

Pro-Apoptotic Activity of 4-Isopropyl-2-(1-Phenylethyl) Aniline Isolated from *Cordyceps bassiana*

Mi Seon Kim^{1,†}, Yunmi Lee^{2,†}, Gi-Ho Sung³, Ji Hye Kim¹, Jae Gwang Park¹, Han Gyung Kim¹, Kwang Soo Baek¹, Jae Han Cho⁴, Jaegu Han⁴, Kang-Hyo Lee⁴, Sungyool Hong¹, Jong-Hoon Kim^{5,*} and Jae Youl Cho^{1,*}

¹Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, ²Department of Chemistry, Kwangwoon University, Seoul 139-701, ³Institute for Bio-Medical Convergence, International St. Mary's Hospital and College of Medicine, Catholic Kwandong University, Incheon 404-834, ⁴Mushroom Research Division, Department of Herbal Crop Research, National Institute of Horticultural & Herbal Science, RDA, Eumseong 369-873, ⁵Department of Veterinary Physiology, College of Veterinary Medicine, Biosafety Research Institute, Chonbuk National University, Jeonju 561-756, Republic of Korea

Abstract

Cordyceps species including *Cordyceps bassiana* are a notable anti-cancer dietary supplement. Previously, we identified several compounds with anti-cancer activity from the butanol fraction (Cb-BF) of *Cordyceps bassiana*. To expand the structural value of Cb-BF-derived anti-cancer drugs, we employed various chemical moieties to produce a novel Cb-BF-derived chemical derivative, KTH-13-amine-monophenyl [4-isopropyl-2-(1-phenylethyl) aniline (KTH-13-AMP)], which we tested for anti-cancer activity. KTH-13-AMP suppressed the proliferation of MDA-MB-231, HeLa, and C6 glioma cells. KTH-13-AMP also dose-dependently induced morphological changes in C6 glioma cells and time-dependently increased the level of early apoptotic cells stained with annexin V-FITC. Furthermore, the levels of the active full-length forms of caspase-3 and caspase-9 were increased. In contrast, the levels of total forms of caspases-3, caspase-8, caspase-9, and Bcl-2 were decreased in KTH-13-AMP treated-cells. We also confirmed that the phosphorylation of STAT3, Src, and PI3K/p85, which is linked to cell survival, was diminished by treatment with KTH-13-AMP. Therefore, these results strongly suggest that this compound can be used to guide the development of an anti-cancer drug or serve as a lead compound in forming another strong anti-proliferative agent.

Key Words: *Cordyceps bassiana*, KTH-13-amine-monophenyl, Anti-cancer activity, Proliferation, Apoptosis, Cell survival

INTRODUCTION

Cordyceps is a genus of insect-parasitizing fungus that is highly regarded as a traditional herbal medicine in Korea and China (Ng and Wang, 2005; Holliday and Cleaver, 2008; Zhou *et al.*, 2009). In traditional medicine, these species have been used not only to alleviate disorders including respiratory, pulmonary, and cardiovascular diseases, but also to recover from various immune diseases (Holliday and Cleaver, 2008; Zhou *et al.*, 2009). It has also been reported that *Cordyceps* have anti-metastatic, immune-modulatory, antioxidant, anti-inflammatory, insecticidal, antimicrobial, hypolipidemic, hypoglycemic, anti-ageing, and neuro-protective effects (Ng and Wang, 2005; Yue *et al.*, 2013; Lee *et al.*, 2014). Furthermore,

this mushroom has been considered as a promising source for anti-cancer drugs due to the anti-metastatic effect against various cancers (Ng and Wang, 2005; Holliday and Cleaver, 2008; Zhou *et al.*, 2009; Jayakumar *et al.*, 2014).

