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조선대학교 대학원

치의학과

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사람 인두 편평세포암종 FaDu에서 bilobalide에 의한 세포성장 억제 및 apoptosis 유도

2018년 2월 23일

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이 논문을 치의학 박사학위신청 논문으로 제출함.

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## 조선대학교 대학원

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# ABSTRACT

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

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Bilobalide isolated from leaves of *Ginkgo biloba* has several pharmacological activities such as neuroprotective, anti-inflammatory and anticonvulsant effects. However, the effect of bilobalide on cancers 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 MTT assay, nuclear DAPI staining, DNA fragmentation analysis and immunoblotting in FaDu cells. The bilobalide inhibited the growth of FaDu cells in the dose- and time-dependent manners. Treatment

with bilobalide resulted in nuclear condensation and DNA fragmentation in the FaDu cells. The bilobalide promoted the proteolytic cleavages of procaspase-3/-7/-8/-9 with increases in the amount of cleaved caspase-3/-7/-8/-9. The bilobalide-induced apoptosis in the FaDu cells was mediated by the expression of Fas and activation of caspase-8, caspase-3 and poly(ADP-ribose) polymerase. Immunoblotting revealed the anti-apoptotic mitochondrial protein Bcl-2 to be downregulated, but the pro-apoptotic protein Bax to be upregulated by bilobalide in FaDu cells. The bilobalide significantly increased the Bax/Bcl-2 ratio. These results suggest that the bilobalide inhibits the cell proliferation and induces the apoptotic cell death in FaDu human pharyngeal squamous cell carcinoma via both the death receptor-mediated extrinsic apoptotic pathway and the mitochondria-mediated intrinsic apoptotic pathway.

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KEY WORDS: Bilobalide, Cell death, Apoptosis, Anti-cancer therapy, Cancer cells

# I. 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 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.

# II. 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 (EMEM, ATCC, Rockville, MD, USA) 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°C in a humidified atmosphere containing 5% $CO_2$.

## 3. Cell viability test (MTT assay)

The FaDu cells were seeded at a concentration of 5 X $10^3$ 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 X $10^3$ cells/well. After 24 hours growth, the FaDu cells were treated with 0, 30 or 300 $\mu$M bilobalide for 72 hours. The FaDu cells were fixed with 1% paraformaldehyde for 30 min and washed twice with PBS. The cells were permeated with ice-cold ethanol for 5 min at room temperature and washed twice with PBS. The fixed FaDu cells were stained with 300 nM DAPI for 5 min at room temperature in the dark and washed twice with PBS. The stained FaDu cells examined by fluorescent inverted microscopy (IX71, Olympus, Japan).

## 5. DNA fragmentation analysis

Following treatment with 0 or 300 $\mu$M bilobalide for 72 hours, approximately 5 X $10^6$ 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°C. 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 $\mu$g/ml ethidium bromide.

## 6. Immunoblotting

The FaDu cells were treated with 0 or 300 $\mu$M 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 (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the primary antibody.

## 7. Data analysis

All experiments were performed at least 4 times. The results were presented as mean ± SEM. 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.

# Ⅲ. 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 $\mu$M 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.

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

| Time (hours) | $IC_{50}$ ($\mu$M) |
|---|---|
| 24 | ND |
| 48 | ND |
| 72 | 223.1 ± 37.2 |
| 96 | 132.6 ± 21.7 |

The $IC_{50}$ values represent the mean ± SEM for four experiments. ND: not detected.

## 2. Changes in the nuclear morphology 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 (Control) had a normal regular and oval shape (Fig. 3A). Treatment with 30 or 300 $\mu$M bilobalide for 72 hours resulted in nuclear condensation and fragmentation, which are the characteristics of apoptosis (Fig. 3A). As quantified in Fig. 3B, 300 $\mu$M bilobalide increased the apoptotic rate of FaDu cells significantly to 65.2 ± 7.9%.

## 3. DNA fragmentation by bilobalide in FaDu cells

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 $\mu$M bilobalide was observed (Fig. 4).

## 4. Activation of caspases by bilobalide in FaDu cells

Treatment with 300 $\mu$M bilobalide for 72 hours significantly promoted proteolytic cleavages of procaspase-3 (Fig. 5) and -7 (Fig. 6) in the FaDu cells, with the increases in the amount of cleaved caspase-3 and -7. Bilobalide also promoted proteolytic cleavages of procaspase-8 (Fig. 7) and -9 (Fig. 8), with the increases in the amount of cleaved caspase-8 and -9 in FaDu cells.

## 5. Apoptosis mediated via Fas/PARP axis by bilobalide in FaDu cells

To determine how bilobalide induce the apoptosis of FaDu cells, immunoblotting was performed to measure the expressions of the Fas and PARP at the protein level. 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. 9). And, the cleaved-PARP was increased by 300 $\mu$M bilobalide compared to the control (Fig. 10).

## 6. Apoptosis-related signal pathways by bilobalide in FaDu cells

The levels of several proteins that are highly relevant to understanding the apoptotic signaling pathways in FaDu cells by bilobalide was measured by immunoblotting analysis. The treatment of FaDu cells with 300 $\mu$M bilobalide for 72 hours increased the level of Bax protein expressions (Fig. 11). On the other hand, the level of Bcl-2 protein expressions in FaDu cells treated with 300 $\mu$M bilobalide for 72 hours decreased (Fig. 12).

# Ⅳ. 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 (Figs. 3 and 4), 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 (Figs. 5, 6, 7 and 8). 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 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. 9). After that, the Fas stimulated by bilobalide induced caspase cascade, which results in the activation of apoptotic factors including cleaved caspase-8 and -3 (Figs. 5 and 7) [23,24]. Finally, activated caspase-3 cleaved the major substrate, PARP, resulting in apoptotic cell death in FaDu cells (Fig. 10) [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. 11), but decreased the level of Bcl-2 protein expression (Fig. 12). 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. 13). Furthermore, the results of this study suggest that the bilobalide can provide a strategy to prevent and treat squamous cell carcinoma.

