

## Anti-Proliferative and Pro-Apoptotic Activities of 4-Methyl-2,6-bis(1-phenylethyl)phenol in Cancer Cells

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### Abstract

It has been found that 4-isopropyl-2,6-bis(1-phenylethyl)phenol (KTH-13), a novel compound isolated from *Cordyceps bassiana*, is able to suppress tumor cell proliferation by inducing apoptosis. To mass-produce this compound, we established a total synthesis method. Using those conditions, we further synthesized various analogs with structural similarity to KTH-13. In this study, we aimed to test their anti-cancer activity by measuring anti-proliferative and pro-apoptotic activities. Of 8 compounds tested, 4-methyl-2,6-bis(1-phenylethyl)phenol (KTH-13-Me) exhibited the strongest anti-proliferative activity toward MDA-MB 231 cells. KTH-13-Me also similarly suppressed the survival of various cancer cell lines, including C6 glioma, HCT-15, and LoVo cells. Treatment of KTH-13-Me induced several apoptotic signs in C6 glioma cells, such as morphological changes, induction of apoptotic bodies, and nuclear fragmentation and chromatin condensation. Concordantly, early-apoptotic cells were also identified by staining with FITC-Annexin V/PI. Moreover, KTH-13-Me highly enhanced the activation of caspase-3 and caspase-9, and decreased the protein level of Bcl-2. In addition, the phosphorylation levels of Src and STAT3 were diminished in KTH-13-Me-treated C6 cells. Therefore, these results suggest that KTH-13-Me can be developed as a novel anti-cancer drug capable of blocking proliferation, inducing apoptosis, and blocking cell survival signaling in cancer cells.

**Key Words:** 4-methyl-2,6-bis(1-phenylethyl)phenol, Apoptosis, Anti-cancer activity, *Cordyceps Bassiana*

### INTRODUCTION

*Cordyceps* is a genus of parasitic fungi. It has been traditionally used as an herbal medicine in Korea and China to enhance longevity and vitality (Siu *et al.*, 2004; Ng and Wang, 2005). In addition to *Cordyceps* species' usage to ameliorate various diseases such as asthma, cardiovascular diseases, skin diseases, and tuberculosis (Zhou *et al.*, 2009), this mushroom has been found to be effective in various cancers by modulation of innate immunity against tumors or by directly targeting tumor cells (Wu *et al.*, 2007; Rao *et al.*, 2010).

Previously, we reported that *Cordyceps bassiana* has vari-

ous pharmacological activities, such as anti-inflammatory, anti-atopic dermatitis, and anti-cancer effects (Byeon *et al.*, 2011a; Byeon *et al.*, 2011b; Wu *et al.*, 2011; Kim *et al.*, 2015a). Moreover, 4-isopropyl-2,6-bis(1-phenylethyl)phenol (KTH-13), which displays anti-tumor activity through inducing apoptosis in various cancer cells (Kim *et al.*, 2015a), was isolated from the butanol fraction of *Cordyceps bassiana*. Based on those results, we established total synthesis conditions to produce KTH-13, and further synthesized several compounds, including 4-methyl-2,6-bis(1-phenylethyl)phenol (KTH-13-Me), and analogs thereof with structural similarity to KTH-13 (Fig. 1). Since these compounds are expected to have anti-cancer ac-

