



# Fluoxetine Simultaneously Induces Both Apoptosis and Autophagy in Human Gastric Adenocarcinoma Cells

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## Abstract

Fluoxetine is used widely as an antidepressant for the treatment of cancer-related depression, but has been reported to also have anti-cancer activity. In this study, we investigated the cytotoxicity of fluoxetine to human gastric adenocarcinoma cells; as shown by the MTT assay, fluoxetine induced cell death. Subsequently, cells were treated with 10 or 20  $\mu$ M fluoxetine for 24 h and analyzed. Apoptosis was confirmed by the increased number of early apoptotic cells, shown by Annexin V- propidium iodide staining. Nuclear condensation was visualized by DAPI staining. A significant increase in the expression of cleaved PARP was observed by western blotting. The pan-caspase inhibitor Z-VAD-FMK was used to detect the extent of caspase-dependent cell death. The induction of autophagy was determined by the formation of acidic vesicular organelles (AVOs), which was visualized by acridine orange staining, and the increased expression of autophagy markers, such as *LC3B*, *Beclin 1*, and *p62/SQSTM 1*, observed by western blotting. The expression of upstream proteins, such as p-Akt and p-mTOR, were decreased. Autophagic degradation was evaluated by using bafilomycin, an inhibitor of late-stage autophagy. Bafilomycin did not significantly enhance *LC3B* expression induced by fluoxetine, which suggested autophagic degradation was impaired. In addition, the co-administration of the autophagy inhibitor 3-methyladenine and fluoxetine significantly increased fluoxetine-induced apoptosis, with decreased p-Akt and markedly increased death receptor 4 and 5 expression. Our results suggested that fluoxetine simultaneously induced both protective autophagy and apoptosis and that the inhibition of autophagy enhanced fluoxetine-induced apoptosis through increased death receptor expression.

**Key Words:** Fluoxetine, Autophagy, Apoptosis, Gastric adenocarcinoma

## INTRODUCTION

Fluoxetine was the first clinically available serotonin reuptake inhibitor (SSRI). It is often used in patients with cancer to treat psychiatric symptoms, such as depression and anxiety. In addition to their antidepressant effects, SSRIs, especially fluoxetine, have been proven to be toxic toward a variety of cancers, including prostate cancer (Abdul *et al.*, 1994), ovarian cancer (Lee *et al.*, 2010), colon cancer (Kannen *et al.*, 2012), breast cancer (Sun *et al.*, 2018), and hepatocellular carcinoma (Mun *et al.*, 2013). Moreover, fluoxetine prevents the growth of tumors *in vivo* (Abdul *et al.*, 1994). In contrast, it has been reported that fluoxetine is safe in non-cancer cell lines, such as HSF (Stepulak *et al.*, 2008), or primary cells,

such as peripheral blood mononuclear cells and B lymphocytes (Serafeim *et al.*, 2003); this suggested that the toxicity of fluoxetine was specific to cancer cells. In addition, fluoxetine can induce the death of cancer cells via different signaling mechanisms, including apoptosis (Krishnan *et al.*, 2008; Cloonan *et al.*, 2010; Choi *et al.*, 2017) and necrosis (Charles *et al.*, 2017). Recently, it was found that fluoxetine induced autophagic cell death in breast cancer cells (Sun *et al.*, 2018). However, to the best of our knowledge, the effect of fluoxetine on gastric cancer cells is still unknown.

Gastric cancer is the second most common cause of cancer-related death and the sixth most common diagnosed carcinoma worldwide, according to global cancer statistics from 2018 (Bray *et al.*, 2018). Worldwide, the incidence and mortality

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ty rates of gastric cancer display broad geographical variations (Sitarz *et al.*, 2018). Of the East Asian countries, the Republic of Korea has the highest incidence rate of gastric cancer, followed by Mongolia and Japan (Bray *et al.*, 2018). Given the oligosymptomatic course of early gastric cancer, most cases are diagnosed in the advanced stages of the disease. Moreover, the curative potential of the current standard treatment continues to be unsatisfactory, despite multimodal approaches involving surgery, chemotherapy, and radiotherapy (Stock and Otto, 2005). Thus, the development of novel anticancer agents or adjuvants for patients with advanced gastric cancer is urgently needed. Recently, targeting of autophagy in gastric cancer treatment has become a hot topic for researchers (Qian and Yang, 2016).

Autophagy is an intracellular homeostatic pathway; that is, it is a recycling mechanism. Under conditions of nutrient depletion or stress, some cells will undergo autophagy to provide energy to other cells through the degradation of damaged intracellular materials and recycling. Thus, autophagy acts as a self-defense mechanism that favors cell survival (Maes *et al.*, 2013). However, when these conditions or stresses are too extreme, aberrant autophagic activity may be initiated, ultimately leading to autophagic cell death (Levine and Kroemer, 2008) as a programmed cell death method. Autophagy may proceed independently or cooperatively with apoptosis. Numerous preclinical reports have suggested that the targeting of autophagy may offer a novel strategy for controlling tumorigenesis and cancer progression.

Therefore, we conducted this study to investigate whether fluoxetine induced cell death in human gastric adenocarcinoma (AGS) cells; and, if so, to explore the apoptotic and autophagic effect of fluoxetine.

## MATERIALS AND METHODS

### Materials

All cell culture reagents were purchased from Invitrogen (Grand Island, NY, USA), Corning Inc (NY, USA), and Welgene Inc (Daegu, Korea). To prepare the cellular extracts, the reagents required for lysis buffer were purchased from Sigma Chemical Co (St. Louis, MO, USA). For the cell viability assay, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. Anti-*LC3B*, anti-*Atg5*, anti-*BECLIN1*, anti-*p62/SQSTM1*, anti-mTOR, anti-p-mTOR, anti-Akt, anti-p-Akt, anti-PARP, DR4, DR5, caspase 3, and caspase 8 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP antibodies were purchased from Bethyl Laboratories (Montgomery, TX, USA). All western blotting reagents were purchased from Elpis Biotechnology (Daejeon, Korea) or Bio-Rad (Hercules, CA, USA). Fluoxetine hydrochloride was purchased from Selleckchem (Houston, TX, USA). The pan-caspase inhibitor, Z-VAD-FMK, was obtained from Enzo Life Sciences (Farmingdale, NY, USA). The autophagy inhibitors, 3-methyladenine (3-MA) and bafilomycin A1, were purchased from Selleckchem and TOCRIS Bioscience (Bristol, UK). 4'-6-Diamidine-2'-phenylindole dihydrochloride (DAPI) was purchased from Roche (Mannheim, Germany) and acridine orange (AO) was pur-

