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Inhibition of cell growth and induction of apoptosis by bilobalide in FaDu human pharyngeal squamous cell carcinoma

Kyung In Jeong, Su-Gwan Kim, Dae-San Go, and Do Kyung Kim*

The Institute of Dental Science, Chosun University, Gwangju 61452, Republic of Korea

Bilobalide isolated from the leaves of *Ginkgo biloba* has several pharmacological activities such as neuroprotective, anti-inflammatory, and anticonvulsant. However, the effect of bilobalide on cancer has not been clearly established. The main purpose of this study was to investigate the effect of bilobalide on cell growth and apoptosis induction in FaDu human pharyngeal squamous cell carcinoma. This was examined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, nuclear 4′,6-diamidino-2-phenylindole dihydrochloride staining, DNA fragmentation analysis, and immunoblotting. Bilobalide inhibited the growth of FaDu cells in dose- and time-dependent manners. Treatment with bilobalide resulted in nuclear condensation and DNA fragmentation in FaDu cells. Furthermore, it promoted the proteolytic cleavage of procaspase-3/-7/-8/-9 with increase in the amount of cleaved caspase-3/-7/-8/-9. Bilobalide-induced apoptosis in FaDu cells was mediated by the expression of Fas and the activation of caspase-8, caspase-3, and poly (ADP-ribose) polymerase. Immunoblotting revealed that the antiapoptotic mitochondrial protein Bcl-2 was downregulated, but the proapoptotic protein Bax was upregulated by bilobalide in FaDu cells. Bilobalide significantly increased Bax/Bcl-2 ratio. These results suggest that bilobalide inhibits cell proliferation and induces apoptosis in FaDu human pharyngeal squamous cell carcinoma via both the death receptor-mediated extrinsic apoptotic pathway and the mitochondrial-mediated intrinsic apoptotic pathway.

Keywords: Bilobalide, Cell death, Apoptosis, Anti-cancer therapy

Introduction

Oral cancer is a cancer occurring in oral mucosa, lips, tongue, periodontal and pharynx, and more than 90% are epithelial cancers [1]. Oral cancer has a low incidence of cancer (about 2%), but it belongs to a cancer with a high risk of metastasis because it is adjacent to important human organs [1,2]. It is widely used as a research model of cancer progression because the progress of cancer can be easily observed with the naked eye and the result of cancer treatment can be easily tracked [3,4]. However, the exact mechanism of oral cancer development is still unknown. The most widely used therapies are topical treatments such as surgery or radiation therapy, but they have not been able to effectively inhibit the growth and metastasis of tumors, and have been using anticancer agents such as cisplatin in combination [3,4]. However, in combination therapy with anticancer drugs is limited due to severe side effects such as gastrointestinal complications, impaired immune function, and decreased bone marrow function, and so on [3,4]. Therefore, as an alternative method, many efforts are being

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***Correspondence to:** Do Kyung Kim, E-mail: kdk@chosun.ac.kr https://orcid.org/0000-0001-6254-946X

made to develop anticancer drugs derived from natural materials that can maintain the effect of anticancer agents while maximally reducing side effects of anticancer agents [5].

Recently, efforts to develop various medicines including anticancer drugs using herbal medicines have been increasing in various industrial fields all over the world [6,7]. Among them, anticancer substances are known to inhibit the growth of cancer cells or induce cancer cell death through various mechanisms in specific cancer cells, and the mechanism of cancer cell growth inhibition plays an important role depending on the kind of cell and stimulation type [8,9]. And also, it should be able to kill cancer cells with minimal side effects [8,9].

Apoptosis is a major form of programmed cell death controlled by genes that plays an important role in regulating tissue development and homeostasis in eukaryotes [10-12]. Most of the anticancer substances cause apoptosis, and thus inhibit cancer cell proliferation, thereby acting as a chemotherapeutic agent for cancer [13,14]. Therefore, the apoptosis of cancer cells caused by the use of these anticancer agents has become an important indicator of the results of cancer treatment [11,12]. Apoptosis, which is one of the important ways of causing cancer cell death, can occur via a death receptor-dependent extrinsic pathway or a mitochondria-dependent intrinsic pathway, which may be induced by a treatment with chemotherapeutic agents in cancer [15,16].

