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# Oleanolic acid induces apoptosis and autophagy via the PI3K/AKT/mTOR pathway in AGS human gastric cancer cells

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ABSTRACT

Oleanolic acid (OA) is widely distributed in food and medicinal plants, it reportedly exerts anti-inflammatory and anti-cancer effects. In this study, we investigated the effect of OA on human gastric cancer cells AGS *in vitro* and *in vivo*. The OA treatment significantly inhibited the AGS cell viability. Apoptosis was confirmed via annexin V/PI staining and 4′,6-diamidino-2-phenylindole (DAPI) staining. The results from western blotting revealed that treatment with OA affected apoptosis-related protein. Meanwhile, OA induced autophagy, characterized by the formation of autophagic vacuoles and acidic vesicular organelles, also increased autophagy-related protein. Inhibition of autophagy further reduced cell proliferation. Moreover, OA treatment decreased phosphorylation of PI3K/AKT/mTOR pathway proteins. Finally, we found that OA reduced tumor volume and weight in xenograft mice via apoptosis without side effects. Overall, our study provides experimental evidence for the anti-cancer action of OA and suggests the possibility of its use as an adjuvant in gastric cancer therapy.

## 1. Introduction

Cancer is a grave and global public health concern and is the leading cause of mortality in South Korea. Coupled with longer lifespans, an increase in the consumption of westernized diet is predicted to increase the incidence of cancer and mortality (Booth et al, 2014; Qiu & Tanaka, 2006). Gastric cancer is the most common type of cancer prevalent in Korea, with an incidence of 13.6%, and its mortality rate is ranked the fourth highest following that of colorectal cancer (Antonioli, 1990; National, 2019). Treatment modalities include surgery and pharmacological therapy; however, the anticancer agents used in these therapies also affect normal cells, leading to the occurrence of adverse reactions a (Kelley & Duggan, 2003; Park et al, 2011). Natural or naturally derived compounds account for approximately half of the anticancer agents developed in recent years, with a majority of those in clinical practice being derivatives of traditional medicinal ingredients. There is mounting interest in the development of novel therapies from natural products, since plant-derived natural products demonstrate fewer side effects compared to those observed with the use of chemical agents (Cragg & Newman, 2005; Feng et al., 2011; Fridlender et al, 2015; Newman & Cragg, 2012).

Oleanolic acid (OA, 3b-hydroxyolean-12-en-28-oic acid), a pentacyclic triterpenoid, is a natural compound and a major component of plants belonging to the family Oleaceae. Triterpenoids, present in the plant epithelium, prevent water loss and are constituents of the defence system against pathogens. OA reportedly confers protection to the liver

*Abbreviations:* AKT, protein kinase B; Atg, autophagy-related genes; Bak, Bcl-2 homologous antagonist killer; Bax, Bcl2-associated X protein; HCQ, hydroxychloroquine; LC3, microtubule-associated protein 1A/1B-light chain 3; mTOR, mammalian target of rapamycin; OA, oleaolic acid; PARP, poly (ADP-ribose) polymerase; PE, phosphatidylethanolamine; PI, propidium iodide; PI3K, phosphatidylinositol-3-kinase; 3-MA, 3-methyladenine.

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and demonstrates antioxidant, antitumor, and anti-inflammatory effects (Pollier & Goossens, 2012). Previous studies have shown the efficacy of OA in controlling breast (Allouche et al., 2011), liver (Shyu et al., 2010; Wang et al., 2013), and lung cancers (Lúcio et al., 2011) however, research on its effects on gastric cancer is lacking.

Apoptosis is defined as the body's defense mechanism that functions to remove damaged, virus-infected, or cancer cells (Han et al., 2008; Nishino et al., 1989). During this process, structural changes occur in the DNA and cells, resulting in the condensation of the nucleus and cytoplasm and the disintegration of cells into small membrane-bound structures (Zaman et al., 2014). There exist intrinsic and extrinsic apoptotic pathways. There are intrinsic and extrinsic apoptotic pathways, which establish interactions with each other and lead to the release of caspases, thereby inducing apoptosis (Fulda & Debatin, 2006). The pro-apoptotic proteins Bax and Bak form oligomers and release cytochrome *c* from the mitochondria, which activates the caspase-9/-3 cascade and triggers apoptosis (Kong et al., 2015; Xiong et al., 2014). On the other hand, anti-apoptotic proteins Bcl-2 and Bcl-xL bind to the pro-apoptotic proteins and inactivate them. An excessive expression of anti-apoptotic proteins causes an imbalance between the pro- and anti-apoptotic proteins, which in turn suppresses apoptotic cell death. Therefore, the regulation of pro- and anti-apoptotic proteins play a key role in overcoming the development of drug resistance of cancer cells (Del Poeta et al., 2003; Minn et al., 1995, Yoshino et al., 2006).

Similar to apoptosis, autophagy is a type of programmed cell death, wherein autophagosomes bind to lysosomes to disintegrate cell components. Autophagy is triggered in response to stress such as cytotoxicity, metabolic stress, atrophy, and chemotherapy (He & Klionsky, 2009). The proteins that trigger autophagy are regulated by autophagy-related genes (Atg), microtubule-associated protein 1A/1B-light chain 3 (LC3), and beclin-1. The LC3-phosphatidylethanolamine conjugate (LC3-II), formed by the binding of LC3-I to phosphatidylethanolamine (PE), is essential for the formation of the double membrane of autophagosomes (Mizushima and Yoshimori, 2007). Autophagy is known to play an important role in cell survival; hence, its suppression can facilitate cell death (Boya et al., 2005). However, a few studies have reported that in addition to its involvement in cell survival, autophagy also plays roles in cell death (Barnard et al., 2007).

The PI3K/AKT/mTOR pathway is involved in cell growth and proliferation, and the activation of PI3K leads to the sequential phosphorylation and activation of AKT and mTOR (Wu et al., 2018; Yu & Cui, 2016). Once activated, AKT inhibits cell cycle arrest, stimulates angiogenesis, and phosphorylates mTOR. The latter is involved in cell growth and survival, and the regulation of growth factors, cell nutrition, and stress in response to cell signaling (Franke et al., 1997; Vanhaesebroeck & Waterfield, 1999). Thus, the intracellular PI3K/AKT/mTOR pathway is a major target considered in the formulation of cancer therapy strategies (Machado et al., 2016).

In this study, we performed *in vitro* experiments to examine the expression of apoptosis and autophagy factors in response to the presence of OA in the gastric cell line AGS. Furthermore, we investigated the relationship between apoptosis and autophagy. Subsequently, the association between these programmed cell deaths and the PI3K/AKT/mTOR pathway was explored. Moreover, we experimented to discover the effect *in vivo*.