# V. REFERENCES

1. Silverman S, Gorsky M. Epidemiologic and demographic update in oral cancer: California and national data-1973 to 1985. J Am Dent Assoc. 1990;120:495-499. doi: org/10.14219/jada.archive.1990.0082.
2. Lee JS, Seo HS, Kim SJ, Kim HJ, Kim J, Lee SH, Park YS, Park BK, Kim BS, Kim SK, Jung JY. Studies on the anticancer effect of apigenin in KB cell xenograft nude mouse model. J Life Sci. 2010;20:1519-1524. doi: 10.5352/JLS.2010.20.10.1519.
3. Sacks PG. Cell, tissue and organ culture as *in vitro* models to study the biology of squamous cell carcinomas of the head and neck. Cancer Metastasis Rev. 1996;15:27-51. PMID: 8842478.
4. Todd R, Donoff RB, Wong DT. The molecular biology of oral carcinogenesis: toward a tumor progression model. J Oral Maxillofac Surg. 1997;55:613-623. doi: 10.1016/S0278-2391(97)90495-X.
5. Links M. Lewis C. Chemoprotectants: a review of their clinical pharmacology and therapeutic efficacy. Drugs. 1999;57:293-308. PMID: 10193684.
6. Park DI, Lee JH, Moon SK, Kim CH, Lee YT, Cheong J, Choi BT, Choi YH. Induction of apoptosis and inhibition of telomerase activity by aqueous extract from *Platycodon grandiforum* in human lung carcinama cells. Pharmacol Res. 2005;51:437-443. doi: 10.1016/j.phrs.2004.11.003.
7. Tan ML, Suaiman SF, Najimuddin N, Smian MR, Tengku Muhammad TS. Methanolic extract of *Pereskia bleo* (Kunth) DC. (Cactaceae) induces apoptosis

in breast carcinama, T47-D cell line. J Ethnopharmacol. 2005;96:287-294. doi: 10.1016/j.jep.2004.09.025.

8. Hoshino T, Hara A, Inoue M, Honda J, Imai Y, Oizumi K, Yokoyama MM. Flow cytometric measurement of NK cell cytotoxicity. J Clin Lab Immunol. 1991;36:39-43. PMID: 1668860.
9. Tian Z, Chen S, Zhang Y, Huang M, Shi L, Huang F, Fong C, Yang M, Xiao P. The cytotoxicity of naturally occurring styryl lactones. Phytomedicine. 2006;13:181-186. doi: 10.1016/j.phymed.2004.07.010.
10. Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998;281:1308-1312. doi: 10.1126/science.281.5381.1309.
11. Hengartner MO. The biochemistry of apoptosis. Nature. 2000;407:770-776. doi: 10.1038/35037710.
12. Kaufmann SH, Hengartner MO. Programmed cell death: alive and well in the new millennium. Trends Cell Biol. 2001;11:526-534. doi: 10.1016/S0962-8924(01)02173-0.
13. Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway. Lancet Oncol. 2003;4:721-729. doi: 10.1016/S1470-2045(03)01277-4.
14. Cohen GM. Caspases: the executioners of apoptosis. Biochem J. 1997;326:1-16. doi: https://doi.org/10.1042/bj3260001.
15. Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. Exp Cell Res. 2000;256:42-49. doi: 10.1006/excr.2000.4838.
16. Reed JC. Apoptosis-regulating proteins as targets for drug discovery. Trends Mol Med. 2001;7:314-319. doi: 10.1016/S1471-4914(01)02026-3.
17. Shi C, Wu F, Yew DT, Xu J, Zhu Y. Bilobalide prevents apoptosis through

activation of the PI3K/Akt pathway in SH-SY5Y cells. Apoptosis. 2010;15:715-727. doi: 10.1007/s10495-010-0492-x.

18. Suzuki R, Kohno H, Sugie S, Sasaki K, Yoshimura T, Wada K, Tanaka T. Preventive effects of extract of leaves of ginkgo (*Ginkgo biloba*) and its component bilobalide on azoxymethane-induced colonic aberrant crypt foci in rats. Cancer Lett. 2004;210:159-169. doi: 10.1016/j.canlet.2004.01.034.
19. Jiang M, Li J, Peng Q, Liu Y, Liu W, Luo C, Peng J, Li J, Yung KK, Mo Z. Neuroprotective effects of bilobalide on cerebral ischemia and reperfusion injury are associated with inhibition of pro-inflammatory mediator production and down-regulation of JNK1/2 and p38 MAPK activation. J Neuroinflammation. 2014;11:167. doi: 10.1186/s12974-014-0167-6.
20. Priyanka A, Nisha VM, Anusree SS, Raghu KG. Bilobalide attenuates hypoxia induced oxidative stress, inflammation, and mitochondrial dysfunctions in 3T3-L1 adipocytes via its antioxidant potential. Free Radic Res. 2014;48:1206-1217. doi: 10.3109/10715762.2014.945442.
21. Yang SS, Kim SG, Park BS, Go DS, Yu SK, Kim CS, Kim J, Kim DK. Effect of β-carotene on cell growth inhibition of KB human oral cancer cells. Int J Oral Biol. 2016;41:105-111. doi: http://dx.doi.org/10.11620/IJOB.2016.41.3.105.
22. Cho JH, Kim SG, Park BS, Go DS, Park JC, Kim DK. MicroRNA-27 promotes odontoblast differentiation via Wnt1 signaling. Int J Oral Biol. 2015;40:197-204. doi: http://dx.doi.org/10.11620/IJOB.2015.40.4.197.
23. Herrnring C, Reimer T, Jeschke U, Makovitzky J, Krüger K, Gerber B, Kabelitz D, Friese K. Expression of the apoptosis-inducing ligands FasL and TRAIL in malignant and benign human breast tumors. Histochem Cell Biol.

2000;113:189-194. PMID: 10817673.

