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ORIGINAL ARTICLE

Apoptotic effect of fluoxetine through the endoplasmic reticulum stress pathway in the human gastric cancer cell line AGS

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Abstract

Gastric cancer is the fourth most common cancer in the world. Fluoxetine (FLX), a selective serotonin reuptake inhibitor, can inhibit the growth of cancer cells by inducing apoptotic cell death through various signaling pathways. This study was aimed to determine the mechanism of apoptotic cell death induced by FLX in AGS cells. MTT assay for cell viability test and colony forming assay was performed for detection of cell proliferation. Western blot analysis was conducted for protein expression. Increased fluorescence intensity and chromatin condensation were observed using DAPI staining. Production of reactive oxygen species (ROS) was measured by DCFDA assay. AGS cell proliferation was remarkedly inhibited by FLX in a dose-dependent manner starting at a concentration of 20 μM. The expression of death receptors was increased, which resulted in elevated expression of activated caspases and cleaved PARP, leading to FLX-induced apoptosis. Moreover, FLX significantly increased production of ROS, and *N*-acetyl cysteine, which scavenges ROS, attenuated the cytotoxic effects of FLX. In addition, treatment with FLX increased the expression of the endoplasmic reticulum (ER) stress marker, CHOP. P53 protein expression in AGS cells also decreased significantly with FLX treatment. Inhibition of ER stress significantly decreased the expressions of death receptor 5 (DR5), cleaved caspase 3, and cleaved PARP, but not to control levels. FLX-induced apoptosis in AGS involved upregulation of death receptors, ROS generation, and activation of ER stress.

Keywords Gastric adenocarcinoma · Fluoxetine · Apoptosis · ROS · Endoplasmic reticulum stress

Introduction

Fluoxetine (FLX), a selective serotonin reuptake inhibitor, has been widely used for treatment of cancer-related depression and panic disorder. In 1992, a study reported that FLX accelerated the growth of malignant tumors in rodents (Brandes et al. 1992). Also, other studies showed that serotonin exhibited a growth stimulatory effect on human tumor cells (Sarrouilhe and Mesnil 2018). However, several clinical studies have demonstrated that there is no association between antidepressants and cancer progression (Coogan et al. 2005; Wernli et al. 2009; Hsieh et al. 2015; Huo et al. 2018; Kiridly-Calderbank et al. 2018).

The effects of FLX on cancer cell growth have been studied, and FLX has been shown to induce cell death in various cancer cell lines such as colon cancer cell line, ovarian cancer cell line, hepatocellular carcinoma cell lines, etc. A previous report showed that FLX ameliorated colitis-associated colon cancer in mice (Koh et al. 2011). In addition, FLX induced apoptosis in cell lines such as ovarian cancer cell line OVCAR-3 (Lee et al. 2010), hepatocellular carcinoma cell line Hep3B (Mun et al. 2013), and neuroblastoma cell line SK-N-BE(2)-M17 (Choi et al. 2017). In colon cancer, FLX exerts apoptosis-independent antiproliferative effects (Kannen et al. 2012). In breast cancer cells, FLX induces autophagic cell death (Sun et al. 2018). FLX induces cell death via different signaling pathways, such as upregulation of reactive oxygen species (ROS) and activation of endoplasmic reticulum (ER) stress. FLX induced ROS production and oxidative stress-mediated apoptosis in both ovarian and hepatocellular carcinomas. In glioma cells, neuroblastoma cells, and breast cancer cells, FLX induced cell death through cytotoxic ER stress (Liu et al. 2015). In addition, in most

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of the published in vitro studies, the apoptotic effects of fluoxetine were induced between 0.1 and 100 μM and varied with the cell type (Charles et al. 2017), which is higher than the plasma concentration of fluoxetine (approximately 1 μM) after treatment at a standard therapeutic dose (40 mg per day for 30 days) as an antidepressant (Cheer and Goa 2001). However, 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 1998a). Still, information regarding the effects of FLX on gastric cancer is limited.

Gastric cancer is the fourth most common cancer worldwide and is a leading cause of cancer-related death (Jemal et al. 2009). Although the incidence of gastric cancer and associated mortality are decreasing worldwide, Korea continues to have one of the highest rates of gastric cancer. According to the Korea Central Cancer Registry and Local Cancer Registries Data, gastric cancer remains the leading malignancy in both sexes, despite a recent decrease in incidence (Shin et al. 2005). Although chemotherapy and surgery improve gastric cancer survival rates, the outcomes of these therapies are often inadequate, and development of drug resistance is a major problem. Therefore, alternative approaches to treat gastric cancer are essential (Lissoni et al. 1993).

FLX is a commonly prescribed antidepressant for cancer patients and may exert anti-cancer effects in several cancer cells (Hsieh et al. 2015). As such, FLX might also be beneficial for treatment of gastric cancer. Thus, this study was conducted to determine the apoptotic effects of FLX on human gastric cancer cells and to examine related pathways and signaling cascades.

Materials and Methods

Materials

Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), ethyleneglycol-bis-(ß-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediamine tetra acetic acid (EDTA), glutathione (GSH), *N*-acetylcysteine (NAC), leupeptin, aprotinin, ß-mercaptoethanol, phenylmethylsulfonylfluoride (PMSF), triton X-100, sodium dodecyl sulfate (SDS), Hank's balanced salt solution-modified (HBSS), KNK437, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Crystal violet was purchased from Selleckchem Korea. Antibiotic-antimyocotic (penicillin, streptomycin, and amphotericin B) and trypsin-EDTA were purchased from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Corning (Corning Inc, NY, USA). 2′,7′-Dichlorofluorescein diacetate (DCFDA) was obtained from Thermo Scientific (Waltham, MA, USA). RPMI 1640, tris-buffered saline (TBS), and phosphate-buffered saline (PBS) were purchased from Welgene Inc. (Daegu, Korea). FLX and salubrinal were purchased from Sigma Chemical Co. (St. Louis, MO, USA). STF-083010 was purchased from Selleckchem Co. (Houston, TX, USA). Anti-BID, anti-caspase-3, anti-cleaved caspase-3, anti-caspase-8, anti-caspase-9, anti-cleaved caspase-9, anti-PARP, anti-BAX, anti-Bcl-2, anti-XIAP, anti-DR4, anti-DR5, anti-FADD, anti-TRAIL, anti-BiP, anti-CHOP, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-JNK, anti-JNK, anti-phospho-ERK, anti-ERK, anti-phospho-MEK, anti-MEK, and anti-p53 antibodies were purchased from Cell Signaling (Danvers, MA, USA).

Paraformaldehyde solution (4%) was purchased from Santa Cruz Biotechnology (Santo Cruz, CA, USA). 4′-6-Diamidine-2′-phenylindole dihydrochloride (DAPI) was purchased from Roche (Manndeim, Germany). Fluorescence mounting medium was purchased from Dako (Santa Clara, CA, USA). Goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, and rabbit anti-goat IgG-HRP were purchased from Bethyl laboratories (Montgomery, TX, USA). Acryl-Bisacrylamide (37.5:1), enhanced chemiluminescence (ECL) agents, and rainbow prestained molecular weight markers were purchased from Elpis Biotechnology (Daejeon, Korea). Ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylendiamine (TEMED), nitrocellulose membranes, tris/glycine/SDS buffer, tris/glycine buffer, and Restore TM Western Blot Stripping Buffer were purchased from BioRad (Richmond, CA, USA).