## 2. Materials and methods

### 2.1. Materials and reagents

Oleanolic acid (purity ≥ 97%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). OA was dissolved in DMSO for further use. The control group was subjected to treatment with DMSO alone. The Fluorescein Isothiocyanate (FITC) Annexin V apoptosis detection kit was purchased from BD Pharmingen™ (San Diego, CA, U.S.A.). Bax, poly (ADP-ribose) polymerase (PARP), LC3B, beclin-1, p-PI3K, p-AKT, p-mTOR, secondary antibody rabbit IgG, and PI3K/AKT inhibitor LY294002 were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Bcl-2 and secondary antibody mouse IgG were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, U.S.A.). 3-Methyladenine (3-MA) and hydroxychloroquine (HCQ) were purchased from Selleck Chemical (Houston, TX, USA).

### 2.2. Cell culture

The gastric cell line AGS was obtained from Korean Cell Line Bank (Seoul, Korea). Rosewell Park Memorial Institute (RPMI-1640) and fetal bovine serum (FBS) were purchased from Welgen (Gyeongsan, Korea), while streptomycin/penicillin was purchased from Gibco BRL (Grand Island, NY, U.S.A.). RPMI-1640 medium containing 5% FBS and 1% streptomycin/penicillin was used for culturing of AGS cells in a $CO_2$ incubator at 37 °C and 5% $CO_2$. The cells were subcultured once they reached a confluence of 80%–90% in the flask, and the medium was replaced with fresh medium every 2–3 days.

### 2.3. MTT assay

MTT assay was performed to observe the suppression of cell viability by OA in AGS cells. The cells were added to a 96-well plate at a density of $2 \times 10^4$ cells/well and incubated for 24 h. Thereafter, the cells were subjected to treatment with various concentrations of OA (0, 20, 40, 60, 80, 100 μM) and were further incubated for 24 h. After removal of the medium, the cells were subjected to treatment with 40 uL of the MTT solution and were incubated for 2 h. The MTT solution was removed, followed by the addition of 100 uL of DMSO to facilitate dissolution of the formazan crystals formed therein. The absorbance was determined at 595 nm using an ELISA reader (Bio-Rad Laboratories Inc).

### 2.4. DAPI staining

DAPI staining was performed to observe the characteristic morphological changes of the nucleus during apoptosis. After incubating cells in a 60-dish at a density of $2 \times 10^5$ cells/mL for 24 h, they were subjected to treatment with various concentrations of OA (0, 40, 60 μM) and maintained in a $CO_2$ incubator for 24 h. After removing the medium containing OA, the cells were subjected to washing steps with PBS and were fixed with 4 % paraformaldehyde for 15 min. Following the removal of paraformaldehyde, the cells were subjected to washing steps with PBS. They were subsequently subjected to treatment with 2 mL of DAPI solution for observation under a fluorescence microscope (Zeiss AG).

### 2.5. Annexin V/PI staining

The degree of apoptosis induced by OA in gastric cancer cells was quantitatively analyzed via annexin V/PI staining, followed by assessment via flow cytometry (FACS). The AGS cells were subjected to treatment with OA (0, 40, 60 μM) and were incubated for 24 h. The cells were suspended in Trypsin-EDTA and centrifuged to obtain cell pellets. The pellets were subjected to washing steps with PBS, followed by centrifugation and resuspension of the cell pellets in 1X binding buffer at a density of 2 × 105 cells/mL. They were mixed with annexin V and propidium iodide (PI) and incubated for 15 min, followed by subjection to measurements using the FACSCalibur™ flow cytometer (BD Biosciences, NJ. USA).

### 2.6. Acridine orange staining

Acridine orange staining was performed to observe acidic vesicular organelles (AVO), a morphological characteristic of autophagy. After incubation in a 60-dish (cell density: $2 \times 10^5$ cells/mL) for 24 h, the cells

