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Authors

Han, Jaeho

Kim, Donghwa

Park, Hyen

et al.

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Antiproliferative Activity of Gibbosic Acid H through Induction of G_0/G_1 Cell Cycle Arrest and Apoptosis in Human Lung Cancer Cells

Jaeho Han[1], Donghwa Kim[1], Hyen Joo Park[1], Hee-Juhn Park[2], Sang Kook Lee[1]

[1]College of Pharmacy, Natural Products Research Institute, Seoul National University, Seoul, [2]Department of Pharmaceutical Engineering, Sangji University, Wonju, Korea

Lung cancer is one of the most common causative cancers worldwide. Particularly, non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases. NSCLC is a serious form of lung cancer that requires prompt diagnosis, and the 5-year survival rate for patients with this disease is only 24%. Gibbosic acid H (GaH), a natural lanostanoid obtained from the *Ganoderma* species (Ganodermataceae), has antiproliferative activities against colon and lung cancer cells. The aim of the present study was to evaluate the antiproliferative activity of GaH in NSCLC cells and to elucidate the underlying molecular mechanisms. GaH was found to induce G_0/G_1 cell cycle arrest and autophagy by activating adenosine monophosphate-activated protein kinase in A549 and H1299 cells. The induction of this cell cycle arrest was associated with the downregulation of cyclin E1 and CDK2. Additionally, the induction of autophagy by GaH was correlated with the upregulation of LC3B, beclin-1, and p53 expression. GaH also induced apoptosis by upregulating cleaved caspase-3 and Bax in the lung cancer cells. These findings suggest that GaH has a potential in the growth inhibition of human lung cancer cells.

Key Words Gibbosic acid H, *Ganoderma* species, Antiproliferation, Cell cycle arrest, Apoptosis

INTRODUCTION

Lung cancer is the leading cause of cancer-associated death and second most commonly occurring malignancy in humans [1]. Non-small cell lung cancer (NSCLC) accounts for the majority of all lung cancers, and the 5-year survival rate associated with this disease is only 24% [2]. Although the current understanding of lung cancer has improved the development of targeted therapies, the side effects and emergence of resistance remain major concerns [3,4]. Therefore, the identification of new effective therapeutic agents from natural products is extremely important to prevent and treat NSCLC.

Ganoderma (Ganodermataceae), a genus of medicinal mushrooms mainly distributed in China and Korea, has been traditionally used for treating insomnia, bronchitis, hepatopathy, diabetes, and cancer [5]. Diverse biological activities including anticancer, anti-inflammatory, antitumor, anti-oxidant, and immunomodulatory effects were exhibited by extracts from diverse *Ganoderma* species [6]. A phytochemical study identified more than 400 secondary metabolites, such as lanostane-type triterpenoids, meroterpenoids, and steroids, isolated from this species [5]. Some of the lanostane-type triterpenoids showed pharmacological activities, such as cell cycle arrest, autophagy, apoptosis, anti-invasion, anti-asthma, and anti-osteoclastogenesis [7]. Recently, lanostane triterpenoids have been considered as potent anticancer agents, and lanostane triterpenoids from *Ganoderma luteomarginatum* exhibited cytotoxicity against human cancer cells [8]. A few articles have reported that lanostane-type triterpenoids possess anti-adipogenic and anti-inflammatory activities [5]. However, the antiproliferative activity of gibbosic acid H (GaH), a lanostane triterpenoid, and precise underlying molecular mechanisms involved in human cancer cells remain unknown.

Adenosine monophosphate-activated protein kinase (AMPK) is a highly-conserved fuel-sensing enzyme that plays a vital role in cellular energy homeostasis [9,10]. AMPK comprises three subunits: α, β, and γ. Activation of AMPK is triggered in response to a shortage of adenosine triphosphate

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Correspondence to Sang Kook Lee, E-mail: sklee61@snu.ac.kr, https://orcid.org/0000-0002-4306-7024

(ATP) production. The effect of AMPK activation is to rewire metabolism to decrease anabolic processes and increase catabolism thereby restoring a suitable energy balance [11]. Once activated, AMPK phosphorylates numerous metabolic enzymes acutely inhibiting pathways that consume ATP and activating pathways that generate ATP. Moreover, activated AMPK induces metabolic tumor suppression by regulating the energy metabolism and enforcing the metabolic checkpoint [10,11]. Further, AMPK plays an essential role in regulating autophagy, a lysosome-dependent catabolic system that mediates cellular material degradation [12]. Typically, autophagy promotes tumor survival and growth, but excessive levels can lead to various forms of cell death, such as autophagy death and apoptosis. Moreover, activation of AMPK induces G_0/G_1 cell cycle arrest via the activation of p53 and its downstream effector p21 [13]. These findings suggest that AMPK activators are important therapeutic candidates for cancer prevention and treatment.

In this study, the antiproliferative effects of GaH were investigated in NSCLC cells. In particular, the underlying mechanisms with a focus on AMPK as a potential target that induces both cell cycle arrest and apoptosis were determined in NSCLC cells.

MATERIALS AND METHODS

Cell lines, reagents, and antibodies

Lung (A594), colorectal (HCT116), breast (MDA-MB-231), and liver (SK-HEP-1) cancer cells were purchased from the American Type Culture Collection. The human NSCLC cell line (H1299) and gastric (SNU-638) were obtained from the Korean Cell Line Bank. The cell lines were cultured in media (A549, HCT116, SNU-638, and H1299 in Roswell Park Memorial Institute [RPMI] 1640; MDA-MB-231 and SK-HEP-1 in Dulbecco's modified Eagle's medium [DMEM]) supplemented with 10% FBS, 100 units/mL penicillin G sodium, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B and incubated in a humidified atmosphere at 37ºC and 5% CO_2.

