Vortioxetine induces apoptosis and autophagy of gastric cancer AGS cells via the PI3K/AKT pathway

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Introduction
With 1,033,701 new cases and 782,685 deaths worldwide annually, gastric cancer (GC) has become the fifth commonly diagnosed cancer, as well as the second leading cause of malignant cancer death, developing a severe worldwide burden [1,2]. Currently, despite advances in diagnostic and treatment options, the rates of recurrence and distant metastasis are high and the prognosis for GC patients is poor with respect to the 5-year survival rate [3–6]. To date, chemotherapy has been suggested as the first-line treatment for patients with GC, although GC cells usually appear to be resistant to chemical drugs, with side effects of cytotoxicity [7,8]. Therefore, the identification of valuable novel drugs is urgently required to improve the curative effect of chemotherapy and the clinical outcome of GC.

As a common form of programmed cell death (PCD), apoptosis has played an important role in regulating the progression of organisms and different cancers [9,10]. Thus, the molecular mechanism of apoptosis induction has generally been explored for anticancer drugs [11]. Autophagy is known to be a lysosomal-dependent degradation process that furnishes energy for

Abbreviations
5-HT, 5-hydroxytryptamine; ANOVA, analysis of variance; CCK-8, cell counting kit-8; GC, gastric cancer; IC₅₀, 50% inhibition; LC3, light chain 3; mTOR, mammalian target of rapamycin; PCD, programmed cell death; PI3K, phosphoinositide 3-kinase.
maintaining metabolism and survival via degradation of long-lived protein and damaged organelles in cells. It comprises not only a conservative self-defense pathway for cells, but also a mechanism to distinguish PCD from apoptosis [12–14]. Usually, autophagy assumes basal levels in cells, although it will be strongly induced under various conditions, such as inflammatory stimulation and hypoxia [15]. Accumulating evidence has demonstrated that autophagy plays an important role in cancer by suppressing the initiation of cancer or promoting cancer growth [16]. Moreover, autophagy is reported to mediate nonapoptotic pathways via a tumor-suppressive or oncogenic role. For example, the disruption of autophagy may promote tumorigenesis [17] and it could also be induced by anticancer cancer treatment [18,19]. Given this, investigating the regulatory role of apoptosis and autophagy appears to be particularly important for identifying valuable anticancer agents.

Vortioxetine, known as a novel antidepressant, is approved in the USA and the European Union for the treatment of major depressive disorder [20–22]. Furthermore, vortioxetine, as a well-documented antagonist, exerts a potent inhibitory effect on 5-hydroxytryptamine (5-HT)3A, 5-HT7 receptors and serotonin transporter, whereas it displays partial agonistic properties with respect to 5-HT1A and 5-HT1B [23,24]. Indeed, the 5-HT receptor is involved in the carcinogenesis of many human tumor types, including bladder, lung, prostate, cholangiocarcinoma and colorectal cancers [25]. 5-HT receptor overexpression has been found to be linked to the pathogenesis of lung complication of signet ring cell carcinoma of the stomach [26]. Fluoxetine, a specific serotonin reuptake inhibitor, has been reported to significantly induce apoptotic GC cell death [27]. However, the functional significance of vortioxetine in tumors, including GC, has not been reported to date.

Therefore, the present study aimed to evaluate the functional implications of vortioxetine in the pathogenesis of GC cells by investigating its effect on the proliferation, invasion, migration, apoptosis and autophagy capabilities of GC cells.

Materials and methods

Cell culture and BafA1 treatment

Human gastric cancer cell line AGS was purchased from the Shanghai Academy of Sciences Cell Bank (Shanghai, China). The cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 100 U·mL⁻¹ penicillin and 0.1 mg·mL⁻¹ streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. After 48 h of culture, AGS cells were treated with vortioxetine and BafA1 (100 nM) in accordance with previous studies [28,29].