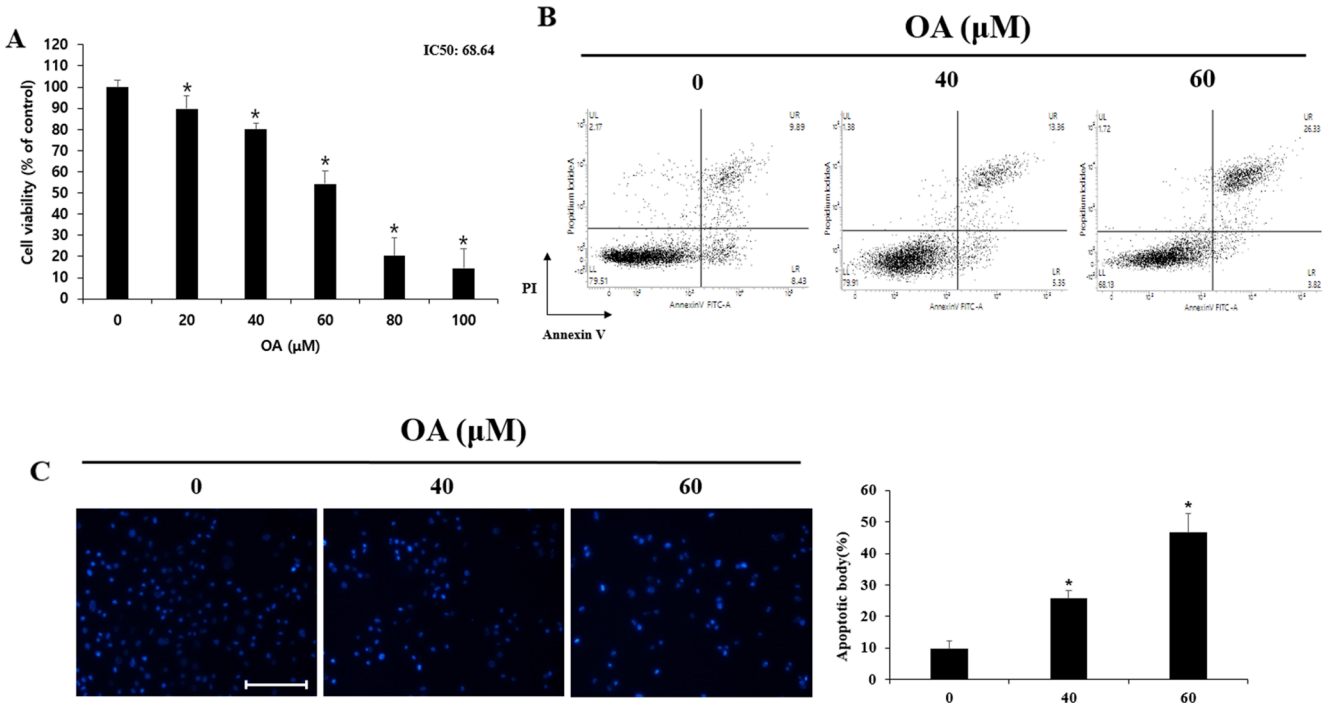


**Fig. 1.** Effect of OA on the inhibition of human gastric cancer (AGS) cell lines. AGS cells were treated with different concentrations of OA (0, 20, 40, 60, 80, and 100 μM) for 24 h, and the cell viability and cell reduction of 50% (IC50) were measured by performing MTT assay. (B) AGS cells were subjected to treatment with 0, 40, and 60 μM OA for 24 h, and the cells were subjected to staining procedures with annexin V/PI double staining, followed by flow cytometry analysis. (C) AGS cells were seeded at a density of $2 \times 10^5$ cells per dish and subjected to treatment with the indicated concentrations of OA (0, 40, and 60 μM) for 24 h. They were subjected to staining procedures with 1 × DAPI solution, and the DAPI-positive cells were analyzed using a fluorescence microscope. Scale bar = 10 μm, the bar graph revealed the proportion of apoptotic body. The control cells were subjected to treatment with DMSO. Data are presented as mean and standard deviation (SD) for three samples. The Student's *t*-test was performed, and $^*p < 0.05$, compared with the control, was considered statistically significant.

were subjected to treatment with OA (0, 40, 60 μM) and were further incubated in a $CO_2$ incubator for 24 h. The medium containing OA was subsequently removed, and the cells were subjected to washing steps twice with PBS. After the completion of washing steps, the cells were fixed with 4% paraformaldehyde for 15 min. They were subjected to washing steps twice with PBS for the removal of paraformaldehyde, followed by treatment with 2 mL of acridine orange solution (5 μg/mL) for observation under a fluorescence microscope.

### 2.7. Western blotting

Western blot was performed to examine the expression of apoptosis-related proteins. AGS cells were added to a 75-$cm^2$ flask and maintained in an incubator at 37 °C and 5% $CO_2$ for 24 h. After completion of the incubation period, the medium was removed and fresh medium containing OA (0, 40, 60 μM) was added to observe another 24 h of incubation. The cells were subsequently suspended in trypsin-EDTA and were centrifuged at 1200 rpm at 4 °C for 5 min. The resultant cell pellet was subjected to treatment with cell lysis buffer and incubated at 4 °C for 20 min. It was subsequently centrifuged at 13000 rpm and 4 °C for 5 min, and the supernatant was used as the cell lysate. The extracted protein was quantified using the Bradford protein assay. The proteins were separated via 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resultant protein bands were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 2 h and was incubated with the primary antibody (1:1000) at 4 °C overnight. Thereafter, secondary antibody (1:1000) incubation was conducted for 2 h. The expression of each protein was observed using enhanced chemiluminescence (ECL) detection reagents (Pierce, Rockford, IL, U.S.A.) and quantified using ImageJ Launcher (provided by NCBI).

### 2.8. Establishment of xenograft models

Female BALB/c nude mice (18–22 g, 4 weeks of age) were purchased from Nara Biotechnology (Seoul, Korea). The animal experiments were approved by the Institutional Animal Care and Use Committee (Approval number: KNU_2021-02, Chungcheongnam-do, Korea) and were performed in adherence to the Committee guidelines and regulations. The mice were reared in a 12-h light/dark cycle at 23 ± 3 °C and 50%±10% humidity. The xenograft ($5 \times 10^6$/mL of AGS) was prepared and injected into both shoulders of the mice. After seven days, the mice that developed tumors were randomized into the saline (Control, n = 5) and OA (10 mg/kg, n = 5) groups (Lúcio et al., 2011). OA was administered intraperitoneally every day for two weeks (10 mg/kg/daily). The tumor volume was measured every three days using Vernier calipers (Mitutoyo Corporation, Tokyo, Japan) and calculated using the formula: volume = $\{(\text{wide} + \text{length}) \times 0.5\}^3$.

### 2.9. Hematoxylin and eosin staining

Hematoxylin and eosin (H&E) staining was performed to examine liver and kidney toxicity caused by OA. After performing the fixation of liver and kidney tissues with 10% formaldehyde, paraffin blocks were prepared and cut into 5 μm-thick blocks. The tissues were subsequently stained with hematoxylin and eosin (H&E) and were observed under an optical microscope at 200x magnification.

### 2.10. Statistical analyses

All experimental results have been presented as mean ± standard deviation. Differences between groups were analyzed with one-way ANOVA and *t*-test. A $p < 0.05$ compared to the control group was deemed statistically significant.

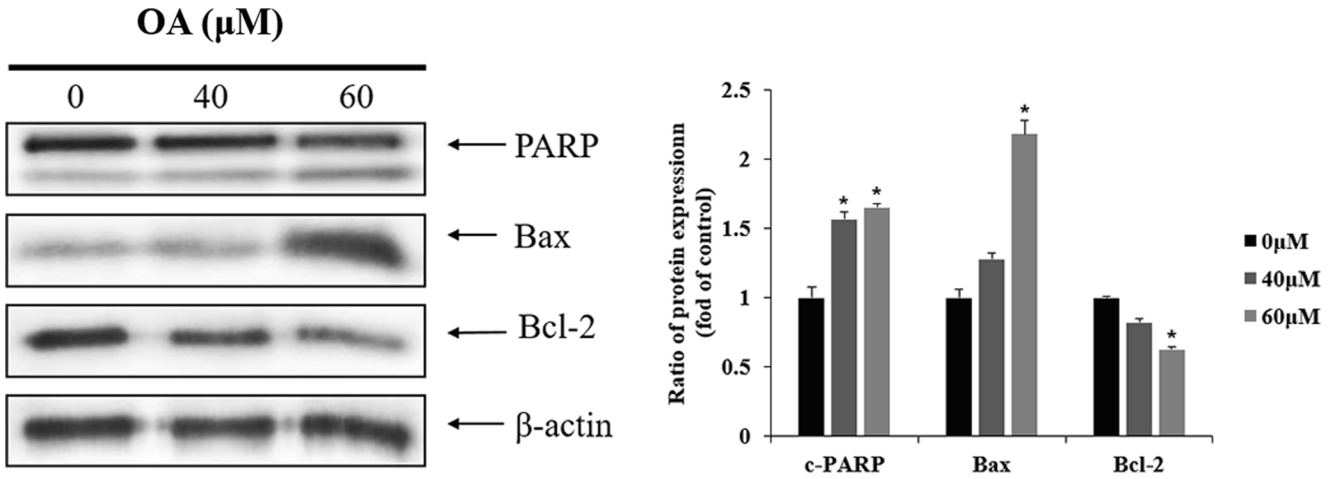


**Fig. 2.** Effects of OA on the expression of the apoptosis-related protein. AGS cells were subjected to treatment with 0, 40, and 60 μM for 24 h, and the protein expression levels of PARP, Bax, and Bcl-2 were observed by western blotting. β-actin was used as loading control, and the quantification was performed using ImageJ. Control cells were subjected to treatment with DMSO. Data are presented as mean and standard deviation (SD) for three samples. The Student's *t*-test was performed, and $*p < 0.05$, compared with the control, was considered statistically significant.