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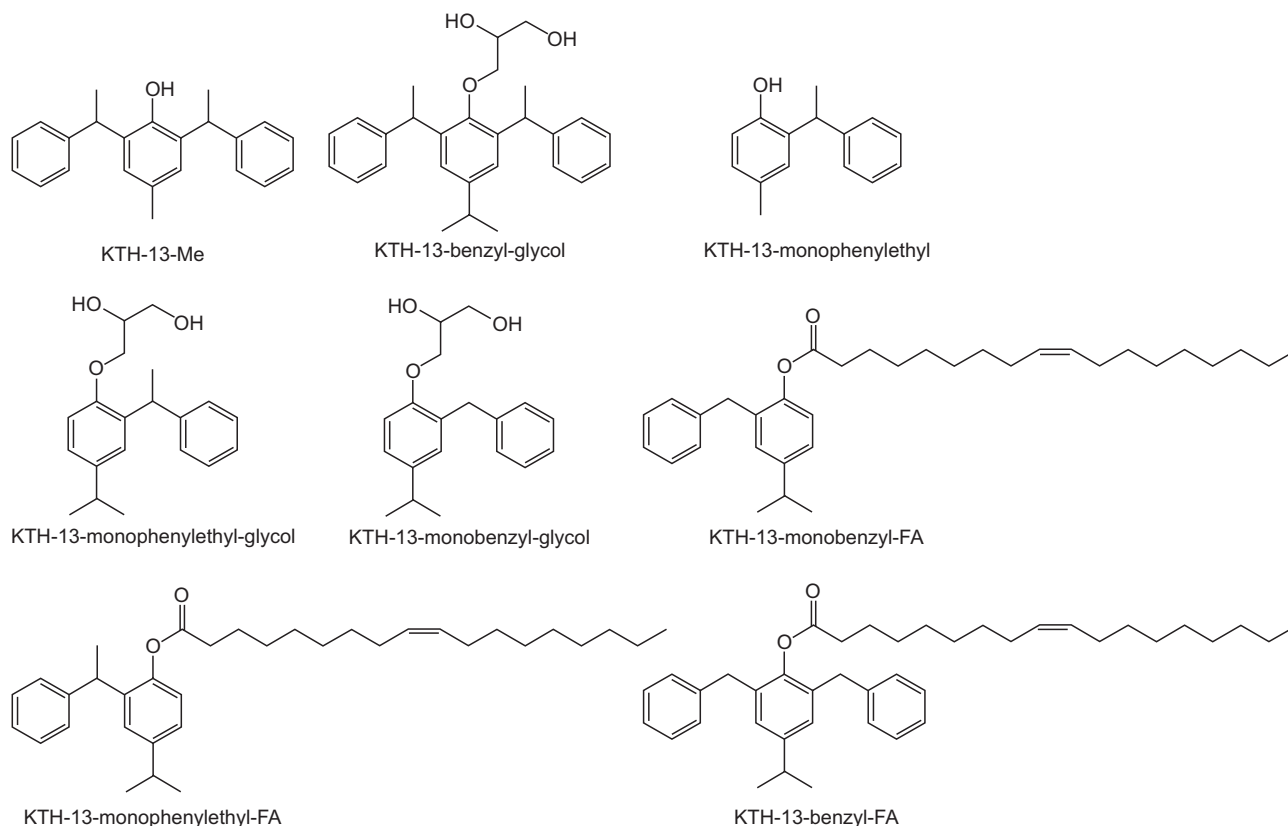
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**Fig. 1.** Chemical structure of KTH-13-Me and its analogs.

tivity, in this study we aimed to investigate the anti-proliferative activities as well as the molecular mechanisms of these compounds.

## MATERIALS AND METHODS

### Materials

4-Methyl-2,6-bis(1-phenylethyl)phenol [KTH-13-Me] and its structural analogs, including KTH-13-benzyl-glycol, KTH-13-monophenylethyl-glycol, KTH-13-monobenzyl-glycol, KTH-13-monophenylethyl, KTH-13-monobenzyl-FA, KTH-13-monophenylethyl-FA, and KTH-13-monophenylethyl-FA (Fig. 1) were supplied by Prof Lee, Yunmi (Kwangwoon University, Seoul, Korea). Hoechst stain solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), staurosporine, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin, and streptomycin were obtained from GE Healthcare Hyclone (Grand Island, NY, USA). C6 glioma, MDA-MB 231, HCT-15, and LoVo cells were purchased from ATCC (Rockville, MD, USA). Antibodies to phospho-, cleaved- or total-protein forms of Src, STAT3, caspase-3, caspase-9, Bcl-2, and  $\beta$ -actin were obtained from Cell Signaling Technology (Beverly, MA, USA).

### Cell culture and drug preparation

C6 glioma, MDA-MB 231, HCT-15, and LoVo cells were maintained in Dulbecco's Modified Eagle's Medium supple-

mented with 100 U/ml of penicillin, 100  $\mu$ g/ml of streptomycin, and 5% heat-inactivated FBS as reported previously (Kim *et al.*, 2014b). The cells were grown at 37°C and 5% CO<sub>2</sub> in humidified air. Stock solutions of KTH-13-Me or its analogs for the experiments were prepared in DMSO.