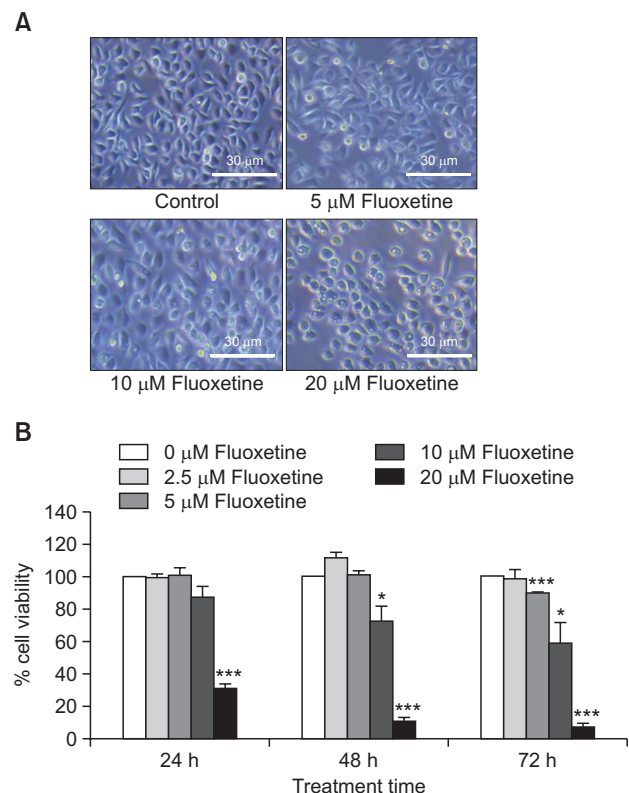
chased from Sigma Chemical Co. The Annexin V-DY-634/PI Apoptosis Detection Kit (ab214484) was purchased from Abcam (Cambridge, UK).

### Cell culture

The AGS human gastric cancer cell line (gastric adenocarcinoma, KCLB 21739) was obtained from the Korean Cell Line Bank (Seoul, Korea). AGS cells were grown in complete medium, consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.1% amphotericin B, at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The cells were seeded and cultured to reach 80% confluency.

### Cell viability measurement (MTT assay)

To determine the cell viability, the MTT assay was performed. AGS cells were plated at a density of  $1 \times 10^5$  cells/well in 24-well plates and maintained in RPMI 1640 supplemented with 10% FBS. When the cells reached 70% confluence, different concentrations of treatment agents were added. After incubation with the treatment agents, the cells were washed twice with PBS, and MTT solution (final concentration, 0.5



**Fig. 1.** Fluoxetine exhibits a potent cytotoxic effect on AGS cells. (A) AGS cells were treated with 5, 10, and 20 μM fluoxetine for 24 h. The changes in cell morphological were observed by using a fluorescence microscope (Leica, Wetzlar, Germany). (B) The cells were treated with the indicated concentrations of fluoxetine for 24, 48, and 72 h, and viable cells were detected by using an MTT assay. The data were collected from three independent experiments (n=3). Student's *t*-test was used to calculate statistical significance. Data are expressed as the mean  $\pm$  SEM (\**p*<0.05 and \*\*\**p*<0.001 compared with the control group).

mg/mL) was added into each well. After incubation for 4 h at 37°C, the medium was slowly removed, and 300 µL DMSO was added. The formazan crystals were dissolved in DMSO and quantified through the measurement of the absorbance at 570 nm by using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

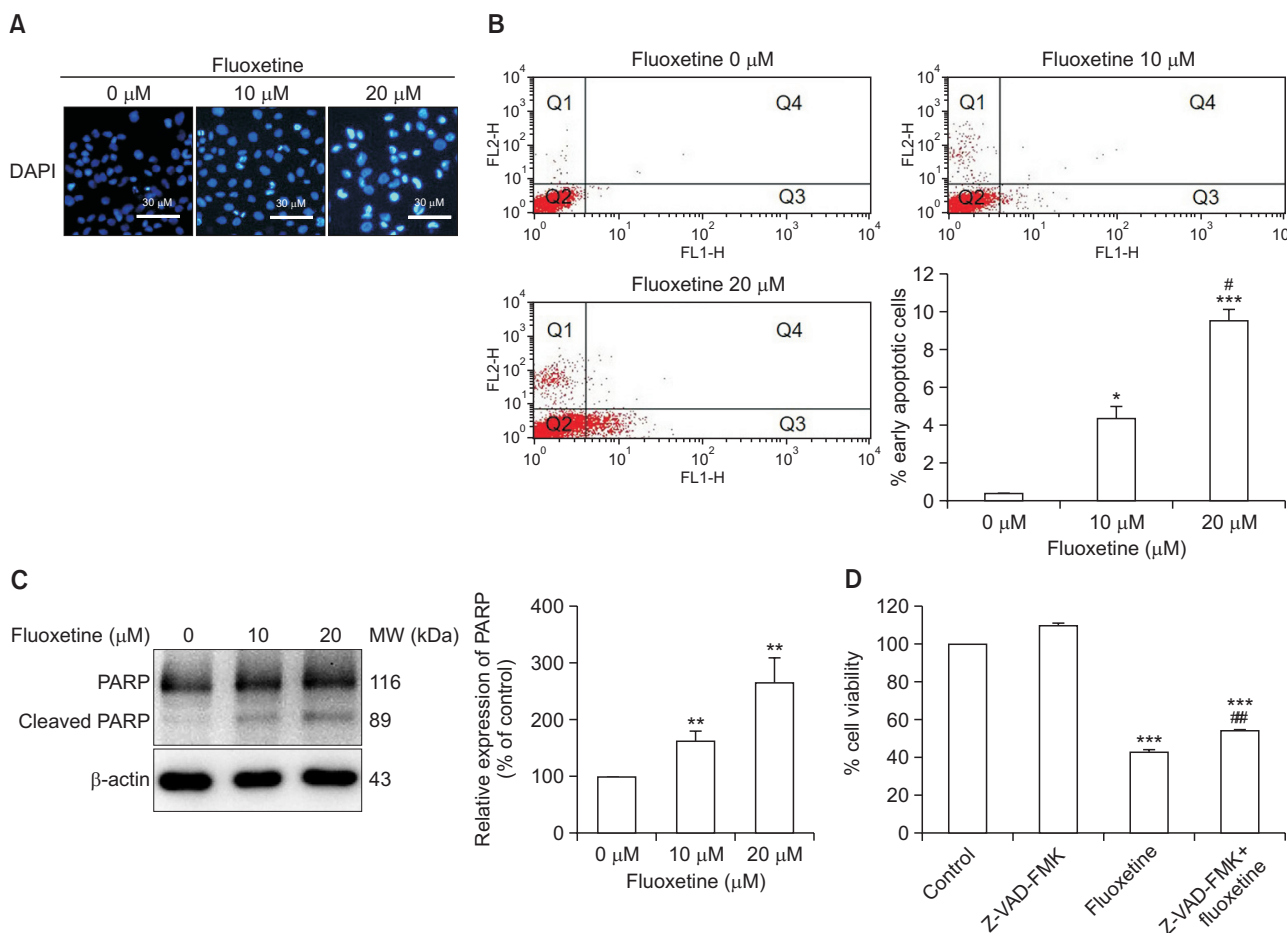
**Preparation of cellular extracts and western blotting analysis**