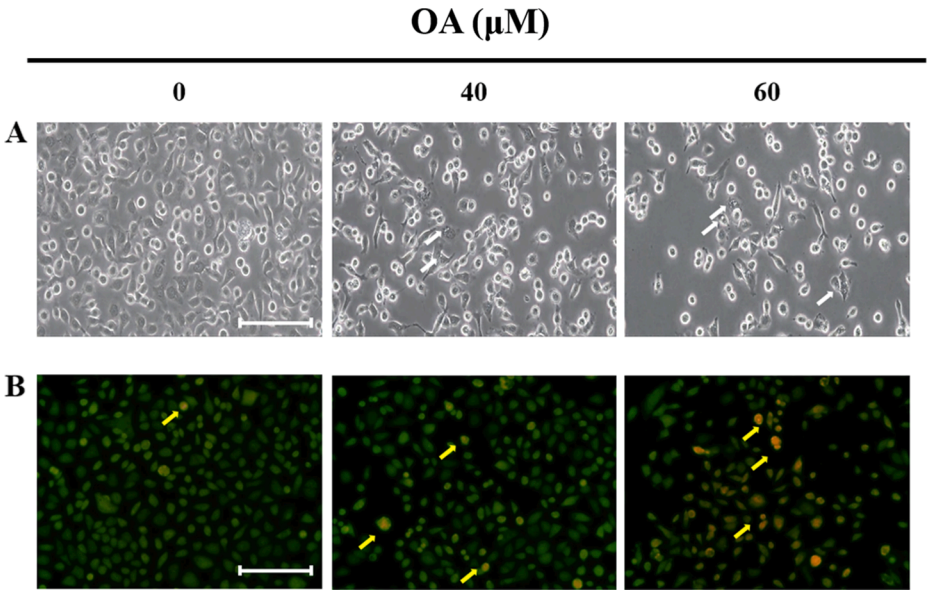


**Fig. 3.** Effect of OA on the induction of autophagy in AGS cells. AGS cells were subjected to treatment with 0, 40, and 60 μM OA for 24 h. (A) Morphologic changes were observed under a phase-contrast microscope. White arrows indicated the autophagic vacuoles. (B) The cells were subjected to staining procedures with 5 μg/ml acridine orange to detect acidic vesicular organelles (AVOs) and were analyzed using a fluorescence microscope. The cytoplasm and nucleus fluoresced green, while AVOS fluoresced red. Yellow arrows indicate AVOs. Scale bar = 10 μm.

## 3. Results

### 3.1. Effects of OA on AGS cell viability

To investigate the effect of OA on AGS cell viability, the cells were incubated with 0, 20, 40, 60, 80, and 100 μM of OA for 24 h, followed by the conduction of MTT assay. Cell viability was reduced to 80.1% when subjected to treatment with 40 uM of OA, and was reduced to 54.2% when subjected to treatment with 60 μM of OA (Fig. 1A). These results show that OA reduces AGS cell viability in a dose-dependent manner.

### 3.2. Effects of OA on AGS apoptosis

Flow cytometry and DAPI staining were performed to determine the role of apoptosis in reduced cell viability. The OA-induced apoptosis was quantified via flow cytometry using annexin V/PI staining. The percentage of early/late apoptosis significantly increased when subjected to treatment with 40 and 60 μM of OA compared to the control group (Fig. 1B). In addition, after incubating the cells with 0, 40, and 60 μM of OA for 24 h, they were subjected to staining procedures with DAPI for the measurement of apoptotic bodies such as DNA fragments and condensed cytoplasm. These apoptotic bodies increased to 9.7%, 25.7%, and 46.7%, respectively, in a dose-dependent manner (Fig. 1C). This confirmed that the reduced AGS cell viability observed after OA administration was due to apoptosis.

### 3.3. Effects of OA on apoptosis-related proteins in AGS cells

Western blotting was performed to examine the expression of apoptosis-related proteins in AGS cells after OA administration. The fragmentation of PARP, a protein related to DNA repair, was observed. The expression of Bax, a pro-apoptotic protein, was found to be upregulated. In contrast, the expression of Bcl-2, an anti-apoptotic protein, decreased with increasing OA concentration (Fig. 2). These results showed that OA induced apoptosis by increasing the ratio of Bax/Bcl-2.