Cell culture

The human gastric cancer cell line AGS (gastric adenocarcinoma, KCL 21739) was obtained from the Korean Cell Line Bank (Seoul, Korea). AGS cells were grown in complete medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. Cells were seeded and cultured to reach 80% confluency.

Analysis of cell viability

For the MTT assay procedure, AGS cells were plated at a density of 1×10^5 cells/well in 6-well plates and maintained in RPMI 1640 containing 10% FBS. When the cells reached 80% confluence, the media was exchanged for serum-free RPMI media and the cells were incubated for 24 h to arrest cell growth and silence gene activity.

Then, the cells were treated as indicated for each experiment. After incubation with FLX, cells were twice washed with PBS, and MTT solution (final concentration, 0.5 mg/ml) was added to each well. After incubation for 4 h at 37 °C, the medium was slowly removed, and 500 μl of DMSO was added. Formazan crystals were dissolved in DMSO and quantified at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Colony forming assay

AGS cells were seeded at a concentration of 1×10^5 cells/well in 6-well plates. Cells were allowed to grow for 24 h. Cells were then treated with FLX for 24 h and washed with PBS. Colonies were fixed with 500 μl of 4% paraformaldehyde per well for 5 min and stained with crystal violet (0.1% w/v) for 2 h at room temperature. Images were acquired using a fluorescence microscope (Leica, Tokyo, Japan)

Protein isolation and Western blot analysis

After incubation with FLX, AGS cells were washed with ice-cold DPBS and 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 ß-mercaptoethanol). The lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes using a Power Pac (Bio-Rad, Melville, NY, USA) power supply, then examined for expression of proteins of interest. Actin was used as a loading control. The results were analyzed using Quantity One analysis software (Bio-Rad Chemical Division, Richmond, CA, USA).

For inhibitor studies, cells were pre-treated for 1 h prior to FLX treatment. Water-insoluble reagents were dissolved in DMSO and a highly concentrated stock solution was prepared. Cells were then incubated in a solution prepared by diluting the stock solution with cell culture media. The final concentration of DMSO in the incubation media was not greater than 0.1%.

Measurement of intracellular ROS accumulation

The fluorescence probe DCFDA was used to monitor intracellular accumulation of ROS. DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS to 2′,7′-dichlorofluorescein (DCF). DCF is a fluorescent compound that can be detected by fluorescence spectroscopy. Cells were seeded in 6-well culture plates and allowed to adhere overnight. After treatment with FLX, AGS cells were loaded with 15 μM DCFDA for 30 min at 37 °C. Stained cells were collected and resuspended in PBS. Then, cells were transferred to 96-well plates for fluorescence detection. Fluorescence intensity was detected using a fluorescence microplate reader at maximum excitation and emission wavelengths of 485 nm and 535 nm, respectively.

DAPI staining

To assess apoptosis, nuclei of AGS cells were stained with DAPI and visualized using fluorescence microscopy. Cells were seeded in 24-well plates and incubated with FLX for 24 h. Following treatment, the cells were fixed with 4% paraformaldehyde for 30 min, then washed with PBS and nuclei were stained with DAPI. Fluorescence signals were detected using a fluorescence microscope at × 200 magnification (Leica, Tokyo, Japan).

Statistical analysis

All data are expressed as means ± S.E.M. of 4–6 independent experiments. Statistical differences among the groups were analyzed by either one-way or two-way ANOVA. Differences were considered significant at $P < 0.05$.

Results

Fluoxetine decreased AGS cell viability

AGS cells were used as a model to study the effects of FLX on apoptosis signaling pathways. To determine the effect of FLX on AGS cells, cells were treated with various concentration of FLX for 24 h. As shown in Fig. 1a, dose-dependent changes in AGS cell morphology and cell population were observed in FLX-treated cells. Colony formation assay demonstrated that treatment with FLX decreased AGS cell proliferation (Fig. 1b). Furthermore, FLX treatment induced cell shrinkage and decreases cell number. AGS cell viability after 24 h FLX treatment was assessed using MTT assay. FLX significantly reduced AGS cell viability to approximately 90%, 80%, 30%, and 15% at the concentration of 10, 20, 30, and 50 μM in a dose-dependent manner (Fig. 1c).

Fluoxetine induced apoptosis in AGS cells

Decreased cell viability in response to FLX may have been due to induction of apoptosis. To characterize cell death induced by FLX, nuclear morphology of dying cells was examined using the fluorescent DNA-binding dye, DAPI. Treatment with various concentrations of FLX for 24 h resulted in induction of apoptosis as evidenced by changes in fluorescence intensity and condensed and fragmented nuclei (indicated by white arrows in Fig. 2a). The number of apoptotic cells increased in a FLX

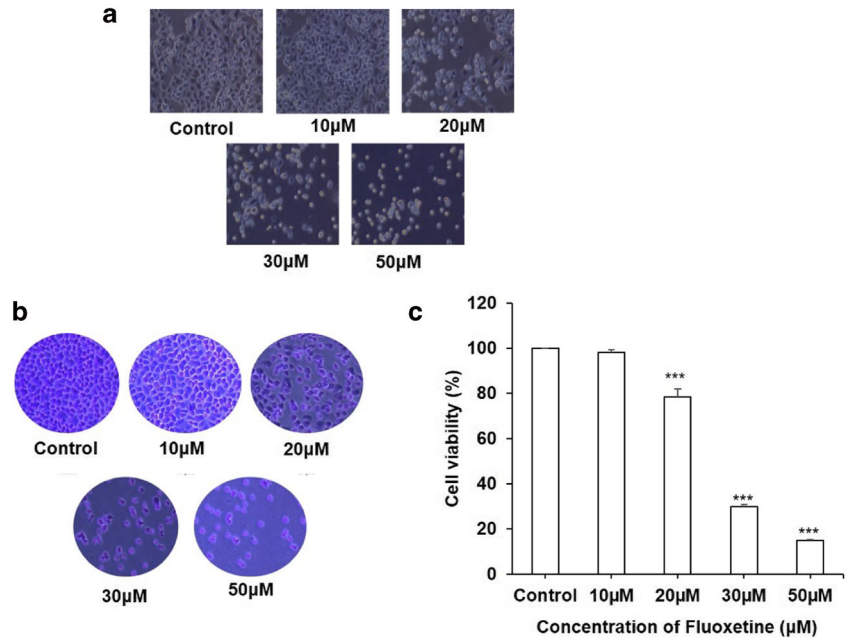

Fig. 1 Fluoxetine decreases the viability of AGS cells. **a** Cells were treated with the indicated concentrations of fluoxetine for 24 h. Cell morphology was assessed by optical microscopy. **b** Colonies of AGS cells were stained with crystal violet after fluoxetine treatment. **c** Cell viability was determined by MTT assay. One-way ANOVA was used to calculate statistical significance. Data were collected from six independent experiments (n = 6). Data are expressed as the mean ± S.E.M. (***P < 0.001 when compared to the control group)

concentration-dependent manner, as indicated by the number of dense fluorescent particles. The increases in apoptosis rates in the 10, 20, 30, and 50 μM groups were significant compared with the untreated control group (P < 0.01; Fig. 2a). To confirm FLX-induced apoptosis, we evaluated the expression of caspase 3 and PARP in FLX-treated cells by Western blot analysis, as these proteins are indicators of apoptosis. As shown in Fig. 2b, the band

