

Mechanism of apoptosis induction associated with ERK1/2 upregulation via goniiothalamine in melanoma cells

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Abstract. The present study aimed to investigate the effect of goniiothalamine on apoptosis induction in the A375 melanoma cell line. Melanoma is a type of skin cancer with increased prevalence and no potential standard treatment. Goniiothalamine is a plant, bioactive styryl-lactone, which has various bioactivities including anti-microbial, anti-inflammatory and anti-cancer. Apoptosis induction by goniiothalamine has been studied in numerous cancer cell lines, however not in the melanoma cell line A375. The results of the MTT assay demonstrated that goniiothalamine induced anti-proliferation in a dose dependent manner. Hoechst staining assay demonstrated that goniiothalamine induced chromatin condensation and apoptotic bodies in A375 treated cells, and JC-1 staining revealed that goniiothalamine induced mitochondrial membrane dysfunction in A375 cells. In addition, goniiothalamine decreased the level of anti-apoptotic proteins myeloid cell leukemia 1, B cell lymphoma (Bcl)-2 and Bcl-extra large, whereas it increased the level of pro-apoptotic proteins, Bcl-2 Associated X, apoptosis regulator, t-BID and Bim in A375 treated cells. In addition, goniiothalamine also increased active caspase-9, -7 and cleaved-poly (ADP-ribose) polymerase expression in A375 treated cells. Furthermore, phosphorylated (p)-pyruvate dehydrogenase kinase (PDK) 1 (Ser241) and p-RAC-alpha serine/threonine-protein kinase (Akt; Ser473) were decreased, however c-Jun and p-extracellular signal-regulated kinase (ERK)1/2 were increased upon goniiothalamine treatment. These results suggest that goniiothalamine has an effect, as anti-proliferation and apoptosis induction in A375 cells were associated with upregulated p-ERK1/2, c-Jun and downregulated p-PDK1 (Ser241), p-Akt (Ser473) in A375 cells. Therefore, goniiothalamine may be a potential candidate for anti-cancer drug development for melanoma treatment.

Introduction

Skin cancer can be divided into two groups, non-melanoma skin cancer (NMSC) and malignant melanoma (MM). MM is the most related death skin cancer; incidence of melanoma is increasing worldwide (1-4). Melanoma developed from melanocyte, which is pigment-producing cell. There are several methods to treat melanoma depending on stage and severity of disease such as surgical excision, chemotherapy, radiotherapy, immunotherapy and biological therapy. All of them kill cancer cells but also kill normal cells, so new therapy is developing everyday (5).

Apoptosis or program cell death is an essential mechanism in multicellular organism. This process plays a critical role including normal cell turnover, development of embryo, functioning of the immune system, tissue homeostasis and elimination of damaged cells. During the early process of apoptosis, cells were initially described by its morphological characteristics including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation. Abnormal apoptosis induces the processes of oncogenesis including initiation, progression and metastasis in abnormal cell. Recently, there are studies about apoptosis with cancer cells, which studied about signaling process in cell (6-10).

Goniiothalamine is extracted from root and bark of plant in family Annonaceae genus *Goniiothalamus*. Studies show effect of goniiothalamine as apoptotic agent and anti-proliferative agent in cancer cell, antibiotics, and antifungal (11,12). Recent research has demonstrated that goniiothalamine showed cytotoxicity and apoptosis induction in various tumor cell lines. However, the study of goniiothalamine in MM has not yet been reported.

In the present study, we found that goniiothalamine inhibited cell proliferation and induce apoptosis associated with mitochondria dysfunction, caspase activation, and the upregulation of c-Jun, p-p38 and p-ERK1/2, but downregulation of Akt signaling pathway in melanoma A375 cells.

Materials and methods

Materials. RPMI-1640 medium were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

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bromide (MTT), JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide) and phenylmethylsulfonyl-fluoride (PMSF) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). DMSO was purchased from Calbiochem (San Diego, CA, USA). Guava Cell Cycle[®] reagent for cell cycle analysis and fetal bovine serum (FBS) were purchased from Merck KGaA. MEK1/2 inhibitor (U0126) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Goniiothalamine was obtained from Assoc. Prof. Wilawan Mahabusarakam, Faculty of Science, Prince of Songkla University (Hat Yai, Thailand) in purified powder form (13).

Cell culture. MM cell line A375 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained as a monolayer in RPMI-1640 supplemented with 10% FBS (GE Healthcare, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories, Pasching, Austria). The cells were cultured in 5% CO₂ at 37°C and subcultured 2-3 times/week.