24. Li HJ, Wang CY, Mi Y, Du CG, Cao GF, Sun XC, Liu DJ, Shorgan B. FasL-induced apoptosis in bovine oocytes via the Bax signal. Theriogenology. 2013;80:248-255. doi: 10.1016/j.theriogenology.2013.04.002.
25. Datta R, Kojima H, Yoshida K, Kufe D. Caspase-3-mediated cleavage of protein kinase C theta in induction of apoptosis. J Biol Chem. 1997;272:20317-20320. doi: 10.1074/jbc.272.33.20317.
26. Liu X, Zou H, Slaughter C, Wang X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell. 1997;89:175-184. doi: 10.1016/S0092-8674(00)80197-X.
27. Yang SJ, Lee SA, Park MG, Kim JS, Yu SK, Kim CS, Kim JS, Kim SG, Oh JS, Kim HJ, Chun HS, Kim YH, Kim DK. Induction of apoptosis by diphenyldifluoroketone in osteogenic sarcoma cells is associated with activation of caspases. Oncol Rep. 2014;31:2286-2292. doi: 10.3892/or.2014.3066.
28. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science. 1997;275:1132-1136. doi: 10.1126/science.275.5303.1132.
29. Kluck RM, Esposti MD, Perkins G, Renken C, Kuwana T, Bossy-Wetzel E, Goldberg M, Allen T, Barber MJ, Green DR, Newmeyer DD. The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. J Cell Biol. 1999;147:809-822. doi: 10.1083/jcb.147.4.809.
30. Yu CS, Huang AC, Lai KC, Huang YP, Lin MW, Yang JS, Chung JG. Diallyl trisulfide induces apoptosis in human primary colorectal cancer cells. Oncol Rep.

2012;28:949-954. doi: 10.3892/or.2012.1882.

31. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 1993;74:609-619. doi: 10.1016/0092-8674(93)90509-O.

# Ⅵ. FIGURE LEGENDS

Fig. 1. Chemical structure of bilobalide.

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 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 ± SEM 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).

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 $\mu$M 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) The percentage of apoptotic FaDu cells was calculated as the ratio of apoptotic cells to total cells.

Fig. 4. Fragmentation of genomic DNA by bilobalide in FaDu cells. The cells were treated with 0 or 300 $\mu$M bilobalide for 72 hours and nuclear DNA was subjected to agarose gel electrophoresis.

Fig. 5. Proteolytic cleavage of caspase-3 by bilobalide treatment in FaDu cells. (A) Activity of cleaved caspase-3 by bilobalide was measured in FaDu cells. The cells were treated with 0 or 300 $\mu$M bilobalide for 72 hours. The cell lysate was prepared and analyzed by immunoblotting as described in "MATERIALS AND METHODS". (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

Fig. 6. Proteolytic cleavage of caspase-7 by bilobalide treatment in FaDu cells. Other legends are the same as in Fig. 5.

Fig. 7. Proteolytic cleavage of caspase-8 by bilobalide treatment in FaDu cells. Other legends are the same as in Fig. 5.

Fig. 8. Proteolytic cleavage of caspase-9 by bilobalide treatment in FaDu cells. Other legends are the same as in Fig. 5.

Fig. 9. Activation of Fas by bilobalide treatment in FaDu cells. Other legends are the same as in Fig. 5.

Fig. 10. Activation of cleaved PARP by bilobalide treatment in FaDu cells. Other legends are the same as in Fig. 5.

Fig. 11. Regulation of Bax level by bilobalide treatment in FaDu cells. Other legends are the same as in Fig. 5.

Fig. 12. Regulation of Bcl-2 level by bilobalide treatment in FaDu cells. Other legends are the same as in Fig. 5.

Fig. 13. Apoptotic signaling pathway induced by bilobalide in FaDu human pharyngeal squamous cell carcinoma.

# Ⅶ. FIGURES

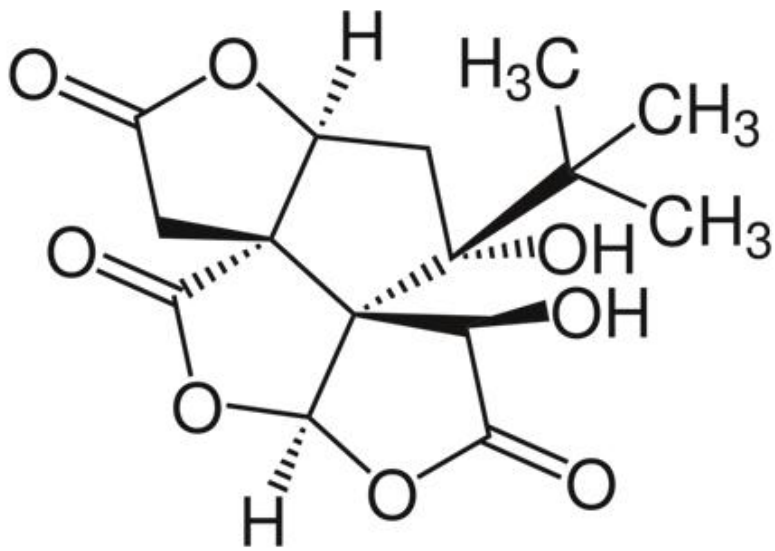


Fig. 1. Chemical structure of bilobalide.