Bilobalide (Fig. 1) is a sesquiterpenoid, which has a 15-carbon skeleton, isolated from leaves of *Ginkgo biloba*, and also exists in minor amounts in the roots [17,18]. It has been substantial experimental evidence indicates that bilobalide possesses several pharmacological activities, such as neuroprotective, anti-inflammatory and anticonvulsant effects in various experimental models [17-20]. However, bilobalide effects on cancers are not clearly established.

In this study, therefore, the effect of bilobalide on cell growth and the mechanism of cell death elicited by bilobalide were examined in FaDu human pharyngeal squamous cell carcinoma.

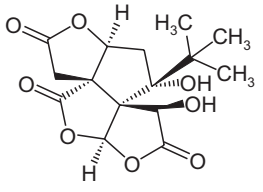

Fig. 1. Chemical structure of bilobalide.

Materials and Methods

1. Materials

Bilobalide (Fig. 1), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were supplied by Sigma (St Louis, MO, USA). Anti-cleaved caspase-3, -7, -8, -9, anti-Fas, anti-cleaved poly (ADP-ribose) polymerase (PARP), anti-Bax and anti-Bcl-2 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Other analytical reagents were purchased based on the analytical grade.

2. Cell line and cell cultures

The FaDu human pharyngeal squamous cell carcinoma was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as according to the cell culture instructions provided by ATCC. Briefly, the FaDu cells were grown in Eagle's Minimum Essential Medium (ATCC) with fetal bovine serum (Invitrogen; Carlsbad, CA, USA) to a final concentration of 10%. The FaDu cells were maintained as monolayers in plastic culture plate at 37℃ in a humidified atmosphere containing 5% CO_2.

3. Cell viability test (MTT assay)

The FaDu cells were seeded at a concentration of 5×10^3 cells/well in 24-well plates. After 24 hours growth, the cells were treated with bilobalide at various concentrations and incubation times. The cell viability test was evaluated using the MTT assay. At least 4 separate experiments were performed on each concentration/exposure time combination.

4. DAPI staining

Nuclear staining with DAPI was performed and the level of apoptosis was examined. The FaDu cells were cultured in 24-well plates at a seeding density of 5×10^3 cells/well. After 24 hours growth, the FaDu cells were treated with 0, 30 or 300 μM bilobalide for 72 hours. The FaDu cells were fixed with 1% paraformaldehyde for 30 minutes and washed twice with phosphate buffered saline (PBS). The cells were permeated with ice-cold ethanol for 5 minutes at room temperature and washed twice with PBS. The fixed FaDu cells were stained with 300 nM DAPI for 5 minutes at room temperature in the

dark and washed twice with PBS. The stained FaDu cells examined by fluorescent inverted microscopy (IX71; Olympus, Tokyo, Japan).

5. DNA fragmentation analysis

Following treatment with 0 or 300 μM bilobalide for 72 hours, approximately 5 × 10^6 cells were collected and transferred to lysis buffer containing 100 mM NaCl, 10 mM EDTA, 300 mM Tris-HCl, pH 7.5, 200 mM sucrose, 0.5% SDS and 0.5 mg/mL proteinase K and incubated at 65℃. The genomic DNA extraction was performed according to the previously described method with minor modifications [21]. The genomic DNA was visualized on 2% agarose gel in the presence of 0.5 μg/mL ethidium bromide.

6. Immunoblotting

The FaDu cells were treated with 0 or 300 μM bilobalide for 72 hours. Immunoblotting was done according to the previously described method with minor modifications [22]. The anti-cleaved caspase-3, -7, -8, -9, anti-Fas, anti-cleaved PARP, anti-Bax or anti-Bcl-2 antibody (1:1,000 dilution, Cell Signaling Technology, Inc.) was used as the primary antibody.