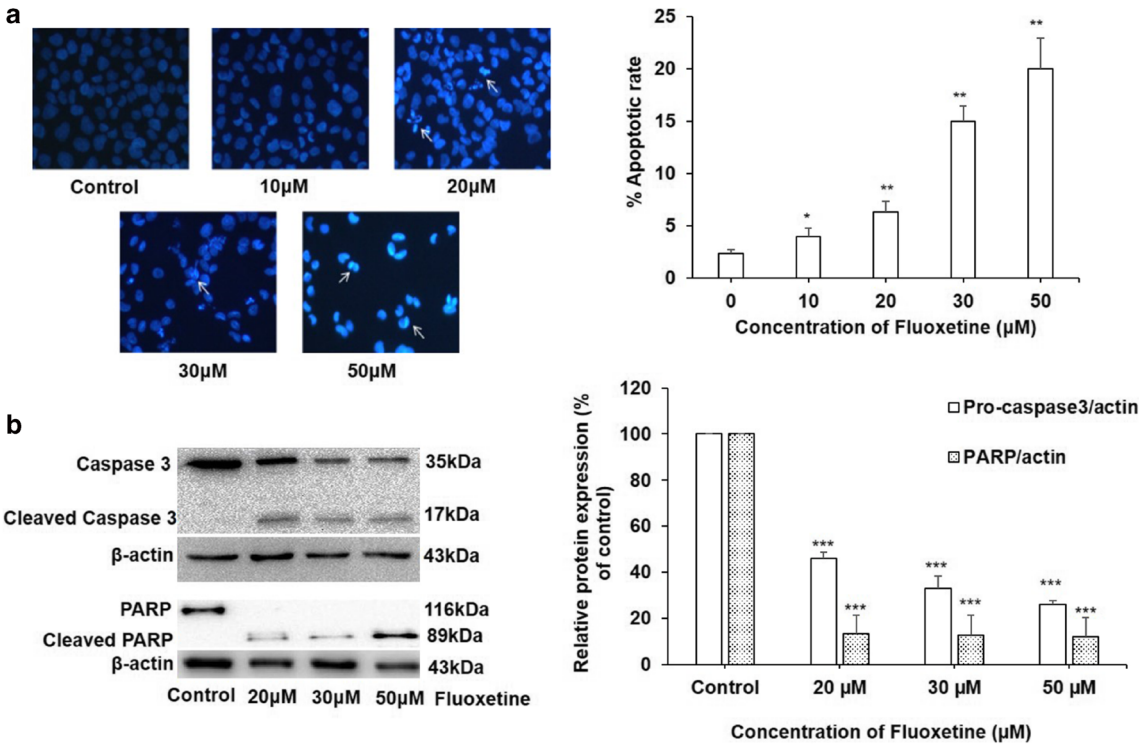

Fig. 2 Fluoxetine induces apoptosis in AGS cells. **a** Representative photomicrographs showing AGS cells. The cells, after treatment with fluoxetine for 24 h, were subjected to DAPI staining for morphological and quantitative analysis of apoptotic changes. **b** Effect of fluoxetine on apoptotic protein activation. AGS cells were treated with the indicated concentrations of fluoxetine for 24 h. Cell lysates were prepared and subjected to Western blotting for caspase 3, cleaved caspase 3, PARP, and cleaved PARP. ß-actin was used as a loading control. The blots were quantified by densitometry. One-way ANOVA was used to calculate statistical significance. Data are presented as means ± S.E.M and are representative of five independent experiments (n = 5). (**P < 0.01, ***P < 0.001 when compared to the control group)

densities of cleaved caspase 3 and cleaved PARP were remarkably increased in FLX-treated AGS cells.

Fluoxetine decreased the expression of XIAP and pro-caspase 9 in AGS cells

Apoptosis involves activation of pro-apoptotic proteins (such as Bax and Bak) and inhibition of antiapoptotic proteins (such as Bcl-2 and Bcl-xl). Changes in interactions of these proteins results in permeabilization of the outer mitochondrial membrane. Therefore, Western blotting was used to detect changes in the ratio of Bax (proapoptotic) and Bcl-2 (antiapoptotic) proteins in FLX-treated cells. As shown in Fig. 3a, FLX significantly increased the ratio of Bax/Bcl-2 in a dose-dependent manner.

To determine whether FLX can induce intrinsic apoptosis, the expression of the initiator caspase of the intrinsic apoptotic pathway, caspase 9, was detected. Treatment with FLX increased the expression of cleavage caspase 9 and inhibited the inhibitor of apoptosis protein (XIAP), which inhibits the activity of caspase 9 (Fig. 3b). These results demonstrated that FLX could induce the intrinsic apoptotic pathway.

Fluoxetine increased the expression of death receptors in AGS cells

BID is a "BH3 domain only" pro-apoptotic protein. It binds either Bcl-2 or Bax and promotes cell death. As FLX increased the expression of BAX, it was assumed that this must have been due to activation of caspase 8 and BID. Therefore, the effect of FLX on caspase 8 and BID expression was evaluated. FLX decreased the band densities of full-length procaspase 8 and BID. This suggested that activation of BAX was due to activation of BID (Fig. 4).

Activation of caspase 8 is triggered by death receptors. As caspase 8 expression was increased by FLX in AGS cells, the expression of death receptors was further investigated to elucidate the signaling pathways involved in FLX-induced cell death. Cells were treated with the indicated concentrations of FLX for 24 h. Then, the protein expression levels of death receptors DR4 (TRAIL-R1), DR5 (TRAIL-R2), Fas-associated death domain (FADD), and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) were determined. Treatment with FLX increased the expression of DR5, FADD, and TRAIL (Fig. 5). The expression of DR4 was increased by FLX treatment, but not significantly. These results suggested that FLX induced apoptosis by upregulating the expression of death receptors in AGS cells.

Fluoxetine induced formation of reactive oxygen species

To study the effect of FLX on reactive oxygen species (ROS) generation, the time course of ROS production in