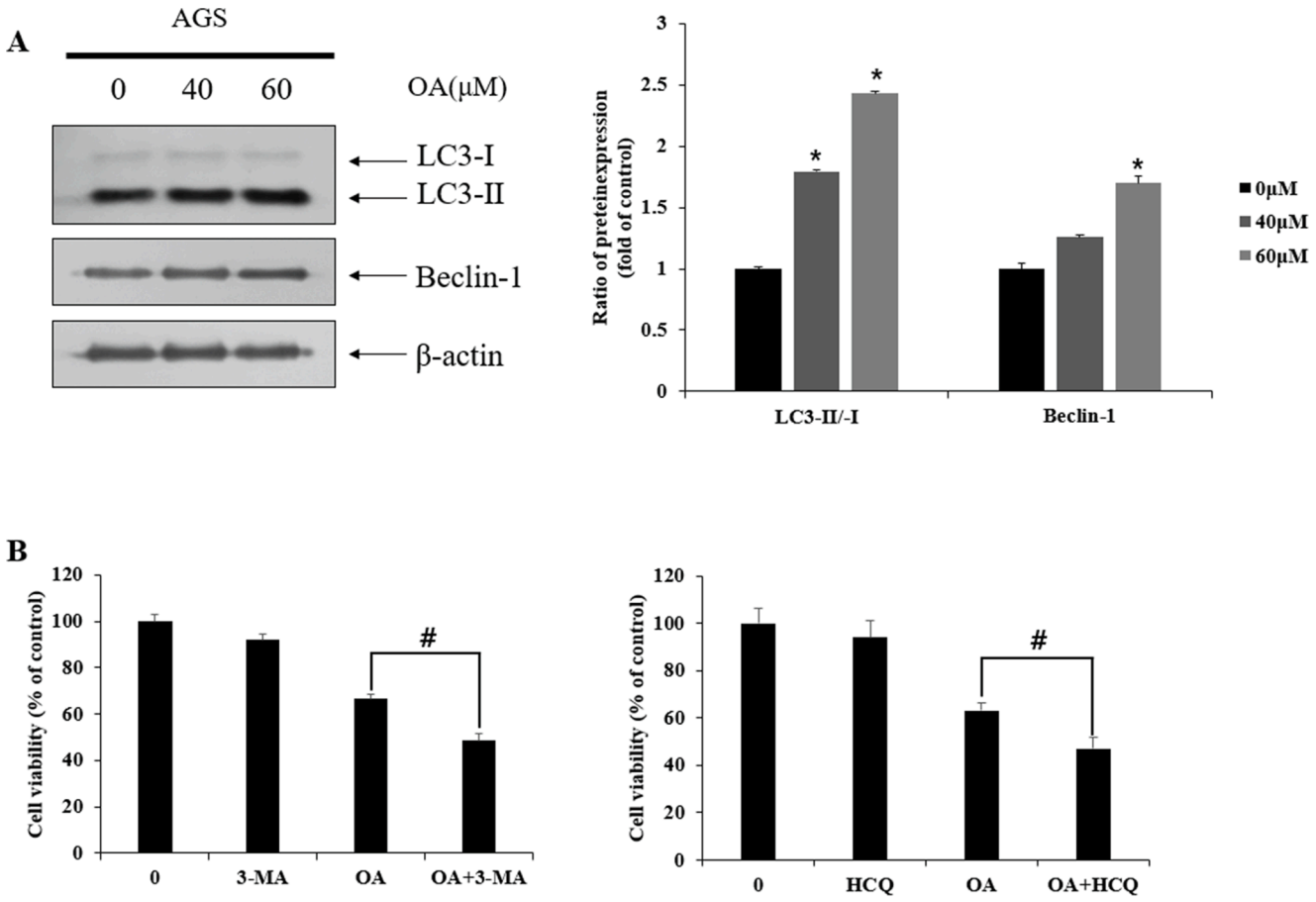


**Fig. 4.** Effects of OA on the autophagy of AGS cells. (A) AGS cells were subjected to treatment with 0, 40, and 60 μM for 24 h, and the protein expression levels of LC3-I, -II, and beclin-1 were observed by western blotting. β-actin was used as loading control, and the quantification was performed using ImageJ. (B) Cells were subjected to pretreatment with 3-MA (1 mM) or hydroxychloroquine (HCQ, 25 μM) for 2 h and subsequently incubated with 60 μM OA for 24 h. Cell viability was measured by performing MTT assay. Control cells were subjected to treatment with DMSO. Data are presented as mean and standard deviation (SD) for three samples. Statistical analyses were performed using the Student's *t*-test, and $*p < 0.05$, compared with the control and $\# p < 0.05$, compared with the OA-treated, were considered statistically significant.

### 3.4. Effects of OA on autophagy in AGS cells

Our next aim was to examine whether OA induced autophagy, a type II programmed cell death, in AGS cells. Upon observing the morphology of the cells by phase-contrast microscope, it was inferred that the OA groups showed an increase in the presence of autophagic vacuoles (Fig. 3A) compared to the control group. The cells were subjected to acridine orange staining and, compared to the control group, the OA groups showed an increase in the formation of acidic vesicular organelles (AVOs) (Fig. 3B). Furthermore, western blotting results showed the presence of autophagy markers LC3 and beclin-1. The results showed that LC3-I was converted to LC3-II and beclin-1 levels increased with increase in OA concentration (Fig. 4A). When OA-induced autophagy was inhibited with application of the widely used autophagy inhibitors 3-Methyladenine (3-MA) and hydroxychloroquine (HCQ), all groups subjected to treatment with these two inhibitors, including OA, showed an increased reduction in AGS cell viability than the group subjected to treatment with only with OA (Fig. 4B). These results suggest that OA induces autophagy in AGS cells and that the OA-induced autophagy exerts a protective effect on these cells.

### 3.5. Effects of OA on the PI3K/AKT/mTOR pathway in AGS cells

Western blotting was performed to examine whether OA inhibited the PI3K/AKT/mTOR pathway. The results showed that the expression levels of p-PI3K, p-AKT, and p-mTOR proteins decreased with increasing OA concentration (Fig. 5A). To examine the apoptosis proteins, the cells were subjected to pretreatment with LY294002, a PI3K inhibitor, followed by treatment with OA. The Bax/Bcl-2 ratio tended to increase in these groups compared to the groups treated with OA alone (Fig. 5B). The AGS cell viability also tended to be lower in the groups treated with LY294002, compared to the groups subjected to treatment with only with OA (Fig. 5C). These results show that OA induces apoptosis by inhibiting expression of the PI3K/AKT/mTOR pathway proteins.

### 3.6. Effects of OA on tumor transplanted using a xenograft

The previously described *in vitro* experiments demonstrated that OA inhibited cell proliferation and induced apoptosis in AGS cells. We attempted to examine whether these results were consistent with the observations in *in vivo* experiments using tumor xenografts. After performing the culture of AGS cells ($1 \times 10^7$), they were subcutaneously injected into both shoulders of mice. After confirming tumor growth, OA was intraperitoneally administered every day (10 mg/kg) for 13 days, while the control group was administered with DMSO. Compared to the control group, the treatment groups showed reduced tumor volume and weight (Fig. 6A, B). Furthermore, we performed western blotting to examine the expressions levels of apoptosis-related proteins and PI3K/AKT/mTOR pathway proteins in tumor tissues after OA administration. Compared to the control group, the treatment groups showed increased Bax expression and reduced Bcl-2 expression. Additionally, the treatment groups also demonstrated reduced expression of p-AKT in the AKT

