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Original Article

Bortezomib induced autophagy to protect esophageal cancer cells from apoptosis through endoplasmic reticulum stress-mediated way

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Abstract: Bortezomib (BZ), a proteasome inhibitor, is a cancer drug for treatment of multiple myeloma and several solid tumors. Studies about BZ therapy on esophageal cancer (EC) are few. Further investigation is necessary. The present study examined the effects of BZ on EC cells and assessed the underlying molecular mechanisms. BZ was observed to inhibit cell viability while promoting cell apoptosis of EC cells, suggesting the anti-tumor effects of BZ for EC therapy. However, BZ may also induce autophagy through increasing the number of $LC3^+$ puncta, Beclin1 expression, and LC3 II/I ratio dose-dependently in EC cells. Activation of autophagy by Rapamycin (Rapa) treatment upregulated cell viability while suppressing cell apoptosis, compared with the BZ treated group, while inhibition of autophagy by Chloroquine (CQ) treatment had the opposite function. Results indicated that BZ could induce protective autophagy, counteracting the anti-tumor effects of BZ in EC cells. Moreover, inhibition of ER stress enhanced the anti-tumor effects of BZ through inhibiting cell viability while promoting cell apoptosis, compared with the BZ treated group. In summary, present results demonstrate that inhibition of ER stress could enhance the anti-tumor effects of BZ via suppressing protective autophagy in EC cells. Cumulative data indicates that BZ exerted anti-tumor effects and induced protective autophagy through ER stress-mediated way in EC cells. Combination of BZ with autophagy inhibitors or ER stress inhibitors could enhance the anti-tumor effects of BZ for EC therapy.

Keywords: Bortezomib, esophageal cancer, endoplasmic reticulum stress, autophagy induction, apoptosis inhibition

Introduction

Esophageal cancer (EC) has been identified as the 8^{th} most common cancer and the 6^{th} most lethal cancer worldwide [1]. There are two main histopathological subtypes of EC, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). Since most patients already have distant metastasis at the time of diagnosis, the prognosis of EC patients remains poor, with an overall 5-year survival rate of only 15%-20% [2]. Currently, more is known about diagnosis and therapy of this disease. However, further research is necessary to take effective steps toward prevention and treatment.

Endoplasmic reticulum (ER) is an important subcellular compartment responsible for protein folding and maturation [3]. Under stress conditions, ER stress is triggered to preserve homeostasis in ER through activation of the subsequent unfolded protein response (UPR) or to induce cell death via activating pro-apoptotic signaling cascades [4].

Autophagy is a highly conserved cellular process in eukaryotes which can recycle cytosolic proteins and organelles. It plays a vital role in maintaining cell survival under both physiological and pathological conditions [5]. Autophagy can maintain cell survival. However, excessive autophagy could disrupt cellular homeostasis and contribute to cell death [6]. Autophagy also contributes to chemoresistance of cancer cells and inhibition of autophagy might augment the anti-tumor effects of chemotherapy agents [7, 8]. It has been reported that ER stress can trig-

ger autophagy. Multiple ER stress associated signaling cascades also take part in the autophagy process [9].

Bortezomib (BZ) is a cancer drug for treatment of multiple myeloma and several solid tumors [10, 11]. BZ can directly induce death of tumor cells through preventing degradation of pro-apoptotic proteins and promoting degradation of anti-apoptotic proteins. Wang reported that BZ sensitizes esophageal squamous cancer cells to radiotherapy by suppressing expression of HIF-1α and apoptosis proteins [12]. Studies about BZ therapy on EC are few. Further investigation is necessary. The present study examined the effects of BZ on EC cells, assessing the underlying molecular mechanisms.

Materials and methods

Reagents

BZ, Rapamycin (Rapa), and Chloroquine (CQ) were purchased from Selleck Chemicals (Shanghai, China). 4-phenylbutyric acid (4-PBA) was purchased from Sigma-Aldrich Inc (St Louis, MO, USA). Antibodies used in this study were as follows: anti-Beclin1 (No.4122), anti-LC3 I/II (No.12741), anti-GRP78 (No.3177), anti-PERK (No.5683), and anti-GAPDH (No.5-174). These were purchased from Cell Signaling Technology (Inc., Danvers, MA, USA).