### Cell proliferation test

After pre-incubation of cancer cells ( $1 \times 10^6$  cells/ml) in 96-well plates overnight, the cells were incubated with KTH-13-Me or its structural analogs for 24 h. The viability of the cells was then measured using a conventional MTT assay. Ten microliters of an MTT solution (5 mg/ml in D.W.) was added to the each well of the plate, and the cells were incubated for 3 h. After the incubation, the reaction was terminated by the addition of 10% sodium dodecyl sulfate into each well, solubilizing the formazan. The absorbance at 570 nm was measured using a Spectramax 250 microplate reader.

### Morphological change test

C6 glioma cells were treated with KTH-13-Me (25 and 50  $\mu$ M). After treatment, images of the cells were obtained using an inverted phase contrast microscope attached to a camera and captured using NIH imaging software, as reported previously (Kim and Cho, 2013).

### Confocal microscopy

C6 cells were plated in 12-well plates containing sterile cover slips at a density of  $5 \times 10^4$  cells/ml and incubated overnight. The cells were incubated with KTH-13-Me or staurosporine for

**Table 1.** Effect of KTH-13-Me and its structural analogs on the proliferation of MDA-MB-231 cells

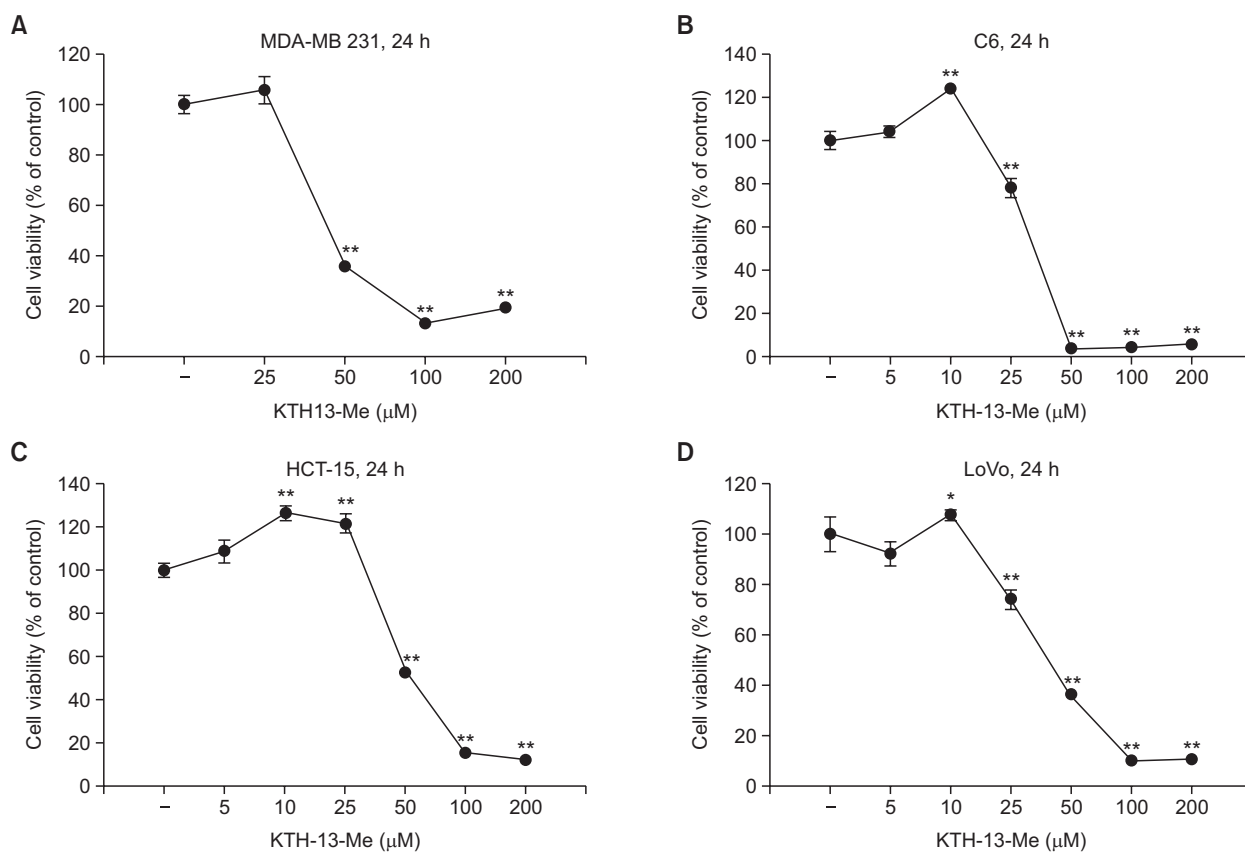
Compound	IC <sub>50</sub> (μM) <sup>a</sup>
KTH-13-benzyl-glycol	78.3
KTH-13-monophenylethyl-glycol	82.5
KTH-13-monobenzyl-glycol	87.4
KTH-13-Me	44.9
KTH-13-monophenylethyl	84.1
KTH-13-monobenzyl-FA	>200
KTH-13-monophenylethyl-FA	>200
KTH-13-benzyl-FA	>200