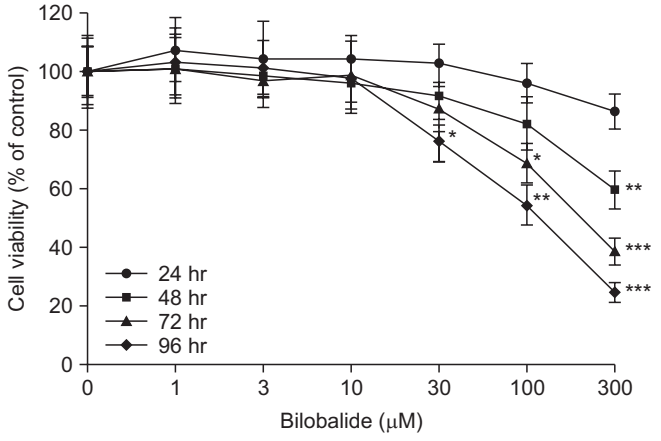

Fig. 2. Effects of bilobalide on cell viability in FaDu human pharyngeal squamous cell carcinoma. The FaDu cells were treated with various concentrations of bilobalide or without bilobalide for 24 (circle), 48 (square), 72 (triangle) and 96 hours (diamond). The cell viabilities were determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays. The percentage of cell viability was calculated as a ratio of $A570_{nms}$ of bilobalide treated cells and untreated control cells. Each data point represents the mean ± standard error of the mean of four experiments.
*p < 0.05 vs. control, **p < 0.01 vs. control and ***p < 0.001 vs. control (the control cells measured in the absence of bilobalide).

7. Data analysis

All experiments were performed at least 4 times. The results were presented as mean ± standard error of the mean. The statistical significance was analyzed by using Student's t-test for two groups and one way analysis of variance for multi-group comparisons. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was considered statistically significant.

Results

1. Cytotoxic effect of bilobalide in FaDu cells

To analyze the effect of bilobalide on the viability of FaDu cells, the cells were treated with bilobalide at various concentrations for 0–96 hours, and then the MTT assay was performed. When the FaDu cells were treated with 1 to 300 μM bilobalide for 0–96 hours, bilobalide inhibited the proliferation of FaDu cells in the dose- and time-dependent manners (Fig. 2), suggesting that bilobalide induces FaDu cell death. The IC_{50} values of bilobalide on the FaDu cell viability are shown in Table 1.

2. Induction of apoptosis by bilobalide in FaDu cells

The nuclear morphological changes were assessed to determine the level of apoptosis by DAPI staining. The nuclei of the control FaDu cells (0 μM) had a normal regular and oval shape (Fig. 3A). Treatment with 30 or 300 μM bilobalide for 72 hours resulted in nuclear condensation and fragmentation, which are the characteristics of apoptosis (Fig. 3A). To determine if apoptosis is indeed the underlying mechanism for the reduced cell proliferation observed, the FaDu cells treated with bilobalide were subjected to DNA fragmentation assay. The formation of DNA ladder in the FaDu cells treated with 300 μM

Table 1. Anti-proliferative effect of bilobalide in FaDu cells

Time (hr)	IC_{50} (μM)
24	ND
48	ND
72	223.1 ± 37.2
96	132.6 ± 21.7

The IC_{50} values represent the mean ± standard error of the mean for four experiments.
ND, not detected.

bilobalide was observed (Fig. 3B).

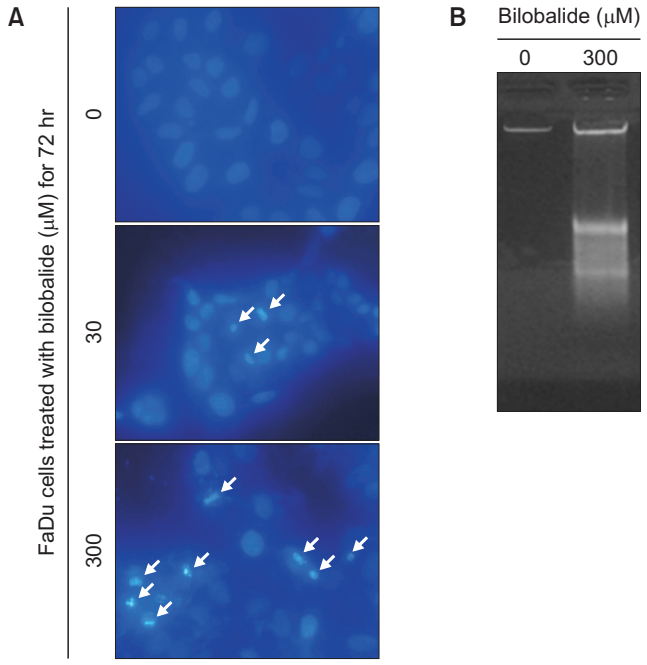

Fig. 3. Induction of apoptosis by bilobalide in FaDu cells. (A) Changes in nuclear morphology by bilobalide. The cells were treated with 0, 30 or 300 μM bilobalide for 72 hours. Representative fluorescence photomicrographs show the nuclei morphology of FaDu cells. The arrows indicate chromatin condensation, reduced nuclear size and nuclear fragmentation typically observed in apoptotic FaDu cells. (B) Fragmentation of genomic DNA by bilobalide in FaDu cells. The cells were treated with 0 or 300 μM bilobalide for 72 hours and nuclear DNA was subjected to agarose gel electrophoresis.