AGS cells were grown to 70% confluence and treated as indicated in the figure legends. After the desired incubation period, the cells were lysed with ice-cold lysis buffer (20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1% (w/v) Triton X-100, 0.01% (w/v) SDS, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mM PMSF, and 0.7 µg/mL β-mercaptoethanol). The lysates were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membranes by using a PowerPac power supply (Bio-Rad, Singapore). Subsequently,

the membrane was incubated with the designated primary antibodies and then with secondary HRP-labeled antibodies to examine the expression of proteins of interest. The results were analyzed by using Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

**Detection of acidic vesicular organelles**

The formation of acidic vesicular organelles (AVOs) such as autolysosomes, a morphological characteristic of autophagy, was detected by acridine orange (AO) staining. Cells were seeded in confocal dishes, grown until 60% confluent, and treated for 3 h. AO was added directly to the growth medium to a final concentration of 2 µg/mL, and then incubated at 37°C for 20 min. The cells were then washed twice with PBS containing 3% fetal bovine serum and immediately visualized in a solution of PBS with 3% fetal bovine serum by using a confocal microscope (LSM 800, Carl Zeiss, Oberkochen, Germany). Single and 3D images were assessed by using the



**Fig. 2.** Fluoxetine induces caspase-dependent apoptosis in AGS cells. (A) AGS cells were treated with fluoxetine 10 and 20 µM for 24 h and the changes in nuclear morphology were visualized by DAPI staining. (B) Apoptotic cell death was evaluated by using Annexin V-DY-634/PI staining. Cells treated with fluoxetine for 24 h were stained with Annexin V-FITC/PI and analyzed by using flow cytometry. Q1=dead cells; Q2=live cells; Q3=early apoptotic cells; Q4=late apoptotic and dead cells. (C) Under the same conditions described above, whole cell lysate was prepared and subjected to western blotting with the PARP antibody, and β-actin was used as the loading control. The blots were quantified by using densitometry. (D) AGS cells were pre-treated with the pan-caspase inhibitor Z-VAD-FMK (50 µM) for 1 h followed by fluoxetine (20 µM) for 24 h. The cell viability was evaluated by using the MTT assay. The data are expressed as the mean ± SEM (n=3). All experiments were repeated at least three times. Student's *t*-test was used for statistical analysis (\**p*<0.05, \*\**p*<0.01 and \*\*\**p*<0.001 compared with the untreated group, and #*p*<0.05, ##*p*<0.01 compared with fluoxetine alone-treated group).

Zen system (Image Software, Carl Zeiss).

### DAPI staining

To assess apoptosis, AGS cells were seeded in 24-well plates and incubated with fluoxetine. After treatment, the cells were fixed with 4% paraformaldehyde for 30 min and then washed with PBS before the nuclei were stained with DAPI for 3 min. Fluorescence signals were detected by using a fluorescence microscope (Leica, Wetzlar, Germany).

### Flow cytometry analysis

For apoptotic assays, AGS cells were treated with fluoxetine and 3-methyladenine, harvested by the addition of 0.25% trypsin without EDTA, washed twice with cold PBS, and re-suspended in 100  $\mu$ L 1 $\times$  binding buffer. The cells were then incubated with 5  $\mu$ L Annexin V-DY-634 and 5  $\mu$ L propidium iodide (ab214484; Abcam) in the dark for 15 min at room temperature. After incubation, 400  $\mu$ L of 1 $\times$  binding buffer was added, and the cells were analyzed by using flow cytometry (BD FACSCalibur™ Flow cytometer, CA, USA).

### Statistical analysis

All data were expressed as the mean  $\pm$  SEM of at least three independent experiments. The statistical significance of differences between groups were analyzed by Student's *t*-test, with values of  $p < 0.05$  considered statistically significant.

