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α-Bisabolol induces dose- and time-dependent apoptosis in HepG2 cells via a Fas- and mitochondrial-related pathway, involves p53 and NFκB

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ABSTRACT

In this study, the apoptotic effect of α-bisabolol, a sesquiterpene, against human liver carcinoma cell line HepG2 was investigated. MTT assay showed α-bisabolol could effectively induce cytotoxicity in several human cancer cell lines (PC-3, Hela, ECA-109 and HepG2). The results of nuclei morphology examination, DNA fragmentation detection, flow cytometry analysis and cleavage of poly(ADP-ribose) polymerase and caspases indicated α-bisabolol might induce dose- and time-dependent apoptosis in HepG2 cells. Western blot data also showed a cascade activation of caspases-8,-9,-3 and promoted expression of Fas, implying caspase-8 might function as an upstream regulator, and the Fas-related pathway might be involved in this process. Preparation of mitochondrial/cytosol fraction followed with immunoblot analysis showed the release of chromosome c from mitochondria, down-regulated expression of Bcl-2 and translocation of Bax, Bak and Bid, suggesting the mitochondrial-related pathway might be involved in α-bisabolol-induced apoptosis either. Detection of accumulation of nuclear wild-type p53 and up-regulated expression of NFκB indicated these two key regulator with transcriptional decision-making function in various signaling pathways might also play a role in α-bisabolol-induced apoptosis in HepG2 cells.

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1. Introduction

Liver cancer (LC) is one of the most common cancers with an annually increasing occurrence worldwide. LC has a two- to three-fold higher incidence rates in developing countries than in developed countries [1,2]. Since most of current therapeutic treatments are ineffective and limited to treat the tumor, great effort has been making to find novel compounds to treat the tumors.

Apoptosis, a basic biological phenomenon, is mostly mediated through extrinsic (death receptor) pathway and/or intrinsic (mitochondrial) pathway [3,4]. The death receptor pathway is stimulated by the binding of cell surface death receptor such as Fas and tumor necrosis factor (TNF) receptor, which leads to the formation of a death-inducing signaling complex and the activation of caspase-8 [5,6]. Activated caspase-8 can not only directly activates downstream effector caspases, but also cleave Bid to tBid, which is important in Fas receptor pathway and can mediates cross-talk between these two pathways [7]. The mitochondrial pathway is dependent on the release of cytochrome c from the mitochondria by mitochondrial outer membrane permeabilization (MOMP) [8]. MOMP is controlled by the BCL-2 family, which is composed of both pro-apoptotic molecules (Bax, Bcl-Xs, Bak, Bid, Bad, Bim, Bik) and anti-apoptotic molecules (Bcl-2, Bcl-XL, Bcl-W, Mcl-1, A1) [9–11]. Once released to cytosol, cytochrome c combines with Apaf-1 and procaspase-9 to form the apoptosome. Activated caspase-9 can cleave and activate downstream effector caspases, such as caspases-3 and -7, leading to apoptotic cell death [12].

Increasing attention has been paid to sesquiterpenes for their potentially useful biological activities, especially the anti-tumor activity [13–15]. α-Bisabolol (Fig. 1), a sesquiterpene alcohol with very low toxicity, has been widely used in fragrances and cosmetic preparations for hundreds of years [16]. Recent years, this oily compound has been studied for its effect of increasing bacterial resistance to antibiotics and antimicrobials [17], antimutagenic activity [18], inhibitory effect on the genotoxic damage [19] and depigmenting effect [20]. Research groups in Italy also have studied the apoptosis-inducing and anti-tumor activities of α-bisabolol, and found that α-bisabolol was able to selectively induce apoptosis in several malignant tumor cells through the mitochondrial pathway, probably by targeting lipid rafts on cell membranes [21–23]. However, the molecular mechanism underlying α-bisabolol cytotoxicity in tumor cells has not been well elucidated.

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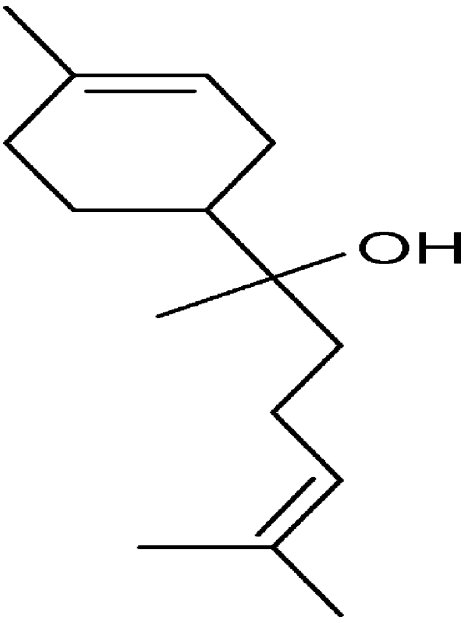

Fig. 1. Molecular structure of α-bisabolol.