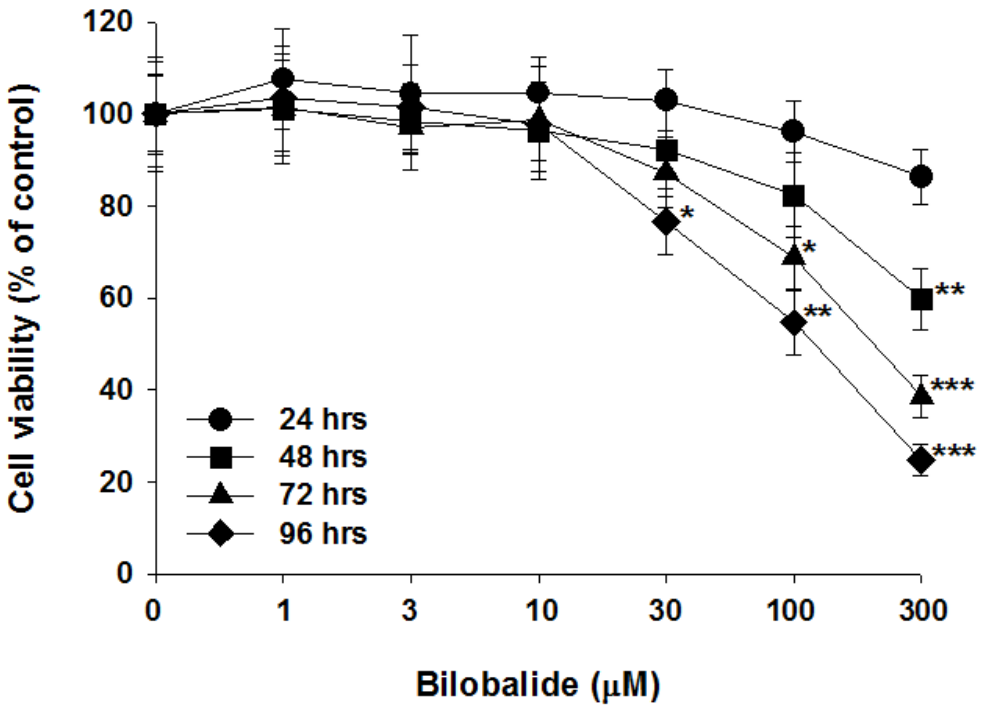


Fig. 2. Effects of bilobalide on cell viability in FaDu human pharyngeal squamous cell carcinoma.

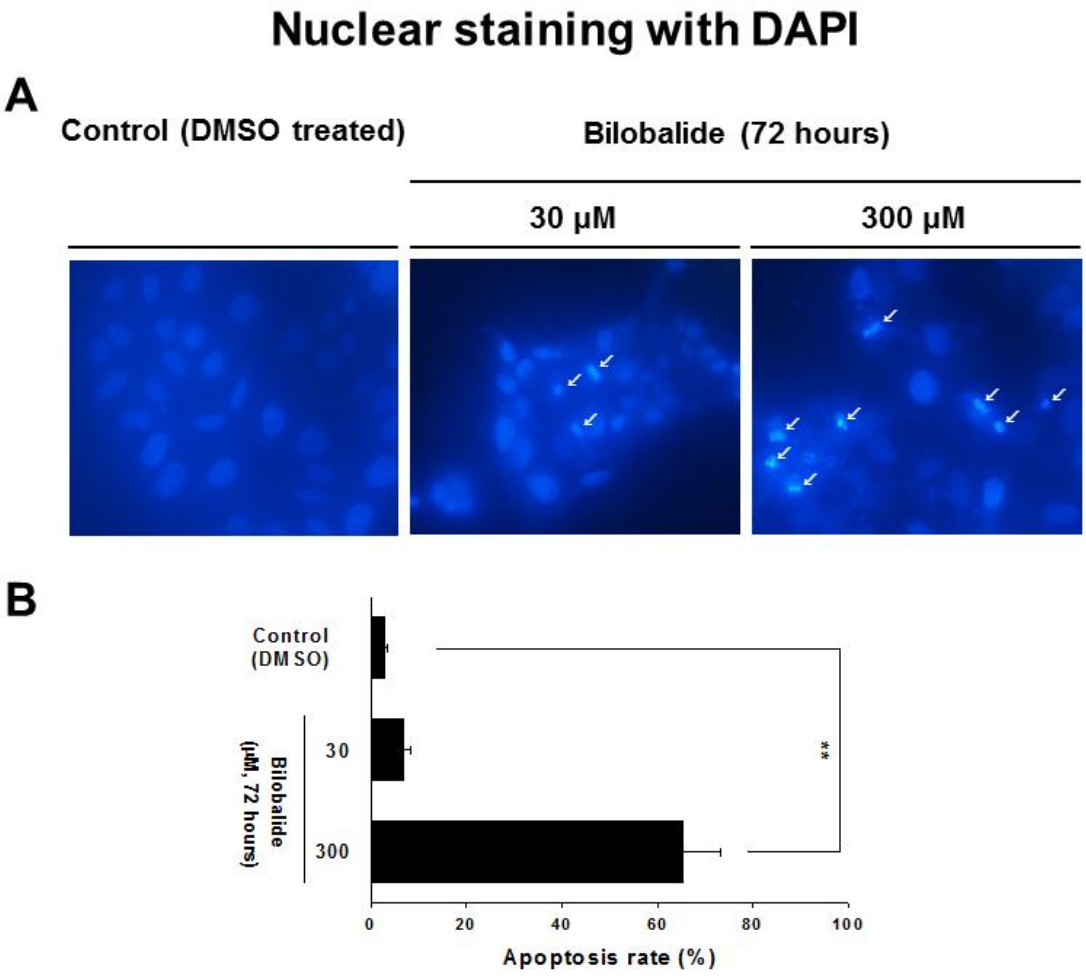


Fig. 3. Induction of apoptosis by bilobalide in FaDu cells.

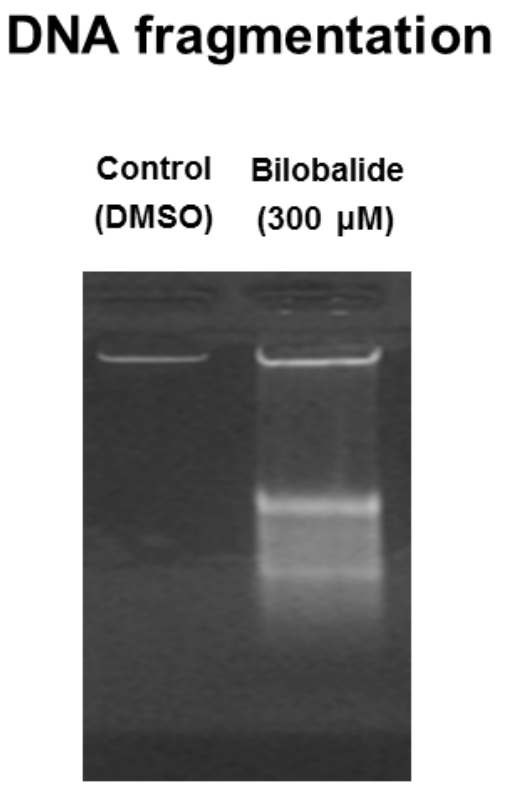


Fig. 4. Fragmentation of genomic DNA by bilobalide in FaDu cells.