Bovine serum albumin (BSA), sulforhodamine B (SRB), etoposide, propidium iodide (PI), anti-β-actin antibody, and dimethyl sulfoxide were purchased from Sigma-Aldrich, Inc. DMEM, RPMI 1640 medium, antibiotic–antimycotic solution (100×), FBS, PBS (1×), and trypsin-EDTA solution (1×) were purchased from HyClone Laboratories, Inc. Goat anti-mouse immunoglobulin (Ig) G-horseradish peroxidase (HRP), goat anti-rabbit IgG-HRP, MDM2, p53, β-actin, p21, p62, Bcl-2, and Bax antibodies were purchased from Santa Cruz Biotechnology, Inc. The cyclin-dependent kinases (CDK) 2, Rb, p-Rb, p-ULK1, LC3B, beclin-1, ACC, p-ACC, AMPKα, and p-AMPKα antibodies were purchased from Cell Signaling Technology. Cyclin D1 and cyclin E1 were purchased from BD Biosciences. Etoposide, an anticancer agent, was used as a positive control in the antiproliferation assay. Gibbosic acids and GaH (purity > 98% by HPLC analysis) were isolated from a $CHCl_3$-soluble methanol fraction of *Ganoderma* species by silica gel column (30 μm, Φ 5 cm × 20 cm; Yamazen) column chromatography as described previously [14]. GaH and its derivatives were dissolved in dimethylsulfoxide (DMSO).

Cell proliferation assay (SRB assay)

MDA-MB-231 (10×10^3 cells/well), SK-Hep-1 (8×10^3 cells/well), HCT116 (8×10^3 cells/well), SNU638 (14×10^3 cells/well), A549 (7×10^3 cells/well), and H1299 (10×10^3 cells/well) cells were seeded in 96-well plates. Cells were treated with different concentrations of test compounds and incubated for either 48 or 72 hours in complete medium; subsequently, the cells were fixed with 10% trichloroacetic acid solution for 30 minutes and washed with tap water. After staining with 0.4% SRB in 1% acetic acid solution, the unbound dye was washed off using 1% acetic acid solution. The plate was washed, and stained cells were mixed with 10 mM Tris (pH 10.0). The absorbance was measured at 515 nm. The concentration for 50% cell growth inhibtion (IC_{50} value) was determined using the Table curve 2D v5.01 software (Systat Software Inc.) using the non-linear regression analysis [15].

Western blot analysis

Western blot analysis was performed according to our previous protocol [16]. The A549 (7×10^4 cells/well) and H1299 (10×10^4 cells/well) cells were seeded in 6-well plates and treated with various concentrations of GaH for indicated times (24-48 hours) in complete medium. The treated cells were lysed by boiling in a 2× sample loading buffer (250 mM Tris-HCl [pH 6.8], 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol, 50 mM sodium fluoride, and 5 mM sodium orthovanadate) and further boiled for 10 minutes at 100ºC. Protein quantification of the samples was performed using the bicinchoninic acid (BCA) assay. Equal amounts of protein were subjected to 6% to 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 5% BSA in TBS containing 0.1% Tween-20 (TBST) for 30 minutes at room temperature and probed with the indicated antibodies overnight. After washing with TBST, the membranes were incubated with the corresponding secondary antibodies mixed in 2.5% BSA in TBST for 1.5 hours at room temperature. The membranes were washed with TBST, and proteins were visualized using an enhanced chemiluminescence detection kit (GE Healthcare).

Cell cycle analysis

The cells (A549 and H1299) were seeded at a density of 4×10^4 cells per 60 mm culture dish. Both cell lines were treated with GaH for 30 hours. After the treatment, the floating cells were collected, washed twice with PBS, fixed with 70% methanol, and incubated with a staining solution (0.2% NP-

40, 50 μg/mL PI, and 50 μg/mL RNase A in phosphate-citrate buffer [pH 7.2]) for 30 minutes. The number of stained cells was counted using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed via histograms using the CELLQuest 3.0.1 software from BD Biosciences [17].

Statistical analysis

Data were collected from at least three independent experiments. The statistical significance ($P < 0.05$) was assessed using Student's *t*-test. All statical analyses were conducted using SigmaStat 3.1 (Systat Software Inc.).

RESULTS

Antiproliferative activity of GaH in a panel of cancer cells

The antiproliferative activities of various gibbosic acids (Fig. 1A) were evaluated in a panel of human cancer cell lines by the SRB assay. Among them, GaH exerted the most effective antiproliferative activities in all of the tested cell lines (Table 1). In particular, GaH displayed high-growth inhibitory activities in the SK-HEP-1 liver cancer cells and H1299 lung cancer cells. Since the incidence of lung and bronchus cancer cases are