We have reported previously that $Cu(OP)_2$ induced G1-phase specific apoptosis in liver carcinoma cell line Bel-7402, and provided evidence for the involvement of copper overload, cellular redox state change and DNA damage in the apoptosis of Bel-7402 cells induced by $Cu(OP)_2$ [24,25]. In the present investigation, we first ascertained that α-bisabolol was able to induce apoptosis in human liver carcinoma cell line HepG2 in a dose- and time-dependent way. We also studied pathways through which α-bisabolol mediates the expression and activation of different proteins involved in the apoptotic cell death. Our data indicated that α-bisabolol-induced apoptosis in HepG2 cells may be through a Fas- and mitochondrial-related pathway, and p53 and NFκB was involved either. The apoptosis induced by α-bisabolol in HepG2 cells may be the consequence of several factors, and the detailed molecular mechanism will be explored in further experiments.

2. Materials and methods

2.1. Materials

α-Bisabolol and ethidium bromide (EB) were purchased from Sigma Chemical Co.(St. Louis, MO, USA). 3-(4,5-Dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258, RNase A, propidium iodide (PI), phenylmethyl sulfonylfluoride (PMSF), trypsin, bovine serum albumin (BSA), Tween-20 and DMSO were all purchased from AMRESCO Inc. (Solon, OH, USA). Dulbecco's modified Eagle medium (DMEM) and newborn bovine serum (NBS) were purchased from GIBCO BRL (Grand Island, NY, USA). Rabbit polyclonal antibodies against poly(ADP-ribose)polymerase (PARP-1), caspase-8, caspase-9, Bcl-2, Bax, Bak, Bid/tBid, cytochrome c, COX IV, p53, Fas, GAPDH and horseradish peroxidase-conjugated secondary antibody were all purchased from ProteinTech Group, Inc. (Chicago, IL, USA); Rabbit polyclonal antibodies against caspase-3 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and rabbit polyclonal antibodies against β-actin were purchased from Beijing CoWin Biotech Co., Ltd. (Beijing, China). Non-fat dry milk was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). All other chemicals and reagents were of the highest quality and obtained from standard commercial sources.

2.2. Cells culture and treatment

Human prostate cancer cell line PC-3, human cervical carcinoma cell line Hela, human esophageal ECA-109, and human liver carcinoma cell line HepG2 were obtained from China Center for Typical Culture Collection (CCTCC). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, GIBCO BRL) supplemented with 10% newborn bovine serum (GIBCO BRL), 1% (w/v) glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were treated with α-bisabolol from a freshly prepared stock solution in absolute ethanol, added to the culture medium to obtain final concentrations. Initial α-bisabolol concentration was 1 mM. The α-bisabolol concentration indicated in cell experiments represents its soluble fraction in culture medium as described [21–23].

2.3. Cell viability assay

Cell viability was measured by MTT assay as described [25]. Cells were plated at 100 μl per well in a 96-well microplate and treated with α-bisabolol at different concentrations (0–20 μM) for 24 h. After treatment, cells were treated with MTT (5 mg/ml) and incubated for 4 h at 37 °C. The reaction was stopped by adding 100 μl DMSO, and the absorbance was measured at 490 nm. Results were expressed as percentage of the untreated controls.

2.4. Morphology examination of nuclei

HepG2 cells were seeded into 24-well culture plates and cultured for 24 h. After α-bisabolol treatment, cells were incubated in fixed buffer ($V_{methanol}$:$V_{acetic\ acid}$ = 3:1) at room temperature for 10 min. The fixative was removed and the cells were washed three times with ice-cold PBS, and then incubated with Hoechst 33258 (5 μg/ml) at room temperature for 45 min in the dark. The cellular fluorescent changes were observed using fluorescence microscope (Leica). Stained nuclei with condensed chromatin or those were fragmented into smaller dense bodies presented bright blue fluorescence were considered as apoptotic. Nuclei with uncondensed chromatin presented dark or no fluorescence were considered as not apoptotic.

2.5. Detection of DNA fragmentation

DNA fragmentation was detected as described method [26] with minor modification. After α-bisabolol treatment, HepG2 cells were harvested with trypsin and washed with ice-cold PBS by centrifugation at 600 × *g* for 5 min. Cell pellets were then treated with lysis buffer at room temperature for at least 5 min (1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5; minimum 50 μl). After centrifugation for 5 min at 1600 × *g* the supernatant is collected and the extraction is repeated with the same amount of lysis buffer. The supernatants were treated for 2 h with RNase A (final concentration, 1 mg/ml) containing 1% SDS, followed by digestion with Proteinase K (final concentration 2 mg/ml) overnight at 56 °C. After the addition of 1/2 vol. 5 M ammonium acetate, the DNA is precipitated with 2.5 vol. ethanol overnight at −20 °C. After centrifugation at 12,000 × *g* for 15 min at 4 °C, the pellets were dried and dissolved in gel loading buffer, separated by 1% agarose gel electrophoresis at 45 V for 1.5 h, and stained with ethidium bromide (EB).