Cell counting kit-8 (CCK-8) assay

AGS cells were seeded in 96-well plates and treated with vortioxetine at a series of concentration gradients (0.1, 1, 10, 20 and 50 µM). After 48 h of incubation, the attenuation of each well at 450 nm (D₄₅₀) was measured using a microplate reader. Then, cells were incubated with vortioxetine (25.1 µM as the IC₅₀) and a normal control (NC, 0.1% DMSO in culture media) for 24, 48 and 72 h.

Colony formation assay

Cells were placed on a 60-mm culture dish (500 cells per well) with or without vortioxetine treatment and cultured at 37 °C for 1–2 weeks. Following the appearance of the visible colonies, the cultures were washed with NaCl/Pi and then fixed with 4% paraformaldehyde for 30 min. After removing the fixing solution, the colonies were stained with 0.1% crystal violet for 30 min and counted visually.

Invasion and migration assays

The invasion and migration assays were analyzed using the transwell assay. For the invasion assay, the upper chamber of 24-well polycarbonate membrane transwell chambers was initially coated with matrigel (1:6; diluted with serum-free Dulbecco’s modified Eagle’s medium). Following treatment with vortioxetine for 24 h, 1 × 10⁵ cells in 100 µL suspension were added to the upper chamber, whereas the lower chamber was filled with 500 µL of complete media. After overnight incubation, the cells that failed to pass through the membrane were removed by cotton swabs and the cells attached to the lower surface were fixed 4% paraformaldehyde and stained with 0.1% crystal violet solution for 20 min. The cells that infiltrated the filter were counted in five random fields under microscopy. For the migration assay, cells were seeded at a density of 5 × 10⁵ and the transwell does not need to be coated with glue.

Flow cytometry analysis

For apoptosis analysis, cells were cultivated in six-well plates and treated with vortioxetine, harvested using trypsin solution, and stained with annexin V-fluorescein isothiocyanate and propidium iodide in accordance with the manufacturer’s instructions. Flow cytometric analysis was performed using FLOWJO (https://www.flowjo.com).

Western blotting

Cells were seeded in six-well plates and then lysed with radioimmunoprecipitation assay buffer in an ice bath. Total
protein concentration was measured using the bicinchoninic acid assay method. The lysates were loaded into SDS-PAGE for electrophoresis and then transferred onto a poly (vinylidene difluoride) membrane. Membranes were blocked with 5% skim milk for 1 h, and incubated with primary antibodies against light chain 3 (LC3), AKT, p-AKT, mTOR, p-mTOR, cyclin D1, Bel-2, Bax, active caspase-3/-9 (all dilution 1:1000) and GAPDH (dilution 1:5000) at 4 °C overnight. After washing with Tris-buffered saline with Tween 20 three times (5 min each time), a secondary goat anti-rabbit antibody (dilution 1:5000) was added to the membrane and incubated for 1 h. Protein bands were visualized by enhanced chemiluminescence.

Statistical analysis

All data are reported as the mean ± SD. The statistical analyses were performed using spss, version 18.0 (SPSS Inc., Chicago, IL, USA) and PRISM, version 5 (GraphPad Software Inc., San Diego, CA, USA). Statistically significant values were compared with Student’s t-tests and analysis of variance (ANOVA) with Dunnett’s and Bonferroni’s post-hoc tests. $P < 0.05$ was considered statistically significant.

Results

Vortioxetine abrogates the proliferative capabilities of GC cells

First, a dose-dependent experiment was performed. As shown in Fig. 1A, vortioxetine reduced the viability of AGS cells in a concentration-dependent manner (the concentration of vortioxetine at 0.1, 1, 10, 20 and 50 µM), with 50% inhibition (IC50) of 25.1 µM, which was used for the subsequent experiments. We next explored the growth inhibitory effect of vortioxetine on AGS cells by a colony formation assay. As shown in Fig. 1B, the number of clones in the vortioxetine-treatment group was significantly lower compared to that in the control group ($P < 0.05$), indicating that vortioxetine could inhibit AGS cell growth.

