. Previous study showed that isoflavones or analogs can induce apoptosis, such as genistein, daidzein, and biochanin A, isoflavone present in soy, have been reported to have significant protective effects against induced and spontaneous cancers [24-26]. Therefore, we assumed barbigerone might inhibit cell proliferation by inducing apoptosis. Our results indicated barbigerone can induce apoptosis on a concentration-dependent manner in LL/2 cells, which consistence to other isoflavone anti-cancer study. To determine the mechanism of apoptosis induced by barbigerone, the proteins involved in apoptosis were analyzed by western

blotting. We found that Barbigerone activated caspase-3, the key molecular in cellular apoptosis procedure. In general, the initiation of apoptosis is through two distinct pathways: one is the extrinsic pathway involved the death receptor signaling, the other is the intrinsic pathway involved the mitochondrial cascades [27]. Activation of caspase-3 is involved in either one of the two pathways and often considered as the point-of-no-return in the apoptotic signaling cascade [28, 29]. To determine the pathway of apoptosis induced by barbigerone, we further analyzed caspase-8 and -9, the primary caspases involved in extrinsic and intrinsic pathway respectively [30, 31]. The results showed caspase-9, but no caspase-8 was activated by barbigerone. It suggested barbigerone induced apoptosis through intrinsic pathway.

The release of mitochondrial cytochrome c is a signal event in the intrinsic apoptotic activation pathway. To confirm this mechanism we next analyzed the levels of cytochrome c in cytosol and mitochondria after treated with barbigerone. Our results showed that the treatment of LL/2 cells with barbigerone released cytochrome c from the mitochondria into the cytosol, suggesting an activation of the intrinsic apoptotic pathway. Mitochondria have been shown to play a central role in the apoptotic process because both the intrinsic and extrinsic pathways can converge at the mitochondrial level and trigger mitochondrial membrane permeabilization [32]. Cytochrome c binds to Apaf-1 and procaspase-9 to form the apoptosome, activating caspase-9, the primary caspase involved in the mitochondrial apoptotic pathway.

Bcl-2 family proteins are central regulators of the intrinsic pathway, which either suppress or promote changes in mitochondrial membrane permeability required for release of cytochrome-c [32, 33]. Bcl-2 and Bax have been identified as major regulators in controlling the release of mitochondrial cytochrome c [34]. Bcl-2 blocks cytochrome c efflux whereas Bax enhances the release of cytochrome c and encourages apoptosis. Overexpression of antiapoptotic Bcl-2 probably occurs in more than half of all cancers [35]. Our results showed that treatment with barbigerone produced down-regulation of Bcl-2 and upregulation of Bax in LL/2 cells. The release of cytochrome c from the mitochondria into the cytosol by barbigerone is resulted from down-regulation of Bcl-2 and/or upregulation of Bax.

Taken above, we concluded that barbigerone induced apoptosis of LL/2 cells by an intrinsic mechanism. This mechanism is regulated via the inhibition of Bcl-2 and a concomitant stimulation of Bax protein expression,

producing mitochondrial disruption and the release of cytochrome c, which leads finally to the activation of caspases-9 and -3.

Moreover, isoflavones have been reported to have various effects on cell cycle progression in different cells, including blocking at various phases of the cell cycle [36-38], for example, genistein promoted a G2-M arrest [39]. Our study also found that barbigerone can induce G2/M arrest of LL/2 cell measured by flow cytometry (data not shown). This data suggested that cell-cycle arrest induced by barbigerone may play a significant role in the antiproliferative effects of this agent. It was reported that genistein could promote apoptosis in mammary epithelial cells by inducing the tumor suppressor PTEN [40]. Furthermore, P38 and P53 involved in cell apoptosis induced by DDTD, an isoflavone derivative, through reactive oxygen species [41]. Therefore, barbigerone may induce apoptosis of LL/2 cells through above mechanisms and further studies are needed.

Besides inducing apoptosis inhibiting cell growth is another important strategy to overcome cancer. The study by Chun et al. showed that Akt/ p44/42 MAPK pathway as important targets could be modulated for lung cancer prevention and treatment [20]. Akt pathway has been an important molecular target for cancer prevention and treatment [42, 43]. Interestingly, we found that barbigerone significantly decreased the phosphorylated levels of both Akt and p44/42 MAPK. The result indicated that barbigerone inhibited cancer cells proliferation by down-regulating activities of Akt and p44/42 MAPK. According to the result together with our other findings, it is suggested that at least two mechanisms are involved in anticancer effect of barbigerone: inducing apoptosis and inhibiting proliferation.

In addition, we founded that barbigerone did not exhibit toxicity to non-cancer cell lines under the conditions used. This is an interesting character, because one reason why a variety of compounds with potent anticancer activity *in vitro* could not be used in clinic is due to their strong toxicities to normal cells. Based on the less toxicity to non-cancer cells, it suggested that barbigerone might be safe *in vivo*.

In conclusion, we first demonstrated barbigerone has anti-tumor activity through inhibition of proliferation and inducing apoptosis via intrinsic pathway, and has a relative few toxicity to non-cancer cells. Delineation of the biological effects of barbigerone on chemotherapeutic potential or chemoprevention of human cancers warrants further investigation.

Acknowledgements

This work was supported by National Key Basic Research Program of China (2004CB518800),

Project of National Natural Sciences Foundation of China (20505006 and 30701027), and National 863 projects (2006AA03Z356).

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