2.6. Flow cytometry analysis

HepG2 cells treated with α-bisabolol at indicated concentration for different time courses were collected and washed twice with ice-cold PBS, and then suspended in 75% ethanol at −20 °C

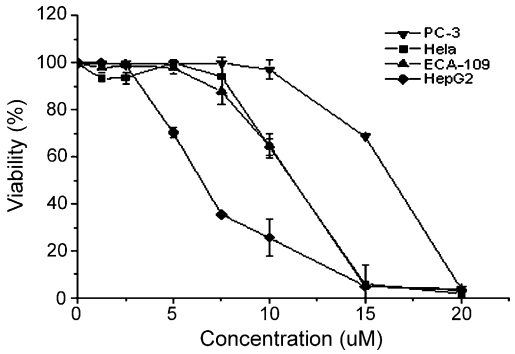

Fig. 2. α-Bisabolol inhibits cell growth in a dose-dependent manner. Cells were plated at 100 μl per well in a 96-well microplate and treated with α-bisabolol at different concentrations (0–20 μM) for 24 h. Cells were treated with MTT (5 mg/ml) and incubated for 4 h at 37 °C and the reaction was stopped by adding 100 μl DMSO. Then the absorbance was measured at 490 nm and the results were expressed as percentage of the untreated controls. Each data represent the mean ± SD from three independent experiments.

overnight. Fixed cells were centrifuged at 600 × *g* for 5 min and washed with ice-cold PBS. For detecting DNA content and cell cycle, cells were incubated with 100 μg/ml RNase A in PBS for 1 h at 37 °C before staining in the dark with PI (20 μg/ml) for 30 min at 4 °C. Samples were applied to a flow cytometer (Becton Dickinson). For each analysis, a minimum of 10,000 cells was counted.

2.7. Preparation of the mitochondrial/cytosol fraction and immunoblot analysis

After drug treatment, both adherent and floating cells were collected and washed with ice-cold PBS, then treated with ice-cold lysis buffer (Beyotime Inst. Biotech, Peking, PR China) with 1% PMSF (phenylmethyl sulfonylfluoride). Cell lysates were centrifuged at 12,000 × *g* at 4 °C for 5 min. Isolation of mitochondrial and cytosolic proteins was performed using the Mitochondria/cytosol Fractionation Kit (Shanghai Xinghan Sci&Tech CO., LTD.). The protein concentration was determined by the BCA Protein Assay Kit (Pierce). Equal amounts of protein were separated by 8–15% SDS-PAGE and were electrotransferred to polyvinylidene difluoride (PVDF; Millipore Corp.) membrane. The membranes were

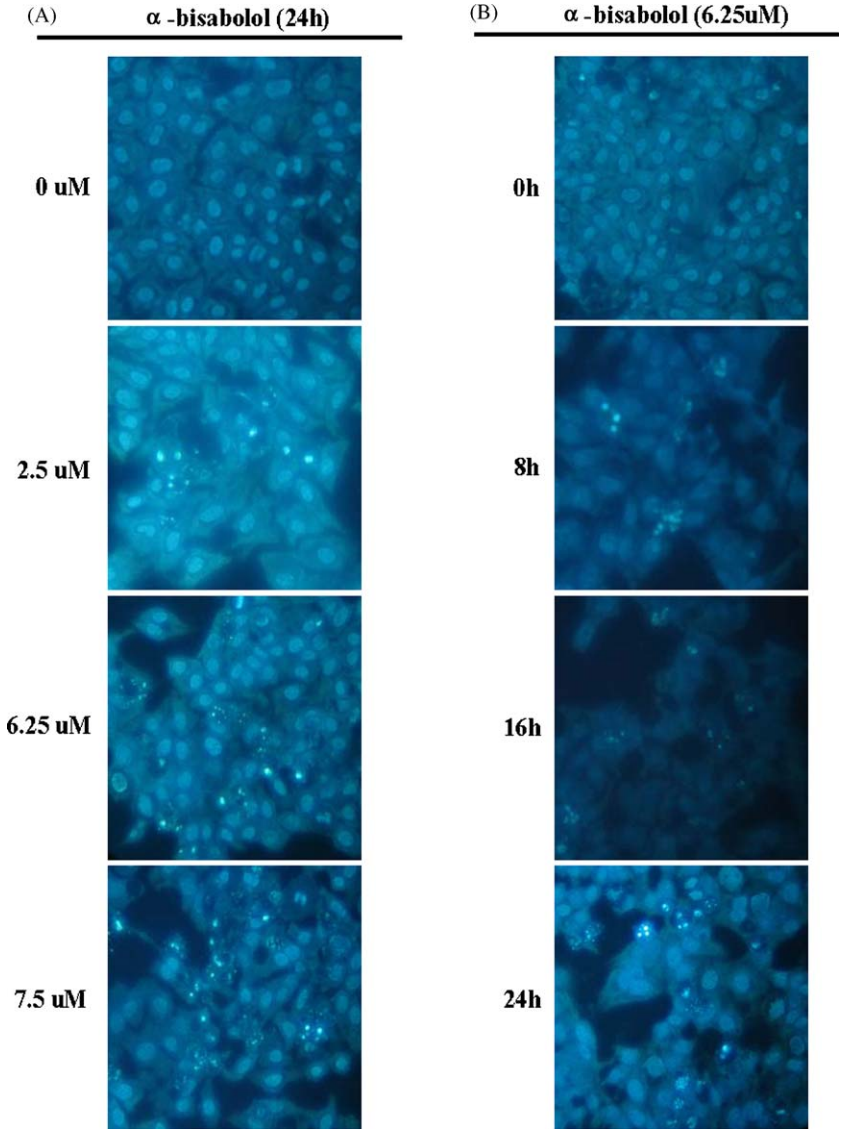

Fig. 3. Cell morphology analysis after HepG2 cells was treated with α-bisabolol. Cells were seeded into 24-well culture plates and treated with α-bisabolol at different concentrations (0–7.5 μM) for 24 h (A) or treated with 6.25 μM α-bisabolol for different times (0–24 h) (B). Hoechst 33258 was used to stain cells at room temperature for 45 min in the dark. The cellular fluorescent changes were observed using fluorescence microscope. Results were obtained from three separate experiments.

soaked in blocking buffer (5% non-fat dry milk) over night at 4 °C, and then incubated with indicated primary antibodies at 37 °C for 1.5 h, followed by horseradish peroxidase-conjugated secondary antibodies at 37 °C for 45 min. Detection was performed using enhanced chemiluminescence Western Blotting Detection Reagents (Millipore).