Vortioxetine impairs the invasiveness and migration abilities of GC cells

Furthermore, to determine the effect of vortioxetine on the migration and invasiveness of GC cells, a transwell assay was performed. The results of the invasion assay revealed that vortioxetine markedly inhibited the cell invasive abilities of vortioxetine-treatment compared to the controls ($P < 0.05$) (Fig. 2A). Similarly, migration assays showed that the number of migrated vortioxetine-treatment cells was significantly lower compared to in controls ($P < 0.05$) (Fig. 2B). Taken together, these results suggested that vortioxetine was cytotoxic to AGS cells and inhibited the tumor progression of GC in vitro.

Vortioxetine induced apoptosis and autophagy of GC cells

To further examine the role of vortioxetine with respect to the suppression of GC cell growth, cell apoptosis was assessed by staining with annexin V-fluorescein isothiocyanate/propidium iodide. As shown in Fig. 3A, the percentage of apoptotic cells in the vortioxetine-treatment group was notably higher than that in the control group ($P < 0.05$). Consistent with this finding, the apoptosis-related proteins levels detected by western blotting suggested that vortioxetine increased the levels of Bax, active caspase-3 and caspase-9, whereas it decreased the level of Bel-2 (all, $P < 0.05$) (Fig. 3B), indicating that vortioxetine could induce apoptosis in AGS cells that have contributed to cell death.

Furthermore, to investigate whether autophagy was activated in response to vortioxetine exposure, we determined the levels of autophagy-related proteins including Beclin-1, P62 and LC3, with/without an autophagy blocker BafA1. The results revealed that the Beclin-1 expression level, as well as the ratio of LC3 II/I and the level of LC3 II, was significantly higher in AGS cells treated with vortioxetine compared to the controls (both, $P < 0.05$) (Fig. 4). By contrast, compared with control cells, P62 expression was obviously decreased in AGS cells treated with vortioxetine ($P < 0.05$) (Fig. 4). In addition, BafA1 treatment could lead to an accumulation of LC3 II and an increase in the LC3 II/I ratio, with an elevation of the P62 level, compared to the NC group ($P < 0.05$) (Fig. 4). Significantly, co-treatment of vortioxetine and BafA1 reinforced the LC3 II level and restored the levels of P62 and Beclin-1 compared to a single treatment group ($P < 0.05$) (Fig. 4). These data indicate that vortioxetine could facilitate AGS cell autophagy.

Vortioxetine suppressed PI3K/AKT signaling

The PI3K/AKT pathway has been identified as the key regulator of apoptosis and autophagy. Therefore, we speculated that vortioxetine might induce apoptosis and autophagy in AGS cells by suppressing the PI3K/AKT signaling pathway. Our results revealed that vortioxetine effectively decreased the phosphorylation levels of AKT and mTOR, as well as cyclin D1 (Fig. 5). The results thus indicate that ectopic
vortioxetine might induce apoptosis and autophagy via the activation of the PI3K/AKT pathway in GC cells.

**Discussion**

Despite the advances made in the treatment of GC, its prognosis still remains frustratingly poor. Hence, novel and effective therapeutic drugs must be identified to enhance the clinical therapy of GC. The results obtained in the present study indicated that vortioxetine could inhibit GC cell proliferation, migration and invasion. Furthermore, vortioxetine suppressed the growth of GC cells by the induction of apoptosis and autophagy through the PI3K/AKT pathway.
To date, vortioxetine has been the novel antidepressant for treatment of major depressive disorder, although its anti-tumor effects on GC have not yet been fully investigated. The main biological characteristics of a developing tumor malignancy, including GC, are the abnormal proliferation, migration and invasion of cells [30,31]. The results of the cell function assay demonstrated that vortioxetine inhibited tumor progression of GC by inhibiting cell proliferation, invasion and migration.