Cell proliferation and cell viability assays. MTT assay was used to determine the cytotoxicity of goniiothalamine. A375 cells were seeded in a 96-well plate at 5x10³ cells/well and allowed to grow for 24 h. Then, cells were treated with goniiothalamine at 0.1, 0.3, 0.5, 1, 3, 5 and 10 µg/ml for 24 h, whereas the control group was treated with 0.5% DMSO. After incubation, 100 µl of 0.5 mg/ml MTT solution was added to each well and incubated for 2-4 h at 37°C, then supernatant was removed and DMSO was added to solubilize the formazan crystals. The absorbance was measured by using a microplate reader at 570 nm (Multiskan EX; Thermo Electron Corp., Vantaa, Finland), and the IC₅₀ value was calculated by using the GraphPad Prism 3.03 (GraphPad Software, Inc., San Diego, CA, USA).

Nuclear morphological staining with Hoechst 33342. A375 cells were seeded at 4x10⁵ cells/well for 24 h. Then, the cells were treated with 3, 5 and 10 µg/ml goniiothalamine for 24 h, while, the control group was treated with 0.5% DMSO. After incubation, cells were stained with 5 µM Hoechst 33342 for 30 min at 37°C and examined under a fluorescence microscope (IX73; Olympus, Tokyo, Japan).

Cell cycle analysis. To examine apoptosis induction via upregulation of sub-G1 population, flow cytometry was carried out. Cells were treated with 3, 5 and 10 µg/ml goniiothalamine for 24 h and 0.5% DMSO was used as the control group. Upon treatment, cells were washed with PBS and fixed with ice cold 70% ethanol at 4°C for more than 1 h. After fixation, cells were stained according to the manufacturer's instructions (Guava Cell Cycle[®] reagent from Merck KGaA). The DNA content was observed by Guava easyCyte[™] flow cytometer and GuavaSoft[™] software (Merck KGaA).

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$). JC-1 was used to determine the function of the $\Delta\Psi_m$, which specific to mitochondria that is incorporated into the mitochondrial membrane. A375 cells were seeded at 3x10⁵/well for 24 h. Then, cells were treated with 3, 5 and 10 µg/ml

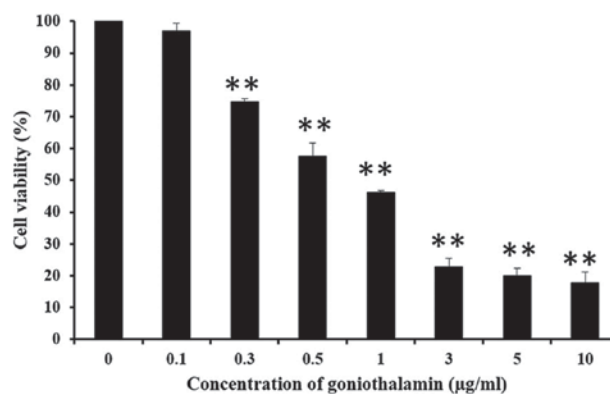


Figure 1. Effect of goniiothalamine on cell viability following treatment with different concentrations of goniiothalamine for 24 h, **P<0.01 significantly compared with the control.

goniiothalamine for 24 h, whereas the control group was treated with 0.5% DMSO. Cells were stained with 5 µg/ml of JC-1 in the dark at 37°C for 10 min and washed with PBS for 3 times before analysis by fluorescence microscopy.

Western blot analysis. A375 cells were seeded at 3x10⁵/well for 24 h. Cells were treated with 3, 5 and 10 µg/ml goniiothalamine for 24 h, whereas the control group was treated with 0.5% DMSO and harvested at designated time points. The pellet cells were lysed with RIPA lysis buffer (50 mM Tris-HCL, pH 7.5, 5 mM EDTA, 250 mM NaCl, 0.5% Triton X-100) supplemented complete mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein expression by using western blot analysis was carried out according to a previously reported protocol (13).

Statistical analysis. All data presented were obtained from at least three independent experiments and were presented as mean ± standard deviation (SD). Statistical significance was assessed by one-way analysis of variance (ANOVA). Statistical analysis was performed by using SPSS statistical software package (version 15; SPSS, Inc., Chicago, IL, USA) also carried out using the software GraphPad Prism 3.03 (GraphPad Software, Inc., La Jolla, CA, USA). The western blotting band intensity was quantified by Image J densitometer.