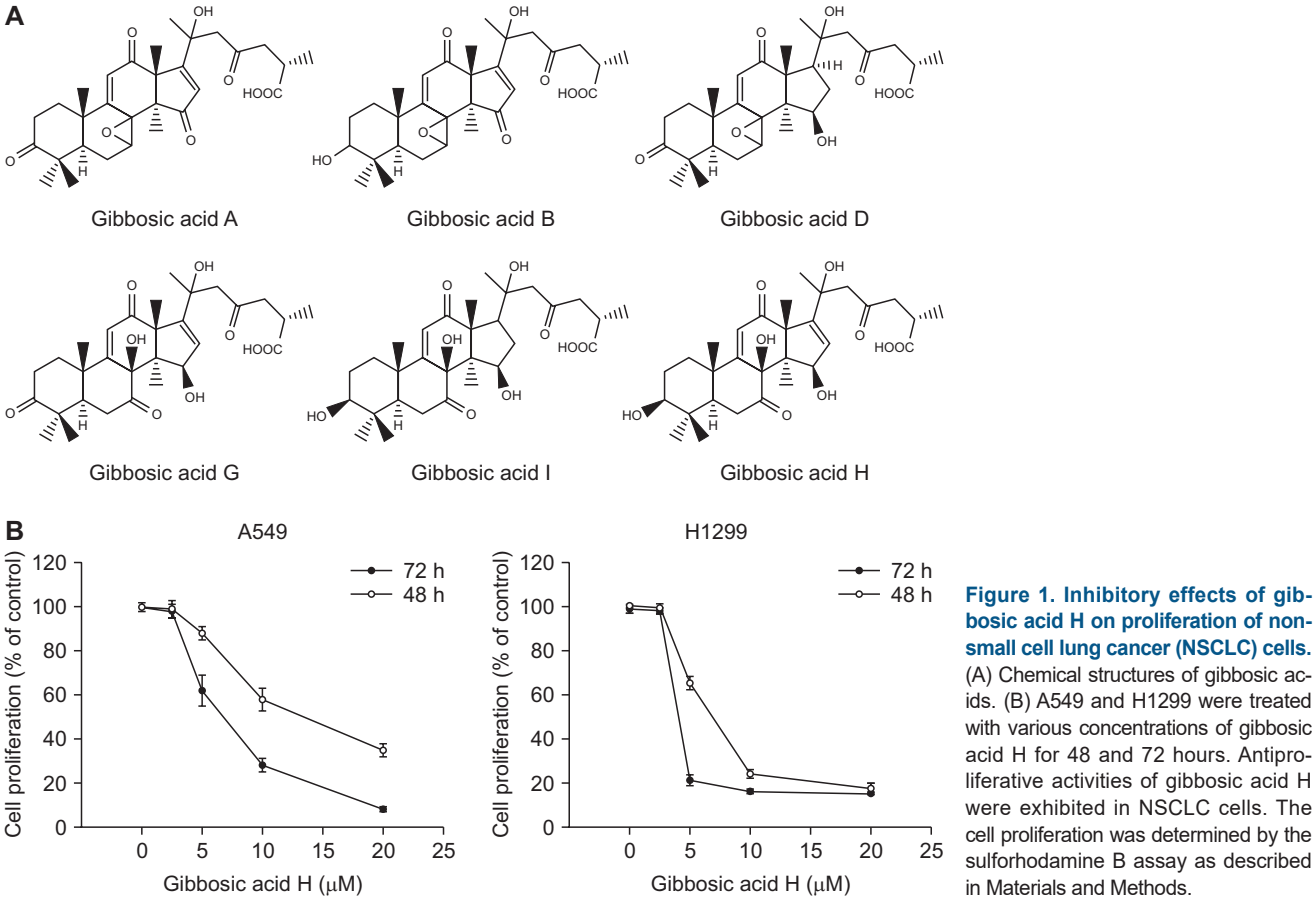

Figure 1. Inhibitory effects of gibbosic acid H on proliferation of non-small cell lung cancer (NSCLC) cells. (A) Chemical structures of gibbosic acids. (B) A549 and H1299 were treated with various concentrations of gibbosic acid H for 48 and 72 hours. Antiproliferative activities of gibbosic acid H were exhibited in NSCLC cells. The cell proliferation was determined by the sulforhodamine B assay as described in Materials and Methods.

Table 1. Antiproliferative activity of gibbosic acids in various cancer cells

Cell line	IC_{50} (μM)[a]					
	MDA-MB-231	SK-Hep-1	HCT116	SNU638	A549	H1299
Gibbosic acid A	>50	30.07	33.36	46.54	35.05	31.64
Gibbosic acid B	>50	>50	>50	>50	>50	>50
Gibbosic acid D	>50	>50	>50	>50	>50	>50
Gibbosic acid G	>50	>50	>50	>50	>50	>50
Gibbosic acid I	7.92	5.36	7.12	18.09	9.42	7.53
Gibbosic acid H	5.13	2.64	6.56	6.14	4.40	3.50
Etoposide[b]	1.39	0.328	0.344	0.668	0.738	4.72

[a]Results are expressed as the calculated half-maximal inhibitory concentration of compounds and etoposide (μM). Data represent mean values ± SD from three independent studies. [b]Etoposide was used as a positive control.

five times higher than those of the liver and intrahepatic bile duct cancers in the United States [3], the detailed mechanism study on the antiproliferation activity of GaH was performed in NSCLC (A549 and H1299) cells. Indeed, GaH inhibited the proliferation of A549 and H1299 cells in a time- and concentration-dependent manners (with an IC_{50} value of 4.4 μM for A549 and 3.5 μM for H1299 at 72 hours, and an IC_{50} value of 13.4 μM for A549 and 9.68 μM for H1299 at 48 hours, respectively) (Fig. 1B).

Induction of the G_0/G_1 phase cell cycle arrest by GaH

To further determine the antiproliferation effect of GaH on cell cycle regulation, A549 and H1299 NSCLC cells were treated with various concentrations of GaH for 24 hours, and flow cytometric analysis was conducted. GaH increased the distribution of cells in the G_0/G_1 phase in both cell lines (Fig. 2). Indeed, the treatment with 10 μM GaH effectively enhanced the G_0/G_1 phase cell cycle progression compared with the vehicle (DMSO)-treated control cells in A549 (control: 63.86% vs. 10 μM GaH: 71.05%) and H1299 (control: 63.12% vs. 10 μM GaH: 72.24%) cells. Additionally, the treatment with a higher concentration of GaH (10 μM) for 24 hours did not show a significant increase in the sub-G_1 peaks compared with the vehicle-treated control cells. These findings suggest that the induction of G_0/G_1 phase cell cycle arrest is predominant compared with the induction of apoptosis in both cells treated with GaH for 24 hours. Further study was designed to determine the expression of checkpoint proteins involved in the progression of G_0/G_1 cell cycle by GaH treatment. Western blot analysis revealed that GaH upregulated the expression of the tumor suppressor, p53, and the CDK inhibitor, p21, in a concentration-dependent manner (Fig. 3). Additionally, the MDM2 protein, which causes p53 proteolysis, was suppressed under GaH treatment in NSCLC cells. p53 is a well-known tumor suppressor that induces cell cycle arrest and apoptosis [18]. p21, a CDK inhibitor, was found as the first transcriptional target of p53 and mediates p53-induced