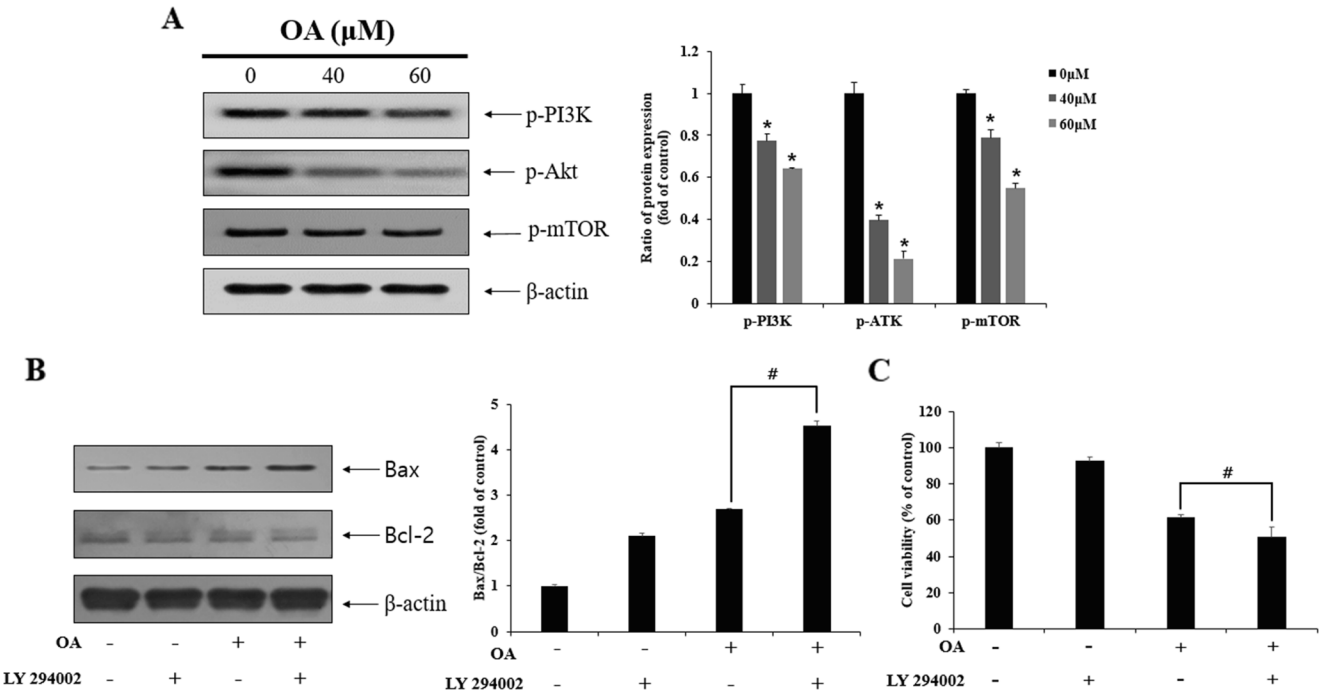


**Fig. 5.** Effect of the OA regulation of the PI3K/AKT/mTOR pathway in AGS cells. (A) AGS cells were subjected to treatment with increasing concentrations of OA (0, 40, and 60 μM) for 24 h, and the protein expression levels of PI3K/AKT/mTOR phosphorylation were observed via western blotting. (B) AGS cells were subjected to pretreatment with LY294002 (25 μM) for 2 h and then incubated with 60 μM OA for 24 h. Protein expression levels of Bax and Bcl-2 were observed via western blotting. β-actin was used as loading control, and the quantification was performed using ImageJ. (C) Cell viability was measured via MTT assay. Control cells were subjected to treatment with DMSO. Data are presented as mean and standard deviation (SD) for three samples. The Student's *t*-test was used for statistical analyses, $^{*}p < 0.05$, compared with the control and $\#p < 0.05$, compared with OA-treated, was considered statistically significant.

pathway involved in apoptosis (Fig. 6C). These results suggest that OA induces apoptosis of AGS tumor in the body by inhibiting p-AKT expression.

### 3.7. *Observation of histopathological changes in the liver and kidney after OA administration*

The histopathological changes induced after OA administration were observed in xenograft model mice through the measurement of body weight and via H&E staining. There was no significant reduction in body weight in the treatment group compared to the control group (Fig. 6D). Additionally, there were no marked differences in the liver and kidneys between the two groups as observed through H&E staining (Fig. 6E). Thus, OA did not seem to induce any adverse reactions in mice.

## 4. Discussion

OA is a pentacyclic triterpenoid widely distributed in the plant kingdom and is present in the plant epidermis It reportedly aids the prevention of water loss defense against pathogens, and possesses hepatoprotective, antioxidant, and anti-inflammatory properties (Pollier & Goossens, 2012). Additionally, OA has been shown to exert anti-tumor effects against various cancers (Allouche et al., 2011; Lúcio et al., 2011; Shyu et al., 2010; Wang et al., 2013). However, research on the anti-tumor effects of OA against AGS, a human-derived gastric cancer cell line, is largely lacking. The present study showed that OA reduced the viability of AGS cells by inducing apoptosis and autophagy. Furthermore, we investigated the relationship between apoptosis and autophagy with OA in AGS cells. We also investigated the anti-tumor effects of OA in *in vivo* models.

To examine the viability of gastric cancer cells after OA administration, cells were incubated with 0, 20, 40, 60, 80, and 100 μM of OA, which does not affect normal cells (Kang et al., 2017), for 24 h, and cell viability was measured via MTT assay. The results showed that cell viability decreased dose-dependently, with significant reductions from a concentration of 40 μM. A previous study reported that incubation of breast cancer cells with OA for 24 h led to a dose-dependent decrease of cell viability (Kayouka et al., 2020). Another study showed that liver cancer cells subjected to treatment with 10, 25, 50, 75, and 100 μM of OA showed significant reductions in cell viability, starting from a concentration of 10 μM (Shyu, Kao & Yen, 2010). The results from the present study suggest that the effects of OA on AGS cells are similar to those reported in previous studies.

When apoptosis occurs, apoptotic bodies are formed and observed. Some apoptotic bodies include condensed nucleus and DNA fragments (Halicka et al., 2000). Additionally, vacuolization of the cell membrane in early apoptosis leads to extracellular exposure of phosphatidylserine (Hammill et al., 1999). We performed DAPI staining and annexin V/PI staining to detect these features and further attempted to investigate whether the OA-induced suppression of AGS cell viability was caused by apoptosis. After incubating AGS cells with OA (0, 40, 60 μM) for 24 h, the cells were subjected to staining procedures with DAPI and were observed. After OA treatment, the cell count decreased in a dose-dependent manner, while the DAPI-positive cell count tended to increase. The DAPI-positive cells were counted from one hundred randomly counted cells. At OA concentrations of 0, 40, and 60 μM, the percentages of DAPI-positive cells were found to be 9.6%, 25.6%, and 46.6%, respectively. Thus, the percentage of DAPI-positive cells increased with an increase in OA concentrations. Treatment of liver cancer cells with OA (15, 30, 60 μM) led to a dose-dependent increase in nucleus fragmentation (Gao et al., 2019). OA-treated cells were analyzed via annexin V/PI staining to quantify OA-induced apoptosis. Compared to the control group, the OA treatment groups tended to show elevated apoptosis. These results show that apoptosis is responsible for reduced cell viability after OA administration.