Results

The effect of goniiothalamine on cell anti-proliferation and apoptosis induction in A375 cells. MTT assay was used to determine the anti-proliferation activity of goniiothalamine in A375 cells. The results showed that goniiothalamine inhibited cell proliferation in a dose-dependent manner in A375 treated cells with the IC₅₀ value was 1.7±0.627 µg/ml (Fig. 1). Hoechst 33342 staining was used to determine the nuclear morphological changes mediated apoptosis. Morphological changes in apoptotic cell include chromatin condensation and apoptotic bodies. Hoechst 33342 is a fluorescence dye and a cell-permeable DNA stain used for labeling DNA, although the dyes can bind to all nucleic acid enhancing fluorescence considerably (14). The results

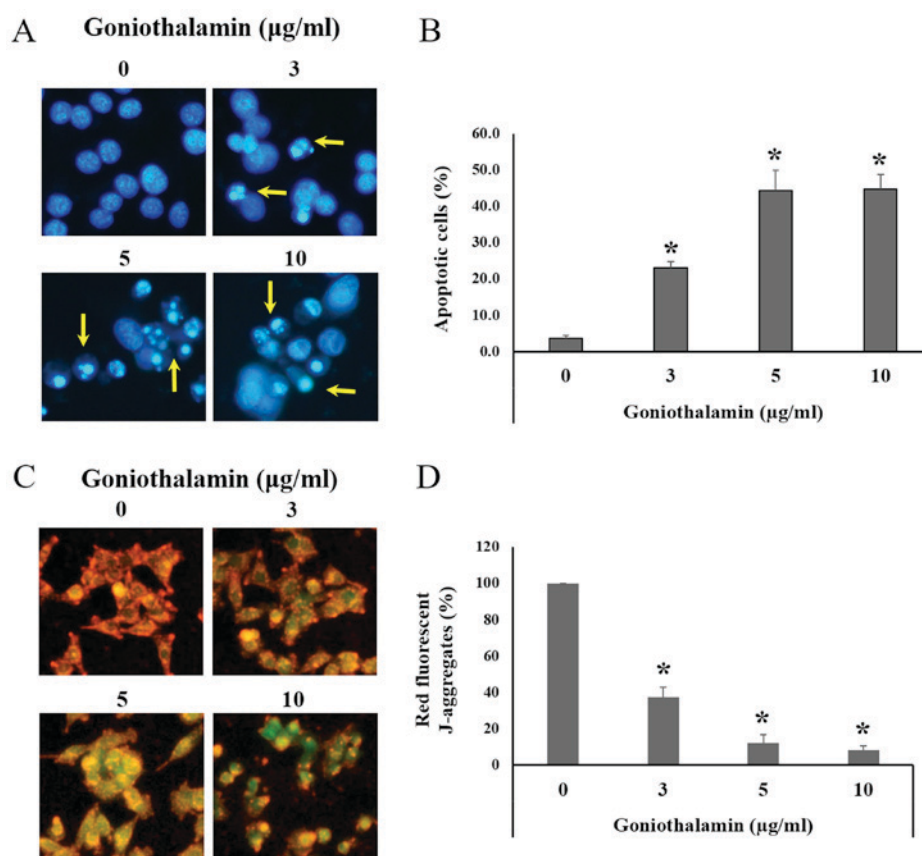


Figure 2. Effect of goniothalamin on apoptosis induction. (A) Effects of goniothalamin on nuclear condensation of A375 cells. Cells were treated with 3, 5, and 10 µg/ml goniothalamin for 24 h and then stained with Hoechst 33342 and examined under a fluorescent microscope (magnification, x40). (B) Percentage of apoptotic cells of A375 goniothalamin treated cells compared with untreated cells. (C) The effect of goniothalamin on mitochondrial membrane potential in A375 cells. Cells were treated with 3, 5 and 10 µg/ml goniothalamin for 24 h. (D) Percentage of red fluorescent J-aggregates of A375 goniothalamin treated cells compared with untreated cells (magnification, x40), *P<0.05 significantly compared with the control.

revealed that goniothalamin induced chromatin condensation and apoptotic bodies in A375 cells following treatment with goniothalamin (Fig. 2A). Our results showed that goniothalamin significantly induced apoptotic cells at 3, 5 and 10 µg/ml (P<0.05; Fig. 2B). This result suggested that goniothalamin could induce apoptosis in A375 cells.