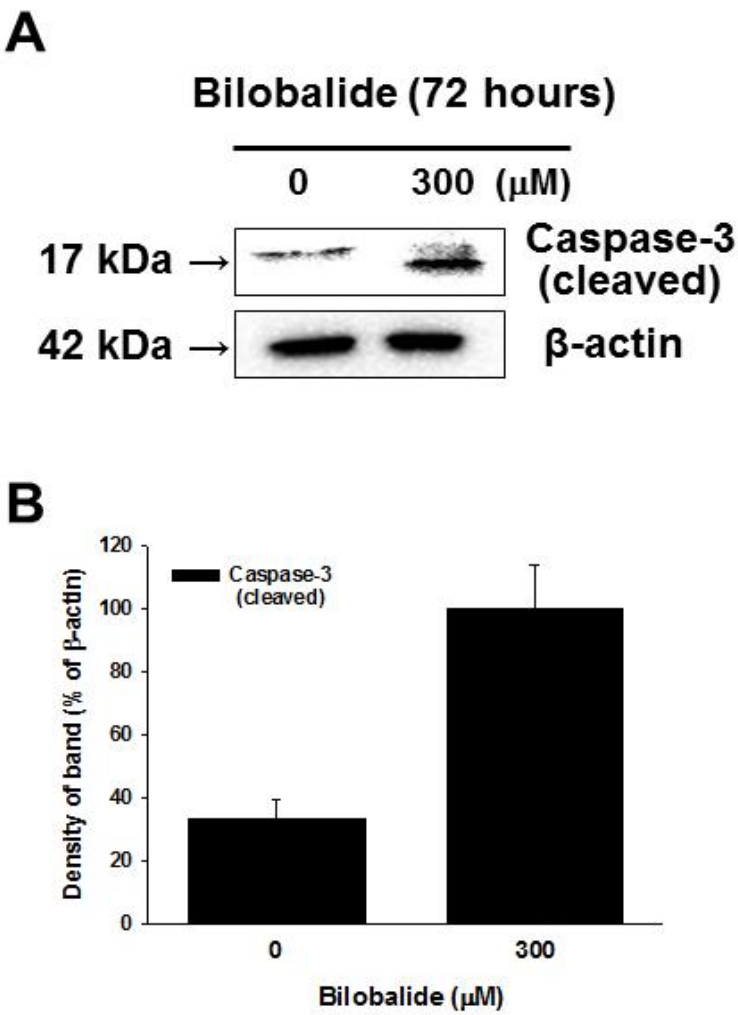


Fig. 5. Proteolytic cleavage of caspase-3 by bilobalide treatment in FaDu cells.

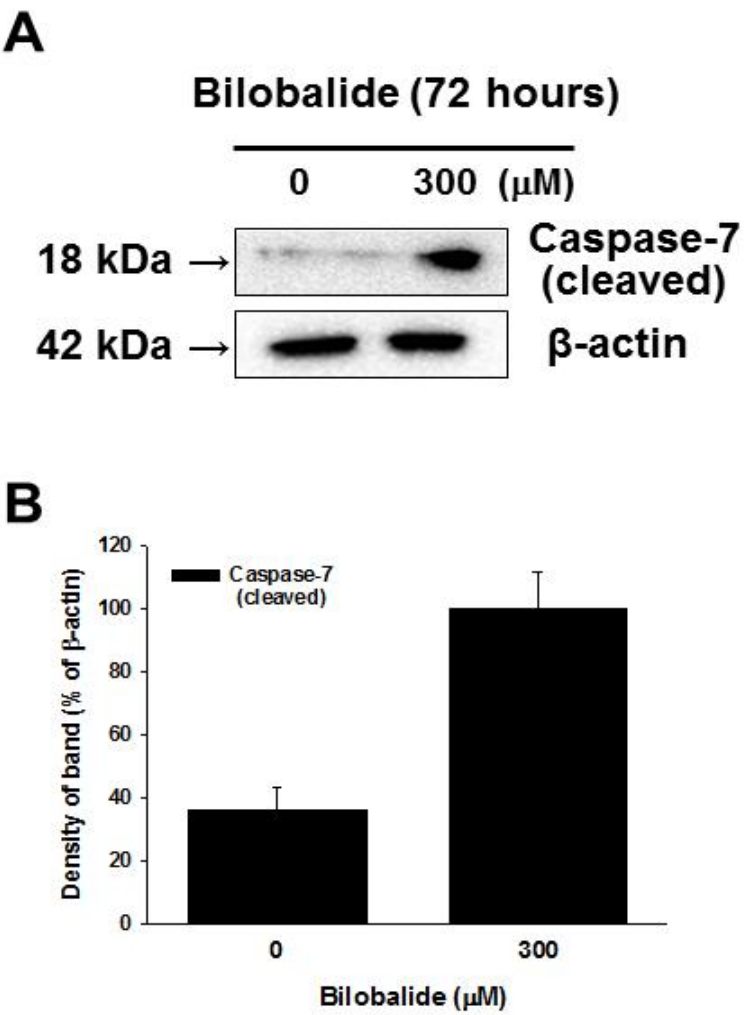


Fig. 6. Proteolytic cleavage of caspase-7 by bilobalide treatment in FaDu cells.