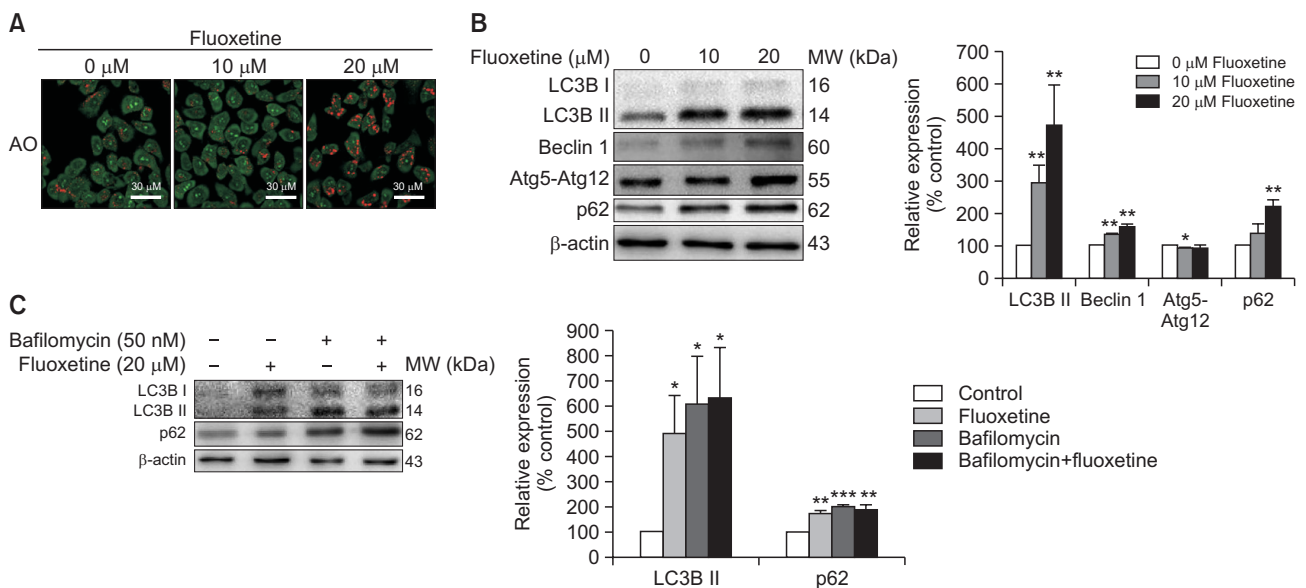
## RESULTS

### Fluoxetine exhibits a potent cytotoxic effect on AGS cells

To determine the cytotoxicity of fluoxetine to AGS cells, we examined the morphological changes in cells by using an optical microscope. After treatment for 24 h with fluoxetine, cell shrinkage and the formation of blebs on the cell surface were observed (Fig. 1A). Then, an MTT assay was performed. Fluoxetine was found to effectively inhibit the growth of AGS cells. The IC<sub>50</sub> values of fluoxetine were approximately 18, 15, and 12  $\mu$ M at 24, 48, and 72 h, respectively (Fig. 1B).

### Fluoxetine induces caspase-dependent apoptosis in AGS cells

To determine the apoptotic effect of fluoxetine, we used DAPI staining to detect nuclear changes. After treatment with fluoxetine for 24 h, AGS cells were fixed, and then stained with DAPI. Fluorescence microscopy revealed the presence of apoptotic bodies with chromatin condensation, nuclear fragmentation, and nuclear condensation in fluoxetine-treated cells (Fig. 2A). The cells were stained with Annexin V-DY-634 and PI to analyze the extent of apoptosis. As shown in Fig. 2B, the number of early apoptotic cells was significantly increased in fluoxetine-treated groups, by approximately 4- and 10-fold, respectively, compared with the untreated cells. As PARP is a selective substrate for caspase-3, we detected the expression of PARP in fluoxetine-treated cells by using western blotting. The expression of cleaved PARP was 1.5-fold and 2-fold higher in the fluoxetine 10  $\mu$ M- and 20  $\mu$ M-treated groups, respectively, than in the untreated group (Fig. 2C). Furthermore,

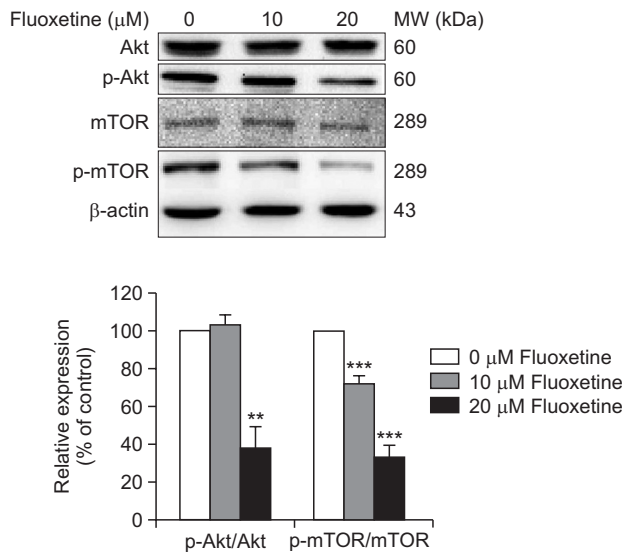


**Fig. 3.** Fluoxetine results in the accumulation of autophagosome formation in AGS cells. (A) Accumulation of acidic vesicular organelles (AVOs) in fluoxetine-treated AGS cells. AGS cells were treated with the indicated concentration of fluoxetine for 3 h and AVOs were stained by acridine orange (AO) and observed by using a confocal microscope. Acidic compartments, such as autolysosomes, fluoresce red; in contrast, other compartments, such as the cytoplasm and nucleus, fluoresce green. (B) Western blotting analysis of autophagosome markers LC3B, *Atg5-Atg12*, *Beclin1*, and p62. The cells were treated with fluoxetine 10 and 20  $\mu$ M for 24 h. Whole cell lysate was prepared and analyzed by western blotting, with  $\beta$ -actin used as the loading control. (C) Cells were pre-treated with bafilomycin for 1 h before fluoxetine treatment for 24 h. The expression of LC3B and p62 was detected by western blotting analysis, and the blots were quantified by using densitometry. The data are expressed as the mean  $\pm$  SEM (n=3). All experiments were repeated at least three times. Student's *t*-test was used to calculate statistical significance (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  compared with the untreated group).