Mitochondrial membrane potential ($\Delta\Psi_m$). During apoptosis, mitochondria was disturbed by pro-apoptotic proteins activation leading to the loss of $\Delta\Psi_m$. JC-1 staining was carried out to measure the $\Delta\Psi_m$. In normal cells with high $\Delta\Psi_m$, JC-1 forms J-aggregates complexes as shown in red fluorescence. Whereas, apoptotic cells with low $\Delta\Psi_m$, JC-1 remains in the monomeric form as shown in green fluorescence (15). Our study showed that goniothalamin significantly induced the loss of $\Delta\Psi_m$ in A375 treated cells showing decreased red fluorescence comparing with the control cells (P<0.05; Fig. 2C and D). This result indicated that goniothalamin induced apoptosis through mitochondria dysfunction in A375 treated cells.

Cell cycle distribution. To further verify the inhibitory effect of goniothalamin on apoptosis induction, cells were stained with PI and histogram analysis-related DNA contents were measured by flow cytometry. The treatment of A375 cells with goniothalamin at 3 µg/ml for 24 and 48 h showed increasing

of sub-G1 population peak to 6.16 and 10.07%, respectively, whereas the control cells showed 0.84% of sub-G1 population (Fig. 3). This result confirmed that goniothalamin induced cell death by apoptosis induction in A375 treated cells.

Bcl-2 family, caspase-7, caspase-9 and cleaved-PARP expression. The results showed that goniothalamin decreased the level of anti-apoptotic proteins Mcl-1, Bcl-2 and Bcl-xL in A375 treated cells (P<0.05; Fig. 4A and B), whereas increased the level of pro-apoptotic proteins Bax, t-BID and Bim in A375 treated cells (P<0.05; Fig. 4A and C). In addition, goniothalamin induced caspase-9, caspase-7 and cleaved-PARP activation in A375 treated cells (P<0.05; Fig. 5A and B). The results indicated that goniothalamin could induce apoptosis via intrinsic pathway.

Effect of goniothalamin on AKT signaling pathway. AKT signaling pathway is one of the most important pathways, which promote cell proliferation, cell growth, transcription and cell migration. The results showed that goniothalamin downregulated p-PDK1 (Ser241) and total Akt indicating that goniothalamin could inhibit A375 cell survival (P<0.05; Fig. 5A and C).

The effect of goniothalamin on MAPK signaling pathway. MAPK signaling pathway is the pathway that involved both apoptosis and cell survival. Upon treatment with