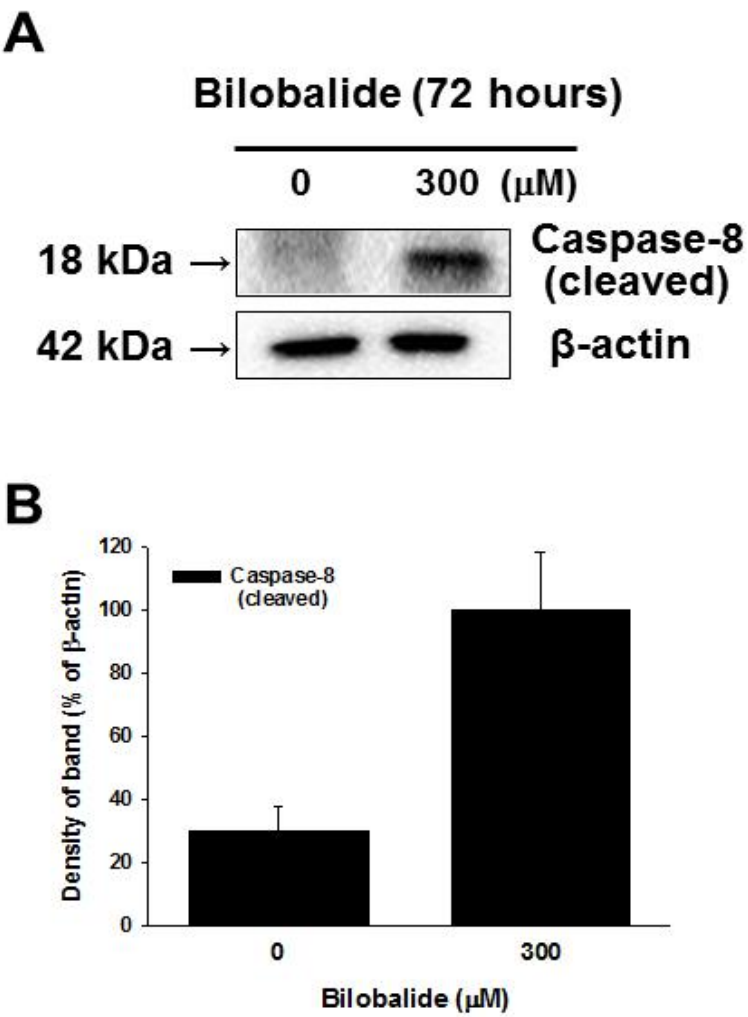


Fig. 7. Proteolytic cleavage of caspase-8 by bilobalide treatment in FaDu cells.

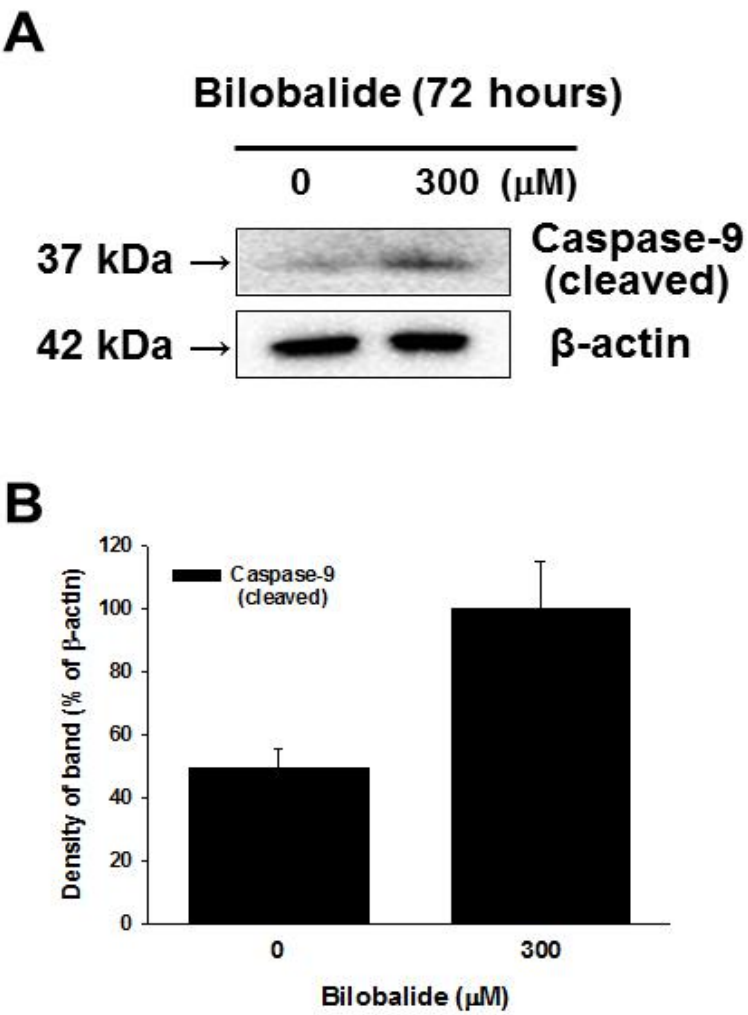


Fig. 8. Proteolytic cleavage of caspase-9 by bilobalide treatment in FaDu cells.

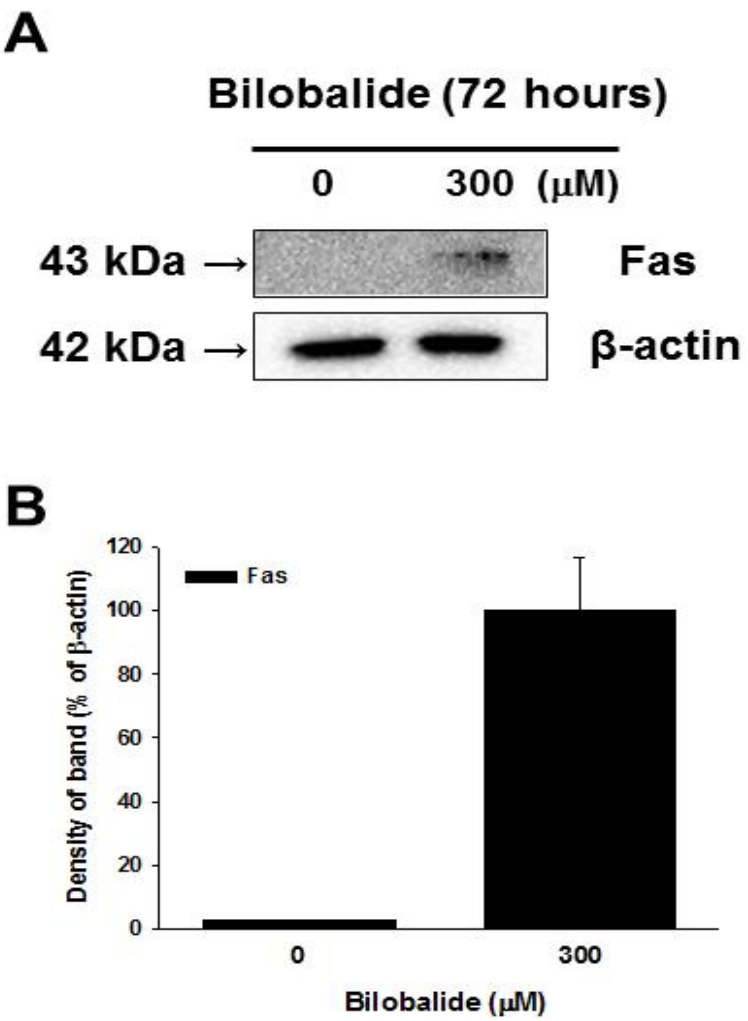


Fig. 9. Activation of Fas by bilobalide treatment in FaDu cells.