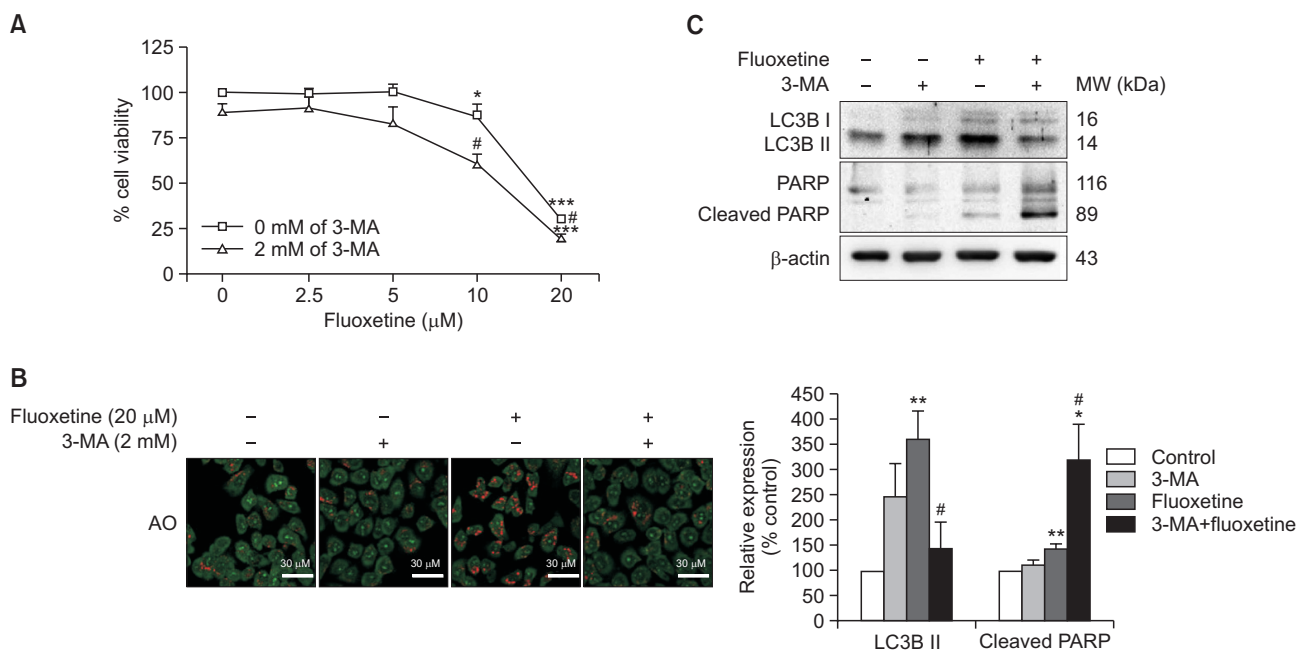
Z-VAD-FMK was used to confirm if fluoxetine induced apoptotic cell death. Fluoxetine-induced cell death was reduced in the cells treated with the combination of fluoxetine and Z-VAD-FMK (Fig. 2D). All these data suggested that fluoxetine-induced AGS cell death involved apoptosis.

### Fluoxetine increases autophagosome formation in AGS cells

To identify whether fluoxetine induced autophagy in gastric adenocarcinoma cells, AO staining was used to visualize AVOs in the untreated and fluoxetine-treated AGS cells. As shown in Fig. 3A, fluoxetine treatment markedly elevated the amount and density of red fluorescence in the AGS cells, suggesting an increase in AVOs. *LC3*, *BECLIN1*, *Atg5-Atg12*, and *p62* are necessary for autophagosome formation. However, *p62* was degraded along with the cargo in the autophagic degradation step. Therefore, to confirm that autophagy was induced by fluoxetine, we analyzed the expression of these proteins. Fluoxetine upregulated the expression of *LC3B II* and *BECLIN1* in a dose-dependent manner, although no significant change in *Atg5-Atg12* expression occurred (Fig. 3B). Unexpectedly, the expression of *p62/SQSTM1* was also elevated in a dose-dependent manner by fluoxetine (Fig. 3B). Bafilomycin, an inhibitor of late-stage autophagy, did not enhance *LC3B II* formation, suggesting that autophagic flux was impaired (Fig. 3C). Based on these data, we assumed that fluoxetine induced autophagosome accumulation in AGS cells through the disturbance of autophagosome degradation.



**Fig. 4.** Fluoxetine decreases the phosphorylation of Akt and mTOR in AGS cells. AGS cells were treated with 10 and 20 μM fluoxetine for 24 h and the expression of Akt, phospho-Akt, mTOR, and phospho-mTOR were determined by using western blotting, with β-actin used as the loading control. The blots were quantified by using densitometry. The data are presented as the mean ± SEM (n=3). All experiments were repeated at least three times. Student's *t*-test was used to calculate statistical significance (\*\**p*<0.01 and \*\*\**p*<0.001 compared with the untreated group).



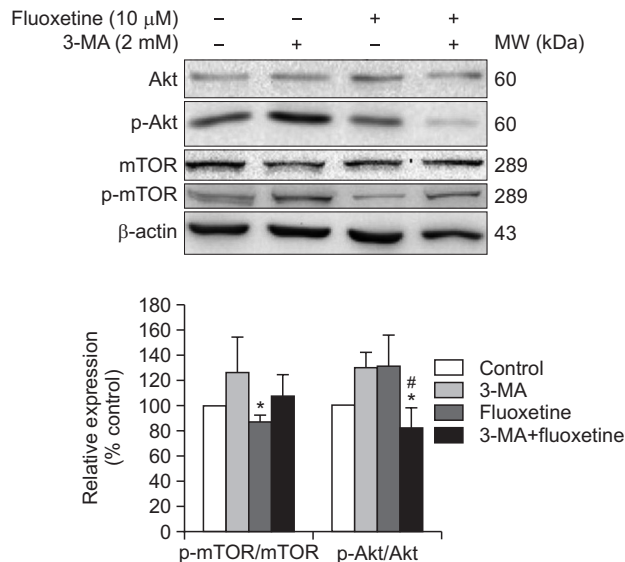
**Fig. 5.** 3-MA enhances the apoptotic effect of fluoxetine in AGS cells. (A) Autophagy inhibitor 3-methyladenine (3-MA) enhanced fluoxetine-induced cell death. AGS cells were pre-treated with 3-MA for 1 h, followed by fluoxetine for 24 h. Cell viability was examined by using an MTT assay. Combination treatment greatly reduced the cell viability compared with the monotherapies. (B) Under the same conditions described above, the cells were stained with acridine orange (AO). Acidic compartments, such as autolysosomes, fluoresce red; in contrast, the others, such as the cytoplasm and nucleus, fluoresce green. Co-treatment with 3-MA decreased the intensity of red fluorescence of acridine orange compared with fluoxetine alone. (C) Subsequently, whole cell lysate was prepared and *LC3B* and *PARP* expression was determined by using western blotting. The blots were quantified by densitometry. The data are presented as the mean ± SEM (n=3). All experiments were repeated at least three times. Student's *t*-test was used to calculate statistical significance (\**p*<0.05, \*\**p*<0.01 and \*\*\**p*<0.001 compared with the untreated group, and #*p*<0.05 compared with fluoxetine alone-treated group).