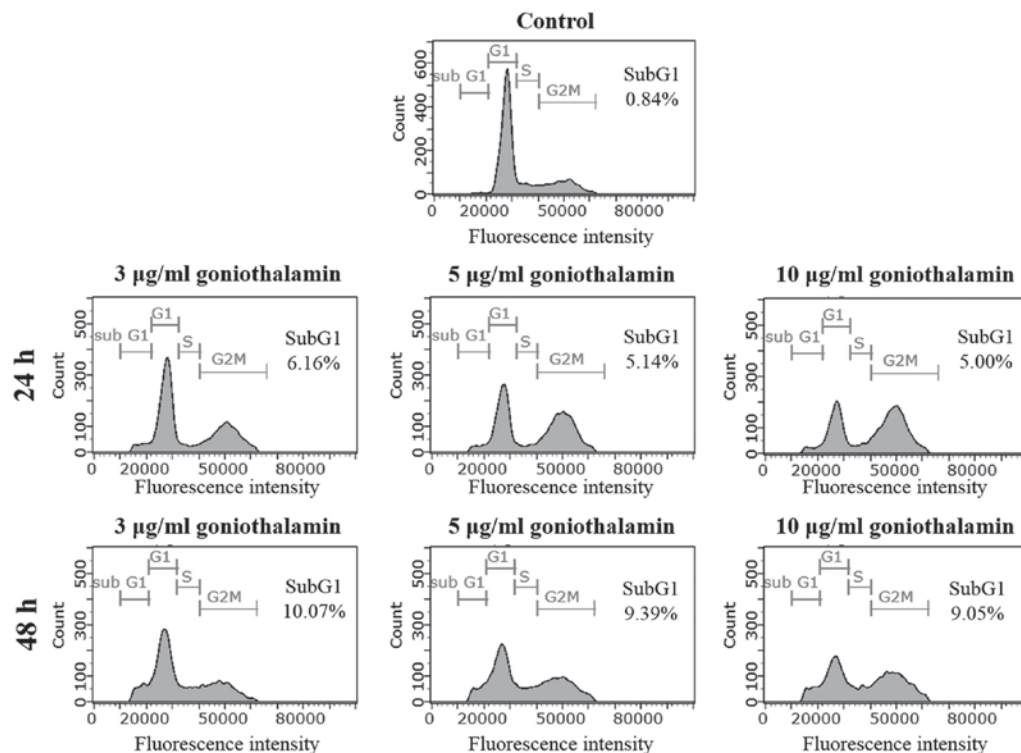


Figure 3. Effect of goniothalamin on cell cycle distribution. The sub G1 peak formed with reduced DNA content represented the presence of apoptotic cells. The mean apoptotic population of A375 cells was 0.84% under the control condition, while increasing to 6.16 and 10.07% upon treatment with 3 µg/ml goniothalamin for 24 and 48 h, respectively.

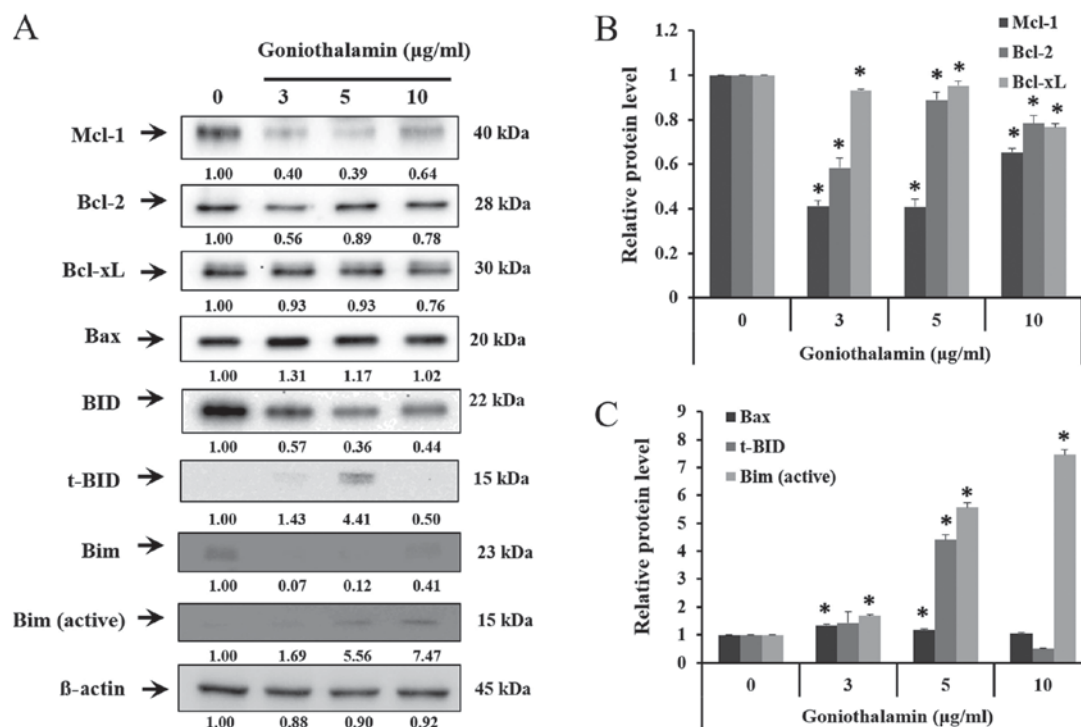


Figure 4. Effect of goniothalamin on Bcl-2 family protein expression. Cells were treated with 3, 5, and 10 µg/ml goniothalamin for 24 h. (A) Effect of goniothalamin on anti-apoptotic and pro-apoptotic proteins in A375 cells were determined by western blot analysis. (B) Relative protein level of anti-apoptotic proteins. (C) Effect of goniothalamin on pro-apoptotic proteins in A375 cells were determined by western blot analysis. β-actin was used as an internal control, *P<0.05 significantly compared with the control.

goniothalamin, the level of c-Jun, p-ERK1/2 and p-p38/p38 ratio were increased (P<0.05; Fig. 6A and B). These results

indicated that goniothalamin induced apoptosis via MAPK signaling pathway. In order to determine the effects of