Cordyceps bassiana is one of the *Cordyceps* species that has been used as an effective herbal remedy. The butanol fraction (Cb-BF) of *Cordyceps bassiana* was reported to interrupt the inflammatory signaling cascade and have a curative effect on atopic dermatitis in systematic studies (Byeon *et al.*, 2011; Wu *et al.*, 2011). Notably, KTH-13 isolated from Cb-BF has been prescribed as an anti-proliferative and pro-apoptotic component in cancer cells (Kim *et al.*, 2015). In our study, we synthesized a new artificial synthetic compound, 4-isopropyl-2-(1-phenylethyl) aniline [KTH-13-amine-monophenyl (KTH-

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*Corresponding Authors

E-mail: jaecho@skku.edu (Cho JY), jhkim1@chonbuk.ac.kr (Kim JH)
Tel: +82-31-290-7868 (Cho JY), +82-63-270-2563 (Kim JH)
Fax: +82-31-290-7870 (Cho JY), +82-63-270-3780 (Kim JH)

[†]These authors equally contributed to this work.

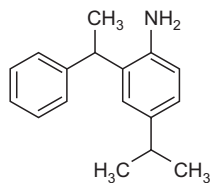


Fig. 1. The chemical structure of KTH-13-AMP.

13-AMP), (Fig 1)], a structural analog of KTH-13, and then examined its anti-cancer activity by analyzing its ability to inhibit proliferation, and checked the pro-apoptosis mechanism in various cancer cells.

MATERIALS AND METHODS

Materials

4-isopropyl-2-(1-phenylethyl) aniline [KTH-13-amine-monophenyl (KTH-13-AMP)] was supplied by Prof Lee, Yunmi (Kwangwoon University, Seoul). The purity of this compound was more than 98% according to HPLC analysis. (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, tetrazole (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). C6 glioma cells, human breast cancer MDA-MB-231 cells, and a human cervical adenocarcinoma (HeLa) cell line were purchased from ATCC (Rockville, MD, USA). A FITC Annexin V Apoptosis Detection Kit I was obtained from BD biosciences (San Diego, CA, USA). Phospho-specific and total antibodies against caspases (3, 8, and 9), Bax, Bcl-2, STAT3, PI3K/p85, Src, and β -actin were obtained from Cell Signaling (Beverly, MA, USA).

Cell culture

C6 glioma cells, MDA-MB-231, and HeLa cells were cultured in DMEM with 5% heat-inactivated FBS and 1% penicillin/streptomycin at 37°C in 5% CO₂. For each experiment, cells were detached with trypsin/EDTA solution. The cell density used in our experiments was 5×10⁵ cells/ml.

Cell viability test

After pre-incubation of C6 glioma, MDA-MB-231, and HeLa cells (5×10⁵ cells/ml) for 18 h, the testing compound was applied to the cells dose-dependently and incubated for 6 h or 24 h under 5% FBS conditions. The effect of KTH-13-AMP on proliferation was then evaluated using a conventional MTT assay (Kim and Cho, 2013a; Twentyman and Luscombe 1987). Ten microliters of MTT solution (10 mg/ml in phosphate-buffered saline (PBS), pH 7.4) was added to the cultures, and the cells were incubated for 3-4 hours. The incubation was stopped by the addition of 15% sodium dodecyl sulphate to each well to solubilize the formazan (Kim and Cho, 2013a). The absorbance at 570 nm (OD₅₇₀₋₆₃₀) was assessed using a Spectramax 250 microplate reader.

Morphological change test

KTH-13-AMP-treated C6 glioma cells were incubated with the indicated doses for the indicated times. Images of the cells

in culture at each time point were obtained using an inverted phase contrast microscope attached to a video camera and captured using NIH imaging software (Kothakota *et al.*, 1997; Kim and Cho, 2013b).

FITC Annexin V-PI staining apoptosis assay

Apoptosis was determined using FITC Annexin V Apoptosis Detection Kit I based on the cell membrane changes (phosphatidylserin-based) (Satzger *et al.*, 2010; Chang *et al.*, 2011). Cells were plated in 12-well culture plates at a seeding density of 4×10⁵ cells/dish (5×10⁵ cells/ml) and KTH-13-AMP (200 μ M) was added to the culture media at a specified concentration. Vehicle alone was added to the culture group serving as the untreated control. The subsequent procedures were conducted, according to the instructions provided by the manufacturer. Briefly, after incubation for each time point, cells were harvested, washed twice with PBS and resuspended in 1X binding buffer. Annexin-V FITC and PI were applied and incubated for 15 min at room temperature (25°C) in the dark. Fluorescence from a population of 1×10⁵ cells was detected using the BD FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and CellQuest Pro (IVD) software (Becton Dickinson, Mountain View, CA, USA). The assays were done in duplicate.