### Fluoxetine decreases the phosphorylation of Akt and mTOR in AGS cells

As various reports have stated that the Akt/mTOR pathway was a negative regulator of the autophagy pathway, we examined the phosphorylation of Akt and mTOR by using western blotting. As shown in Fig. 4, fluoxetine dose-dependently reduced the phosphorylation of Akt and the phosphorylation of mTOR, which suggested that fluoxetine-induced autophagosome accumulation may be not only due to disturbance of the degradation step, but also due to the induction of autophagosome formation through mTOR inhibition.

### 3-MA enhances the apoptotic effect of fluoxetine in AGS cells

Subsequently, to elucidate if the autophagosome accumulation induced by fluoxetine in AGS cells was pro-survival or pro-death, the autophagy inhibitor 3-MA was used, with an MTT assay performed to examine cell viability. The inhibition of autophagy by 3-MA significantly enhanced cell death compared with fluoxetine alone (Fig. 5A), whereas the formation of AVOs was significantly inhibited by 3-MA, as observed by AO staining (Fig. 5B). Subsequently, we performed western blotting to measure the expression of cleaved PARP, which is an "executioner compound" in apoptosis, and LC3B II. As shown in Fig. 5C, the cells were co-treated with fluoxetine and 3-MA exhibited twice the amount of cleaved PARP compared with cells treated with fluoxetine alone, but half the expression of LC3B II induced by fluoxetine treatment alone.



**Fig. 6.** Effect of 3-methyladenine on the Akt/mTOR pathway. AGS cells were pre-treated with 3-methyladenine (3-MA) for 1 h, followed by 10  $\mu$ M fluoxetine for 24 h. Whole cell lysate was prepared and western blotting was used to determine the protein expression of Akt, phospho-Akt, mTOR, and phospho-mTOR, with  $\beta$ -actin used as the loading control. The blots were quantified by using densitometry. The data are presented as the mean  $\pm$  SEM (n=3). All experiments were repeated at least three times. Student's *t*-test was used to calculate statistical significance (\**p*<0.05 compared with the untreated group, and \*\**p*<0.05 compared with fluoxetine alone-treated group).

### Effect of 3-methyladenine on the Akt/mTOR pathway

To detect if the enhancement of cell death by 3-MA was related to Akt expression, western blotting was performed. As shown in Fig. 6, pre-treatment of 3-MA markedly reduced the expression of Akt in fluoxetine-treated AGS cells, whereas mTOR level was restored.

### 3-MA enhances fluoxetine-induced apoptosis through the death receptor pathway

The morphological characteristics of apoptosis were visualized by DAPI staining using a fluorescent microscope. Nuclear condensation was markedly increased by the combination treatment of fluoxetine and 3-MA compared with the monotherapy (Fig. 7A). Apoptotic cell death was also examined by Annexin V-DY-634/PI staining. The percentage of early apoptotic cells was significantly increased, from 4.38% to 12.17%, in the presence of 3-MA (Fig. 7B). In addition, 3-MA enhanced the protein expression of death receptor 4 and 5 (DR4, DR5), and the expression of pro-caspase 8 and pro-caspase 3 was reduced (Fig. 7C).

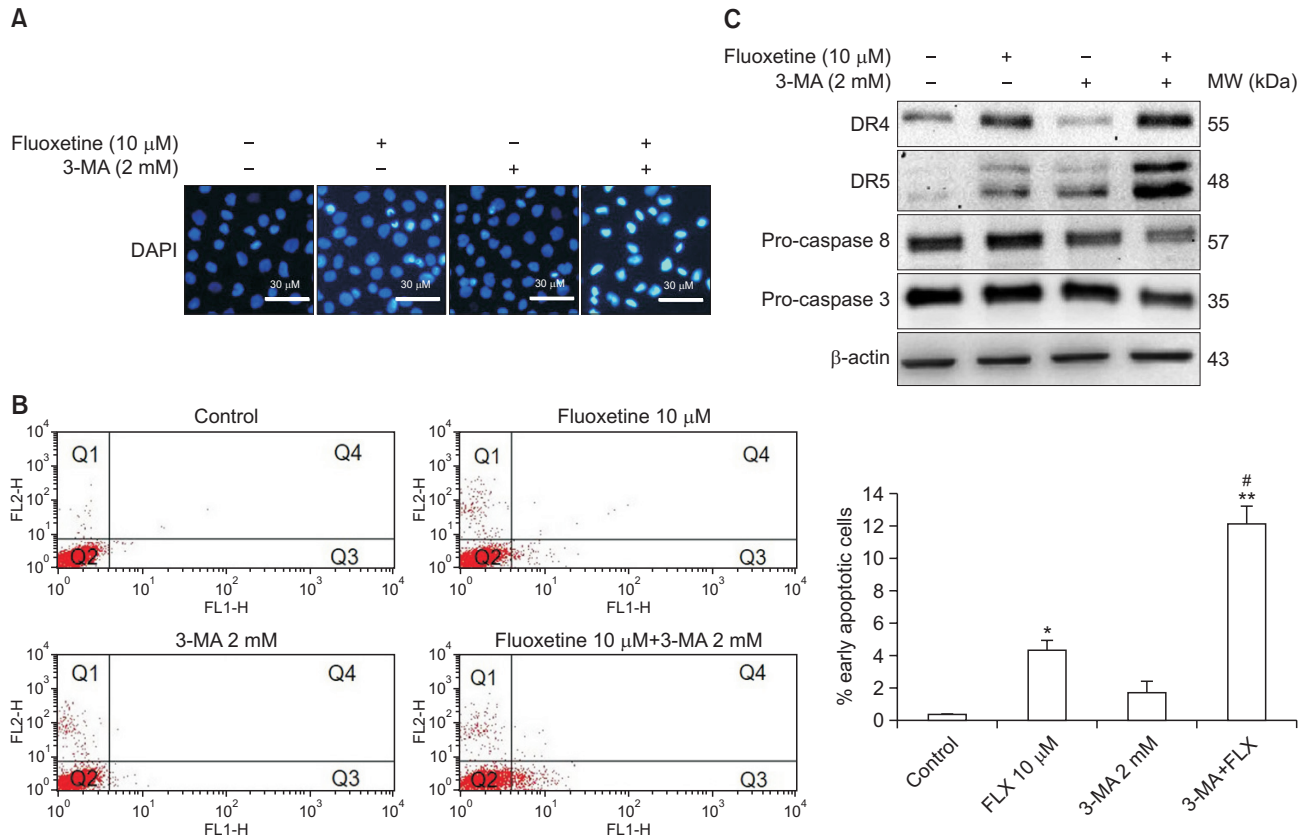
### Summary of proposed mechanism of fluoxetine-induced autophagy and apoptosis in AGS cells