2.8. Data analysis

Data are expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) with a Student–Newman–Keuls follow-up test. Significance was declared when $P < 0.05$.

3. Results

3.1. α-Bisabolol inhibits cell growth in a dose-dependent manner

In this study, the effect of α-bisabolol on the viability of PC-3, Hela, ECA-109, and HepG2 cells was examined by MTT assay. Cells were treated with α-bisabolol at different concentrations (0–20 μM) for 24 h. Our data indicated that α-bisabolol exhibited a cytotoxic effect in a dose-dependent manner. As shown in Fig. 2, 70% of HepG2 cells was killed after 24 h of treatment with 10 μM α-bisabolol, only 30% of Hela and ECA-109 cells were killed, whereas PC-3 cell line appeared to be much more resistant to α-bisabolol. The results showed that α-bisabolol seemed to have a stronger death effect towards human liver carcinoma cell line HepG2.

3.2. Effect of α-bisabolol on cell morphology and DNA fragmentation

The abnormalities of cell morphology were examined using a fluorescence microscope. Cells treated with α-bisabolol at different concentrations (0, 2.5, 6.25, 7.5 μM) for 24 h (Fig. 3A) or treated with 6.25 μM α-bisabolol for different times (0, 8,16, 24 h) (Fig. 3B) showed significant morphological changes. When the drug concentration gets higher or the treatment time gets longer, more and more cells exhibited morphological characteristic of apoptosis, such as chromatin condensation and formation of apoptotic bodies. Besides cell morphology, fragmentation of chromatin into units of single or multiple nucleosomes was also examined in this study. After treated with α-bisabolol, cell extracts were analysed by electrophoresis to observe the formation of DNA ladder. As shown in Fig. 4A, no evidence of oligonucleosomal DNA ladder was present in control group (0 μM), when the concentration of α-bisabolol raised, DNA ladder became more evident, which indicated an increasing amount of apoptotic cells. We obtained a similar result in time assay, as shown in Fig. 4B.

3.3. Effect of α-bisabolol on sub-G1 peak measured by PI-staining and flow cytometric analysis

To evaluate whether α-bisabolol-caused HepG2 cell death occurs via apoptosis, we used PI-staining and flow cytometry. The induction of apoptosis was confirmed by the presence of the sub-G1 cell population in flow cytometric analysis. As demonstrated in Fig. 5A, when HepG2 cells were treated at different concentrations (0, 2.5, 5, 7.5, 10 μM) for 48 h, the sub-G1 peak increased from 3.5% of the control to 30.5%. The result of time-dependent assay shown in Fig. 5B also present an evident increase of the sub-G1 peak (from 1.2% to 37.2%) following the treatment with a fixed concentration of α-bisabolol (10 μM) for indicated time (0, 12, 24, 36, 48 h). The results we obtained corresponded very well to that of cell morphology observation and DNA fragmentation, indicating a dose- and time-dependent apoptosis-inducing effect of α-bisabolol on HepG2 cells.

3.4. The cleavage of poly(ADP-ribose) polymerase and the activation of caspases

The effect of α-bisabolol on poly(ADP-ribose) polymerase (PARP-1) and caspases were examined by western blot analysis. PARP-1, an intracellular substrate of caspase-3, was shown to be cleaved from 116- to 85-kDa fragments in cells treated with α-bisabolol for 48 h at 7.5 μM and higher concentration (Fig. 6).

Since caspases activation plays a very important role in apoptosis, we investigated the activation of caspases-3, -8 and -9 after cells were treated with α-bisabolol at different concentrations (0, 2.5, 5, 7.5, 10 μM) for 48 h. As shown in Fig. 7A, the active form of caspases-8 and -9 was observed. Similarly, the activation of caspase-3 was determined by the reduction of procaspase-3. These results indicated that α-bisabolol activates initiator and executioner caspases involved in both the extrinsic and the intrinsic pathways. Since caspase-8 functions as an initiator in the death receptor pathway, we next attempted to characterize the hierarchy between these three caspases. As shown in Fig. 7B, the active form of caspases-8 and -9 was observed rather earlier than caspase-3, which suggested that caspase-8 may functions as an upstream regulator in α-bisabolol-induced apoptosis.