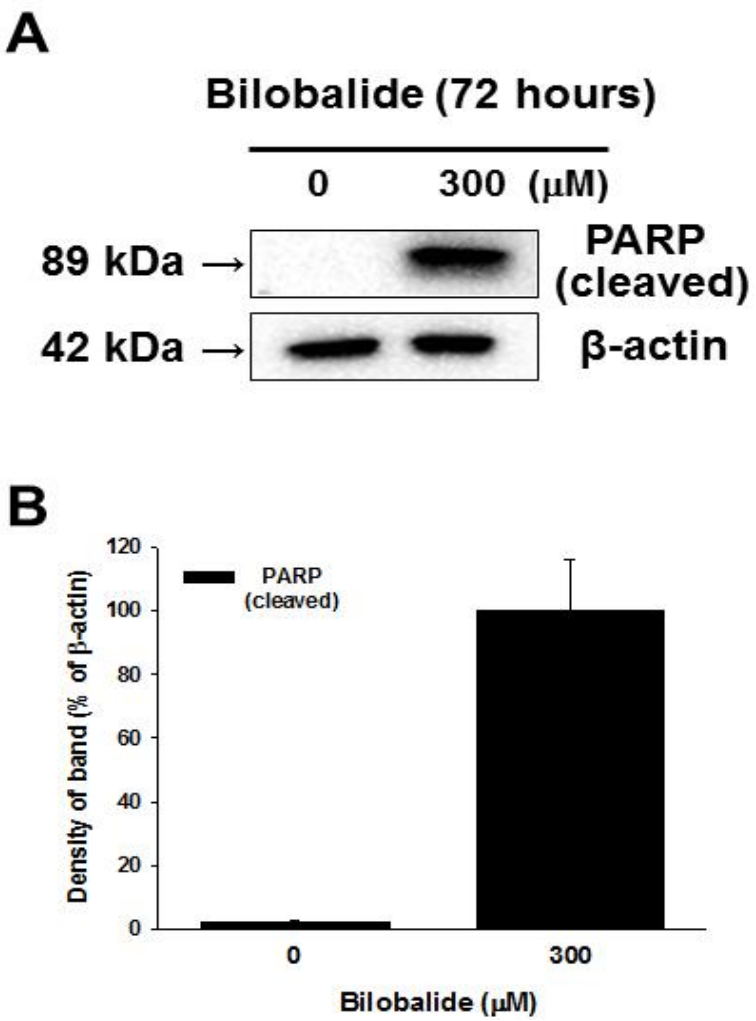


Fig. 10. Activation of cleaved PARP by bilobalide treatment in FaDu cells.

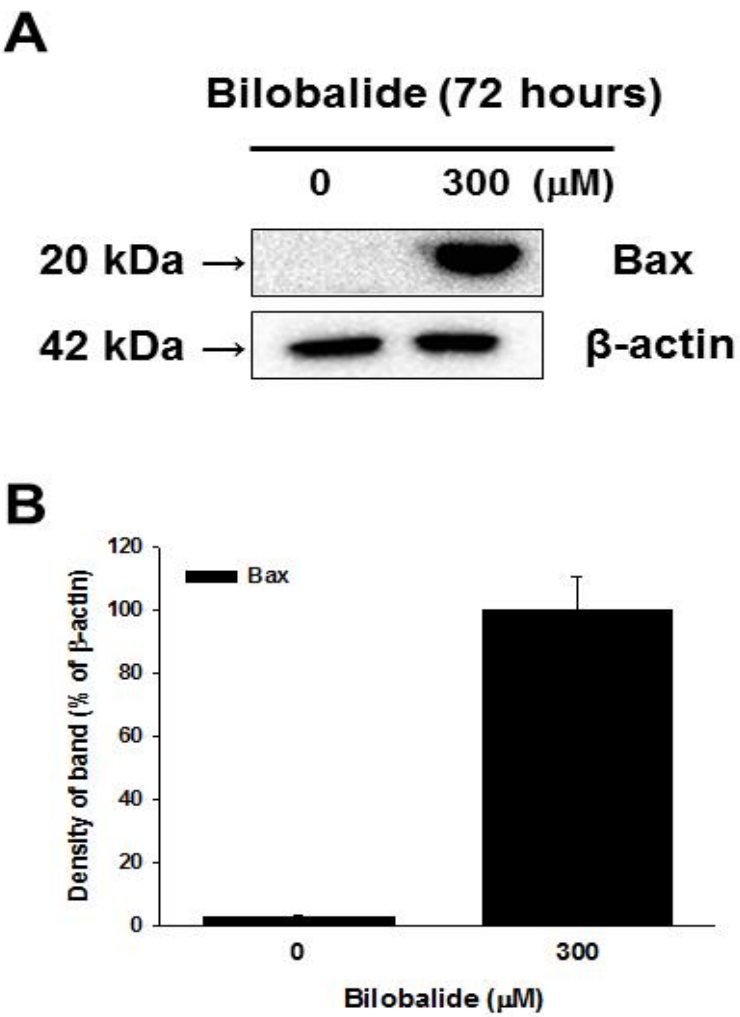


Fig. 11. Regulation of Bax level by bilobalide treatment in FaDu cells.