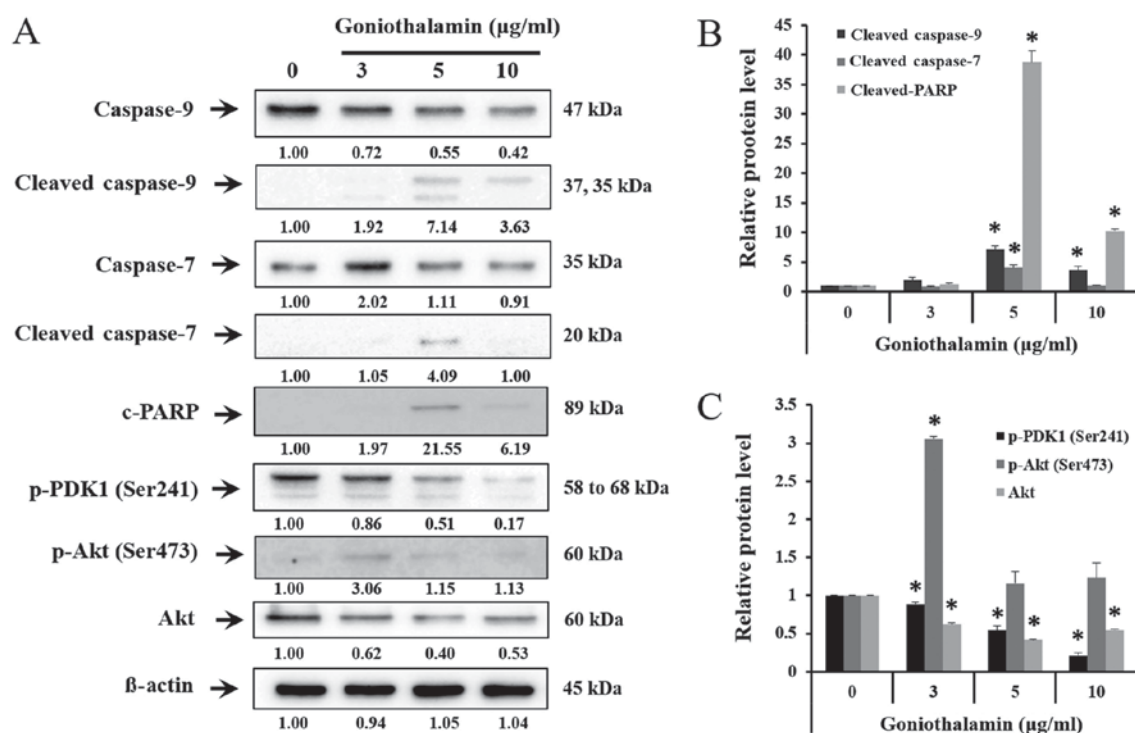


Figure 5. Effect of goniiothalamine on caspase, cleaved-PARP induction and Akt signaling molecules. Cells were treated with 3, 5, and 10 $\mu\text{g/ml}$ goniiothalamine for 24 h. (A) Effect of goniiothalamine on caspase-9, -7, cleaved-PARP, p-PDK1, p-Akt at Ser473 and total Akt proteins in A375 cells were determined by western blot analysis. (B) Relative protein level of cleaved-caspase-9, cleaved-caspase-7 and cleaved-PARP proteins. (C) Relative protein level of p-PDK1, p-Akt and Akt proteins. β -actin was used as an internal control, * $P < 0.05$ significantly compared with the control.