3. Extrinsic death receptor-dependent and intrinsic mitochondria-dependent apoptotic signaling pathways induced by bilobalide in FaDu cells

To determine the cellular apoptotic pathways associated with bilobalide-induced FaDu cell death, immunoblotting was performed and the expression levels of apoptotic proteins were evaluated. Fas, which is an apoptotic ligand that triggers the death receptor-dependent extrinsic apoptotic pathway in cancer cells [23,24], was induced significantly by bilobalide (Fig. 4A). The level of cleaved caspase-8, the downstream target of pro-apoptotic factor Fas, increased following bilobalide treatment (Fig. 4A). These results suggest the involvement of the extrinsic death receptor-mediated apoptosis pathway in bilobalide-induced FaDu cell apoptosis.

The expression level of Bcl-2, anti-apoptotic factors associated with the intrinsic mitochondria-dependent apoptosis pathway, was downregulated by bilobalide, while that of mitochondria-dependent pro-apoptotic factor such as Bax was upregulated by bilobalide in FaDu cells (Fig. 4B). And also, bilobalide treatment increased the expression level of cleaved caspase-9 in FaDu cells (Fig. 4B). This result indicates that bilobalide-induced FaDu cell death involves the intrinsic mitochondria-dependent apoptosis pathway.

Both cleaved caspase-8 and caspase-9, acting in the extrinsic death receptor-mediated and intrinsic mitochondria-dependent apoptosis pathways, induced the expression of cleaved caspase-3/-7 and PARP in FaDu cells following bilobalide treatment (Fig. 4C). Therefore, bilobalide-induced FaDu cell death is thought to be mediated by both the extrinsic

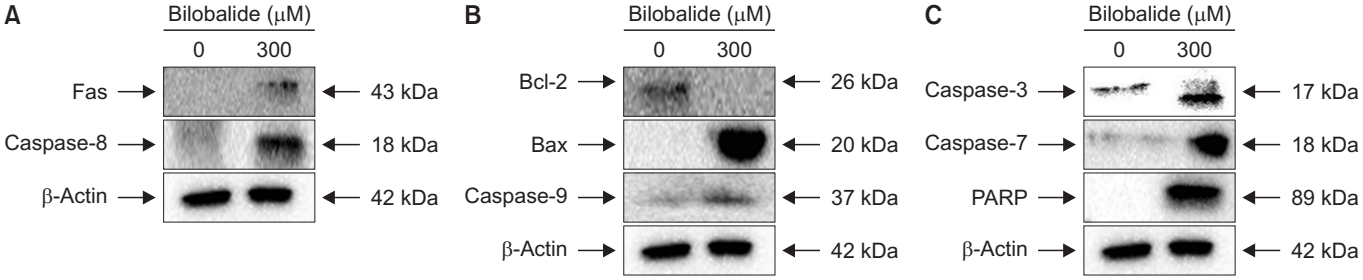

Fig. 4. Extrinsic death receptor-mediated as well as intrinsic mitochondria-dependent apoptotic signaling pathways induced by bilobalide in FaDu cells. The FaDu cells were treated with 0 or 300 μM bilobalide for 72 hours. The cell lysate was prepared and analyzed by immunoblotting as described in "Materials and Methods". (A) Extrinsic death receptor-mediated apoptotic signaling pathway induced by bilobalide. Bilobalide upregulated the expression level of the death receptor ligand Fas and subsequently activated the extrinsic death receptor-mediated apoptotic signaling pathway through the cleavage of caspase-8 in FaDu cells. (B) Intrinsic mitochondria-dependent apoptotic signaling pathway induced by bilobalide. Bilobalide downregulated anti-apoptotic factors Bcl-2 associated with the intrinsic mitochondria-dependent apoptotic pathway and upregulated the mitochondria-dependent pro-apoptotic factor Bax in FaDu cells. (C) Extrinsic death receptor-mediated and intrinsic mitochondria-dependent apoptosis signaling pathways via the activation of caspase-3/-7 and poly (ADP-ribose) polymerase (PARP) induced by bilobalide. Cleaved caspase-8 and cleaved caspase-9 induced the activation of caspase-3/-7 and PARP in FaDu cells treated with bilobalide.

death receptor-dependent and intrinsic mitochondria-dependent apoptotic signaling pathways.