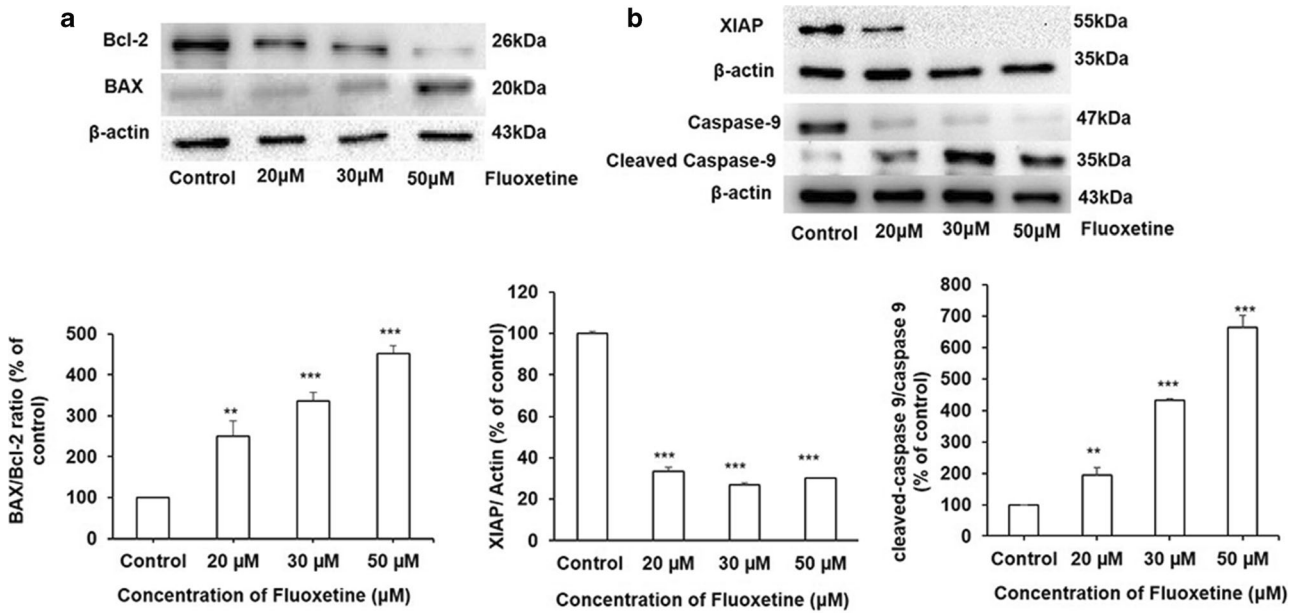

Fig. 3 Fluoxetine decreased the expression of XIAP and pro-caspase 9 in AGS cells. **a** Serum-starved AGS cells were incubated with various concentrations of fluoxetine for 24 h. Western blotting was performed against Bcl-2 and BAX. The ratio of BAX to Bcl-2 was displayed as a percent ratio. **b** Under same condition as above, XIAP, caspase 9, and cleaved caspase 9 were detected by Western blot. ß-actin was used as a loading control. The blots were quantified by densitometry. One-way ANOVA was used to calculate statistical significance. Data are presented as means ± S.E.M and are representative of four independent experiments ($n = 4$). (**$P < 0.01$ and ***$P < 0.001$ when compared to the control group)

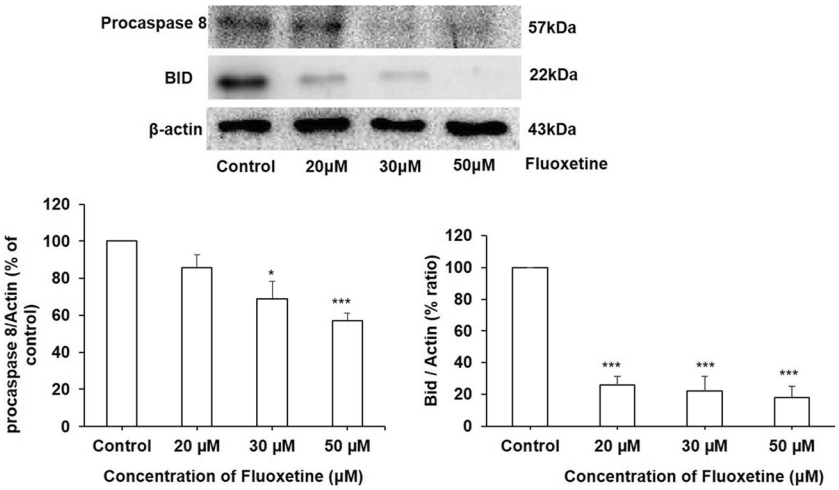

Fig. 4 Fluoxetine inhibits the expression of pro-caspase 8 and BID in AGS cells. Serum-starved AGS cells were incubated with various concentrations of fluoxetine for 24 h. Cell lysates were prepared and subjected to Western blotting for procaspase 8 and BID. ß-actin was used as a loading control. One-way ANOVA was used to compare statistical significance. Data are presented as means ± S.E.M and are representative of four independent experiments ($n = 4$). ($^{*}P < 0.05$ and $^{***}P < 0.001$ when compared to the control group)

response to FLX treatment was evaluated. After incubation of cells with the indicated concentration of FLX for various times, intracellular ROS levels were measured using the cell-permeable molecule, DCFDA. We found that ROS levels increased 30 min after treatment in a dose-dependent manner (Fig. 6a). To confirm FLX-induced ROS generation, AGS cells were pretreated with the antioxidant, NAC (10 mM), 1 h before FLX treatment. Pre-incubation with NAC blocked increased FLX-induced DCF fluorescence (Fig. 6b). These results demonstrated that FLX increased intracellular ROS generation.

To further examine the involvement of reactive oxygen species in the apoptotic effects of FLX, AGS cells were exposed to FLX in the absence or presence of the antioxidant NAC. As shown in Fig. 6c, pretreatment with NAC partially reversed FLX-induced decreases in cell number. This result suggested that the cell death induced by FLX in AGS cells was partially due to ROS signaling.

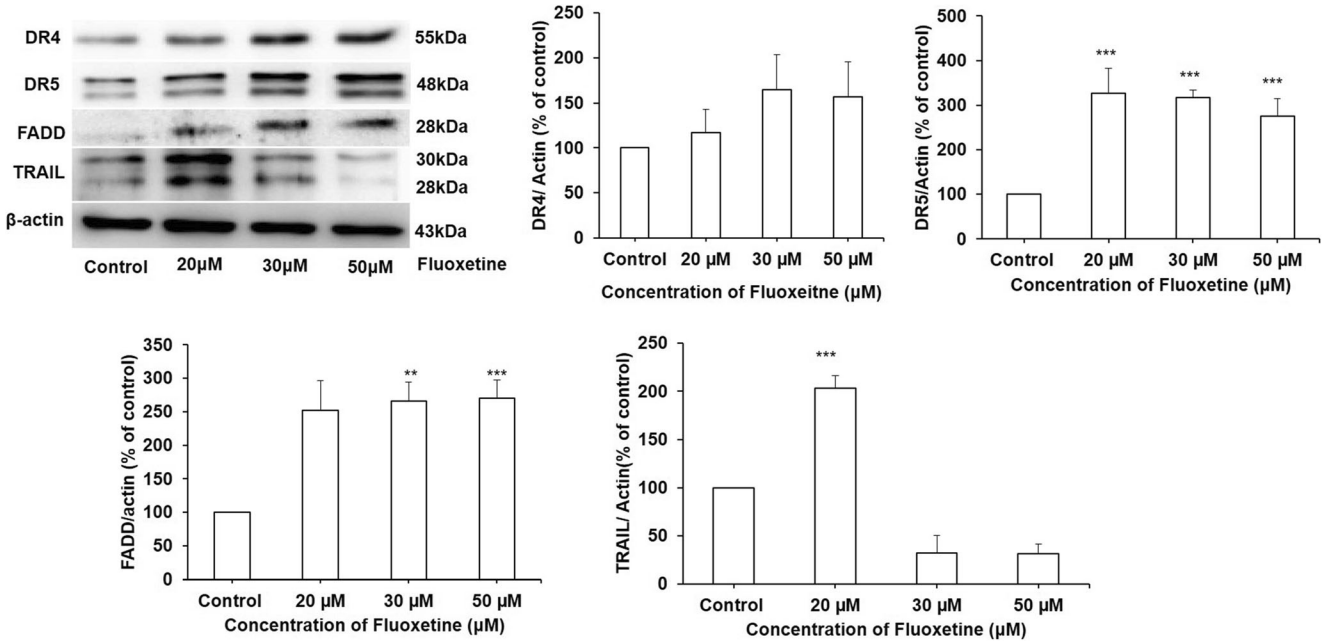

Fig. 5 Fluoxetine increases the expression of death receptors in AGS cells. Western blot analysis of DR4, DR5, FADD, and TRAIL in AGS cells treated with fluoxetine for 24 h. ß-actin was used as a loading control. The blots were quantified by densitometry. One-way ANOVA was used to calculate statistical significance. Data are presented as means ± S.E.M and are representative of four independent experiments ($n = 4$). ($^{**}P < 0.01$ and $^{***}P < 0.001$ when compared to the control group)