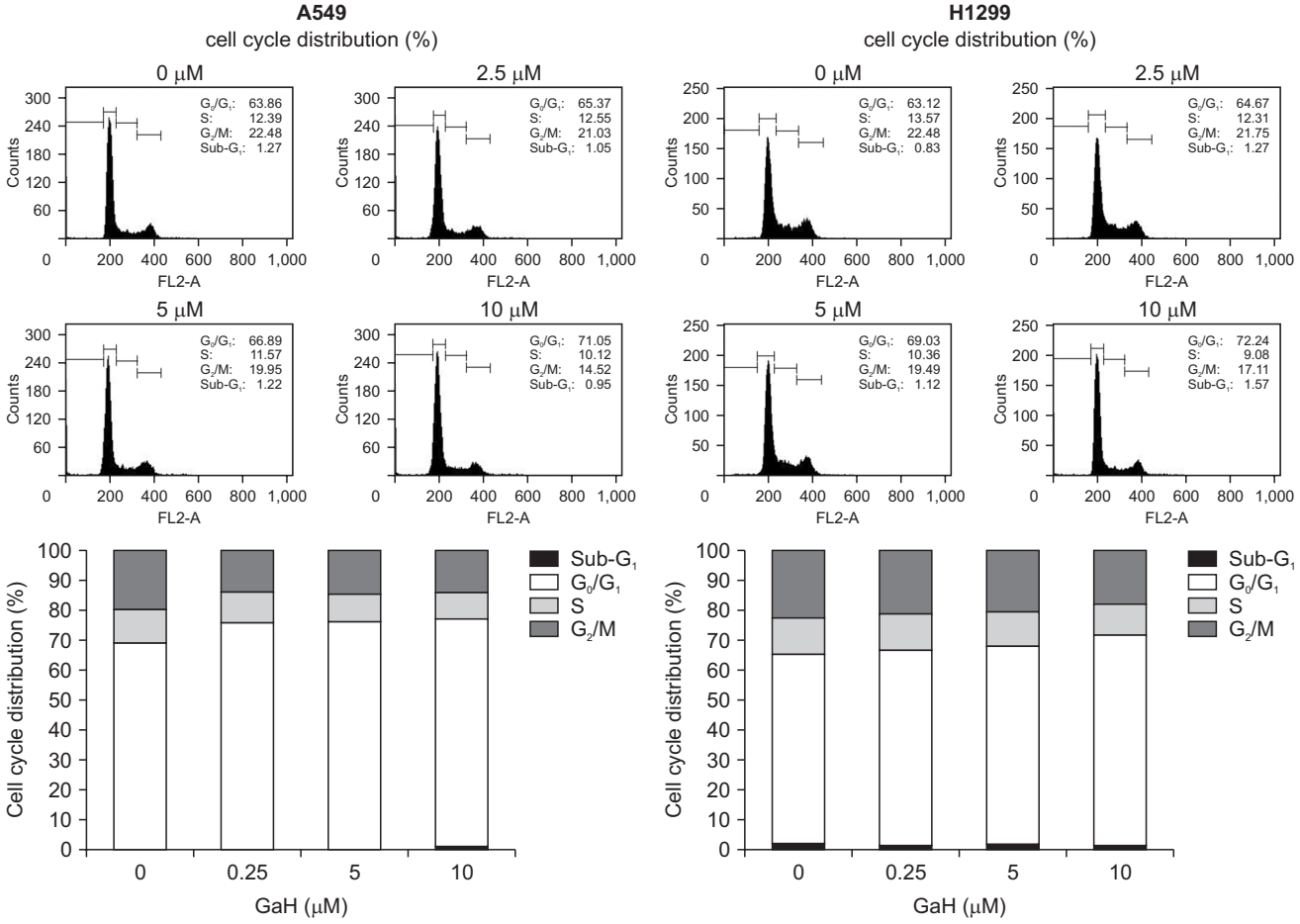

Figure 2. Regulation of cell cycle progression by GaH. A549 and H1299 cells were treated with or without various concentrations of GaH for 24 hours, and fixed with 70% cold ethanol overnight. The cells were then incubated with RNase A and propidium iodide for 30 minutes, and the cell cycle distribution was analyzed by flow cytometry. The bar graph depicts the cell cycle distributions (bottom panels). Data are representative of three independent experiments. GaH, gibbosic acid H.