In the present study, we first confirmed that vortioxetine could induce the apoptosis of AGS cells by regulating apoptosis-related proteins. Bcl2 protein has been reported to regulate the programmed death of cells as an anti-apoptotic gene [32]. Furthermore, Bax could also downregulate apoptosis and the mitochondrial membrane in mitochondria as a pro-apoptotic gene [33]. Caspases, such as cleaved caspase-3 and caspase-9, comprise a family of cysteine proteases that contribute to apoptosis and ultimately cell death [34,35]. and Bax and Bcl2 are effective factors for the downstream activation of caspase protein [36]. In the present study, we found that Bax and active caspase-3/-9 were highly expressed, whereas Bcl-2 was decreased in AGS cells treated with vortioxetine.

Accumulating convincing evidence over several decades has shown that the effectiveness of anti-cancer therapies is not only caused by apoptosis, but also involves autophagy [37,38]. Therefore, detecting autophagy-associated regulators may be a potential strategy for cancer therapy. In the present study, vortioxetine induced autophagy in AGS cells by increasing LC3 and Beclin-1, as well as decreasing of P62. Microtubule-associated protein LC3 and P62 are regarded as important markers of autophagy, usually being employed to evaluate the autophagy level [39]. Beclin-1 is involved in the nucleation of the autophagic vesicles and could be regulated by autophagy during tumor progression as the tumor suppressor gene [40,41].

Accumulating evidence has demonstrated that the PI3K/AKT pathway serves as the major negative regulator of autophagy [42], and several drugs can induce...
Fig. 3. Vortioxetine induces apoptosis in AGS cells. Apoptosis was evaluated by a flow cytometry assay (A) and the protein levels of Bcl-2, Bax and active caspase-3/-9 were measured by western blotting (B). Student’s t-test was used to test differences between groups. *P < 0.05 compared to the untreated control. Data are reported as the mean ± SD (n = 3).

Fig. 4. Vortioxetine induces autophagy in AGS cells. Autophagy-related proteins of Beclin-1, P62 and LC3 were measured in AGS cells by western blotting. ANOVA with Bonferroni’s post-hoc test was used to test differences between multiple groups. *P < 0.05 compared to the NC group. #P < 0.05 compared to vortioxetine or BafA1. Data are reported as the mean ± SD (n = 3).

Fig. 5. Vortioxetine inhibits the PI3K/Akt pathway in AGS cells. Protein expression was determined by a western blot assay. Student’s t-test was used to test differences between groups. *P < 0.05 compared to the untreated control. Data are reported as the mean ± SD (n = 3).
autophagy at the early stage of disease, contributing toward resistance to apoptotic death [43]. AKT and downstream mTOR are critical factors in the antagonism of autophagy [44]. Thus, inhibition of PI3K/Akt signaling may represent a potential approach for anti-tumor therapy. The data obtained in the present study suggested that vortioxetine inactivated the PI3K/AKT pathway by decreasing the phosphorylation levels of AKT and mTOR, as well as cyclin D1. Indeed, correlations have been demonstrated between apoptosis and autophagy; for example, the promotion of autophagy could enhance the apoptosis of GC cells [45,46]. Hence, we speculated that vortioxetine induced apoptosis and autophagy via activation of the PI3K/AKT pathway in GC cells.

In conclusion, our data show that vortioxetine played a significant role in the proliferation, migration and invasion of GC, as well with respect to inducing apoptosis and autophagy via the PI3K/AKT pathway. The results of the present study demonstrate that vortioxetine might become a new therapeutic strategy for GC. However, the work described comprises a preliminary study conducted at the cellular level, and therefore further investigations regarding the functional mechanism of vortioxetine in different cell lines and animals are still warranted.

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Conflict of interests
The authors declare that they have no conflicts of interest.

Author contributions
LFZ and GBL contributed to the conceptualization and design of the study, as well as to the writing of the article. GBL, TTW, HLZ, HKW and WS were responsible for performing the experiments and analyzing the related data. All authors read and approved the final manuscript submitted for publication.