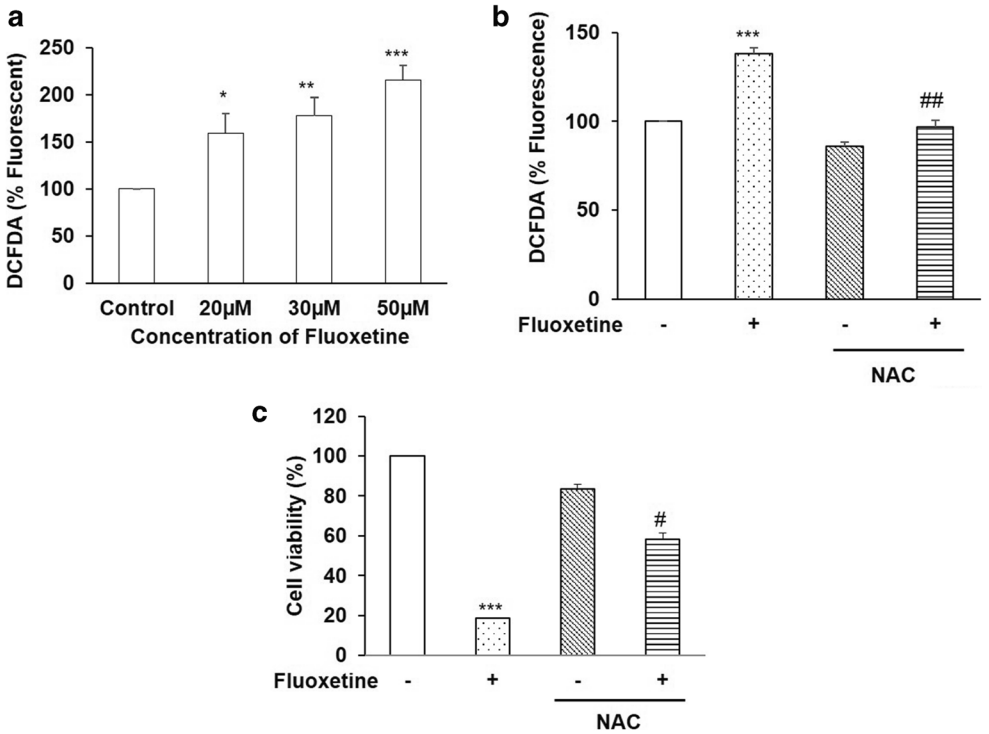

Fig. 6 Fluoxetine induces formation of reactive oxygen species (ROS). **a** AGS cells were treated with the indicated concentrations of fluoxetine for 30 min and ROS generation was determined by measuring changes in 2′,7′-dichlorofluorescin (DCF) fluorescence. **b** The cells were pre-treated with or without 10 mM NAC for 1 h, then treated with 50 μM FLX for 30 min. Fluoxetine induced increased ROS production and NAC attenuated increased ROS levels. **c** The cells were treated with 50 μM of fluoxetine with or without NAC and cell viability was determined by MTT assay. The data are expressed as the mean ± S.E.M and representative of four independent experiments ($n = 4$). One-way and two-way ANOVA were used to calculate the statistical significance. (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ when compared with control group and #$P < 0.05$, ##$P < 0.01$ when compared with fluoxetine-treated group)

Fluoxetine induced endoplasmic reticulum stress in AGS cells

To see whether FLX can induce endoplasmic reticulum (ER) stress in AGS cells, cells were treated with the indicated concentrations of FLX and Western blot analysis was performed to determine the expression of ER stress-associated proteins such as BiP (GRP 78) and CHOP. FLX decreased the expression of BiP and increased the expression of CHOP, respectively (Fig. 7).

Fluoxetine decreased the expression of p53 in AGS cells

The effect of FLX on p53 expression was examined. p53 expression was decreased by FLX-treated cells (Fig. 8a). To determine whether reduction of p53 expression in response to FLX was due to ER stress, a chemical inhibitor of ER stress, salubrinal, a selective inhibitor of p-eIF2α, was used. AGS cells were pre-treated with 10 μM salubrinal for 1 h before treatment with 20 μM FLX for 24 h. The expression level of p53 did not change significantly following treatment with salubrinal (Fig. 8b). Therefore, we determined that reduction of p53 expression by FLX was not due to ER stress.

Fluoxetine increase the phosphorylation of JNK and p38 MAPK in AGS cells

The effect of FLX on MAPK expression was examined to determine the role of MAPK in FLX-induced apoptosis in gastric cancer cells. Levels of phosphorylated and total MAPK were determined in AGS cells treated with FLX. As shown in Fig. 9a, FLX induced significant increases in p-JNK and p-38 MAPK expression.

As p-38 phosphorylation is also activated by the ER stress signaling protein IRE1α, IRE1α inhibitor, STF-083010 was used to see if whether phosphorylation of p-38 MAPK by FLX was related to IRE1 α. AGS cells were pretreated with 2 μM STF-083010 1 h prior to FLX treatment (20 μM). Pretreatment with STF-083010 inhibited FLX-induced p-38 phosphorylation (Fig. 9b).

Fluoxetine-induced DR5 expression was partially due to ER stress in AGS cells

To determine whether FLX-induced increase in DR5 expression was related to increased CHOP expression, an inhibitor of ER stress, salubrinal, was used. Cells were pre-treated with 10 μM salubrinal for 1 h prior to treatment with FLX (20 μM)

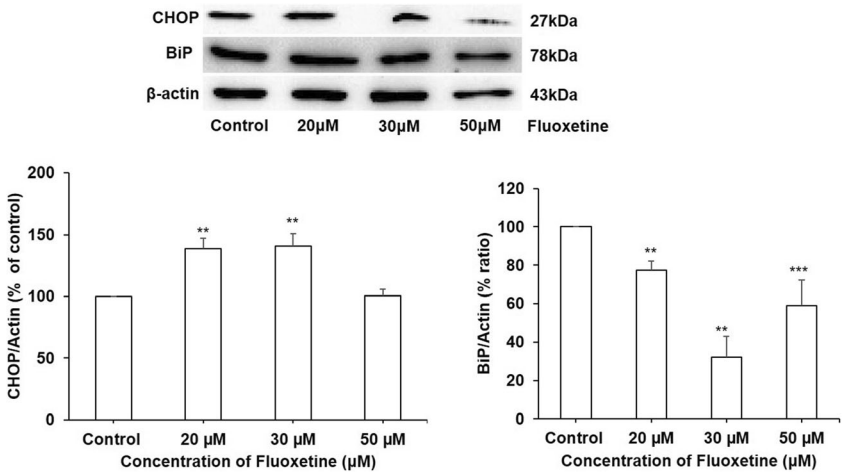

Fig. 7 Fluoxetine induces endoplasmic reticulum (ER) stress in AGS cells. Serum-starved AGS cells were incubated with various concentrations of fluoxetine for 24 h. Whole-cell extracts were prepared and subjected to Western blot analysis using anti-BiP, CHOP, and actin antibodies. The blots were quantified by densitometry. One-way ANOVA was used for statistical analysis. Data presented as means ± S.E.M are representative of four independent experiments ($n = 4$). (**$P < 0.01$ and ***$P < 0.001$ when compared to the control group)

for 24 h. Pre-treatment with salubrinal inhibited the FLX-induced CHOP expression (Fig. 10a). Furthermore, salubrinal reduced FLX-induced DR5 expression (Fig. 10b).