3.5. Involvement of mitochondrial pathway in α-bisabolol-induced apoptosis

The mitochondrial apoptotic pathway is dependent on the release of cytochrome c from the mitochondria to cytosol, which is believed to be an initiator of the caspase cascade. Pro-apoptotic members of the Bcl-2 family such as Bax, Bak and Bid play key roles in a variety of drug-induced cytochrome c release. To investigate

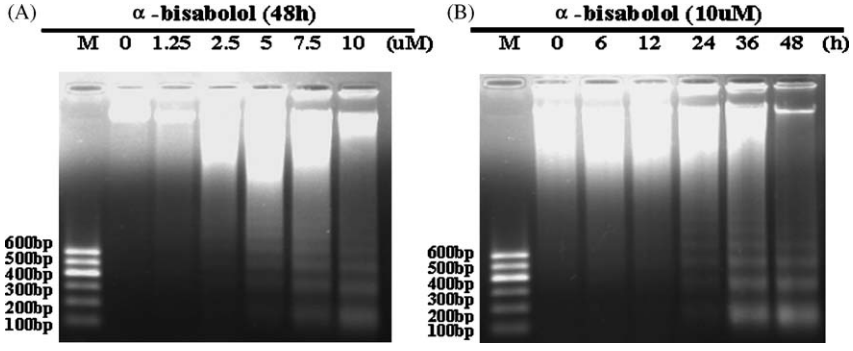

Fig. 4. DNA fragmentation after exposure of HepG2 cells to α-bisabolol. After treated with α-bisabolol for 48 h at indicated concentration (A) or with 10 μM α-bisabolol for indicated time (B), cells were treated with lysis buffer, centrifuged for 5 min at 1600 × *g*, the extraction is repeated with the same treatment and the supernatant is collected. The supernatants were treated for 2 h with RNase A (final concentration, 1 mg/ml) containing 1% SDS, followed by digestion with Proteinase K (final concentration 2 mg/ml) overnight at 56 °C. After the addition of 1/2 vol. 5 M ammonium acetate, the DNA is precipitated with 2.5 vol. ethanol overnight at −20 °C. After centrifugation at 12,000 × *g* for 15 min at 4 °C, final DNA pellets were dried and dissolved in gel loading buffer, separated by 1% agarose gel electrophoresis at 45 V for 1.5 h, and stained with ethidium bromide (EB).