Preparation of cell lysates and immunoblotting analysis

KTH-13-AMP-treated C6 glioma or MDA-MB-231 cells (5×10⁵ cells/ml) were washed three times in cold PBS and then lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycotetraacetic acid, 50 mM β -glycerophosphate, 0.1 mM sodium vanadate, 1 mM dithiothreitol, 2% Triton X-100, 10% glycerol, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin, 2 μ g/ml leupeptin, 1 mM benzimidazole, 1.6 mM pervanadate, 20 mM NaF and 50 μ M PMSF) for 2 h on ice. The lysates were clarified by centrifugation at 12,000×rpm for 10 min at 4°C and then stored at -20°C until used.

Whole cells were then analyzed using immunoblotting (Kim *et al.*, 2013). Proteins were separated on 10%, 12%, or 15% SDS-polyacrylamide gels and transferred by electroblotting onto a polyvinylidenedifluoride (PVDF) membrane. Membranes were blocked for 60 min in Tris-buffered saline containing 3% FBS, 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20 at room temperature. The membranes were incubated for 60 min with specific primary antibodies at 4°C, washed three times with the same buffer, and then incubated for an additional 60 min with HRP-conjugated secondary antibodies. The total, active, and phosphorylated levels of the signaling enzymes and transcription factors were visualized using an ECL system (Amersham, Little Chalfont, Buckinghamshire, UK) (Shinohara *et al.*, 1996).

Statistical analysis

All data presented in this paper are the mean \pm standard deviation (SD) of an experiment performed with six numbers of samples. For statistical comparisons, the results were analyzed using ANOVA/Scheffe's post hoc test and Kruskal-Wallis/Mann-Whitney tests. A *p*-value<0.05 was considered statistically significant. All statistical tests were performed using SPSS software (SPSS Inc., Chicago, IL, USA). Similar experimental data were also obtained in an additional independent set of experiments performed with the same number of samples.

RESULTS AND DISCUSSION

Several previous studies have shown that the *Cordyceps* species have anti-cancer activity (Park *et al.*, 2009; Jayakumar *et al.*, 2014). It has been found that the aqueous extract of *Cordyceps militaris* (AECM) induces apoptosis of MDA-MB-231 cells by activation of caspases (Jin *et al.*, 2008). The *Cordyceps pruinosus* butanol fraction (Cp-BF) also has been reported to inhibit the proliferation of HeLa cells via inducing pro-apoptotic activity (Kim *et al.*, 2010). In addition, a recent study showed that KTH-13 isolated from Cb-BF suppressed the growth and viability, and induced apoptosis in C6 glioma cells and MDA-MB-231 cells (Kim *et al.*, 2015). These results imply that the structurally similar derivative from KTH-13 might also have anti-proliferative and pro-apoptotic effects. To test this hypothesis, we synthesized KTH-13-AMP and tested whether this derivative can lower the viability of several types of cancer cells including MDA-MB-231, HeLa, and C6 glioma cells.

As expected, KTH-13-AMP strongly inhibited the cell viability of MDA-MB-231, HeLa, and C6 glioma cells in a dose-dependent manner at concentrations over 100 μM (Fig. 2). The IC_{50} values of KTH-13-AMP are presented in Table 1. Also, these results led us to further investigate the mechanism underlying the compound's anti-cancer effect.