To determine the relationship between FLX-induced apoptotic cell death and ER stress, salubrinal was used to inhibit ER stress and the expression of the apoptotic marker proteins cleaved caspase 3 and PARP were measured. Salubrinal reduced FLX-induced expression of cleaved PARP (Fig. 10c).

Summary of the proposed mechanism by which fluoxetine exerts apoptosis in AGS cells

According to the data generated in this study, we concluded that FLX induced apoptosis in AGS cells. FLX increased the expression of DR5, a TRAIL binding receptor. Binding of TRAIL to DR5 activated caspase 8 and led to activation of caspases. Increases in the ratio of BAX to Bcl-2 led to the

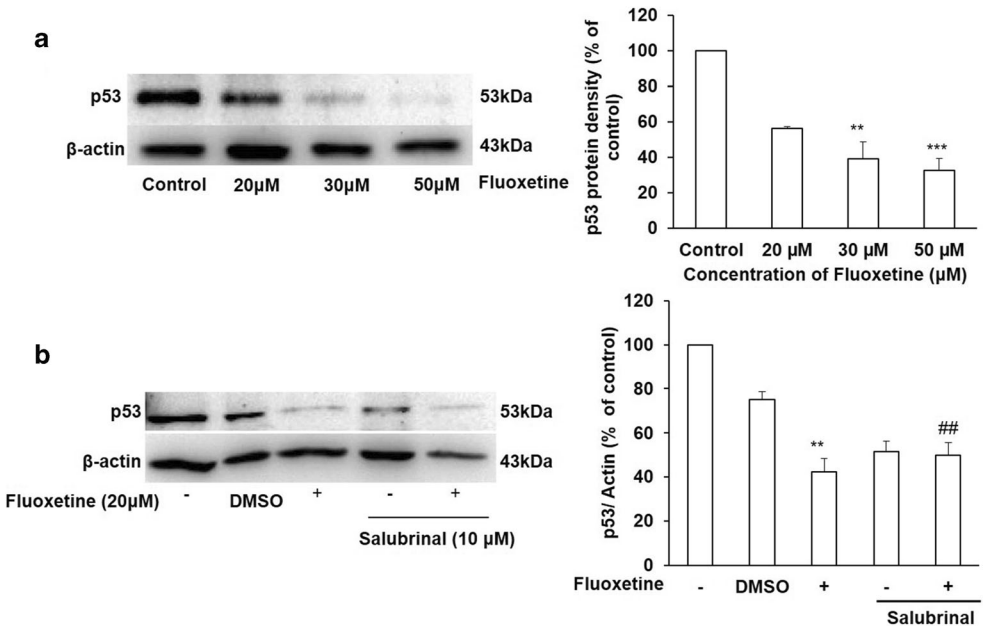

Fig. 8 Fluoxetine decreases the expression of p53 in AGS cells. **a** Serum-starved AGS cells were incubated with various concentrations of fluoxetine for 24 h. The whole cell lysate was prepared and subjected for Western blot analysis against p53 and ß-actin antibodies. **b** Western blot analysis of p53 with or without the co-treatment of salubrinal (10 μM). The cells were pre-treated with salubrinal (10 μM) for 1 h followed by 20 μM of fluoxetine for 24 h. Then, p53 was analyzed by Western blotting. ß-actin was used as a loading control. The blots were quantified by densitometry. One-way and two-way ANOVA were used for statistical analysis. Data presented as means ± S.E.M are representative of four independent experiments ($n = 4$). (**$P < 0.01$ and ***$P < 0.001$ when compared with control group and ##$P < 0.01$ when compared with fluoxetine-treated group)

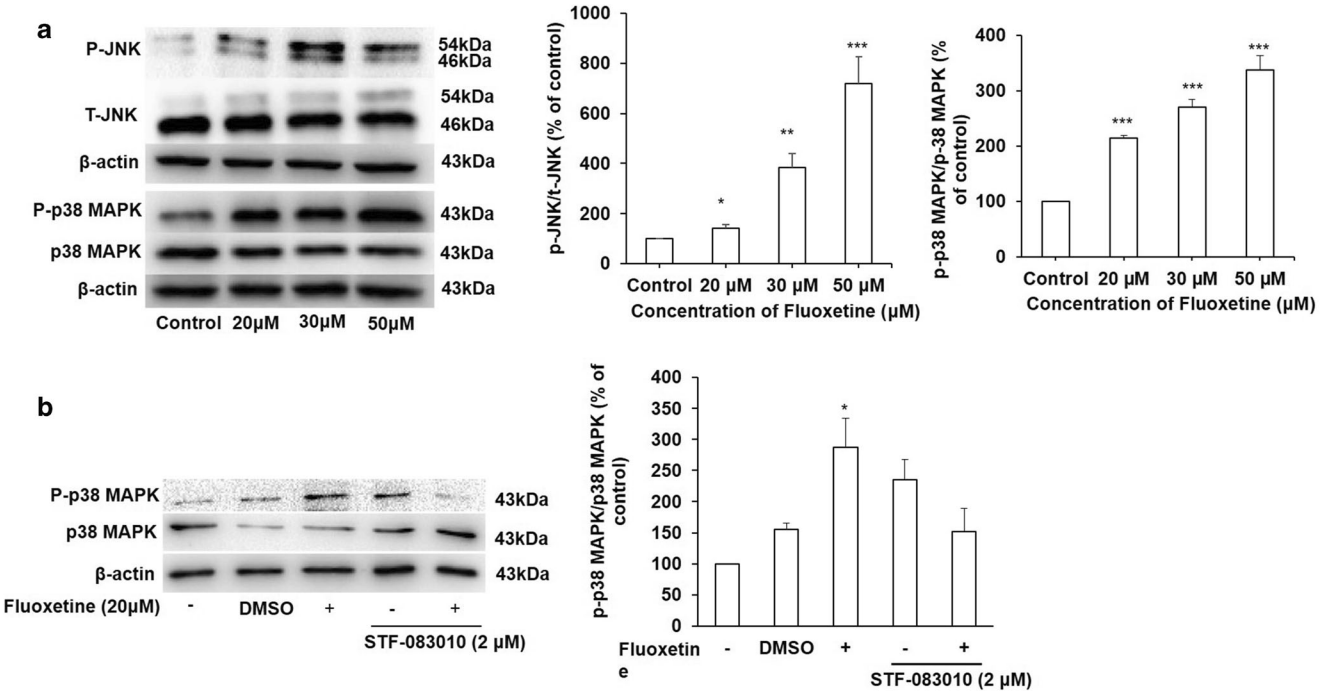

Fig 9: Fluoxetine increases the phosphorylation of JNK and p38 MAPK through IRE1α in AGS cells. **a** Serum-starved AGS cells were incubated with various concentrations of fluoxetine for 24 h. Whole cell lysates were subjected to Western blotting for p-JNK, JNK, p-p38 MAPK, and p38 MAPK. **b** Serum-starved AGS cells were incubated with the IRE1α inhibitor STF-083010 (2 μM) 1 h prior to 20 μM fluoxetine treatment for 24 h, followed by cell lysate preparation and western blotting for phospho-p38 and p-38. ß-actin was used as a loading control. The blots were quantified by densitometry. The data are presented as means ± S.E.M. experiments and representative of four independent experiments ($n = 4$). One-way and two-way ANOVA were used to compare statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ when compared to the control group, $\#P < 0.05$ when compared with fluoxetine-treated group)