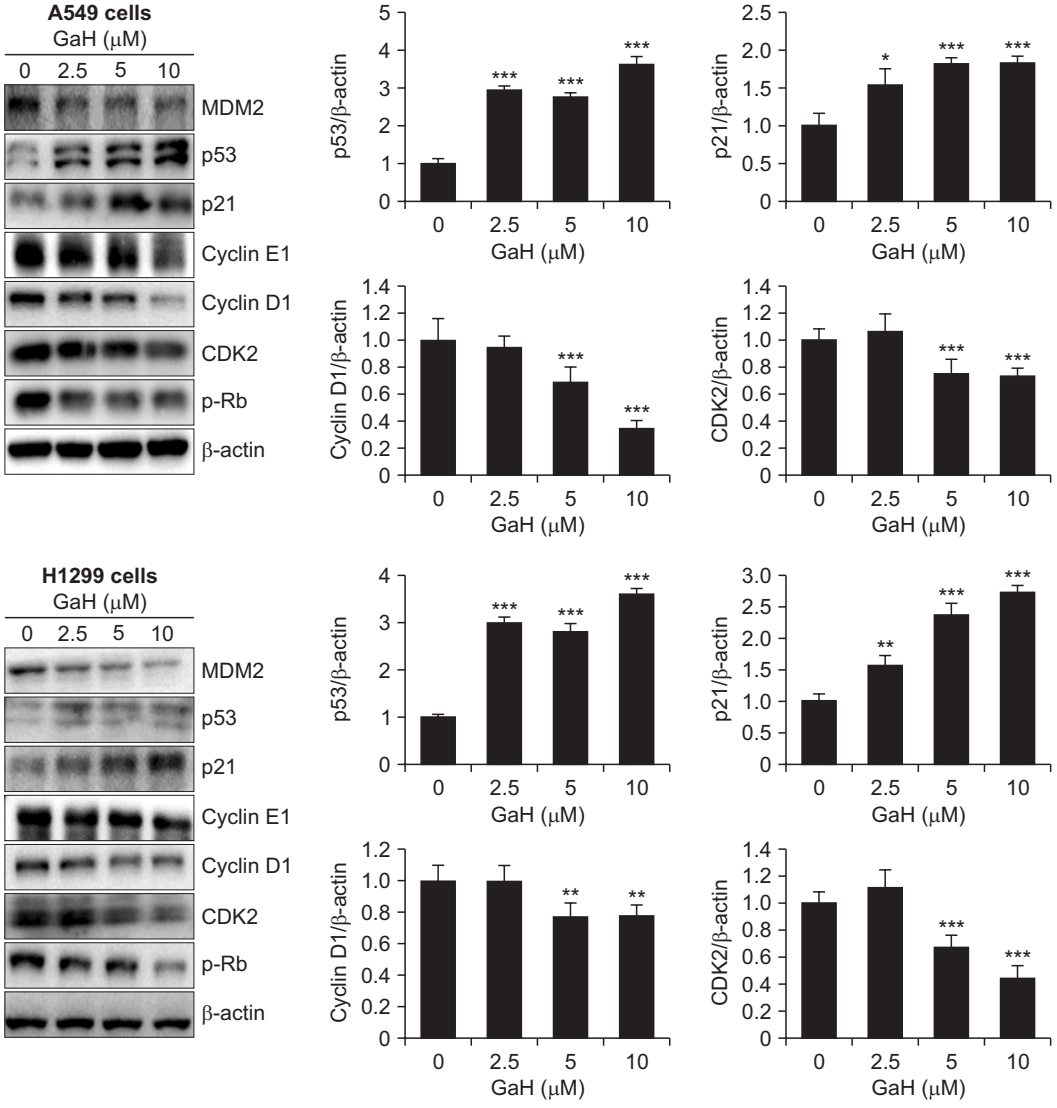

Figure 3. The effects of GaH on expression of cell cycle-related proteins. A549 and H1299 cells were treated with various concentrations of GaH for 24 hours. The protein expression level was analyzed by Western blotting (left panel). β-actin was used as a loading control. Protein expression was quantified by Image J (right panel). GaH, gibbosic acid H. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$ indicate statistically significant differences compared with the vehicle-treated control group.

cell cycle arrest [19]. Furthermore, the expression of the G_1 cell cycle checkpoint proteins, such as cyclin D1 and CDK2, and G_1/S checkpoint proteins, cyclin E1 and Rb, were down-regulated (Fig. 3). These data indicate that the induction of G_0/G_1 cell cycle arrest by GaH is partly associated with the regulation of the expression of cell cycle checkpoint proteins in A549 and H1299 NSCLC cells.

Activation of the AMPK pathway by GaH in NSCLC cells

It is known that the activation of AMPK signaling is correlated with the antiproliferation of cancer cells achieved by cell cycle regulation and fatty acid oxidation. To determine whether GaH could activate the AMPK pathway, the phosphorylation levels of AMPK in NSCLC cells were measured by Western blot analysis in NSCLC cells. As shown in Fig. 4, the treatment of GaH (10 μM) significanlty increased the expression of phsophorylated form (activated) of AMPK protein in NSCLC cells. However, the expression of total AMPK was not affected by GaH treatment, indicating the phosphorylation-dependent activation of AMPK by GaH in both cells. Futher, we determined the correlation between the activation of AMPK and induction of fatty acid oxidation by GaH treatment in NSCLC cells. As a result, GaH effectively upregulated the expression of the phosphorylated (activated) form of ACC protein (p-ACC) compared with the expression of total ACC protein in both NSCLC cells. These findings sugget that the antiproliferation activity of GaH is partly associated with the

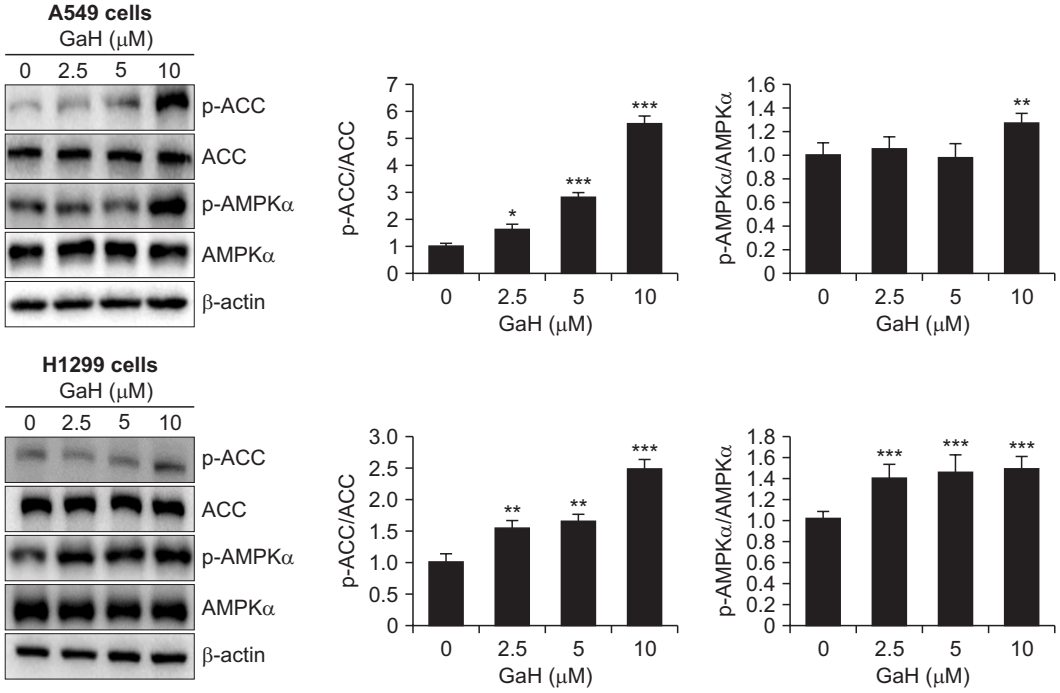

Figure 4. Upregulation of adenosine monophosphate-activated protein kinase (AMPK) by GaH in non-small cell lung cancer cells. A549 and H1299 cells were lysed after treatment with indicated concentrations of GaH for 24 hours, and the expression levels of AMPK and ACC were determined by Western blot analysis (left panel). β-actin was used as a loading control. Protein expression was quantified by Image J (right panel). GaH, gibbosic acid H. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$ indicate statistically significant differences compared with the vehicle-treated control group.

activation of the AMPK pathway in NSCLC cells.