Cell culture

Human EC cell line TE-13 was purchased from Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China). Cells were cultured in DMEM media (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

CCK-8 assay was performed for cell viability assay. Cells were seeded into 96-well cell culture plates at a density of 3×10^4 cells/well and cultured at 37°C for 24 hours. After co-culturing with different concentrations of BZ for another 24 hours, 10 μL of the CCK8 reagent was added to each well. After 2 hours at 37°C, absorbance was measured at 450 nm using a microplate reader to indicate cell viability. All experiments were performed in triplicate.

Flow cytometric analysis of apoptosis

Cell apoptosis rates were measured using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Multisciences, Shanghai, China), according to manufacturer protocol. Briefly, cells were washed with ice-cold PBS twice. Cells were then resuspended and incubated with 5 μL of Annexin V-FIFC and 10 μL of PI. Cell apoptosis was analyzed in a flow cytometer (BD Biosciences).

GFP-LC3 analysis

Cells were transfected with a GFP-LC3-expressing plasmid. After 24 hours, cells were fixed in 3.7% formaldehyde for 20 minutes, washed with PBS, mounted, and inspected using a fluorescence microscope. A minimum of 150 GFP-positive cells were counted under each condition. Graphs were plotted as a percentage of GFP-LC3-positive cells over total transfected cell population.

Western blot assay

Total proteins were extracted from cultured cells by SDS Lysis Buffer (Beyotime) with concentrations quantified by BCA assay (Beyotime). The same amount of proteins were separated by SDS-PAGE gel and transferred to PVDF membranes. These membranes were blocked, incubated with primary antibodies at 4°C overnight, and incubated with conjugated secondary antibodies for 1 hour at room temperature. Immunoblotted proteins were visualized by enhanced chemiluminescence.

Statistical analysis

Data are presented as mean ± standard deviation (SD) from independent experiments performed in triplicate. Results were evaluated by one-way ANOVA and Student's t-test using SPSS software (version 19). Values of $P < 0.05$ are considered statistically significant.

Results

BZ inhibits cell viability while promoting cell apoptosis in EC cells

First, the effects of BZ on EC cell viability and apoptosis were detected. It was observed that BZ treatment suppressed cell viability while