To determine whether the anti-proliferation activity of this compound was based on apoptotic or necrotic effects, we confirmed the morphological changes of KTH-13-AMP-treated C6 glioma cells. In previous studies, it was reported that the actin cytoskeleton was cleaved by caspases during cell apoptosis (Kim *et al.*, 2013; Foerster *et al.*, 2014; Kim *et al.*, 2014). When actin or its binding partners such as c-gelsolin and α -fodrin, regulating actin cytoskeleton, are cleaved, the truncated proteins are not able to support structural functions (Maravei *et al.*, 1997; Martin *et al.*, 2010). The appearance of these truncated proteins leads to morphological changes seen in pro-apoptotic cells (Mashima *et al.*, 1999; Utsumi *et al.*, 2003). As Fig. 3A shows, actin cytoskeleton-related morphological changes appeared at 150 μM from 3 h and 200 μM from 1 h. These results reflect the differentiation and apoptosis of cancer cells. Fig. 3B depicts the time-dependent increase in Annexin V-FITC staining level from 2.05 to 25.3% between 0 and 2 h at 200 μM . C6 glioma cells stained with Annexin V-FITC demonstrated that KTH-13-AMP induces a pro-apoptotic effect in cancer cells because Annexin V-FITC is an early apoptotic marker (Vermees *et al.*, 1995; Zhang *et al.*, 1997).

Next, we examined the molecular mechanism underlying the pro-apoptotic effect of KTH-13-AMP. As caspases are well known as key enzymes regulating apoptosis (Nuñez *et al.*, 1998), we verified the effect of KTH-13-AMP on caspase-3, -8, and -9. To determine which mediates KTH-13-AMP-induced apoptosis between extrinsic and mitochondrial-dependent intrinsic pathways, patterns of total and active forms of caspase-8 and -9 were measured in KTH-13-AMP-treated C6 glioma cells. As shown in Fig. 4A, levels of both full-length caspase-8 and -9 were decreased dose-dependently, while the cleaved forms of caspase-8 and -9 were increased. These results indicate that KTH-13-AMP induces apoptosis through both extrinsic and mitochondria-dependent intrinsic pathways. In addition, the level of cleaved caspase-3, an executive caspase, was also elevated in KTH-13-AMP-exposed both C6 glioma (Fig. 4A left panel) and MDA-MB-231 (Fig. 4A right panel)