Inducton of autophagy pathway by GaH in NSCLC cells

It is known that AMPK is able to induce autophagy in cancer cells [20,21]. To further elucidate whether the antiproliferation activity of GaH is associated with autophagy induction via AMPK activation, the NSCLC cells were treated with GaH, and activation of AMPK-mediated autophagic biomarker expressions was determined by Western blot analysis. As shown in Fig. 5, the treatment of NSCLC cells with GaH for 24 hours effectively upregulated the expression of p-ULK, beclin-1, and LC3B proteins. The phosphorylation of beclin-1 (Ser14), which can be determined by the activation of ULK, is important for the induction of autophagy. Furthermore, GaH treatment for 24 hours induced the downregulation of p62, which is an autophagy substrate used as an autophagic cargo adaptor. p62 is degraded by autophagy and serves as a link between LC3B and ubiquitinated proteins for the autophagic machinery to enable the degradation in autolysosomes [22]. The upregulation of LC3B and downregulation of p62 indicate that GaH affects an activated status of autophagy and autophagy flux. These findings suggest that the antiproliferative activity of GaH is also partly correlated with the effective induction of AMPK-mediated autophagy signalings in NSCLC cells.

Induction of the switch from autophagy to apoptosis by prolonged exposure of NSCLC cells to GaH

To further determine whether the long-term treatment of GaH is able to induce apoptotic cell death, A549 and H1299 cells were treated with GaH for 48 hours, and the regulation of apoptosis-related proteins was analyzed by Western blotting. GaH affected the caspase 3 activity, a key enzyme in apoptotic process, with effective upregulation of cleaved caspase-3 (active form) but downregulation of pro-caspase 3 in both cell lines. Consequently, the anti-apoptotic biomarker Bcl-2 expression was downregulated, whereas the pro-apoptotic biomarker Bax expression was upregulated under GaH treatment in both cell lines (Fig. 6A). To further analyze whether GaH treatment can switch from autophagy to apoptosis in a time-dependent manner, NSCLC cells were treated with GaH (10 μM) for 24 or 48 hours, and subsequently the expression of beclin-1, LC3B, and cleaved caspase-8 was determined by Western blot analysis. As shown in Fig. 6B, the expression of autophagy biomarkers, beclin-1 and LC3B, peaked at 24 hours and then decreased at 48 hours, but the expression of apoptosis biomarker cleaved capsape-8 showed a gradual increase from 24 hours to 48 hours after GaH treatment. These data suggest that prolonged exposure to GaH is able to induce the switch from autopaghy to apotosis by modulating caspase-8-mediated cleavage of beclin-1 in NSCLC cells.

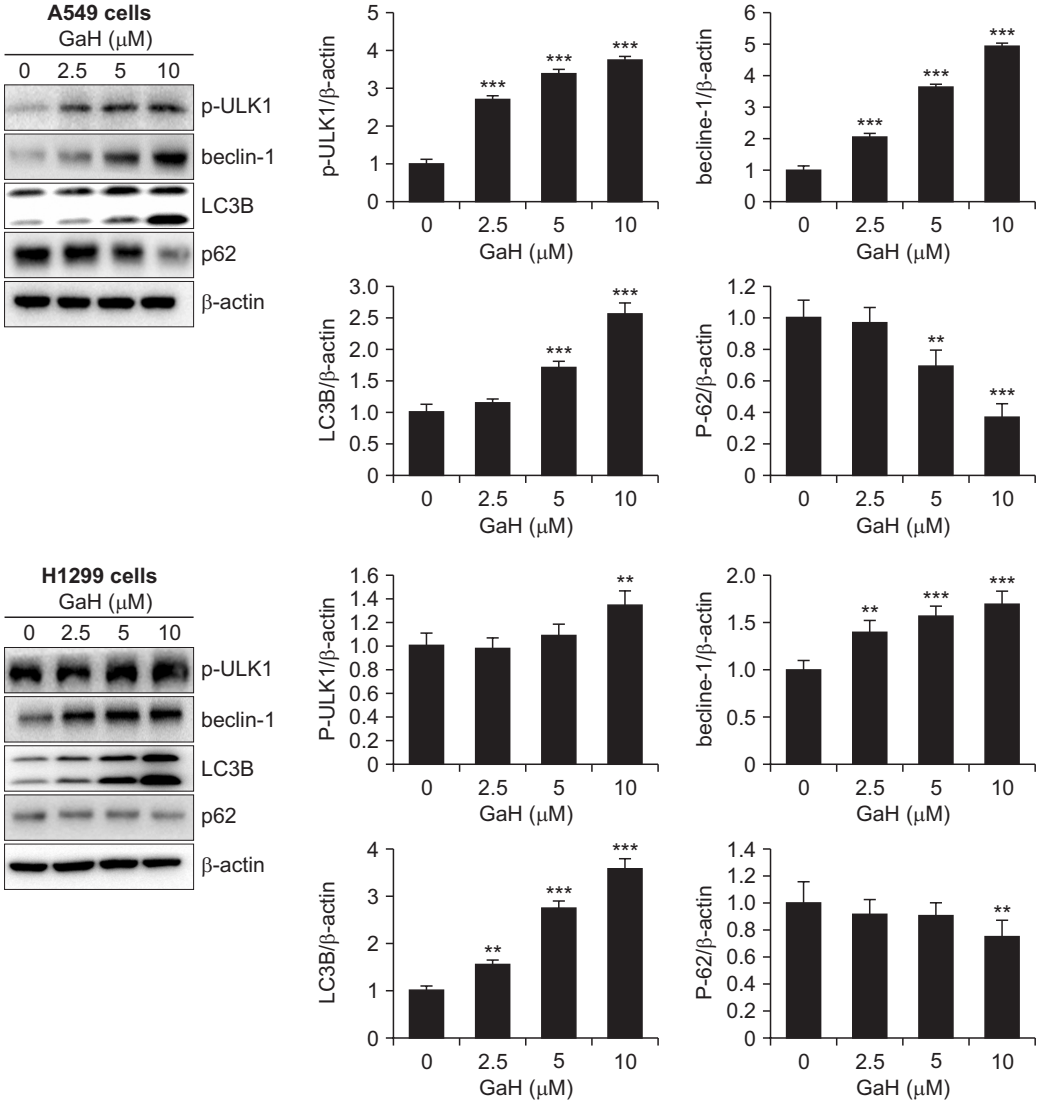

Figure 5. Induction of autophagy by GaH in non-small cell lung cancer cells. A549 and H1299 cells were treated with GaH for 24 hours, and the expression levels of autophagy regulators and activators were determined by Western blot analysis (left panel). β-actin was used as a loading control. Protein expression was quantified by Image J (right panel). GaH, gibbosic acid H. **P* < 0.05, ***P* < 0.01, and ****P* < 0.005 indicates indicate statistically significant differences compared with the vehicle-treated control group.