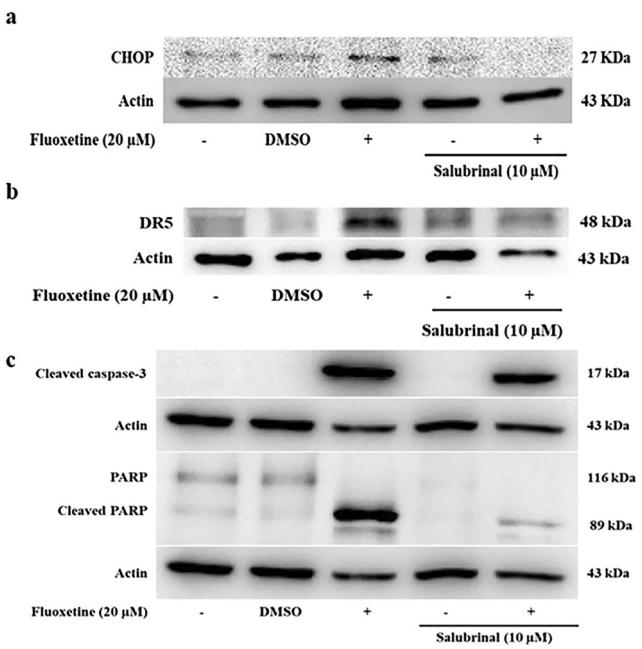

Fig. 10 Fluoxetine-induced DR5 expression is partly due to ER stress in AGS cells. Western blot analysis in serum-starved AGS cells for **a** CHOP, **b** DR5, **c** cleaved caspase 3, and PARP after treatment with fluoxetine for 24 h. ß-actin was used as a loading control. The blot figures are the representative of four independent Western blot experiments ($n = 4$)

release of apoptotic mediator proteins from the mitochondria. Changes in the equilibrium of the outer mitochondrial membrane led to generation of ROS and induction of apoptosis. FLX also induced ER stress, and inhibition of ER stress reduced DR5 expression. Thus, FLX-induced DR5 expression may have been due to ER stress. In summary, FLX-induced apoptosis occurred through increased expression of TRAIL, DR5, and ROS (Fig. 11).

Discussion

FLX acts as a specific inhibitor of serotonin uptake in neural tissues. It is beneficial for treatment of patients with depression, anxiety, and insomnia (Santarsieri and Schwartz 2015). FLX is a lipophilic weak base and quickly diffuses through multiple body tissues (Lefebvre et al. 1999). Despite its antidepressant effects, a number of studies have focused on the ability of FLX to slow tumor metabolism and cancer cell proliferation. FLX may promote tumor growth by increasing extracellular serotonin level in cultures. However, several reports have shown that FLX inhibited cancer cell proliferation in a variety of cancer cells, including prostate carcinoma cells, colon carcinoma, breast cancer cells, and ovarian cancer cells.

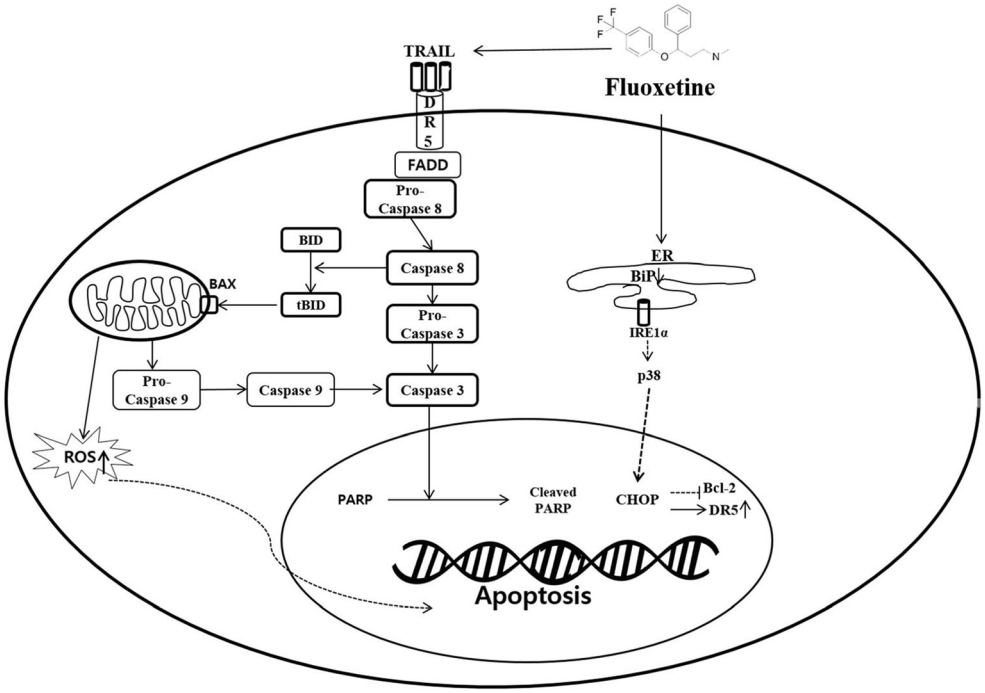

Fig. 11 Summary of proposed mechanisms by which fluoxetine exerts apoptosis in AGS cells. Fluoxetine upregulated the expression of death receptors, leading to activation of caspases, and increased ROS generation. Fluoxetine regulated ER stress-related protein activation and induced DR5 expression. The solid arrow indicates pathways investigated in the present study. The dashed arrow indicates pathway for which there is suggestive evidence

It has also been demonstrated that the antiproliferative effect of FLX is not related to the action of serotonin reuptake inhibition (Stepulak et al. 2008). FLX has a broad safety range and less adverse effects than other antidepressants (Barbey and Roose 1998b).

In cell culture models, FLX inhibited cell growth and proliferation and induced autophagic or apoptotic cell death by promoting cytotoxic ER stress, increasing MAPKs expression, and increasing intracellular ROS levels (Zhai et al. 2009; Lee et al. 2010; Mun et al. 2013; Choi et al. 2017). Furthermore, FLX reduced Bcl-2 expression and dissipated the mitochondrial membrane potential, which induced DNA fragmentation and apoptosis in lymphoma cells (Serafeim et al. 2003).

In this study, we evaluated the effects of FLX on human gastric cancer cells, as FLX has been shown to induce gastroprotective effects and SSRIs have been shown to decrease the risk of gastric cancer (Hsieh et al. 2015; Salem Sokar et al. 2016). We showed that FLX significantly inhibited AGS cell proliferation in a dose-dependent manner. DAPI staining demonstrated nuclear changes such as chromatin condensation. These results suggested that FLX-induced cell death was due to apoptosis. To confirm this finding, changes in apoptotic proteins expression were investigated by Western blot. Treatment with FLX increased the expression of cleaved caspase 3 and cleaved PARP. PARP is cleaved by caspase 3 during apoptosis and cleavage of PARP prevents repair of DNA strand breaks during apoptosis (Boulares et al. 1999). These results confirmed that FLX induced apoptosis in AGS cells.