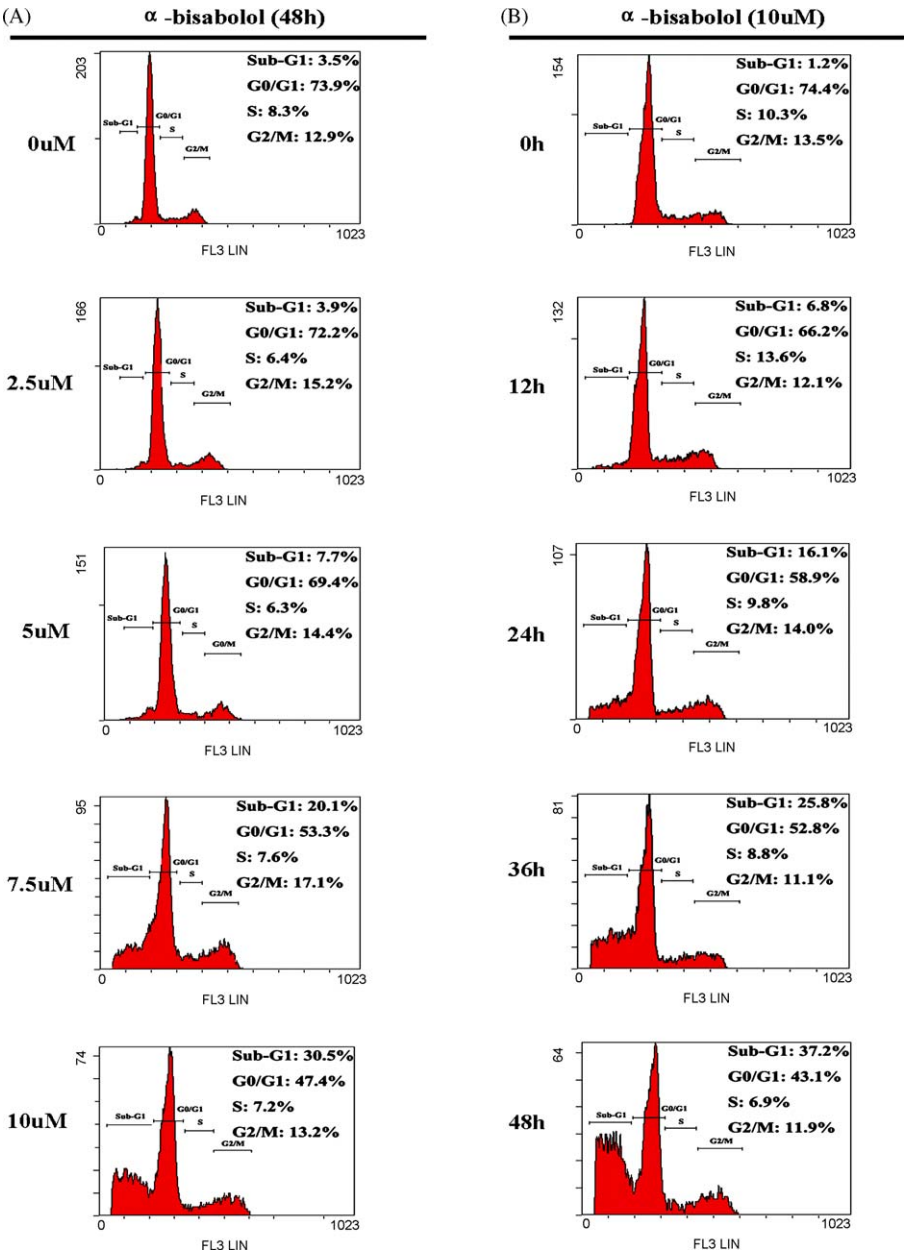

Fig. 5. Effect of α-bisabolol on cell cycle. HepG2 cells were incubated with α-bisabolol for 48 h at indicated concentration (A) or incubated with 10 μM α-bisabolol for indicated time (B), then were collected and suspended in 75% ethanol at −20 °C overnight. Fixed cells were centrifuged at 600 × *g* for 5 min and washed with ice-cold PBS. Cells were incubated with 100 μg/ml RNase A in PBS for 1 h at 37 °C before staining in the dark with PI (20 μg/ml) for 30 min at 4 °C. Samples were applied to a flow cytometer (Becton Dickinson). For each analysis, a minimum of 10,000 cells was counted.

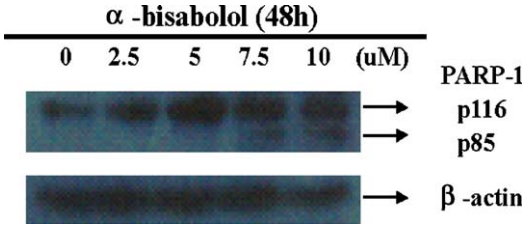

Fig. 6. Cleavage of PARP-1 by treatment with α-bisabolol. HepG2 cells were treated with α-bisabolol for 48 h at indicated concentration. Cells were collected and washed with ice-cold PBS, then treated with ice-cold lysis buffer with 1% PMSF. Cell lysates were centrifuged at 12,000 × *g* at 4 °C for 5 min and equal amounts of protein was separated by 8% SDS-PAGE, electrotransferred to PVDF membrane and then immunoblotted with the corresponding antibodies. β-Actin was used for loading control. Data were representative of three different experiments.

whether the mitochondrial apoptotic pathway is also involved in α-bisabolol-induced apoptosis, the effect of α-bisabolol on Bax, Bak, Bid and cytochrome c was monitored. Fig. 8 shows that α-bisabolol treatment caused a decrease in mitochondrial cytochrome c and a concurrent increase in cytosolic cytochrome c, as well as Bak. On the contrary, after α-bisabolol treatment, a translocation of full length Bax and Bid from cytosol to mitochondria was observed.

3.6. Characterization of several signal molecules involved in apoptosis induced by α-bisabolol

Based on the results obtained above, we further used western blot to identify several signaling molecules that might be involved in this apoptotic cascade. As demonstrated in Fig. 9, after

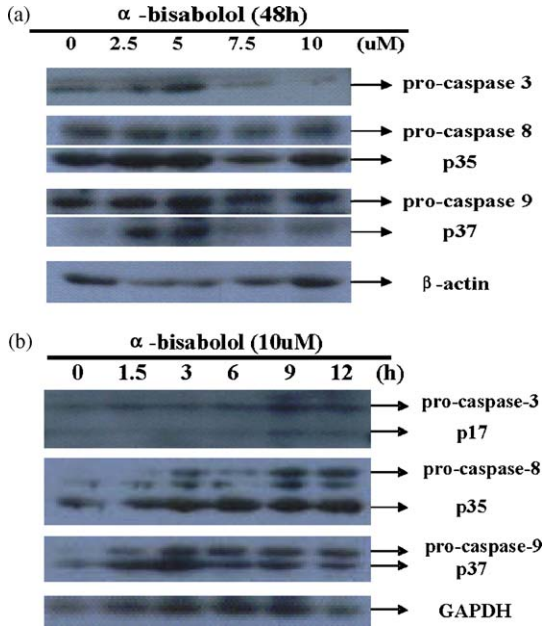

Fig. 7. Effect of α-bisabolol on the activation of caspases. (A) After treated with α-bisabolol at indicated dose for 48 h, cells were collected and washed with ice-cold PBS, then treated with ice-cold lysis buffer with 1% PMSF. Cell lysates were centrifuged at 12,000 × *g* at 4 °C for 5 min and equal amounts of protein was separated by 12% SDS-PAGE, electrotransferred to PVDF membrane and then immunoblotted with the corresponding antibodies. (B) After treated with 10 μM α-bisabolol, the hierarchy between caspases-3, -8 and -9 was characterized. The active forms of caspase-8 (p35) and caspase-9 (p37) were observed rather earlier than that of caspase-3 (p17). β-Actin and GAPDH were used for loading control. Data were representative of three different experiments.

α-bisabolol treatment, the expression of Bcl-2, one of the anti-apoptotic members of the Bcl-2 family, was significantly suppressed. While the results of p53, NFκB and Fas we observed here were quite different. It can be seen in Fig. 9 that α-bisabolol strongly increased the expression of p53 and NFκB, as well as Fas. We also observed that the activation of Fas was much earlier than that of p53 and NFκB.