DISCUSSION

Natural products have played an important role in the development of antitumor agents. Parituclarly, plant-derived compounds have been explored as major lead candidates for cancer therapeutic agents. Li et al. [13] demonstrated that Mogrol, an aglycon of the Luo Han Guo ingredient mogroside, inhibits the growth of NSCLC cells by activating AMPK and inducing apoptosis. Recent findings suggest that AMPK plays a complex role in tumorigenesis and by regulating autophagy [23]. Additionally, G_0/G_1 cell cycle arrest and autophagic death induced by AMPK activation are related to the inhibition of cancer cell proliferation [24]. In this study, we identified GaH, a natural lanostanoid, as a potential antiproliferative agent which activates the AMPK signaling pathway in NSCLC cells. AMPK is known to play as a metabolic tumor suppressor in various cancers, including colorectal cancer and liver cancer, by regulating energy levels and inhibiting cell growth [11]. AMPK activation was also associated with a significant decrease in cancer-specific mortalities [25]. Since GaH exhibits antiproliferative effects on various cancers, GaH may predict the potential antiproliferative activity against other cancer cell types beyond NSCLC cells by targeting the AMPK pathway.

Cell cycle is finely regulated in normal cell proliferation; however, abnormal activation of cell proliferation is generally found in cancer cells as a reuslt of escape from normal regulation of cell cycle checkpoints [26]. Therefore, the modula-

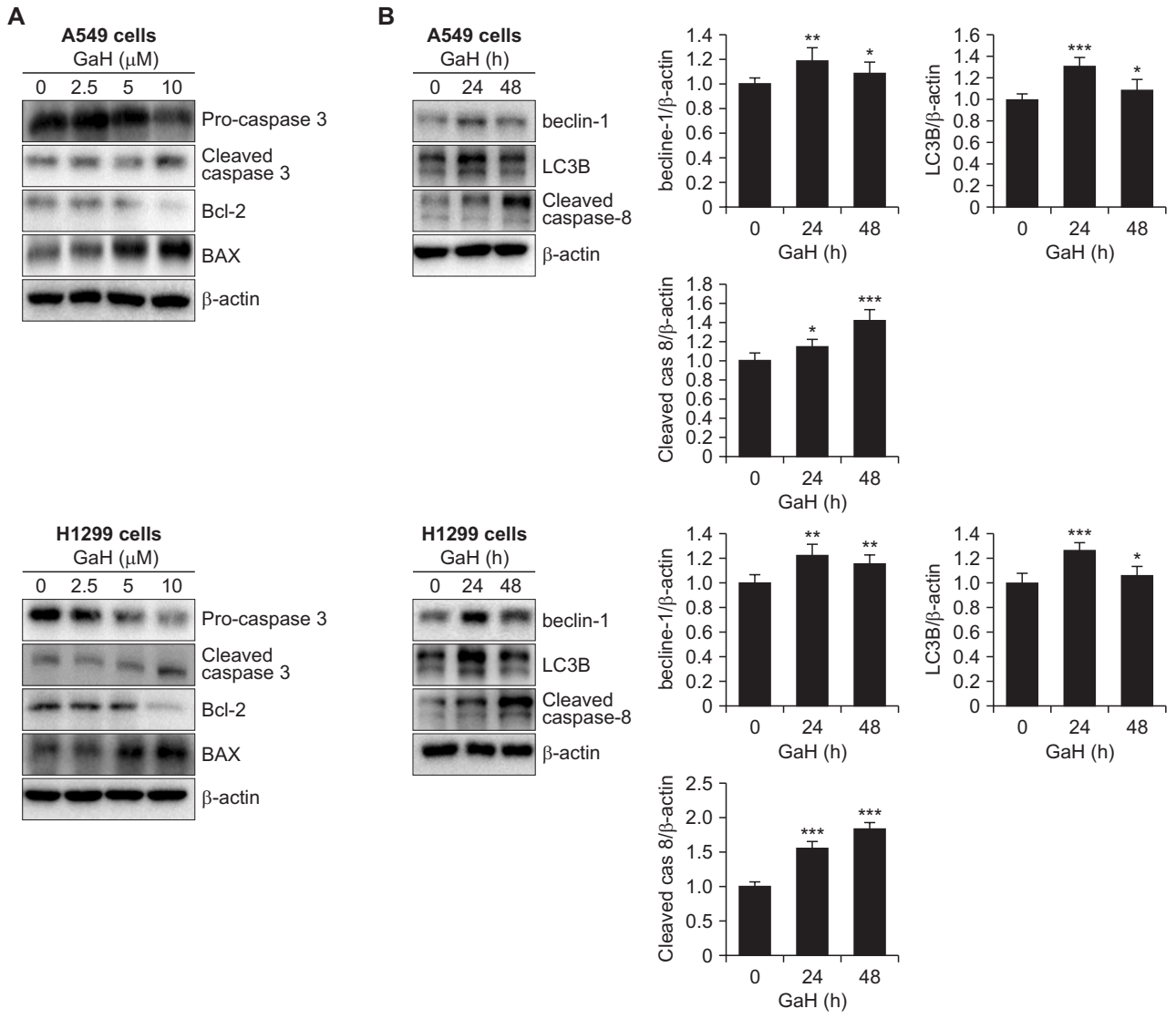

Figure 6. GaH-induced switching from autophagy to apoptosis in non-small cell lung cancer (NSCLC) cells. (A) Effect of GaH on the expression of apoptosis-related proteins in NSCLC cells. A549 and H1299 cells were treated with indicated concentrations of GaH for 48 hours. The expression levels of pro-caspase-3, cleaved caspase-3, Bcl-2, and Bax were determined by Western blot analysis (left panel). β-actin was used as a loading control. (B) A549 and H1299 cells were treated with 10 μM of GaH for 24 and 48 hours. The protein expression levels of autophagy markers (LC3B and beclin-1) and apoptotic maker (cleaved caspase-8) were determined by Western blot analysis (left panel). β-actin was used as a loading control. Protein expression was quantified by Image J (right panel). GaH, gibbosic acid H. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$ indicate statistically significant differences compared with the vehicle-treated control group.

tion of cell cycle distribution may provide clues about the antiproliferative activity of bioactive compounds in cancer cells. Our present study revealed that GaH effectively induced G_0/G_1 cell cycle arrest and thus evoked the decrease of S phase cell population in NSCLC cells. Further investigation confirmed the induction of the G_0/G_1 phase cell cycle arrest by analyzing the expression levels of the cell regulatory proteins. In NSCLC cells, GaH-induced G_0/G_1 phase cell cycle arrest, which is associated with activation of p53 and its downstream effector, p21, a cell cycle inhibitor. p53 is a transcription factor that plays an essential role in inducing cell cycle arrest and regulates transcription of various genes [27]. The upregulation of p53 affects p21, a CDK inhibitor, to mediate G_0/G_1 cell cycle arrest in lung cancers [28].