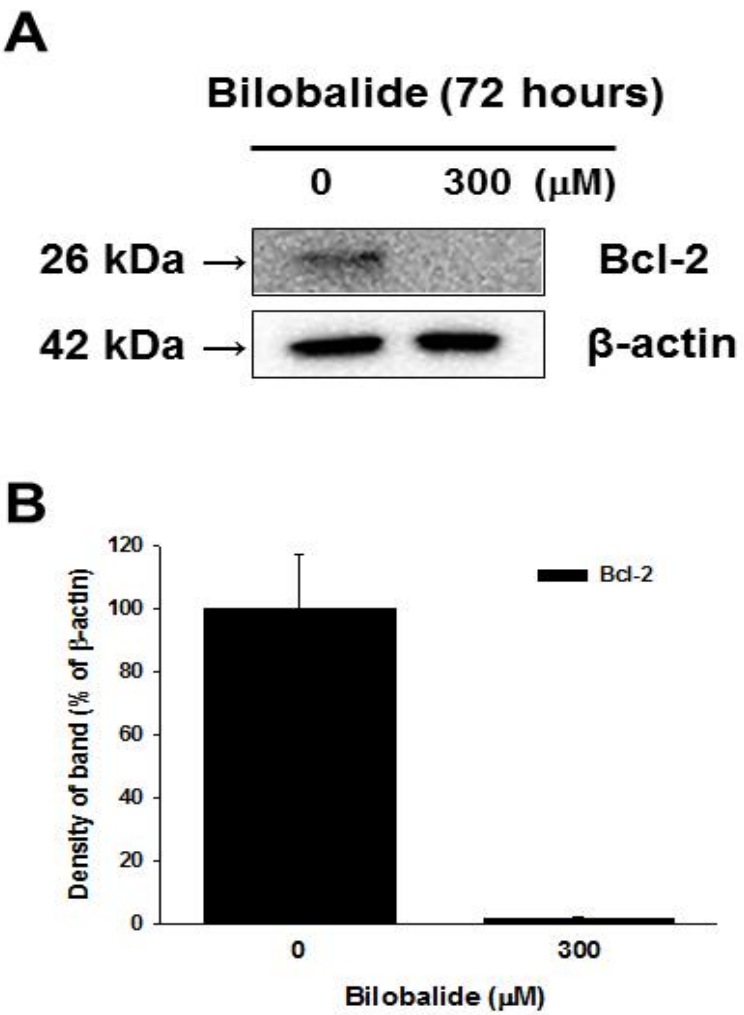


Fig. 12. Regulation of Bcl-2 level by bilobalide treatment in FaDu cells.

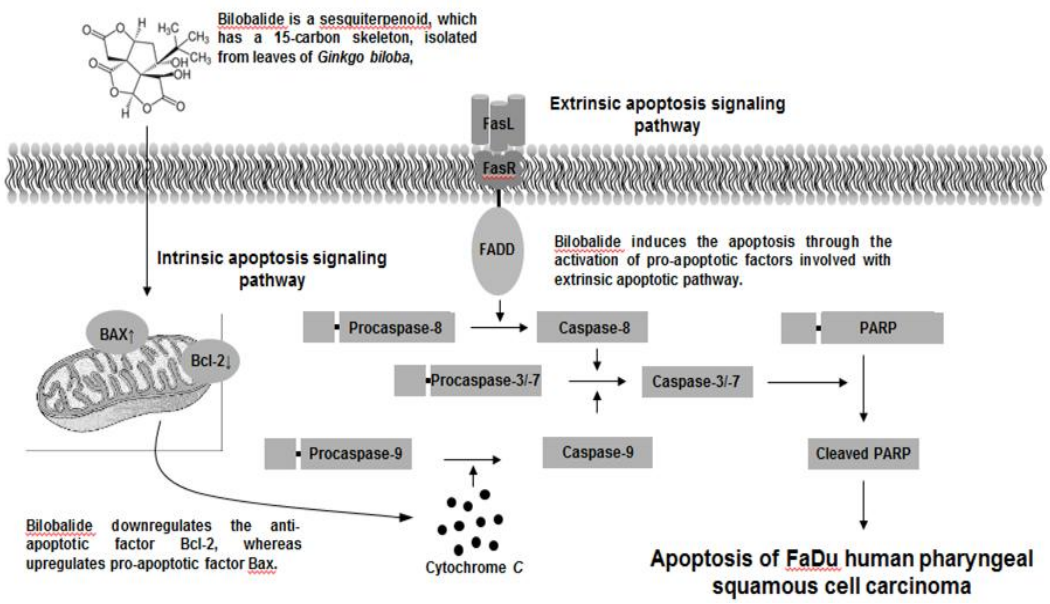


Fig. 13. Apoptotic signaling pathway induced by bilobalide in FaDu human pharyngeal squamous cell carcinoma.

# ABSTRACT in KOREAN

## 사람 인두 편평세포암종 FaDu에서 bilobalide에 의한 세포성장 억제 및 apoptosis 유도

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은행나무 잎에서 분리한 bilobalide는 신경보호, 항염증, 항경련 등의 약리적 효능을 있다고 보고되었다. 그러나 암과 관련된 bilobalide에 관한 자료는 거의 없다. 따라서 본 연구에서는 사람 인두 편평세포암종 FaDu 세포주를 이용하여 bilobalide의 암세포 성장억제에 미치는 효과와 세포성장 억제기전을 분석하였다.

본 연구에서 bilobalide에 의한 암세포 성장억제와 그 기전을 조사하기 위해, FaDu 세포주에서 bilobalide를 이용하여 MTT 분석, DAPI를 이용한 핵 염색 분석, DNA fragmentation 분석 및 immunoblotting 등을 시행하였다.

사람 인두 편평세포암종 FaDu에서 bilobalide는 암세포의 성장을 농도와 시간에 의존적으로 억제하였다. FaDu 세포에서 bilobalide는 핵의 응집과 파쇄 및 분절을 유도하였다. FaDu 세포에 bilobalide를 처리한 실험군에서 procaspase-3, -7, -8 및 -9의 proteolytic cleavage 현상을 확인할 수 있었다. FaDu 세포에서 bilobalide는 Fas의 발현을 증가시켰으며, poly(ADP-ribose) polymerase의 proteolytic cleavage를 촉진시켰다. Bilobalide 처리에 의해 Bax의 발현은 증가되고 Bcl-2의 발현은 억제되었으며, Bax/Bcl-2의 비율은 유의하게 증가되었다.

본 연구의 결과로 bilobalide는 사람 인두 편평세포암종 FaDu의 apoptosis를 유도하여 암세포 성장을 억제시키는 것으로 사료된다. 또한 본 연구의 결과로, bilobalide를 이용한 암세포 성장억제에 관한 하나의 방향을 제시할 수 있을 것으로 사료된다.

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중심어: Bilobalide, 세포사, Apoptosis, 항암치료제, 암세포