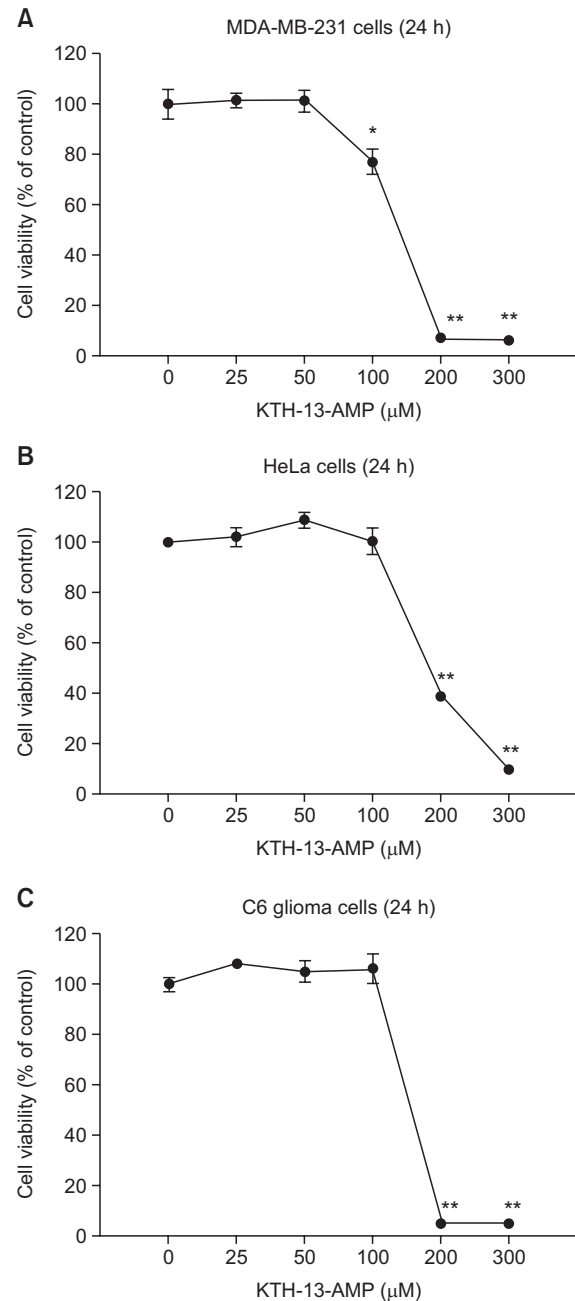


Fig. 2. The effect of KTH-13-AMP on the proliferation of cancer cells. (A, B, C) Viability of MDA-MB-231, HeLa, and C6 glioma cells treated with KTH-13-AMP for 24 h was assessed by a conventional MTT assay. * $p < 0.05$ and ** $p < 0.01$ compared to the control group.

Table 1. Effect of KTH-13-AMP on inhibiting the proliferation of MDA-MB-231, HeLa, and C6 glioma cells

Cell line	Treatment time (h)	IC_{50} (mM)
MDA-MB-231 cells	24	135.1
HeLa cells	24	181.6
C6 glioma cells	24	155.6