There exist intrinsic and extrinsic pathways of apoptosis. The

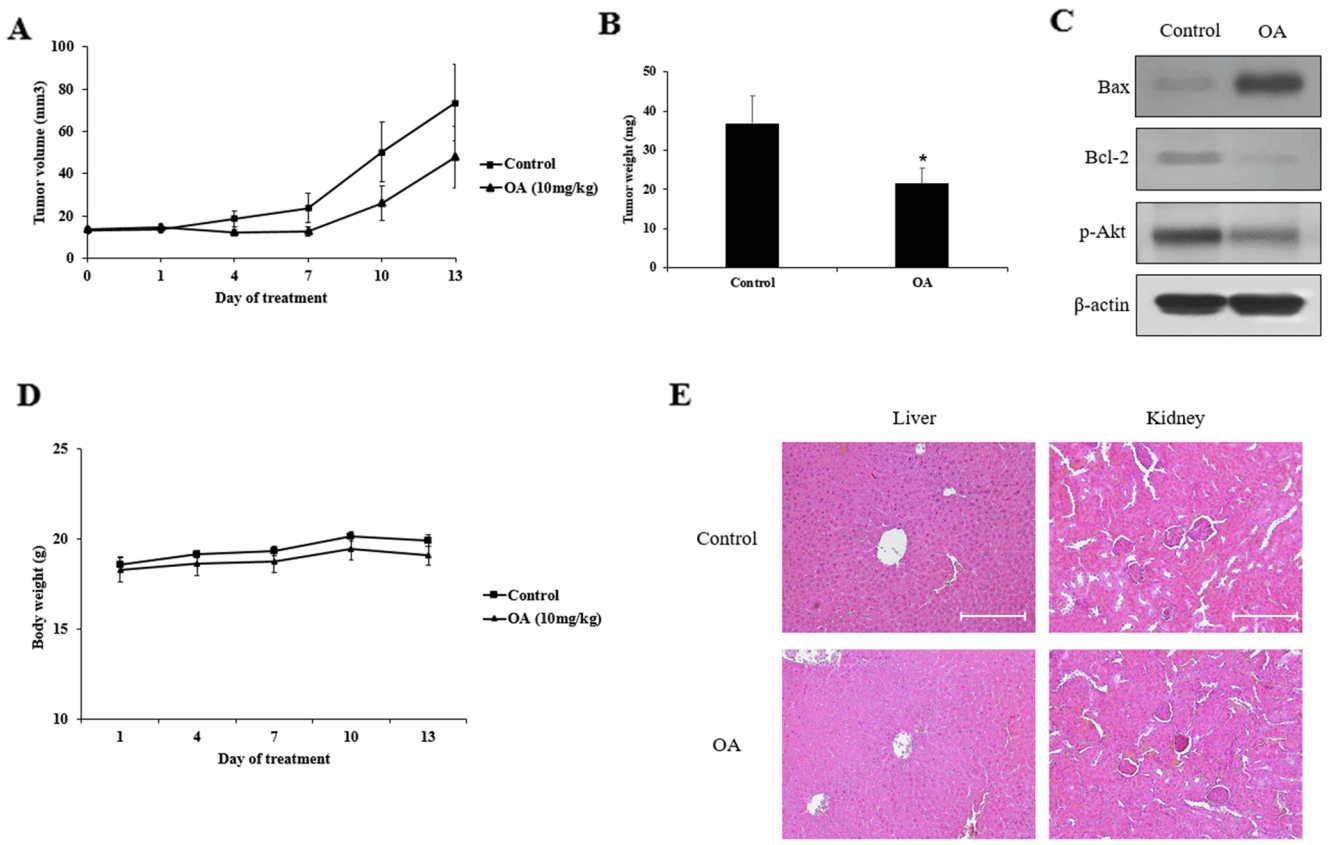


**Fig. 6.** Effects of the OA inhibits tumor growth in xenograft models without side effects. AGS cells ($5 \times 10^6$ cells) were injected subcutaneously into both shoulders of mice. After 7 days, the mice were randomly divided into two groups, namely control or OA (10 mg/kg). They were intraperitoneally injected with vehicle or OA daily for 14 d. Subsequently, the tumor volume (A) and the tumor weight (B) were measured and the protein expression levels for Bax, Bcl-2, and p-AKT were observed via western blotting (C). β-actin was used as loading control. (D) Body weight of mice was measured every 3 days. (E) Results of hematoxylin and eosin staining performed for the liver and kidney. Slides were observed under a light microscope. Scale bar = 10 μm. The Student's *t*-test was performed, and $^*p < 0.05$, compared with the control, was considered statistically significant.

intrinsic pathway is regulated by the mitochondria, while the extrinsic pathway is regulated by cell death receptors (Fulda & Debatin, 2006). Particularly, the Bax/Bcl-2 ratio is an important factor in the mitochondria-mediated apoptotic pathway (Del Poeta et al., 2003). Previous studies have reported that OA treatment increases Bax expression and decreases Bcl-2 expression in SW-579 thyroid cancer cells and PC-3 prostate cancer cells (Duan et al., 2019; Li et al., 2016). Thus, based on the fact that apoptosis is induced through an increase in the Bax/Bcl-2 ratio, we hypothesized that Bax and Bcl-2 are involved in OA-induced apoptosis. As hypothesized, the OA-treated AGS cells showed increased Bax expression and a decline in Bcl-2 expression compared to the control group. Moreover, PARP fragments, which are formed in response to DNA injury, were observed (Bouchard et al., 2003). These results suggest that OA induces mitochondria-mediated apoptosis by increasing the Bax/Bcl-2 ratio, thereby diminishing cell viability.

Autophagy demonstrates two functions, namely cell protection and cell death. These effects vary depending on the cell type and stimuli (Yun & Lee, 2018). Our experiment showed that OA-treated AGS cells presented with a dose-dependent increase of autophagic vacuoles, and acridine orange staining also confirmed that these cells tended to possess increased AVOs. Western blotting confirmed elevated levels of autophagy markers LC3-I, LC3-II, and beclin-1, suggesting that OA induced autophagy in AGS cells. There is a complex relationship between autophagy and apoptosis, which can suppress or facilitate cell death. Ascertainment of the fact whether autophagy plays protective or cytotoxic roles, depending on the cell type or signaling pathway, is crucial. Thus, additional analysis is warranted to examine the interaction between apoptosis and autophagy (Bhutia et al., 2013; Yonekawa & Thorburn, 2013). Accordingly, we attempted to examine this interaction based on cell viability using autophagy inhibitors 3-MA and HCQ. Both compounds further reduced cell viability compared to that observed with OA treatment alone. Moreover, the Bax/Bcl-2 ratio also increased compared to that observed with OA treatment alone. These results show that OA-induced autophagy exerts a protective effect on AGS cells, and inhibition of autophagy induces increased apoptosis.