<sup>a</sup>IC<sub>50</sub> values were calculated with dose-responsive anti-proliferative activity of each compound treated to MDA-MB-231 cells for 24 h. Their viability was measured using a conventional MTT assay.

**Table 2.** Effect of KTH-13-Me and its structural analogs on the proliferation of MDA-MB-231, C6 glioma, HCT-15, and LoVo cells

Cell line	IC <sub>50</sub> (μM) <sup>a</sup>
MDA-MB 231	44.9
C6	34.48
HCT-15	53.37
LoVo	40.96

<sup>a</sup>IC<sub>50</sub> values were calculated with dose-responsive anti-proliferative activity of KTH-13-Me treated to MDA-MB-231, C6 glioma, HCT-15, and LoVo cells for 24 h. Their viability was measured using a conventional MTT assay.



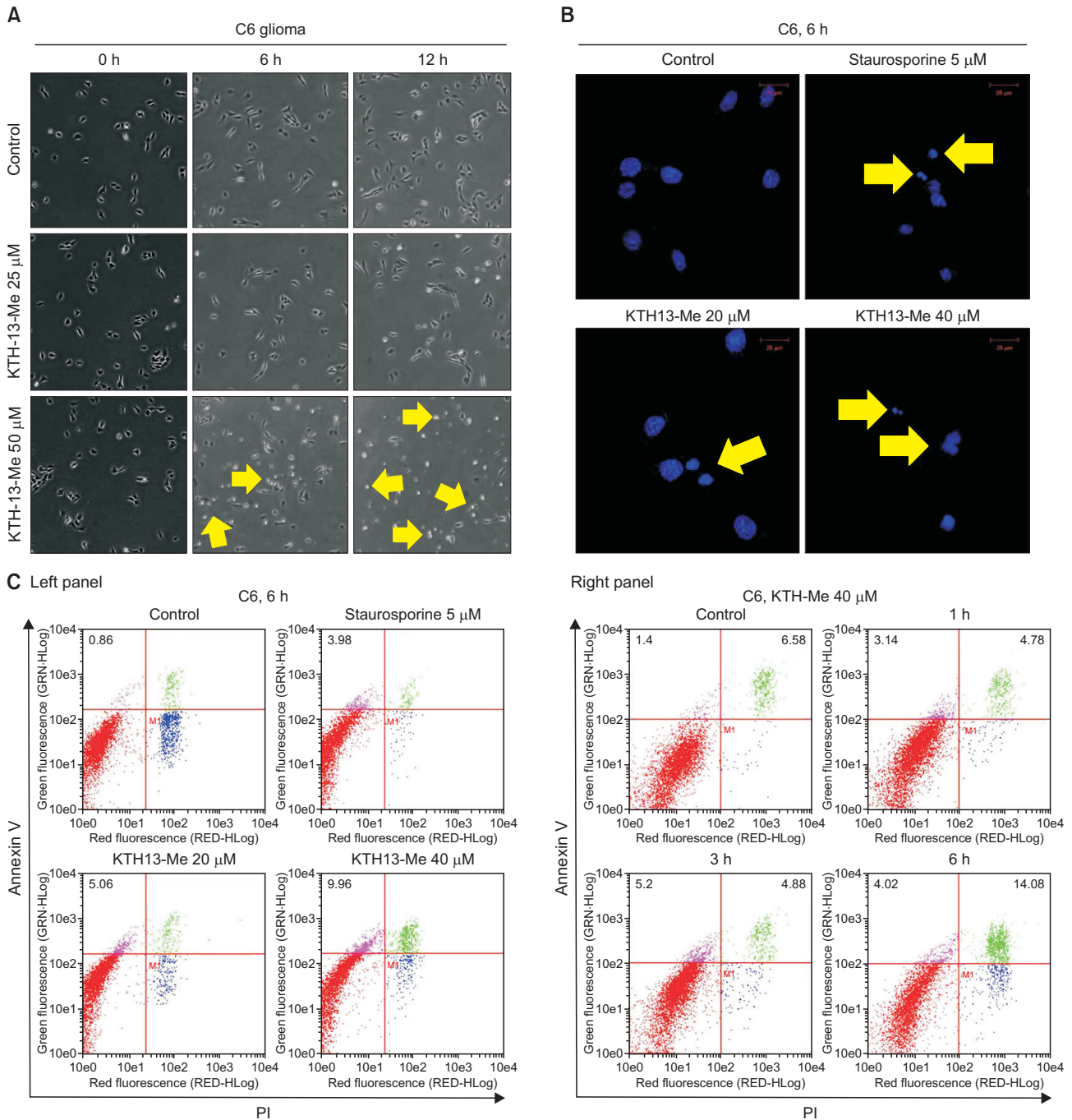
**Fig. 2.** Anti-cancer activity of KTH-13-Me. (A, B, C, and D) C6 glioma, MDA-MB-231, HCT-15, and LoVo cells were incubated with KTH-13-Me for 24 h. The viability of cancer cells was measured by MTT assay. \**p*<0.05 and \*\**p*<0.01 compared to normal group.