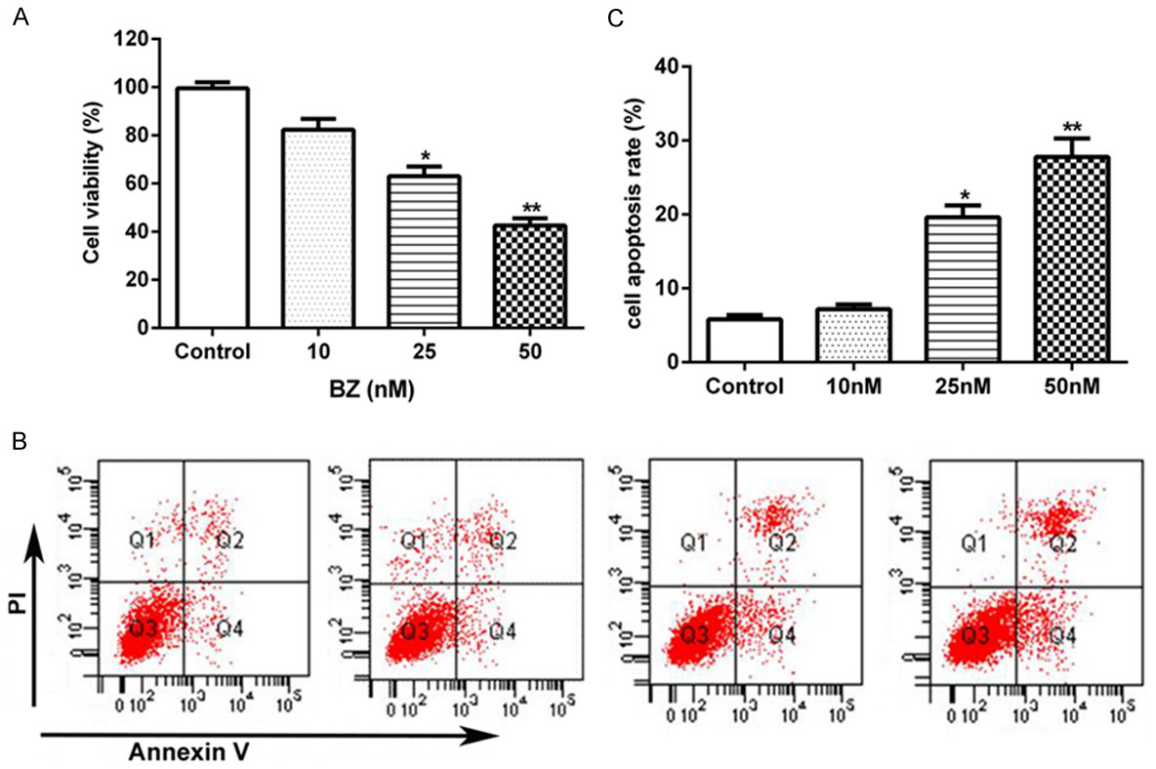

Figure 1. BZ inhibits cell viability while promoting cell apoptosis in EC cells. EC cells were treated with different concentrations of BZ. A. Cell viability was detected by CCK-8 assay. B, C. Cell apoptosis rates were measured by flow cytometric analysis. All data are represented as the mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group.

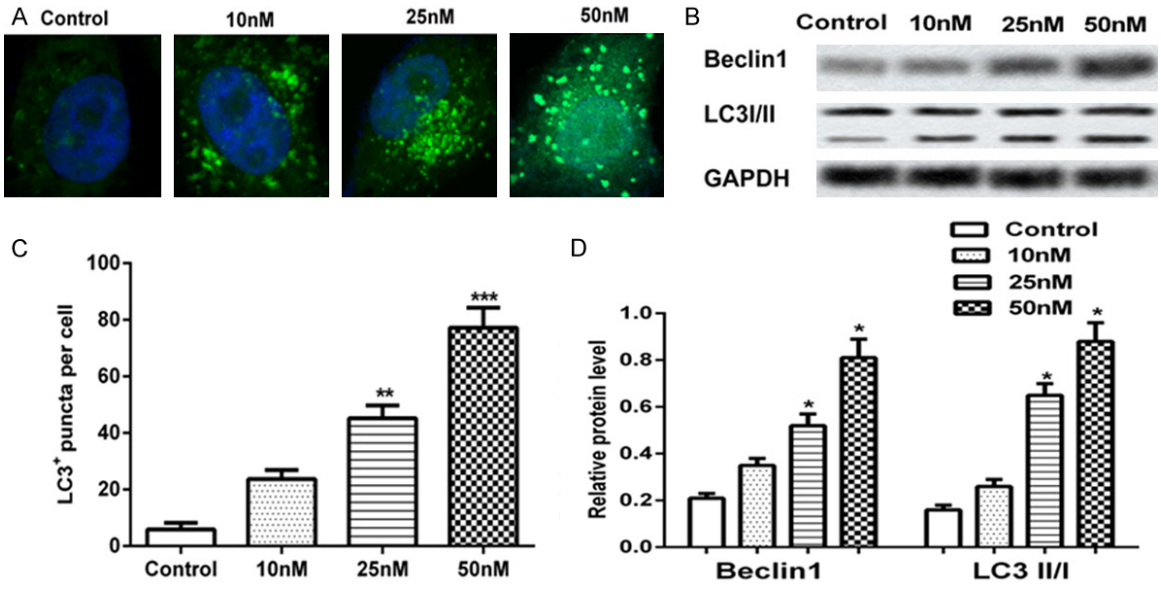

Figure 2. BZ induces autophagy in EC cells. EC cells were treated with different concentrations of BZ. A, B. $LC3^{+}$ puncta per cell was detected by GFP-LC3 analysis. C, D. Relative expression of Beclin1 and LC3 I/II was measured by Western blot. All data are represented as the mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group.