The PI3K/AKT/mTOR pathway plays an important role in the coordination of biological processes such as cell proliferation, survival, and angiogenesis. Thus, inhibition of this pathway is an important strategy for inducing apoptosis and for suppressing cell viability (Xu et al., 2020). A previous study reported that OA increased PI3K/AKT activity in SW-579 thyroid cancer cells (Duan et al., 2019). In contrast, our results showed that the AKT proteins were inhibited in AGS gastric cancer cells upon treatment with OA, along with the downregulation of mTOR expression. These results are similar to a previous observation indicating that OA induces apoptosis by inhibiting the PI3K/AKT pathway in PC-3 prostate cancer cells (Li et al., 2016). When the cells were subjected to pretreatment with PI3K inhibitor LY294002, further decrease was observed in cell viability along with an increase in the Bax/Bcl-2 ratio, when compared to treatment with OA alone. These results highlight the possibility that OA treatment induces apoptosis by inhibiting the PI3K/AKT/mTOR pathway in AGS cells.

We conducted an *in vivo* experiment using a xenograft model to examine the effects of OA on gastric cancer. In a previous study, the OA group treated for colorectal cancer exhibited a significant decrease not only in weight but also in volume (Li et al., 2015). However, the present

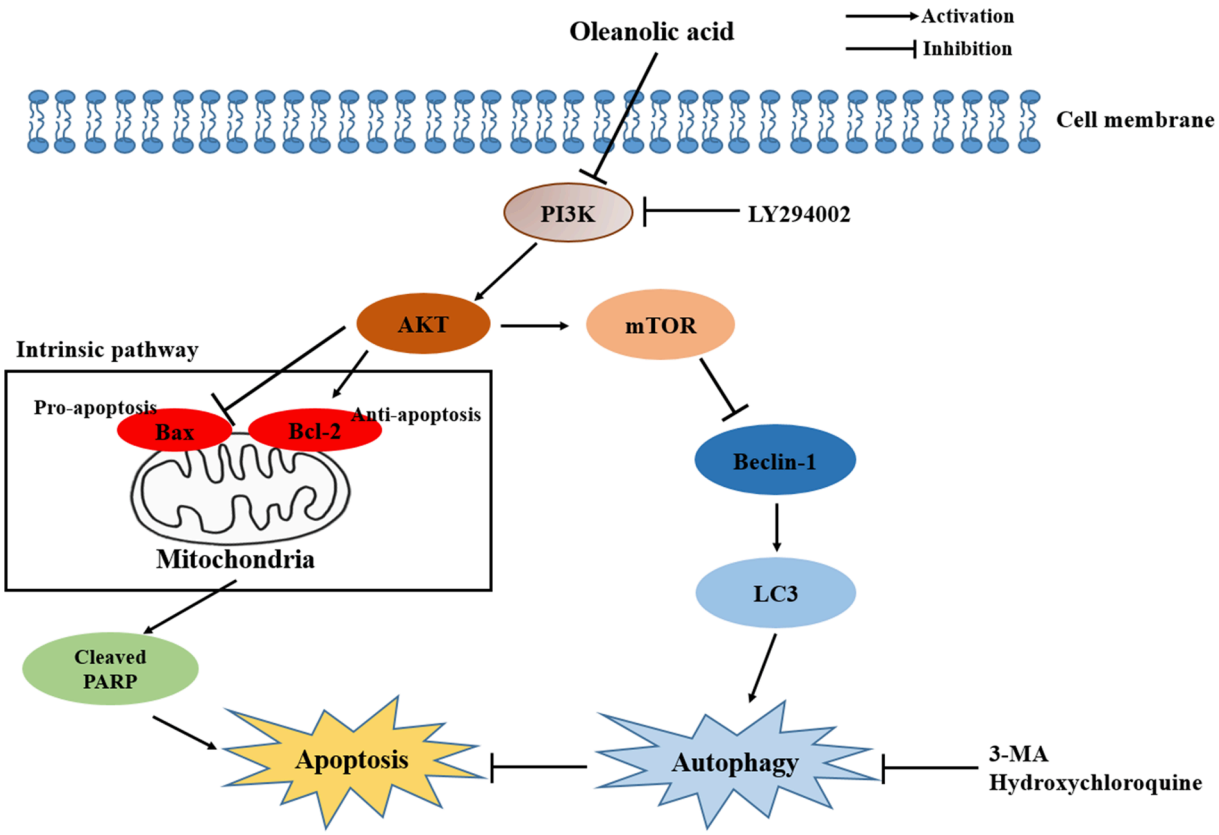


**Fig. 7.** Schematic of OA-induced apoptosis and autophagy in AGS cells. OA-induced apoptosis through the intrinsic pathway by suppressed PI3K/AKT signaling, and autophagy through mTOR signaling in AGS cells. Arrows represent activation, whereas bar represents inhibition.

results showed that OA decreased the volume and weight of AGS tumors compared to the controls but did not exhibit a significant decrease in volume. Consistent with the results of the *in vitro* experiment, western blotting showed an increase in the expression of bax and reduced expression levels of bcl-2 and p-AKT. These results indicated that apoptosis was induced due to the suppressed p-AKT in tumor cells, and the volume will decrease significantly when the period of OA administration increases. However, additional studies are warranted to examine whether OA induces autophagy as well as apoptosis in the body and to examine the relationship between the two.

Taken together, OA seems to reduce AGS cell viability via apoptosis. As shown in the diagram, the effect of OA not only suppressed PI3K/AKT pathway to affect the intrinsic pathway but also inhibited the mTOR pathway to increase autophagy. In addition, autophagy induced by OA in AGS cells protects the cells (Fig. 7). We also confirmed that OA induced apoptosis in the body using a xenograft model and that this was mediated by the inhibition of p-AKT. These results suggest that OA demonstrates the potential to be developed as a natural anti-cancer agent against gastric cancer.

*CRediT authorship contribution statement*

**Jae-Han Lee:** Investigation, Visualization, Writing – original draft. **Eun-Seon Yoo:** Investigation, Data curation. **So-Hee Han:** Investigation, Formal analysis. **Gi-Hwan Jung:** Validation. **Eun-Ji Han:** Data curation. **Soo-Hyun Jung:** Validation, Formal analysis. **Bum Seok Kim:** Supervision, Conceptualization. **Sung-Dae Cho:** Supervision, Writing – review & editing. **Jeong-Seok Nam:** Supervision, Methodology. **Changsun Choi:** Supervision, Conceptualization. **Jeong-Hwan Che:** Supervision, Writing – review & editing. **Ji-Youn Jung:** Conceptualization, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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