6 h and washed twice with PBS then fixed to the cover slips by incubating in 3.7% formaldehyde for 10 min. After fixing, the cells were washed with PBS 3 times, and blocked using 1% BSA in PBS for 30 h. For nuclear staining, cells were incubated with Hoechst stain solution (1:1000) for 20 min in the dark. The coverslips were then washed 3 times and mounted on slide glasses using fluorescent mounting medium (DakoCytomation, Carpinteria, CA, USA). Fluorescence images were captured using an inverted microscope (DMI 4000B; Leica,

Buffalo Grove, IL, USA).

**FITC-annexin V/PI staining apoptosis assay**

The percentage of apoptotic cells was determined using a FITC-Annexin V Apoptosis Detection Kit (BD Bioscience, San Jose, CA, USA) based on cell membrane changes (phosphatidylserine-based). C6 glioma cells (5×10<sup>5</sup> cells/ml) were plated in 12-well plates and incubated overnight. KTH-13-Me was then added to the cells at the indicated doses. At the spe-



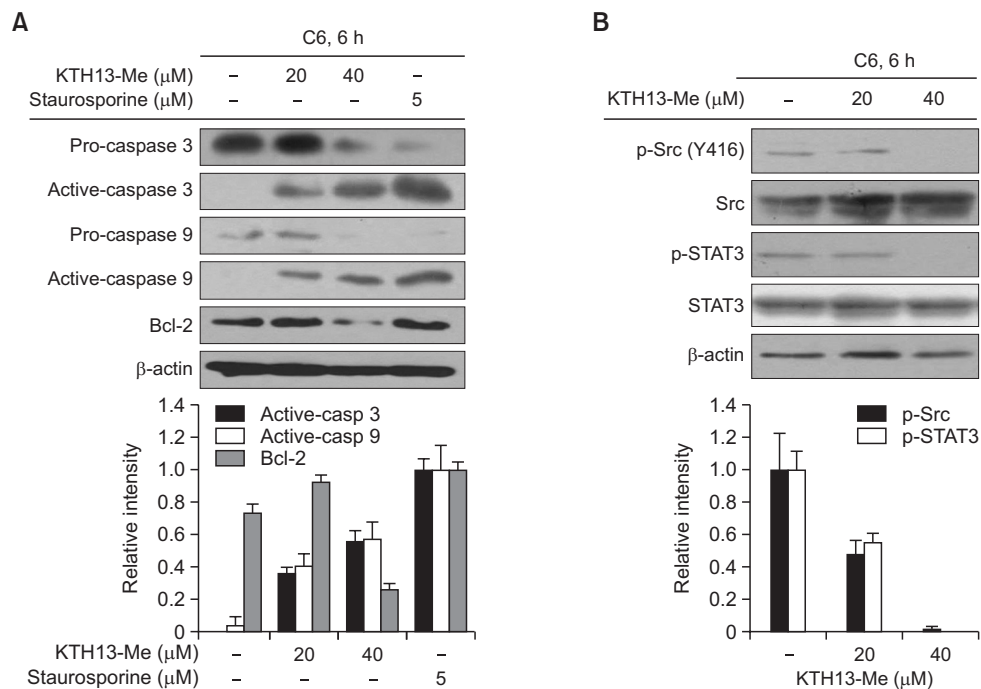
**Fig. 3.** Apoptosis-inducing activity of KTH-13-Me. (A) C6 glioma cells were incubated with KTH-13-Me for 0, 6, and 12 h, and their morphological changes were observed by microscopic analysis. (B) C6 glioma cells were treated with KTH-13-Me for 6 h, and their nuclei were visualized by confocal microscope after staining with Hoechst dye. (C) C6 glioma cells were stained with FITC-Annexin V and PI after KTH-13-Me was administered in varying doses and for varying times. The percentages of early-apoptosis (Annexin V-positive / PI-negative) populations were measured with a flow cytometer.