Discussion

Bilobalide (Fig. 1) is a biologically active sesquiterpenoid present in *Ginkgo biloba* [17,18]. Recently, it has received attention because of its effect on the central nervous system [19,20]. Actually, it has been demonstrated its neuroprotective effects based on *in vitro* and *in vivo* experimental models [17-20]. However, the bilobalide effects on cancer cells are not clearly established. In this study, therefore, the cytotoxic activity of bilobalide and the mechanism of cell death exhibited by bilobalide were examined in FaDu human pharyngeal squamous cell carcinoma. The present study demonstrated that the bilobalide can act as apoptotic inducer in human pharyngeal squamous cell carcinoma.

In cell viability test, the bilobalide inhibited growth of FaDu cells in a concentration- and a time-dependent manner (Fig. 2). These results speculated that the bilobalide has cytotoxicity for pharyngeal squamous cell carcinoma and potential value for anti-cancer drug discovery.

Induction of apoptosis in cancer cell growth inhibition process is a useful strategy for the development of anticancer drugs from herbal medicines [8,9]. Therefore, researchers have conducted studies to induce apoptosis of cancer cells from a variety of natural products, including herbal medicines [8,9]. In this study, we examined the nuclear morphological changes with DAPI staining and DNA fragmentation analysis to confirm whether apoptosis is involved in the inhibition of FaDu cell growth by bilobalide. The treatment with bilobalide induced the formation of apoptotic bodies, nuclear condensation and DNA fragmentation in FaDu cells (Fig. 3), suggesting apoptotic cell death by bilobalide. Therefore, these results indicated that bilobalide-induced FaDu cell death is mediated via the apoptotic signaling pathway.

The caspase-3, -7, -8, and -9 can serve as effector caspases of apoptotic cell death in mammalian cells [14,25-27]. The caspase-3, -7, -8, and -9 are synthesized as inactive proenzymes, which require proteolytic activation to cleaved enzymes by a range of stimuli [14,25-27]. The results in this study show that low levels of cleaved capase-3, -7, -8, and -9 were present in bilobalide-untreated FaDu cells, and the amount of cleaved enzymes was dramatically increased after bilobalide treatment in FaDu cells (Fig. 4). These data suggest that the bilobalide induces apoptotic cell death through both the death receptor-mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway by the activation of