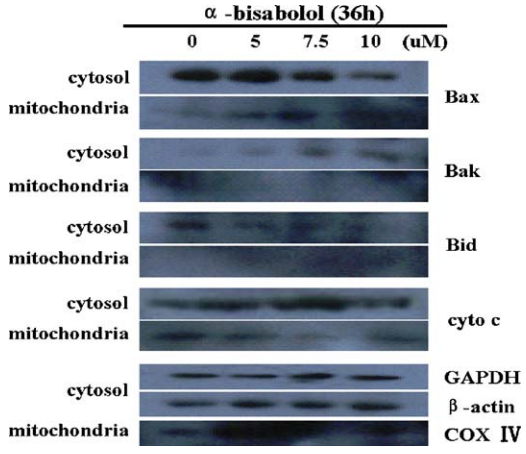

Fig. 8. Effect of α-bisabolol on the translocation of Bax, Bak and Bid, and the release of cytochrome c(cyto c) from mitochondria. After treated with α-bisabolol at indicated dose for 36 h, cells were collected and the mitochondrial and cytosolic proteins were isolated using the Mitochondria/cytosol Fractionation Kit. Equal amounts of protein were separated by 8–15% SDS-PAGE, electrotransferred to PVDF membrane and then immunoblotted with the corresponding antibodies. β-Actin, GAPDH and COX IV were used for loading control. Data were representative of three different experiments.

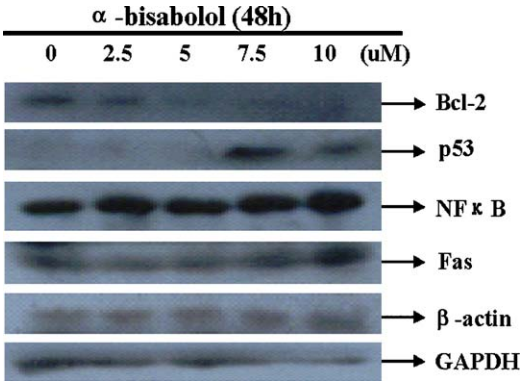

Fig. 9. Effect of α-bisabolol on the expression of Bcl-2, p53, NFκB and Fas in HepG2 cells. Cells were seeded into 6-well culture plates and treated with α-bisabolol at indicated dose for 48 h. Cells were collected and washed with ice-cold PBS, then treated with ice-cold lysis buffer with 1% PMSF. Cell lysates were centrifuged at 12,000 × *g* at 4 °C for 5 min and equal amounts of protein was separated by 10–12% SDS-PAGE, electrotransferred to PVDF membrane and then immunoblotted with the corresponding antibodies. β-Actin and GAPDH were used for loading control. Data were representative of three different experiments.

4. Discussion

In the present study, we have found that α-bisabolol was cytotoxic to several human cancer cell lines (PC-3, Hela, ECA-109 and HepG2), and could effectively induce apoptosis in HepG2 cells in a dose- and time-dependent manner, which extends the previous notion on the cytotoxic effect of α-bisabolol on malignant cells [21–23]. Moreover, we have described the signaling pathway that involved in α-bisabolol-induced apoptosis in HepG2 cells, which was composed of both an extrinsic and an intrinsic pathway, p53 and NFκB were involved as well.

In the cell viability assay (Fig. 2), we observed cytotoxic effect of α-bisabolol on several human cancer cell lines different from those mentioned in previous studies [21–23], which indicated that the cytotoxic action of α-bisabolol can be applied to a broad spectrum of tumor cell species. We next examined the cell morphology abnormalities (Fig. 3A and B), formation of DNA fragmentation (Fig. 4A and B), increase in sub-G1 peak and the cleavage of PARP-1, and came to the conclusion that α-bisabolol-induced apoptosis in HepG2 cells in a dose- and time-dependent manner.

Our study is the first to prove the extrinsic pathway might be involved in α-bisabolol-induced apoptosis, as demonstrated by evident caspase-8 activation at earlier time points than caspase-3 (Fig. 7B), and the increase expression of Fas (Fig. 9). Caspases have been known to play a central role during apoptosis induced by a variety of stimuli [27]. The hierarchic activation of caspases can be decisive in the determination of whether an extrinsic or an intrinsic apoptotic pathway to be [28]. We observed the activation of both caspases-3, -8 and -9, which indicated the caspases functions in both the extrinsic and the intrinsic were involved in α-bisabolol-induced apoptosis (Fig. 7A). In the short-time course assay (Fig. 7B), we detected the active forms of caspase-8 rather earlier than caspase-3 and -9, which clearly demonstrated that caspase-8 might play a role as an upstream initiator, and the extrinsic pathway might be involved in α-bisabolol-induced apoptosis. This speculation could be further supported by the result of the increasing expression of Fas (Fig. 9). Fas, also called CD95 or APO-1, is a cell surface receptor belonging to the TNF receptor superfamily, can mediates apoptosis by ligation with an agonistic anti-Fas antibody or Fas ligand. Stimulation of Fas results in the aggregation of its intracellular death domains, leading to the formation of the death-inducing signaling complex (DISC) [29]. DISC recruits and activates caspase-8, resulting in the release of the

active forms of caspase-8 into the cytosol [30]. The hierarchic activation of caspases, together with the up-regulated expression of Fas we observed clearly indicated that Fas-mediated signaling pathway might play an important role in α-bisabolol-induced apoptosis in HepG2 cells.