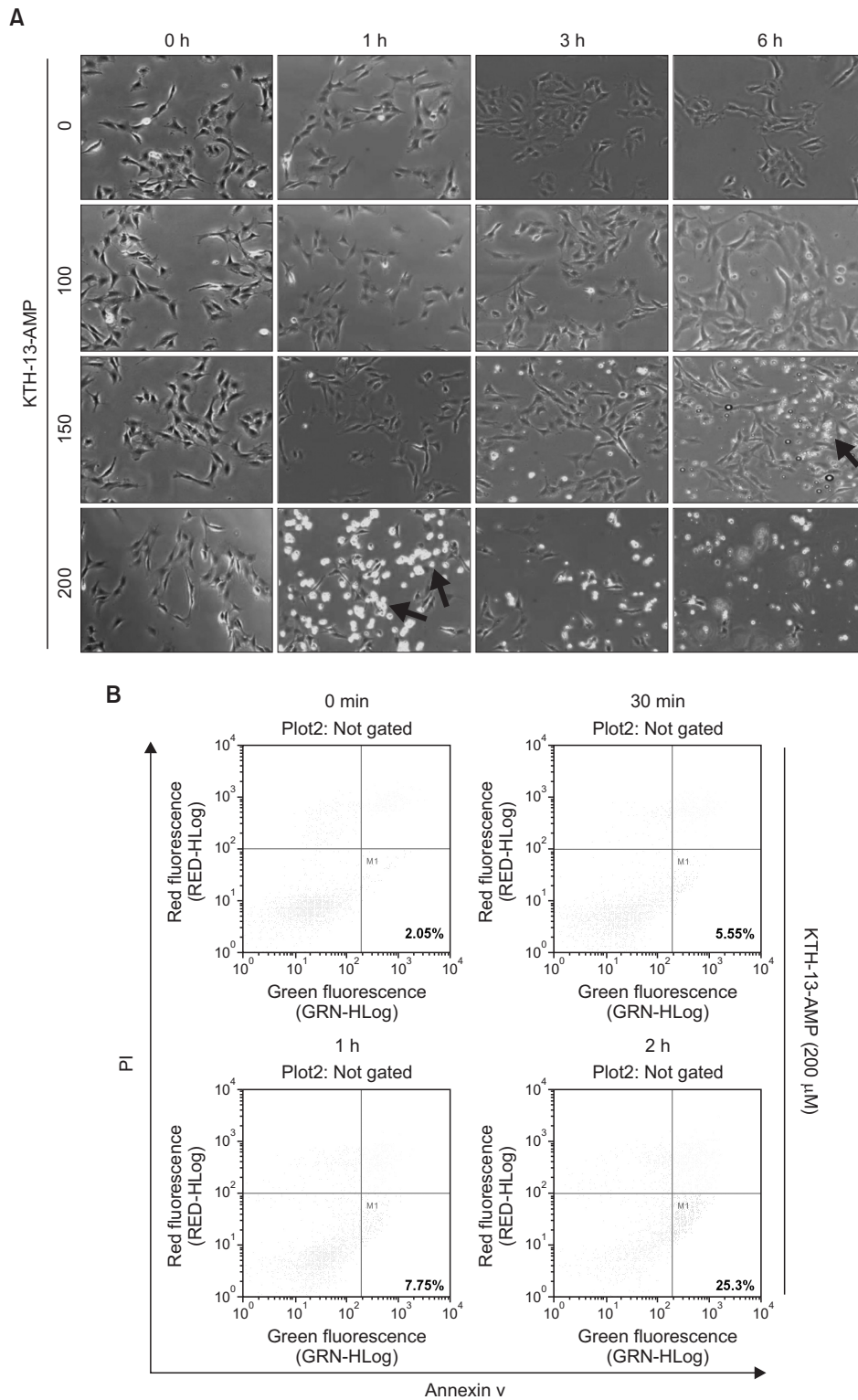


Fig. 3. Pro-apoptotic effect of KTH-13-AMP in C6 glioma cells. (A) KTH-13-AMP-treated C6 glioma cells (5×10^5 cells/ml) were incubated for 0, 1, 3, and 6 h. Morphological changes were detected at each time point by microscopic analysis. (B) Early apoptosis-inducing effect of KTH-13-AMP was examined by FITC Annexin V-PI staining assay. Annexin V and PI were treated in C6 glioma cells treated with 200 μM of KTH-13-AMP for indicated times. Each staining cell was detected by flow cytometer.

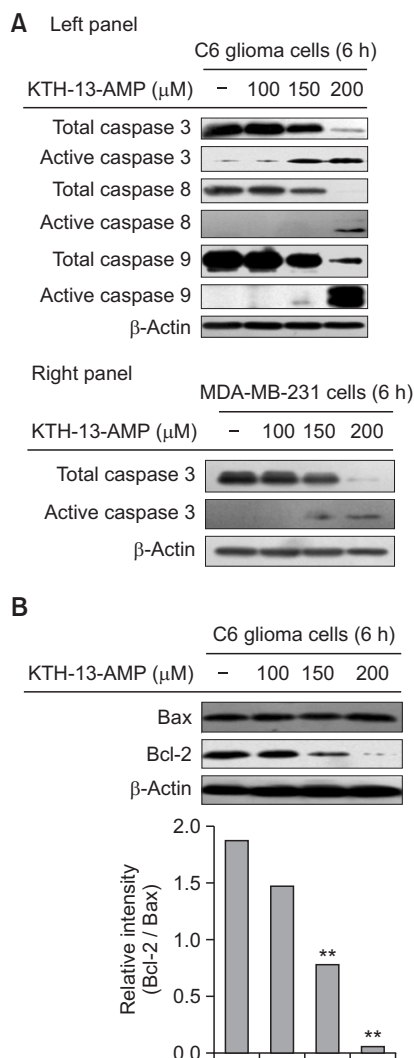


Fig. 4. Dose-dependent effect of KTH-13-AMP on induction of pro-apoptosis related proteins. (A and B) C6 glioma and MDA-MB-231 cells (5×10^6 cells/ml) treated with KTH-13-AMP for 6 h were introduced to prepare whole cell lysates and total and active form levels of caspase 3, 8, 9, Bax, Bcl-2, and β -actin were then detected by immunoblotting analysis. Relative intensity was calculated using total levels by the DNR Bio-imaging system. ** $p < 0.01$ compared to the control group.

cells in a dose-dependent manner. Then, the ratio of Bcl-2 (an apoptosis promoter)/Bax (an apoptosis inhibitor) was observed, because the ratio between these two molecules is an important index indicating the sensitivity of cells to a death signal (Oltval *et al.*, 1993). KTH-13-AMP treatment reduced the Bcl-2 expression in C6 cells, but the level of Bax was consistent, resulting in a decreased Bcl-2/Bax ratio (Fig. 4B).