References
1 Charalampakis N, Economopoulou P, Kotsantis I, Tolia M, Schizas D, Liaakos T, Elimova E, Ajani JA and Psyrri A (2018) Medical management of gastric cancer: a 2017 update. Cancer Med 7, 123–133.
2 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68, 394–424.
3 Akhondi-Meybodi M, Ghane M, Akhondi-Meybodi S and Dashti G (2017) Five-year survival rate for gastric cancer in Yazd Province, Central Iran, from 2001 to 2008. Middle East J Digest Dis 9, 39–48.
4 Veisani Y and Delpisheh A (2016) Survival rate of gastric cancer in Iran; a systematic review and meta-analysis. Gastroenterol Hepatol Bed Bench 9, 78–86.
5 Nanthanangkul S, Suwanrungruang K, Wiangnon S and Promthet S (2016) Survival of stomach cancer cases in Khon Kaen, Thailand 2000–2012. Asian Pacific J Cancer Prevent 17, 2125–2129.
6 Li Z, Shan F and Wang Y (2018) Correlation of pathological complete response with survival after neoadjuvant chemotherapy in gastric or gastroesophageal junction cancer treated with radical surgery: a meta-analysis. PLoS One 13, e0189294.
7 Oba K, Paoletti X, Bang YJ, Burzykowski T, Fuse N, Michiels S, Morita S, Ohashi Y, Pignon JP et al. (2013) Role of chemotherapy for advanced/recurrent gastric cancer: an individual-patient-data meta-analysis. Europ J Cancer (Oxford, England: 1990) 49, 1565–1577.
8 Liu JB, Jian T, Yue C, Chen D, Chen W, Bao TT, Liu HX, Cao Y, Li WB, Yang Z et al. (2019) Chemoresistant gastric cancer associated gene expression signature: bioinformatics analysis based on gene expression Omnibus. Anticancer Res 39, 1689–1698.
9 Gamage CDB and Park SY.(2019) Deoxypodophyllotoxin exerts anti-cancer effects on colorectal cancer cells through induction of apoptosis and suppression of tumorogenesis. 20, 2612.
10 Lowe SW and Lin AW (2000) Apoptosis in cancer. Carcinogenesis 21, 485–95.
11 Wong RS (2011) Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res 30, 87.
12 Booth LA, Tavallai S, Hamed HA, Cruickshanks N and Dent P (2014) The role of cell signalling in the crosstalk between autophagy and apoptosis. Cell Signal 26, 549–555.
13 Filippi-Chiela EC, Viegas MS, Thome MP, Buffon A, Wink MR and Lenz G (2016) Modulation of autophagy by calcium signalosome in human disease. Mol Pharmacol 90, 371–384.
14 Rabinowitz JD and White E (2010) Autophagy and metabolism. Science (New York, NY). 330, 1344–8.
15 Liu J, Zhang Y, Qu J, Xu L, Hou K, Zhang J, Qu X and Liu Y (2011) beta-Elemene-induced autophagy protects human gastric cancer cells from undergoing apoptosis. BMC Cancer 11, 183.
Yang X, Yu DD, Yan F, Jing YY, Han ZP, Sun K, Liang L, Hou J and Wei LX (2015) The role of autophagy induced by tumor microenvironment in different cells and stages of cancer. *Cell Bioloc* 5, 14.

Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y et al. (2003) Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Investig* 112, 1809–20.

Wang Y and Xie W. (2020) Autophagy induction by thiostrepton improves the efficacy of immunogenic chemotherapy. *J ImmunoTherapy Cancer* 8, e00462.

Jian S, Chen L, Minxue L, Hongmin C, Ronghua T, Wang Y and Xie W. (2020) Autophagy induction by tumor microenvironment in different cells and stages of cancer. *Autophagy* 71, 158–169.

Liu JJ, Liu JY, Chen J, Wu YX, Yan P, Ji CD, Wang YX, Xiang DF, Zhang X, Zhang P et al. (2016) Scinderin promotes the invasion and metastasis of gastric cancer cells and predicts the outcome of patients. *Cancer Lett* 376, 110–117.