cific time points, the cells were harvested, washed twice with PBS, and resuspended in  $1 \times$  binding buffer. FITC-annexin V and PI were applied to the cells, which were then incubated for 15 min at room temperature in the dark. Fluorescence signals from the cells were measured using a BD FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and

CellQuest Pro (IVD) software (Becton Dickinson).

#### Immunoblotting analysis

C6 glioma cells ( $5 \times 10^6$  cells/ml) were washed twice in cold PBS and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycoltetraacetic acid, 50 mM  $\beta$ -glycero-



**Fig. 4.** Effect of KTH-13-Me on the expression of apoptosis-related and cell survival regulatory proteins. (A and B) Enhanced levels of active apoptosis-related proteins (cleaved caspases-3 and -9) and activated cell survival-regulatory proteins (phospho-Src and phospho-STAT-3) in C6 glioma cells incubated with KTH-13-Me for 6 h were detected by immunoblotting analysis. Relative intensity was calculated using total levels with the DNR Bio-Imaging system. All of the data are expressed as the means  $\pm$  SD of experiments that were performed with six or three samples. \* $p < 0.05$  and \*\* $p < 0.01$  compared to the control group.

phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimidazole, and 2 mM PMSF) for 1 hour, with rotation, at 4°C. The lysates were clarified by centrifugation at 12,000×g for 10 min at 4°C. Proteins were separated on 10% or 12% SDS-polyacrylamide gels and transferred by electroblotting to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 60 min in Tris-buffered saline containing 3% BSA and 0.1% Tween 20 at room temperature. The membranes were incubated overnight with specific primary antibodies at 4°C, washed 3 times with Tris-buffered saline containing 0.1% Tween 20, and incubated for an additional 120 min with HRP-conjugated secondary antibodies. The total and phosphorylated levels of each protein were visualized using an ECL system (Amersham, Little Chalfont, Buckinghamshire, UK).

**Statistical analysis**

All of the data presented in this paper are expressed as means  $\pm$  SD. For statistical comparisons, results were analyzed using either ANOVA/Scheffe’s *post-hoc* test or the Kruskal-Wallis/Mann-Whitney test. A  $p$ -value  $< 0.05$  was considered to be a statistically significant difference. All statistical tests were carried out using the computer program, SPSS (SPSS Inc., Chicago, IL, USA).

**RESULTS**

**Anti-cancer activity of KTH-13-Me**

First, we compared the anti-cancer activities of 8 deriva-

tives by determining the anti-proliferative activity of each compound toward MDA-MB-231 cells. Of these, 4-methyl-2,6-bis(1-phenylethyl)phenol (KTH-13-Me) showed the strongest activity, with an  $IC_{50}$  value of 44.9 μM (Table 1).

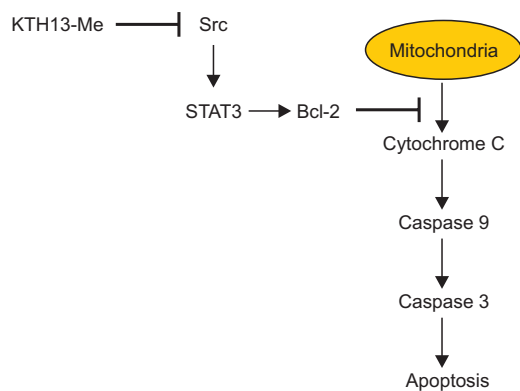
To further investigate the cytotoxic activity of KTH-13-Me, we tested the inhibitory effect of this compound on the proliferation of various cancer cell lines. As Fig. 2 shows, KTH-13-Me strongly suppressed the viability of MDA-MB-231 (human breast cancer cell line), C6 glioma (rat glioma cell line), HCT-15 (Human colon cancer cell line), and LoVo (human colon cancer cell line) cells in a dose-dependent manner. It was determined that KTH-13-Me exerted a similar cytotoxicity toward all of these cancer cells, with  $IC_{50}$  values ranging from 34.48 to 53.37 μM (Table 2).