Given the linkage to apoptotic activity, cell survival signaling is also associated with molecular mechanisms of the anti-apoptotic pathway. For example, it has been reported that PI3K exerts its anti-apoptotic effects via various downstream targets, including STAT-3 (Zhang *et al.*, 2005). Therefore, we examined the effects of KTH-13-AMP on PI3K/STAT-3 signaling in C6 glioma cells. Fig. 5 shows that KTH-13-AMP strongly suppressed phosphorylation of PI3K/p85 and STAT-3 in a

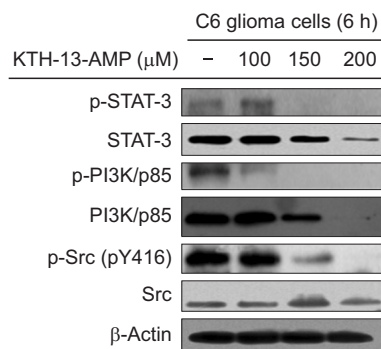


Fig. 5. Dose-dependent effect of KTH-13-AMP on cell survival signaling pathway. C6 glioma cells (5×10^6 cells/ml) were cultured with KTH-13-AMP for 6 h and whole cell lysates were prepared. The phospho- or total protein levels of STAT3, Src, and PI3K/p85 were analyzed by immunoblotting analysis.

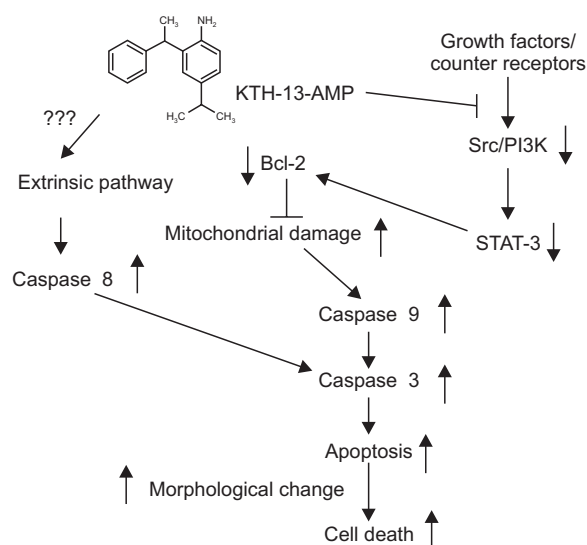


Fig. 6. Schematic apoptosis-inducing mechanism of KTH-13-AMP in cancer cells.

dose-dependent manner, although total forms of these proteins were markedly decreased at 200 μM . Also, Src phosphorylation, an upstream enzyme of PI3K, was suppressed by KTH-13-AMP without altering total forms of Src (Fig. 5). These results indicate that KTH-13-AMP blocks the anti-apoptotic pathway by inhibiting the Src-PI3K-STAT3 signal cascade. Furthermore, it was reported that the phosphorylation of Src affects the activity of Bcl-2 and cell apoptosis through STAT-3 regulation (Kundu *et al.*, 2014). Thus, it is likely that inhibition of Src-PI3K-STAT3 signaling by KTH-13-AMP also induces the pro-apoptotic pathway by activating Bcl-2. However, since the causes of suppression of PI3K expression and STAT-3 expression by KTH-13-AMP (Fig. 5) remains unclear, we will now address the reason for the decreased total level of PI3K/p85 and STAT-3 by KTH-13-AMP in terms of apoptosis.

In conclusion, we have demonstrated that KTH-13-AMP is able to suppress the cell viability of cancer cells through activation of apoptotic pathway, as summarized in Fig. 6. The apoptotic activity of this compound was induced by activation

of pro-apoptotic signaling mediated via caspases and the Bcl family. Moreover, it seems that the Src-PI3K-STAT3 signal, a representative anti-apoptotic signal cascade, is also suppressed by KTH-13-AMP treatment. Our results show that KTH-13-AMP possesses anti-cancer activity and has the potential to be used for cancer treatment. In addition, this study implies the possibility of developing new cancer therapeutics designed from *Cordyceps bassiana*-derived anti-cancer compounds.

CONFLICT OF INTEREST

The authors have no conflicts of interest to report. The authors alone are responsible for the content and writing of the paper.

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