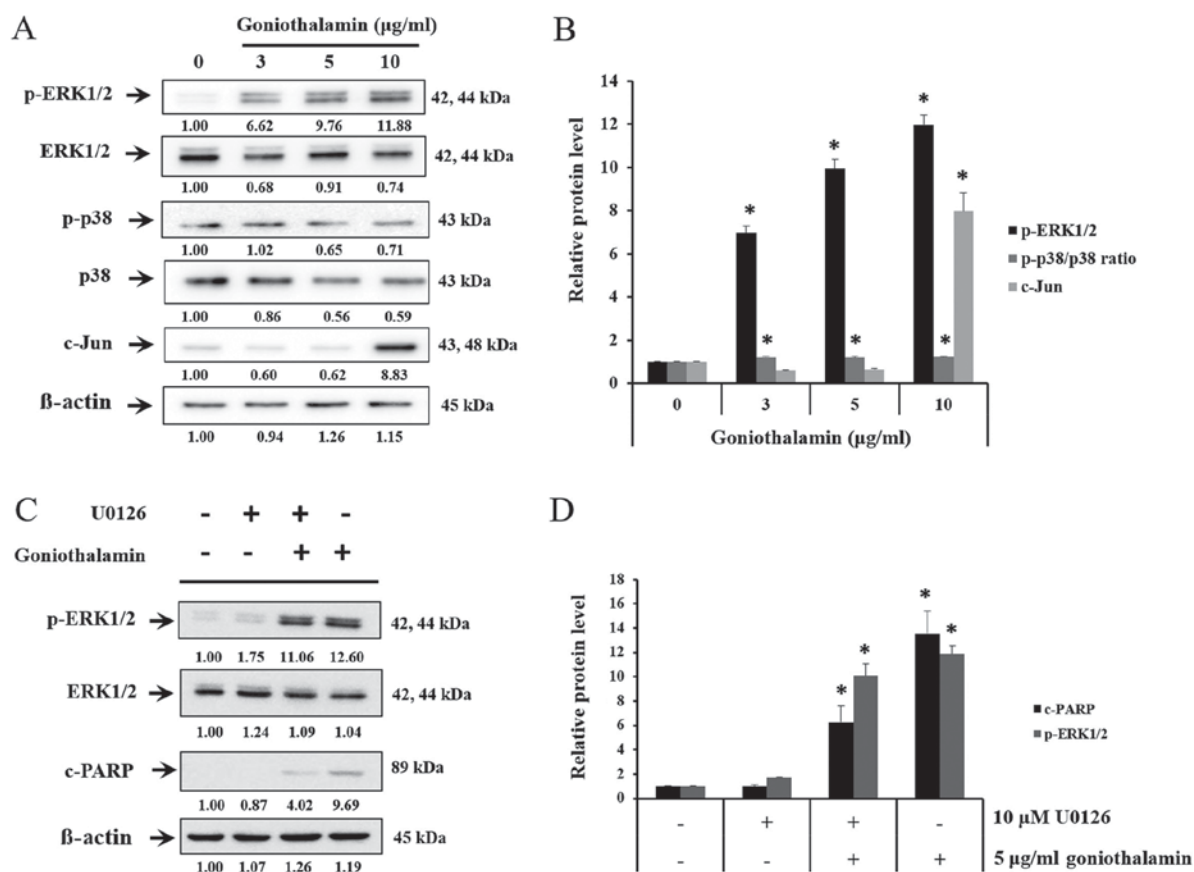


Figure 6. Effects of goniiothalamine on phosphorylation of MAPK signaling in A375 cells. (A) Cells were treated with 3, 5, and 10 $\mu\text{g/ml}$ goniiothalamine for 24 h and examined by Western blot analysis. (B) Relative protein level of p-ERK1/2, c-Jun and p-p38/p38 ratio (C) U0126, MEK1/2 inhibitor was used as a positive control compared with the goniiothalamine treatment. (D) Relative protein level of p-ERK1/2 and cleaved-PARP. β -actin was used as an internal control, * $P < 0.05$ significantly compared with the control.

p-ERK1/2 on apoptosis induction by goniothalamin in A375 cells, MEK1/2 inhibitor (U0126) was used. The result showed that U0126 simultaneously blocked p-ERK1/2, in contrast, co-treatment between U0126 and goniothalamin showed upregulation of p-ERK1/2 and cleaved-PARP indicating that goniothalamin induced apoptosis through ERK1/2 signaling ($P < 0.05$; Fig. 6C and D). These results suggested that goniothalamin induced apoptosis in A375 cells by promoting cell death through p-ERK1/2 activation.

Discussion

In this study, A375 cell line was used as a model for MM. MM is one of the most related death skin cancer which originated from melanocyte (pigment cells). The treatments of MM depending on stage and severity of disease, surgical excision is the main therapy of MM. However adjuvant therapy is needed for advanced stage disease but most of therapy have side effects and not significantly increase survival rate in patients.

Recent research group has demonstrated that goniothalamin showed cytotoxicity and apoptosis induction in various tumor cell lines, H1299 (non-small cell lung cancer) (16), HepG2 (human hepatoblastoma) (17), SK-BR-3 (human breast cancer) (13), A549 (lung carcinoma) (18), HL60 (promyelocytic leukemia) (19,20), SGC7901 (stomach cancer) (21), HT29 (colon cancer) (22,23), HeLa (cervical cancer) (24), and MDA-MB231 (invasive breast cancer) (25). However, the study of goniothalamin in MM have not yet been reported.