CDKs play a critical role in regulating the cell cycle along with corresponding regulatory cyclins by controlling the progression of cells through the different stages of the cell cycle [29,30]. In particular, cyclin D1 is involved in the G_0/G_1 cell cycle arrest, while cyclin E1 and CDK2 control the progression from the G_1 to the S phase. Therefore, the downregulation of cyclin D1, cyclin E1, and CDK2 by GaH is considered to contribute to G_0/G_1 cell cycle arrest in the NSCLC cells. The CDK

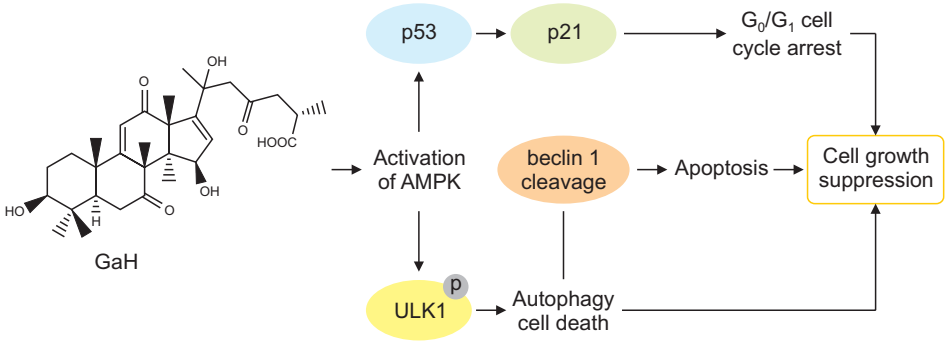

Figure 7. Proposed mechanisms underlying the antiproliferative activity of GaH in non-small cell lung cancer cells. GaH, gibbosic acid H; AMPK, adenosine monophosphate-activated protein kinase.

inhibitor, p21, can negatively regulate cell cycle progression by blocking the kinase activities of CDK/cyclin complexes [31]. Additionally, Rb is a tumor suppressor that plays a crucial role in regulating the cell cycle and preventing uncontrolled cell division by phosphorylating Rb at the serine site [32]. We found that GaH upregulated p21 and downregulated p-Rb, suggesting the involvement of G_0/G_1 cell cycle arrest in NSCLC cells.

Autophagy is a self-degradative process resulting in the removal of unnecessary cells to balance the sources of energy. However, excessive levels of autophagy can induce autophagic cell death in NSCLC cells [33,34]. Activation of AMPK directly affects and phosphorylates the autophagic protein ULK1, which is a significant upstream marker of the autophagy signaling pathway [35]. Activated AMPK upregulates the expression of beclin-1 and LC3B, the key players in the activation of autophagy. Furthermore, p62 directly binds to LC3B and interacts with autophagy machinery as a key adaptor of target cargo. p62 is a polyubiquitin-binding protein which has been implicated in cancer promotion and is degraded by autophagy [22]. The present findings revealed the downregulation of ULK1 and p62, and upregulation of beclin-1 and LC3B expression. These results suggest the induction of autophagic cell death by GaH in NSCLC cells.

Further study was designed to determine whether prolonged exposure of GaH could induce apoptosis in NSCLC cells. Apoptosis usually occurs via either the death receptor (extrinsic) pathway or the mitochondrial (intrinsic) pathway with the activation of caspase-3 [36]. In this study, we found that GaH induced the regulation of pro-caspase 3 and cleaved caspase 3, suggesting an induction of the intrinsic pathway of apoptosis. Bcl-2, a member of the BCL family, induces changes in the mitochondrial potential upon apoptotic stimuli leading to the activation of other pro-apoptotic proteins [37]. In addition, Bax, a member of the Bcl-2 family of proteins, is known to play a central role in regulating apoptosis. GaH was found to induce Bax and suppress Bcl-2, leading to the induction of apoptosis in NSCLC cells.

Since GaH seems to induce autophagy and apoptosis sequentially, the critical biomarker expression was analyzed at different time points following the treatment of NSCLC cells with 10 μM GaH. Consequently, the expression of autophagic proteins, LC3B and beclin-1, reached a peak at 24 hours, but was downregulated at 48 hours. Moreover, the level of cleaved caspase-8, an apoptotic protein, was continuously upregulated up to 48 hours. The activation of autophagy and induction of apoptosis by GaH are simultaneous processes in early stages, and the cleavage of beclin 1 is mediated by caspase-8 in GaH-treated NSCLC cells. Caspase-8-mediated cleavage of beclin 1 regulates cytochrome c, which is important in inducing the switch from autophagy to apoptosis [38]. These data indicate that the extended treatment of GaH leads to the switch from autophagy to apoptosis in NSCLC cells.

In conclusion, the present study demonstrates the antiproliferative activity of GaH in NSCLC cells. A plausible mechanism of action involved in this activity could be related to the activation of AMPK as schematically represented in Fig. 7. The findings of this study suggest that GaH might be considered as a potential lead candidate for the development of cancer chemotherapeutic agents against NSCLC cells.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

ORCID

Jaeho Han, https://orcid.org/0009-0009-9437-425X
Donghwa Kim, https://orcid.org/0000-0003-1425-213X
Hyen Joo Park, https://orcid.org/0000-0001-8262-3903
Hee-Juhn Park, https://orcid.org/0000-0003-4328-9038
Sang Kook Lee, https://orcid.org/0000-0002-4306-7024

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