Besides caspases, the Bcl-2 family members are also playing critical role in the regulation of apoptosis. BCL-2 family, which is composed of both pro-apoptotic molecules (Bax, Bcl-Xs, Bak, Bid, Bad, Bim, Bik) and anti-apoptotic molecules (Bcl-2, Bcl-XL, Bcl-W, Mcl-1, A1), control the release of mitochondrial cytochrome c by modulating the permeability of the outer mitochondrial membrane [31,32]. Here we have shown that α-bisabolol treatment induced cytochrome c release from mitochondria to cytosol (Fig. 8). Immunoblotting for Bax, Bak and Bid was also carried out in both the mitochondrial and the cytosol fractions, and we observed Bax as well as Bid translocated from cytosol to mitochondria, whereas Bak exhibited an very unlike trend (Fig. 8). Effect of α-bisabolol on expression of Bcl-2 was also shown in Fig. 9, and we can see the expression of Bcl-2 was down-regulated, which corresponded well with the standpoint that anti-apoptotic molecules such as Bcl-2 and Bcl-XL can inhibit the activation of Bax and Bak through interacting with them, and ultimately resulted in the mitochondrial dysfunction [33,34]. These results might suggest that the mitochondrial pathway also play important role in α-bisabolol-induced apoptosis in HepG2 cells. What is interesting is the observation of translocation of Bak from mitochondria to cytosol, which is quite different from that of Bax (Fig. 9). The role of Bak might play in α-bisabolol-induced apoptosis in HepG2 cells remains to be further studied.

Besides caspases and Bcl-2 family members, α-bisabolol also can activates various signaling molecules such as p53 and NFκB. As shown in Fig. 9, after α-bisabolol treatment, the expression of both p53 and NFκB were up-regulated, which was similar with the results of several reported researches on other anticancer drugs [35,36]. As we know, p53 functions as a central mediator for organizing cell responses to various stress and anticancer drugs with apoptosis, G1-phase arrest, and DNA repair [37]. p53, as a transcription factor, exerts its effects on many genes whose products are involved in apoptosis [38–40]. In recent years, the potential relationship between Fas and p53 expression has been studied. Owen-Schaub LB et al. argued that Fas/APO-1 might be a target gene for transcriptional activation by p53 [41]. Petak I et al. found that elevating Fas expression in a p53-dependent manner might enhance the sensitivity of tumors to Fas-mediated apoptosis [42]. Monte SMdl et al. observed significantly increased expression of p53 and Fas and widespread, intense p53/Fas immunoreactivity in Alzheimer's disease [43], and Muller M et al. also found that bleomycin up-regulated expression of p53 and Fas in cells carrying wild-type p53 and increased sensitivity towards Fas-mediated apoptosis, but had no same effect on cells carrying mutated p53 [44]. Nuclear factor-κB (NFκB) is also known for its anti-apoptotic function of transcriptional regulating of various anti-apoptotic genes involved in survival signaling [45,46], and NFκB-linked pathway was reported to be involved in various drugs induced apoptosis [47–50]. Studies on the relationship between p53 and NFκB have been widely carried out in recent years [51–53]. Ryan KM et al has shown that induction of p53 might activate NFκB and inhibition or loss of NFκB activity abrogated p53-induced apoptosis [51], whereas Webster and Perkins argued that both NFκB and p53 can inhibit each other to stimulate gene expression, shedding light on the regulation of the transcriptional deciding mechanisms that govern apoptosis [52], and Tergaonkar V et al. also demonstrated that p53 stabilization is decreased upon NFκB activation, implying a role that NFκB might play in blocking chemotherapy-induced p53 activation [53]. Besides p53, the role for NFκB in regulating Fas-mediated apoptosis was also examined by Marusawa H et al, suggesting NFκB was involved in suppression of Fas-mediated apoptosis in human hepatocyte cell lines [54]. In our study, we speculated that α-bisabolol might first stimulate Fas-mediated apoptosis, then elevating expression of Fas could intrigued the activation of p53 and NFκB. Since p53 and NFκB both can play anti- or pro-apoptotic role in drug-induced apoptosis, the virtual apoptotic effect of α-bisabolol should mainly depend upon the balance of their activities. These data and speculations might be helpful for understanding the molecular mechanism underlying α-bisabolol cytotoxicity in tumor cells, while the role that p53, Fas and NFκB might play and the relationship between them in anticancer drug-induced apoptosis are still unknown. Further studies are still necessary to explain the definite role of these signaling molecules in chemotherapy to optimize future cancer therapy.

In conclusion, our findings demonstrate that α-bisabolol was toxic to several human carcinoma cell lines (PC-3, Hela, ECA-109 and HepG2), and can efficiently inhibit cell growth. Our further studies have shown that α-bisabolol might induce apoptosis in HepG2 cell dose- and time-dependently manner through both an extrinsic and an intrinsic pathway, and p53 and NFκB were involved either. These results provide further insight into α-bisabolol-induced apoptosis and deepen our previous cognition of the toxicity and anticancer activity of α-bisabolol, and can increase the possibility of developing α-bisabolol to be a promising future chemotherapeutic agent.

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