FLX readily penetrates cells by crossing the plasma membrane, allowing it to influence mitochondrial function (Mukherjee et al. 1998). FLX interacts with mitochondria and triggers apoptosis by altering mitochondrial functions (de Oliveira 2016). The mitochondrial apoptotic pathway is largely mediated by the Bcl-2 family of proteins, which include both pro-apoptotic proteins such as Bax and Bak, and antiapoptotic proteins such as Bcl-2 and Bcl-xl. Changes in equilibrium between these pro- and antiapoptotic proteins can increase mitochondrial permeability, resulting in release of apoptotic mediators (Bagci et al. 2006; Salakou et al. 2007). We showed that FLX increased the ratio of Bax to Bcl-2 in AGS cells. The ratio of Bax to Bcl-2 acts as a sensor which determines cellular susceptibility to apoptosis (Khodapasand et al. 2015).

Increased mitochondrial membrane permeability can promote release of apoptotic mediators such as cytochrome c and procaspase 9. Cytochrome c binds to cytosolic Apaf-1 (apoptotic protease-activating factor 1) and forms the apoptosome complex, which recruits procaspase 9. Procaspase 9 then undergoes autocatalytic cleavage (Zou et al. 2003; Li et al. 2017b).

We measured the expression of caspase 9, which is the initiator caspase of the intrinsic apoptosis pathway (Li et al. 2017a). Cleaved caspase 9 expression was increased in response to FLX treatment. Moreover, FLX inhibited the expression of X-linked inhibitor of apoptosis (XIAP). XIAP inhibits apoptosis by binding to caspase 9 under normal conditions. However, when second mitochondrial-derived activator (Smac)/DIABLO is released from mitochondria, it binds to

XIAP, thus competing with preventing interaction with caspase 9 (Martinez-Ruiz et al. 2008). We showed that FLX upregulated the expression of cleaved caspase 9 and inhibited XIAP expression. Therefore, treatment with FLX may lead to release of other apoptotic mediators from mitochondria.

In ovarian cancer cell lines, FLX-induced apoptosis was regulated by inhibiting BID (a BH-3 domain-containing proapoptotic Bcl2 family member). BID is a caspase 8 substrate. Caspase 8 cleaves BID, leading to the release of a truncated form (tBID) which has the capacity to accumulate at the mitochondria and oligomerize BAX on the mitochondrial membrane. Caspase 8 is an initiator caspase. It is the first link between the death receptor and apoptotic proteases. When ligands bind to death receptors, these receptors undergo conformational changes and recruit procaspase 8. Molecules of procaspase 8 become activated by autolytic cleavage, which initiates a cascade of caspase activation (McIlwain et al. 2015). FLX decreased the expression of full-length procaspase 8 and BID. These results suggested that an increase in the ratio of BAX to Bcl-2 by FLX may be due to increased expression of caspase 8 and BID.

In cancer cells, death receptor upregulation has been shown to trigger receptor clustering on the cell membrane. This clustering enhances activation of caspases and subsequent apoptosis (Wagner et al. 2007). Caspase 8 activation is triggered by death receptors. A previous study showed that FLX induced ER stress in various cancer cell lines by increasing CHOP expression. CHOP is the transcription factor for DR5 (Yamaguchi and Wang 2004). DR5 is a cell surface receptor of the TNF-receptor that binds TRAIL. In our study, upregulation of DR5, FADD, and TRAIL was observed in FLX-treated AGS cells. However, the expression of DR4 only increased in response to high concentrations of FLX, but not significantly. This may have been due to the fact that the expression of DR4 is very low in AGS cells compared to the expression of DR5 (Li et al. 2016). Treatment with a chemical inhibitor of ER stress, salubrinal, decreased the expression of DR5 and cleaved PARP, suggesting that ER stress was also involved in the apoptotic effects of FLX.

It has been described that in hepatocellular carcinoma and ovarian cancer cell lines, FLX induced apoptosis through ROS formation and changing of mitochondria membrane potential (MOMP) (Lee et al. 2010; Mun et al. 2013). Therefore, to see whether FLlX induced cell-death in AGS cells is due to ROS formation or not, the effect of FLX on ROS generation was investigated. ROS generation was increased by FLX treatment in AGS cells. Treatment with the antioxidant, *N*-acetyl cysteine (NAC), reduced FLX-induced ROS generation and FLX-induced decreased cell number was reversed by NAC. This suggested that generation of ROS was involved in FLX-induced apoptosis in AGS cells.

GRP78, also known as BiP, plays a major role in ER stress. Elevated expression of BiP is associated with pro-survival and cytoprotective responses in cancer cells (Wang et al. 2014). CHOP expression and accumulation increase in the nucleus during apoptosis induced by ER stress (Lu et al. 2014). In our study, we showed that FLX decreased the expression of BiP and increased the expression of CHOP. These results agree with the results of previous studies. In glioma cells, FLX acted synergistically with temozolomide and induce ER stress-related apoptosis by increasing CHOP expression (Ma et al. 2016). Moreover, a previous study showed that lower levels of CHOP and overexpression of BiP are related to poor prognosis in gastric cancer patients (Zheng et al. 2008).

Cells are required to integrate external stress signals to determine whether to initiate pro-death or pro-survival signaling cascades on an ongoing basis. The mitogen-activated protein kinases (MAPKs) are a family of kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli, including stress (Seger and Krebs 1995). The MAPK signaling pathways modulates gene expression, mitosis, proliferation, motility, metabolism, and apoptosis (Waskiewicz and Cooper 1995; Tibbles and Woodgett 1999). P38-MAPK and JNK are stress kinases. When these stress kinases are induced by stimuli, they activate other MAPK signaling cascades. The p38-MAPK and JNK signaling pathways also contribute to multiple cellular functions such as differentiation, proliferation, cytokine secretion, and cell death (Wada and Penninger 2004). A study showed that FLX induced apoptosis by increasing phosphorylation of MAPKs (Mun et al. 2013). In this study, we showed that FLX significantly increased phosphorylation of JNK and p-38 MAPK. During ER stress, p38 phosphorylation by IRE1α also activates the transcription factor CHOP (Sano and Reed 2013). A previous study showed that FLX increased the expression of CHOP. Therefore, to determine whether p38 phosphorylation was related to IRE1α, the IRE1α inhibitor STF-083010 (Ming et al. 2015) was used to determine the effects of FLX on p38 phosphorylation. Pre-treatment with STF inhibited FLX-induced phosphorylation of p38. This suggested that p38 phosphorylation was related to IRE1α activation.

Conclusion

The present study demonstrated that FLX reduced the viability of AGS cells by triggering apoptosis. Based on the results of the present study and previous studies, it can be assumed that FLX induced apoptosis in AGS cells by upregulating death receptors and their related proteins and by increasing ROS production. Furthermore, FLX activated the ER stress-related protein, CHOP, and inhibition of this protein partially suppressed the apoptotic effects of FLX. These results suggest that the apoptotic effect of FLX in AGS cells was partly due to the ER stress. To sum up, the mechanism of FLX-induced

apoptosis in AGS cells was mediated by internal, external pathway, and ER stress. In addition, since FLX can enhance the expression of DR5 through CHOP activation and increase TRAIL activation, a combination treatment with FLX and a chemotherapeutic drug that can activate TRAIL might provide therapeutic benefits against gastric cancer.

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Author contribution PPK and UDS conceived and designed research. PPK and WWP conducted experiments. PPK and WT analyzed data. PPK wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare that they have is no conflict of interest.

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