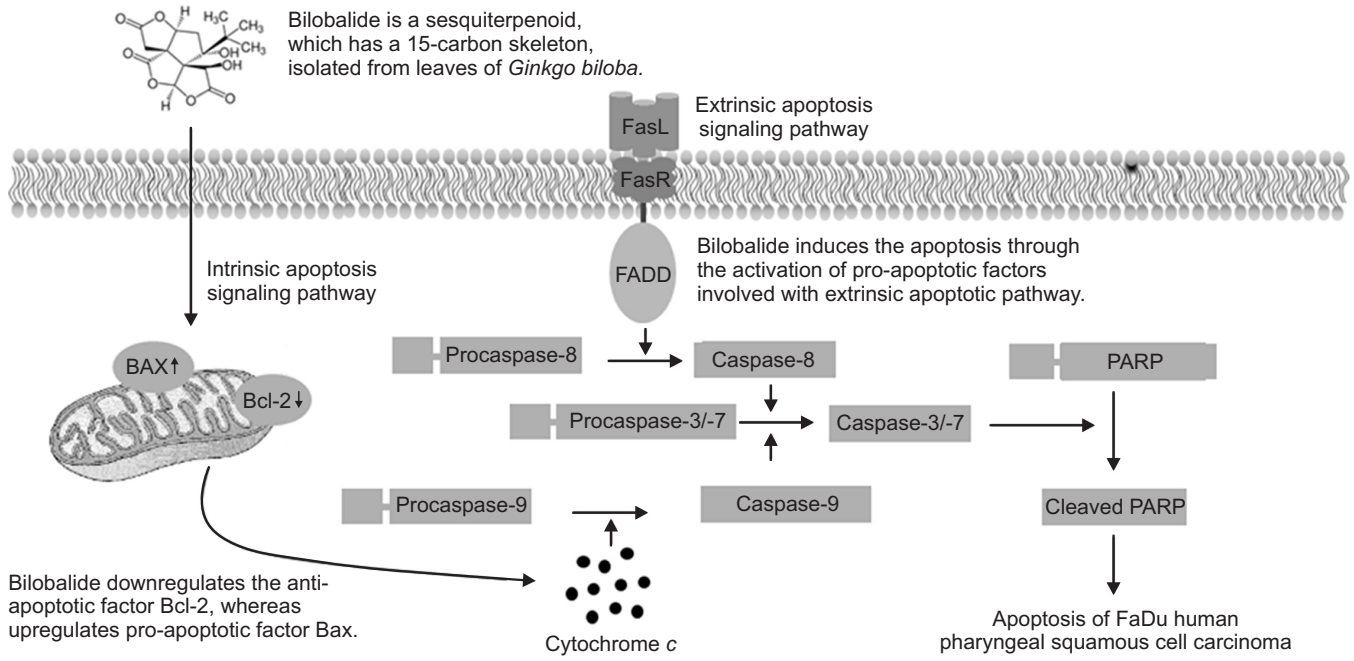

Fig. 5. Apoptotic signaling pathway induced by bilobalide in FaDu human pharyngeal squamous cell carcinoma.
PARP, poly (ADP-ribose) polymerase.

caspases-3/-7/-8/-9 in FaDu cells.

The Fas, an important regulator of apoptosis, binds to the receptor FasR that spans the surface of target cells and then initiates the death receptor-mediated extrinsic apoptotic pathway through the activation of caspase-8, -3 and PARP [23,24]. In this study, the level of Fas protein expression was significantly elevated by bilobalide in FaDu cells (Fig. 4A). After that, the Fas stimulated by bilobalide induced caspase cascade, which results in the activation of apoptotic factors including cleaved caspase-8 and -3 (Fig. 4A and 4C) [23,24]. Finally, activated caspase-3 cleaved the major substrate, PARP, resulting in apoptotic cell death in FaDu cells (Fig. 4C) [23,24]. Therefore, these results suggest that bilobalide-induced apoptosis in FaDu cells is mediated by the death receptor-mediated extrinsic apoptotic pathway through the Fas/PARP axis.

In sequence, we evaluated the effect of bilobalide on the expressions of Bax and Bcl-2 proteins in FaDu cells. The pro-apoptotic protein Bax and the anti-apoptotic mitochondrial protein Bcl-2 are important regulators of cytochrome *c* release from the mitochondria [27-29]. In addition, the Bcl-2 is localized to the mitochondrial membrane and regulates apoptosis by permeabilizing the mitochondrial membrane, leading to the release of cytochrome *c* [30]. In this study, treatment of FaDu cells with bilobalide increased the level of Bax protein expression (Fig. 4B), but decreased the level of Bcl-2 protein expression (Fig. 4B). The ratio of Bax/Bcl-2 is one of the indicators of the mitochondria-mediated intrinsic apoptotic pathway [31]. The bilobalide-induced apoptosis appears to involve Bax/Bcl-2 signal transduction because the bilobalide has increased this ratio in FaDu cells [31]. Thus, it is suggested that the bilobalide induces apoptosis in FaDu cells involving the death receptor- and mitochondrial-signal transduction pathways. On the other hand, the mechanisms of cell apoptosis induced by bilobalide in FaDu cells were not fully understood. Therefore, further studies are needed to investigate the precise cellular and molecular mechanisms of cell apoptosis induced by bilobalide.

In conclusion, these results suggest that the bilobalide inhibits cell proliferation and induces apoptotic cell death in FaDu human pharyngeal squamous cell carcinoma through both the death receptor-mediated extrinsic apoptotic pathway and the mitochondria-mediated intrinsic apoptotic pathway (Fig. 5). Furthermore, the results of this study suggest that the bilobalide can provide a strategy to prevent and treat squamous cell carcinoma.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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