To be brief, fluoxetine induced apoptosis in AGS cells through the death receptor pathway. Fluoxetine increased the death receptors 4 and 5 expression and PARP. On the other hand, fluoxetine accumulated the formation of autophagosomes not only by increasing LC3B II expression through Akt/mTOR pathway but also by inhibiting the autophagic degradation step. In addition, inhibition of autophagy by 3-methyladenine (3-MA) enhanced the fluoxetine-induced apoptosis by decreasing Akt expression and by supporting the fluoxetine-induced death-receptor-related apoptosis (Fig. 8).

## DISCUSSION

Despite the development of new therapeutics and advances in diagnostic technology, gastric cancer remains the second most common cancer worldwide, and the demand for novel and alternative approaches for gastric cancer therapy is still high. Recently, researchers have focused on the relationship between the autophagy and gastric cancer and found that autophagy-promoters and autophagy-inhibitors showed promise in gastric cancer therapy (Qian and Yang, 2016). Fluoxetine has been shown to have anticancer potential in a variety of cancer cells, but not in gastric cancer cells. Recently, it was reported that fluoxetine induced autophagy in breast cancer cells and glioblastoma cells (Cloonan and Williams, 2011; Sun *et al.*, 2018). As fluoxetine has been used widely in patients with gastric cancer as an antidepressant, if it is able to induce cell death in gastric cancer cells, it may lead to a new alternative approach for gastric cancer therapy. However, autophagy is a complicated and controversial process and it can be either tumor-promoting or tumor-suppressing in gastric cancer, as shown in previous findings (Qian and Yang, 2016). This study was designed to investigate the cytotoxic effect of fluoxetine in human gastric adenocarcinoma (AGS) cells.

In the present study, we found that fluoxetine concentrations from 10  $\mu$ M induced the death of AGS cells. Admittedly, this is higher than the plasma concentration of fluoxetine (approximately 1  $\mu$ M) after treatment at a standard therapeutic



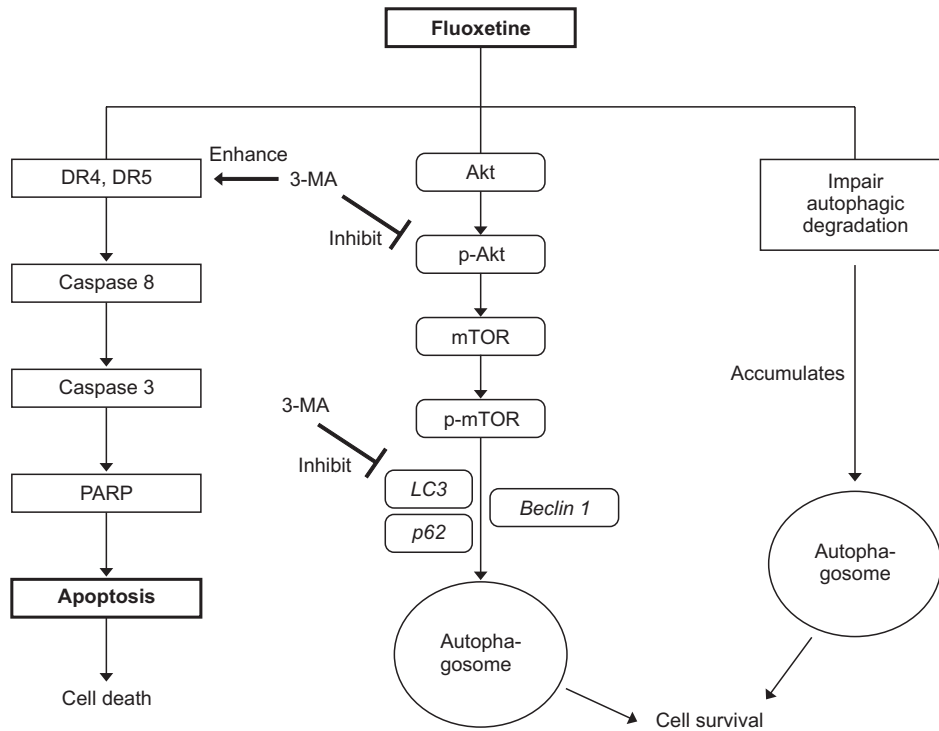
**Fig. 7.** 3-Methyladenine enhances the fluoxetine-induced apoptosis through the death receptor pathway. (A) Cells were pre-treated with 3-MA for 1 h, followed by 10  $\mu$ M fluoxetine for 24 h, and then stained with DAPI to visualize the apoptotic bodies. (B) Under the same conditions described above, cells were stained with Annexin V-DY634 and PI and analyzed by using flow cytometry. Q1=dead cells; Q2=live cells; Q3=early apoptotic cells; Q4=late apoptotic and dead cells. (C) Western blotting analysis of death receptor (DR)4, DR5, caspase 8, and caspase 3 protein expression was conducted, with  $\beta$ -actin used as a loading control. The blots were quantified by densitometry. The data are presented as the mean  $\pm$  SEM of three independent experiments (n=3). Student's *t*-test was used to calculate statistical significance (\**p*<0.05 and \*\**p*<0.01 compared with the control group, and #*p*<0.05 compared with fluoxetine alone-treated group).

dose (40 mg per day for 30 days) as an antidepressant (Cheer and Goa, 2001). However, fluoxetine tissue levels were up to 20-fold higher than in the plasma, because of its lipophilic properties (Bolo *et al.*, 2000). It also has a wide safety range; serious side effects are observed in humans when administered at more than 75 times the therapeutic dose for depression (Barbey and Roose, 1998). In addition, in most of the published *in vitro* studies, the apoptotic effects of fluoxetine were induced between 0.1 and 100  $\mu$ M and varied with the cell type (Charles *et al.*, 2017). Based on these references, we decided to use 10 and 20  $\mu$ M fluoxetine in this study.