Nakahata AM, Mayer B, Neth P, Hansen D, Sampaio MU and Oliva ML (2013) Blocking the proliferation of human tumor cell lines by peptidase inhibitors from Bauhinia seeds. *Planta Med* 79, 227–235.

Bang-Andersen B, Ruhland T, Jorgensen M, Smith G, Sanchez C, Asin KE and Artigas F (2015) Vortioxetine, a novel antidepressant with multimodal activity: review of preclinical and clinical data. *Revista de psiquiatria y salud mental*. 11, 48–59.

Sanchez C, Asin KE and Artigas F (2015) Vortioxetine, a novel antidepressant with multimodal activity: review of preclinical and clinical data. *Pharmacol Ther* 145, 43–57.

Fiorino F, Severino B, Magli E, Ciano A, Caliendo G, Frederiksen K, Jensen KG, Zhong H, Nielsen SM, Hogg S, Mork A and et al. (2011) Discovery of 1-[2-(4-dimethylphosphonyl)phenyl]piperazine (Lu AA21004): a novel multimodal compound for the treatment of major depressive disorder. *J Med Chem* 54, 3206–3221.

Sanchez C, Asin KE and Artigas F (2015) Vortioxetine, a novel antidepressant with multimodal activity: review of preclinical and clinical data. *Pharmacol Ther* 145, 43–57.

Khin PP, Po WW, Thein W and Sohn UD (2020) Apoptotic effect of fluoxetine through the endoplasmic reticulum stress pathway in the human gastric cancer cell line AGS. *Naunyn Schmiedebergs Arch Pharmacol* 393, 537–549.

Liu J, Liu W, Lu Y, Tian H, Duan C, Lu L, Gao G, Wu X, Wang X and Yang H (2018) Piperlongumine restores the balance of autophagy and apoptosis by increasing BCL2 phosphorylation in rotenone-induced Parkinson disease models. *Autophagy* 14, 845–861.

Utaipan T, Ashipornchai A, Suksamrarn A, Jirachotikoon C, Yuan X, Lertcanawanichakul M and Chunglok W (2017) Carbazole alkaloids from Murraya koenigii trigger apoptosis and autophagic flux inhibition in human oral squamous cell carcinoma cells. *J Nat Med* 71, 158–169.
Rubinsztein DC, Codogno P and Levine B (2012) Autophagy modulation as a potential therapeutic target for diverse diseases. *Nat Rev Drug Discovery* **11**, 709–730.

Yang ZJ, Chee CE, Huang S and Sinicrope FA (2011) The role of autophagy in cancer: therapeutic implications. *Mol Cancer Ther* **10**, 1533–1541.

Seitz C, Hugle M, Cristofanon S, Tchoghandjian A and Fulda S (2013) The dual PI3K/mTOR inhibitor NVP-BEZ235 and chloroquine synergize to trigger apoptosis via mitochondrial-lysosomal cross-talk. *Int J Cancer* **132**, 2682–2693.

Fu L, Kim YA, Wang X, Wu X, Yue P, Lonial S, Khuri FR and Sun SY (2009) Perifosine inhibits mammalian target of rapamycin signaling through facilitating degradation of major components in the mTOR axis and induces autophagy. *Can Res* **69**, 8967–8976.

Feng H, Cheng X, Kuang J, Chen L, Yuen S, Shi M, Liang J, Shen B, Jin Z, Yan J and *et al.* (2018) Apatinib-induced protective autophagy and apoptosis through the AKT-mTOR pathway in anaplastic thyroid cancer. *Cell Death Dis* **9**, 1030.

Zhu Y, Cheng J, Min Z, Yin T, Zhang R, Zhang W, Hu L, Cui Z, Gao C, Xu S, Zhang C and Hu X (2018) Effects of fucoxanthin on autophagy and apoptosis in SGC-7901 cells and the mechanism. **119**, 7274–7284.