**Apoptosis-inducing activity of KTH-13-Me**

To determine whether the anti-proliferative activity of KTH-13-Me is due to an apoptosis- or necrosis-inducing activity, we first looked for several markers indicating apoptosis in KTH-13-Me-treated C6 glioma cells. As Fig. 3A depicts, C6 glioma cells incubated with KTH-13-Me showed apoptosis-specific cellular features, such as actin cytoskeleton disruption and appearance of apoptotic bodies. In addition, KTH-13-Me treatment for 6 h also induced nuclear condensation, another indicator of apoptotic death, in C6 glioma cells (Fig. 3B).

To obtain further evidence of apoptotic activity of KTH-13-Me, we employed Annexin V/PI staining assay conditions using C6 cells treated with KTH-13-Me at varying doses for various times (Fig. 3C). The percentage of early-apoptotic cells, which show higher FITC-Annexin V and lower PI staining, was increased according to the concentration of KTH-13-Me (Fig.





**Fig. 5.** Schematic pathway of apoptosis-induction by KTH-13-Me.

3C left panel). The cell population in an early apoptotic stage was also increased time-dependently by exposure to KTH-13-Me up until 3 h, with a maximal percentage of late-apoptotic population (area of FITC-Annexin V-positive and PI-positive) at 6 h (Fig. 3C, right panel).

#### Effect of KTH-13-Me on the expression of apoptosis-related and cell survival regulatory proteins

Based on a variety of evidence regarding the apoptosis-inducing activity of KTH-13-Me, we investigated the mechanism underlying its pro-apoptotic activity. As Fig. 4A indicates, caspase-3 and -9, well-known apoptosis-inducing factors (Orrenius, 2007), were cleaved into active forms in C6 glioma cells incubated with KTH-13-Me for 6 h. In addition, treatment with KTH-13-Me (40  $\mu$ M) decreased the protein level of Bcl-2, an apoptosis-preventing protein (Igney and Krammer, 2002), in C6 glioma cells. Moreover, the activities of Src and STAT3 (which are proteins related to the expression of Bcl-2) were strongly suppressed by 40  $\mu$ M KTH-13-Me, as indicated by their phosphorylation levels (Fig. 4B).

## DISCUSSION

It has been previously reported that *Cordyceps* species exert anti-cancer activity via pro-apoptotic activity (Lee *et al.*, 2006; Jin *et al.*, 2008; Kim *et al.*, 2010). Previously, we discovered that 4-isopropyl-2,6-bis(1-phenylethyl)phenol, known as KTH-13, is one of the components which contributes to the apoptosis-inducing activity of *Cordyceps bassiana* (Kim *et al.*, 2015a), similarly to other naturally occurring anti-cancer compounds, such as ginsenoside Rg3 epimers, ginsenoside Rh2, and resveratrol (Ali and Braun, 2014; Kim *et al.*, 2014a; Park *et al.*, 2014). Thus, we synthesized derivatives of KTH-13 with similar molecular structures to the parent compound, expecting these to have modified apoptosis-inducing activities. We previously reported that one of the derivatives, KTH-13-AMP, is able to induce pro-apoptotic activity in cancer cells, although it displayed less activity than did its parent substance (Kim *et al.*, 2015b). To improve upon the activity of KTH-13, we further developed another 8 derivatives of KTH-13 and tested their anti-cancer activities.

Of these 8 compounds, it was found that KTH-13-Me, synthesized substituting isopropyl group of KTH-13 to methyl

group, is the strongest inhibitor blocking the proliferation of cancer cells, with  $IC_{50}$  values of 30 to 50  $\mu$ M (Fig. 2, Table 1). Compared to that of KTH-13 (53.3  $\mu$ M in C6 glioma cells, with 48 h incubation) (Kim *et al.*, 2015a), the inhibiting activity (44.9  $\mu$ M with 24 h incubation) of KTH-13-Me (Table 1) seems to be greater. These results imply that molecular modification from KTH-13 to KTH-13-Me might enhance the cytotoxic activity of the KTH-13.