The results showed goniothalamin inhibited A375 cell growth in a dose-dependent manner. Innajak *et al* demonstrated that goniothalamin inhibited SK-BR-3 cell growth in a time- and dose-dependent manner with an IC_{50} value of $10 \pm 0.45 \mu\text{g/ml}$ (13). At 72 h, goniothalamin completely inhibited cell viability in MDA-MB-231 with an IC_{50} value of about $1.46 \mu\text{M}$ (25).

Hoechst staining was used to confirm nuclear morphological changes via apoptosis induction. Hoechst staining showed condensed chromatin and apoptotic bodies in the A375 cells after treatment with goniothalamin (Fig. 2A). In other cell line, Chen *et al* reported that after treat MDA-MB-231 cells with $30 \mu\text{M}$ goniothalamin for 48 h, chromatin condensation and nuclear fragmentation were detected (25).

Moreover the JC-1 staining assay showing significantly decreased red fluorescence while increased green indicating that the loss of $\Delta\Psi\text{m}$ and leading to apoptosis induction (Fig. 2C and D).

To confirm signaling pathway of apoptosis induction, Bcl-2 family proteins, caspase proteins, Akt and MAPK pathway were analyzed by western blotting.

The anti-apoptotic proteins Bcl-2 family protein, Bcl-2, Mcl-1 and Bcl-xL was decreased (Fig. 4A) whereas pro-apoptotic proteins Bax, t-BID and Bim were increased upon treatment with goniothalamin (Fig. 4A). In addition, there are two types of caspase, initiator and effector caspase, caspase-9 (initiator caspase) can activate caspase-7 (effector caspase) and deactivate PARP, which is DNA repairing protein. The results showed that caspase-7 and caspase-9 were increased which then induced cleaved-PARP activation (Fig. 5A). These results correlated with previous study revealing that goniothalamin induced apoptosis in different cancer cell types including HeLa (26), SK-BR-3 (13), Colo 205, SW480 and LoVo cells (27).

Akt is signaling pathway that promotes cell growth and anti-apoptosis. From previous studies, goniothalamin down regulated phosphorylated Akt at Ser473, Thr308 and total Akt in SK-BR-3 cells leading to apoptosis induction (13). These studies showed the decrease of p-PDK1 (Ser241) and total Akt (Fig. 5A) indicating that goniothalamin induced apoptosis and inhibited cell proliferation.

Another group is protein in MAPK signaling pathway playing important role both in cell survival and cell death. Conventional MAPKs in mammalian include the ERK1/2, JNK1/2 and p38. ERK1/2 activates Bax protein and caspase then deactivates Akt pathway, which leads to apoptosis. JNK1/2 can activate the transcriptional factors including c-Jun, which express Bim. p38 is tumor suppressor, which induce apoptosis and inhibit cell proliferation. p38 can activate p53, which is tumor suppressor (Fig. 6A). This result showed that goniothalamin induced p-ERK1/2, p-p38/p38 ratio and c-Jun upregulation in A375 treated cells leading to apoptosis. In general, ERK is important in cell proliferation, cell differentiation, cell growth or cell survival, however, we found that goniothalamin induced p-ERK1/2 upregulation in A375 treated cells. These results correlated with previous report by Tan *et al*, that ERK1/2 could activate caspase and pro-apoptotic protein in Bcl-2 family, moreover ERK1/2 could deactivated Akt signaling pathway leading to apoptosis induction (28). Therefore, the results showed that c-Jun and p-ERK1/2 were increased implying that goniothalamin induced apoptosis in A375 cell.

Indeed, we also confirmed the effect of goniothalamin on apoptosis induction through p-ERK1/2 activation using U0126. The results showed that goniothalamin induced apoptosis in A375 cells via ERK1/2 signaling (Fig. 6C). Our study correlated with previous report that ERK is involved in apoptosis induction of *Moringa oleifera* fruit (MOF) extract in human melanoma A2058 cells (29).

In summary, goniothalamin has an effect as anti-proliferation and apoptosis induction in A375 cells associated with upregulated p-ERK1/2, c-Jun and downregulated p-PDK1 (Ser241), total Akt in A375 cells. Studying the effect of Goniothalamin in other MM cell lines. In the future, the effect of goniothalamin in primary epidermal melanocytes (normal) will be studied to confirm that this compound could affect melanoma but not the normal cell. Furthermore the effect of goniothalamin should be studied in animal model.

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