Then, we examined the apoptotic and autophagic effect of fluoxetine. Remarkable upregulation of both apoptotic proteins, such as cleaved PARP, and autophagic proteins, such as *BECLIN1*, *LC3-II*, and *p62*, was observed in fluoxetine-treated AGS cells, consistent with the previous studies (Liu *et al.*, 2015; Sun *et al.*, 2018). In addition, Annexin V/PI staining showed an increase in apoptotic cells. We also observed the accumulation of apoptotic bodies and AVOs in fluoxetine-treated cells by using DAPI and AO staining. Moreover, the negative regulator of the autophagy protein p-mTOR was decreased as the fluoxetine concentration was increased. We assumed that fluoxetine induced both apoptosis and autophagy.

gosome formation in AGS cells at the same time.

However, autophagy is a dynamic and complex process that may be regulated in multiple steps. A series of reports has shown that the accumulation of the autophagosomes may be indicative of either the activation of autophagy or blockade of the degradation step of the autophagy (Levine and Kroemer, 2008; Wong and Cuervo, 2010). Moreover, *p62* is an autophagy receptor that interacts with specific autophagy targets in the autophagosome through the *LC3-II* protein. Eventually, it was enclosed by the autolysosome and degraded by lysosomal enzymes (Vallecillo-Hernández *et al.*, 2018). However, in the current study, both *LC3 II* and *p62* levels were increased by fluoxetine. Therefore, we evaluated the autophagic degradation activity by using the late-stage autophagy inhibitor, bafilomycin. Bafilomycin is a vacuolar-type H(+) ATPase (V-ATPase) inhibitor that impairs the fusion of autophagosomes and lysosomes, which leads to the inhibition of autophagy (Yamamoto *et al.*, 1998). We discovered that a non-significant increase in *LC3 II* and *p62* accumulation occurred in fluoxetine-treated cells in the presence of bafilomycin (Fig. 4), which suggested that autophagic degradation activity may also be affected by fluoxetine. The accumulation of autophagosomes may be due to not only to the induction of membrane forma-



**Fig. 8.** Summary of proposed mechanism of fluoxetine-induced autophagy and apoptosis in AGS cells. Fluoxetine induces apoptosis through the death receptor pathway. However, it also results in the accumulation of autophagosome formation by decreasing mTOR and inhibiting the degradation step. Herein, inhibition of autophagy by 3-methyladenine (3-MA) enhances fluoxetine-induced apoptosis by decreasing Akt expression and by increasing the death receptor expression.

tion, but also through disturbance of the degradation activity.

This assumption was supported by a recent study, in which it was reported that fluoxetine decreased mTORC1 activity and increased lysosomal biogenesis, but resulted in the abundance of *p62* mRNA and protein in a dose-dependent manner. They reported that despite the induction of lysosomal biogenesis, fluoxetine clearly disturbed the lysosomal function (Lu *et al.*, 2017).

A growing body of evidence has supported that the cross-talk between autophagy and apoptosis plays a role in the regulation of cell death or cell survival (Su *et al.*, 2013). Chemotherapeutic agents that kill cancer cells primarily act through the induction of apoptosis. To defend against this process, the cancer cell may itself activate the autophagy as a protective mechanism (Li *et al.*, 2017). Autophagy has been shown to favor tumor cell survival by acting as an oncogenic pathway, and inhibition of autophagy has been reported to increase cell death induced by various stimulants (Shin *et al.*, 2012; He *et al.*, 2018). Therefore, we speculated that fluoxetine-induced autophagy may also affect the extent of apoptosis induced by fluoxetine. We also observed that fluoxetine-induced autophagy had a pro-survival role in AGS cells, and that the autophagy inhibitor 3-MA could enhance fluoxetine-induced cell death through the increased expression of death receptor and through a reduction in Akt level. This was in contrast to a study of fluoxetine in breast cancer cells, in which fluoxetine induced autophagic cell death (Sun *et al.*, 2018). However, our data were consistent with other previous studies, in which it was reported that the inhibition of autophagy enhanced the apoptosis-inducing effect of MHY218 in AGS cells (Choi *et*

*al.*, 2015), and that the addition of autophagy inhibitors, such as chloroquine and bafilomycin A1, sensitized gastric cancer cells to the cytotoxic effects of anticancer drugs (Zhang *et al.*, 2015; Li *et al.*, 2016), and that the inhibition of autophagy enhanced 5FU-induced cell death in human gastric carcinoma (He *et al.*, 2018). Hence, the role of the autophagy may differ depending on the type and developmental stage of tumor; it may contribute to the anticancer efficacy of the drug, as well as drug resistance. In accordance with our data, we assumed that AGS cells activate autophagy as a resistance mechanism to fluoxetine-induced cell death.

In summary, fluoxetine induces both apoptosis and autophagosome formation simultaneously in gastric adenocarcinoma cells. In addition, the autophagy inhibitor 3-MA enhanced fluoxetine-induced apoptosis. Based on these results, we suggested that fluoxetine has a promising therapeutic effect for the treatment of gastric adenocarcinoma, and that the inhibition of autophagy may be a good approach for gastric cancer therapy. However, it is still necessary to determine, in detail, the interaction between apoptosis and autophagy.

## CONFLICT OF INTEREST

There is no conflict of interest.

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