After confirmation of its anti-cancer activity, we next examined whether KTH-13-Me could induce apoptosis or necrosis in cancer cells. The specific features of apoptosis have been well characterized. The actin cytoskeleton is cleaved during apoptosis, and thus is unable to maintain its structural function (Mashima *et al.*, 1997; Häcker, 2000). Activation of proteins such as ROCK1 by caspases have been reported to lead to the induction of blebbing and apoptotic bodies (Coleman *et al.*, 2001; Sebbagh *et al.*, 2001; Wi and Lee, 2014). Caspases also activate nuclear factors and DNases, resulting in DNA fragmentation and chromatin condensation (Liu *et al.*, 1998). Apoptotic cells lose their membrane phospholipid asymmetry, so they can be identified by detecting the exposure of phosphatidylserine using Annexin V (Koopman *et al.*, 1994). Based on these facts, we further explored the apoptosis-inducing activity of KTH-13-Me. As shown in Fig. 3A, KTH-13-Me treatment of C6 cells led to morphological changes, followed by the appearance of apoptotic bodies. Next, we observed their chromatin structure using Hoechst dye. Incubation of C6 glioma cells with KTH-13-Me for 6 h led to nuclear shrinkage and chromatin condensation (Fig. 3B). Similar phenomena also can be observed in C6 cells treated with staurosporine, which is a well-known apoptosis-inducing drug (Bertrand *et al.*, 1994). To check early apoptosis levels in KTH-13-Me-treated cells, we stained C6 glioma cells with FITC-Annexin V and PI after treatment with KTH-13-Me in varying doses and for varying times. As shown in Fig. 3C, KTH-13-Me significantly increased the population of Annexin V-positive C6 cells. These results strongly suggest that KTH-13-Me could inhibit the viability of cancer cells by upregulation of a pro-apoptotic pathway.

Since it is known that caspases are key proteins regulating apoptosis (Cohen, 1997), we evaluated the levels of active caspases in KTH-13-Me-treated C6 glioma cells. As Fig. 4A shows, protein levels of inactive caspase-3 and -9 were decreased dose-dependently with administration of KTH-13-Me to C6 cells, while the levels of the active forms were highly increased. Since caspase-9 is involved in the mitochondrion-dependent intrinsic pathway in apoptosis, we also determined the protein level of Bcl-2, which prevents the release of cytochrome C from mitochondria (Yang *et al.*, 1997; Kim *et al.*, 2014c). As we expected, KTH-13-Me reduced the protein level of Bcl-2 in C6 cells, implying that the mitochondrial pro-apoptotic pathway could be stimulated by KTH-13-Me.

STAT3 and Src, two major oncogenes in cancer cells, are known to be among the key molecules involved in the expression of Bcl-2 proteins (Bromberg *et al.*, 1999; Niu *et al.*, 2002). Survival signaling composed of Src, phosphatidylinositol 3-kinase (PI3K), and AKT, which is linked to the activation of transcription factors including NF- $\kappa$ B and AP-1 required to express additional survival proteins, is needed to maintain cell viability (Byeon *et al.*, 2012; Khanna *et al.*, 2013). Interestingly, it was found that the phosphorylation levels of Src and STAT3 in cancer cells were suppressed by KTH-13-Me (Fig. 4B), indicating

that the activity of those proteins was blocked by KTH-13-Me. It is thus reasonable that the Src-linked survival pathway is negatively regulated by KTH-13-Me. Interestingly, considering that the original compound, KTH-13, of KTH-13-Me did not block the phosphorylation of Src (Kim *et al.*, 2015a), it seems to be suggested that KTH-13-Me might have different pharmacological property due to chemical change. Although it has not yet been fully explained, replacement of 4-isopropyl group to methyl group might alter the non-polarity of this compound, affecting to drug's molecular structure to interact with target protein. Which pathways involved in cell survival signaling or target(s) are directly modulated by this compound and how the methyl group-induced molecular change in KTH-13-Me affects its alteration of molecular structure will be further examined in subsequent studies.

In summary, here we show that KTH-13-Me strongly suppresses the proliferation of cancer cells by inducing apoptosis and suppressing cell survival signaling. The pro-apoptotic and anti-proliferative activities of KTH-13-Me appear to result from decreases in Bcl-2 and phospho-Src levels, caused by suppression of Src and STAT3 activity (Fig. 5).

Therefore, our results suggest that KTH-13-Me compounds have the potential to be developed as anti-cancer drugs, inducing apoptosis in cancer cells.

## ACKNOWLEDGMENTS

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