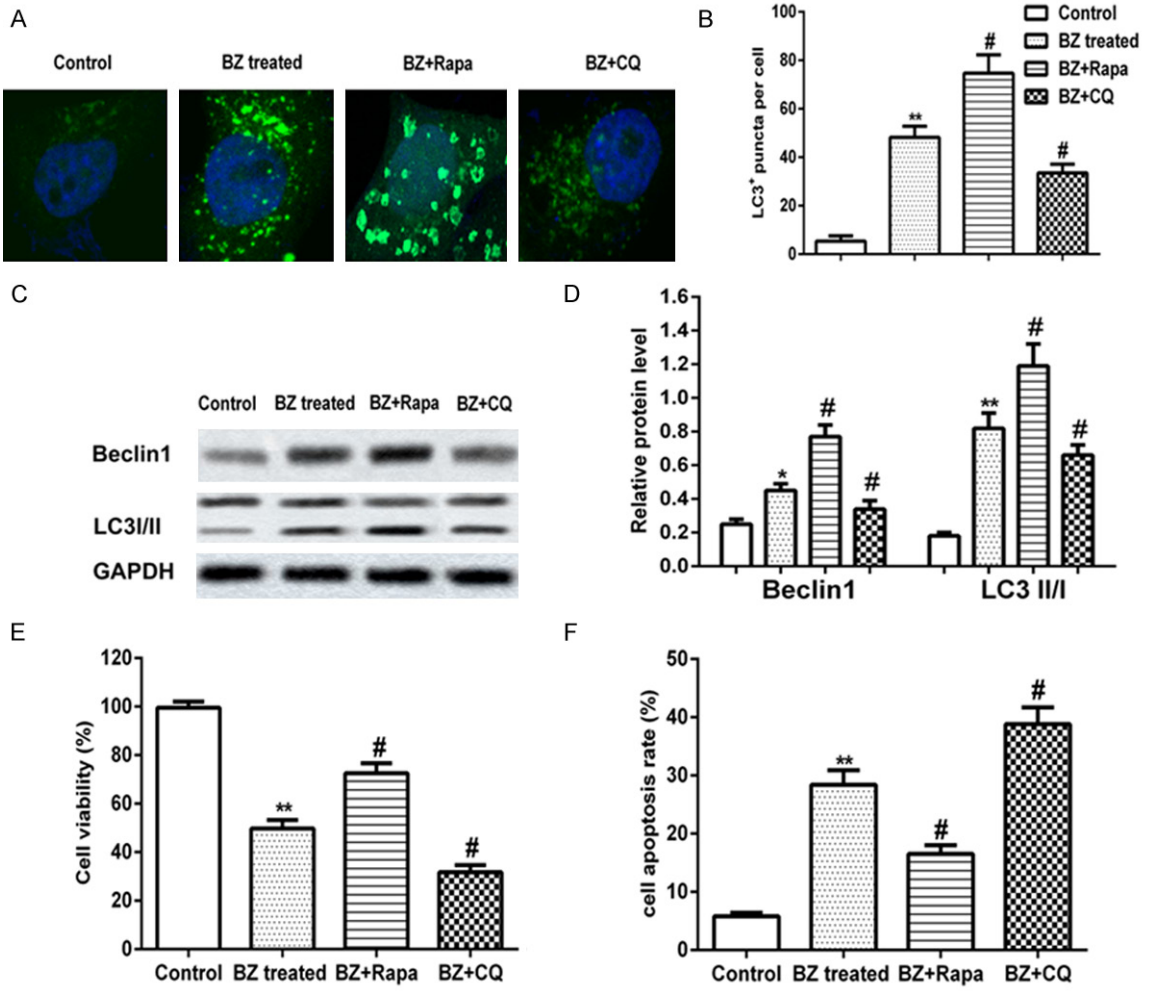

Figure 3. Activation of autophagy inhibits BZ's anti-tumor effects while inhibition of autophagy has opposite effects. EC cells were treated with BZ with or without Rapa, CQ, respectively, with non-treated cells as control. A, B. $LC3^+$ puncta per cell was detected by GFP-LC3 analysis. C, D. Relative expression of Beclin1 and LC3 I/II was measured by Western blot. E. Cell viability was detected by CCK-8 assay. F. Cell apoptosis rates were measured by flow cytometric analysis. All data are represented as the mean ± SD from three independent experiments. $^{**}P < 0.01$ compared with control group, $^{\#}P < 0.05$ compared with BZ treated group.

promoting cell apoptosis rates in a dose-dependent manner (**Figure 1A-C**, $^{*}P < 0.05$, $^{**}P < 0.01$). Results indicate that BZ acted as an anti-cancer agent in EC.

BZ induces autophagy in EC cells

Autophagy plays a regulatory role in the chemoresistance of cancer cells. Thus, this study detected autophagy levels of BZ treated EC cells. It was observed that BZ treatment increased the number of $LC3^+$ puncta dose-dependently (**Figure 2A**, **2B**, $^{**}P < 0.01$, $^{***}P < 0.001$). Moreover, results from Western blotting showed that BZ increased expression of Beclin1 and the ratio of LC3 II/I in a dose-dependent manner (**Figure 2C**, **2D**, $^{*}P < 0.05$). Data indicates that BZ could induce autophagy in EC cells.

Activation of autophagy inhibits BZ's anti-tumor effects while inhibition of autophagy has opposite effects

To explore the influence of autophagy on the anti-tumor effects of BZ, autophagy activator Rapa and autophagy inhibitor CQ were combined with BZ for treatment of EC cells, re-

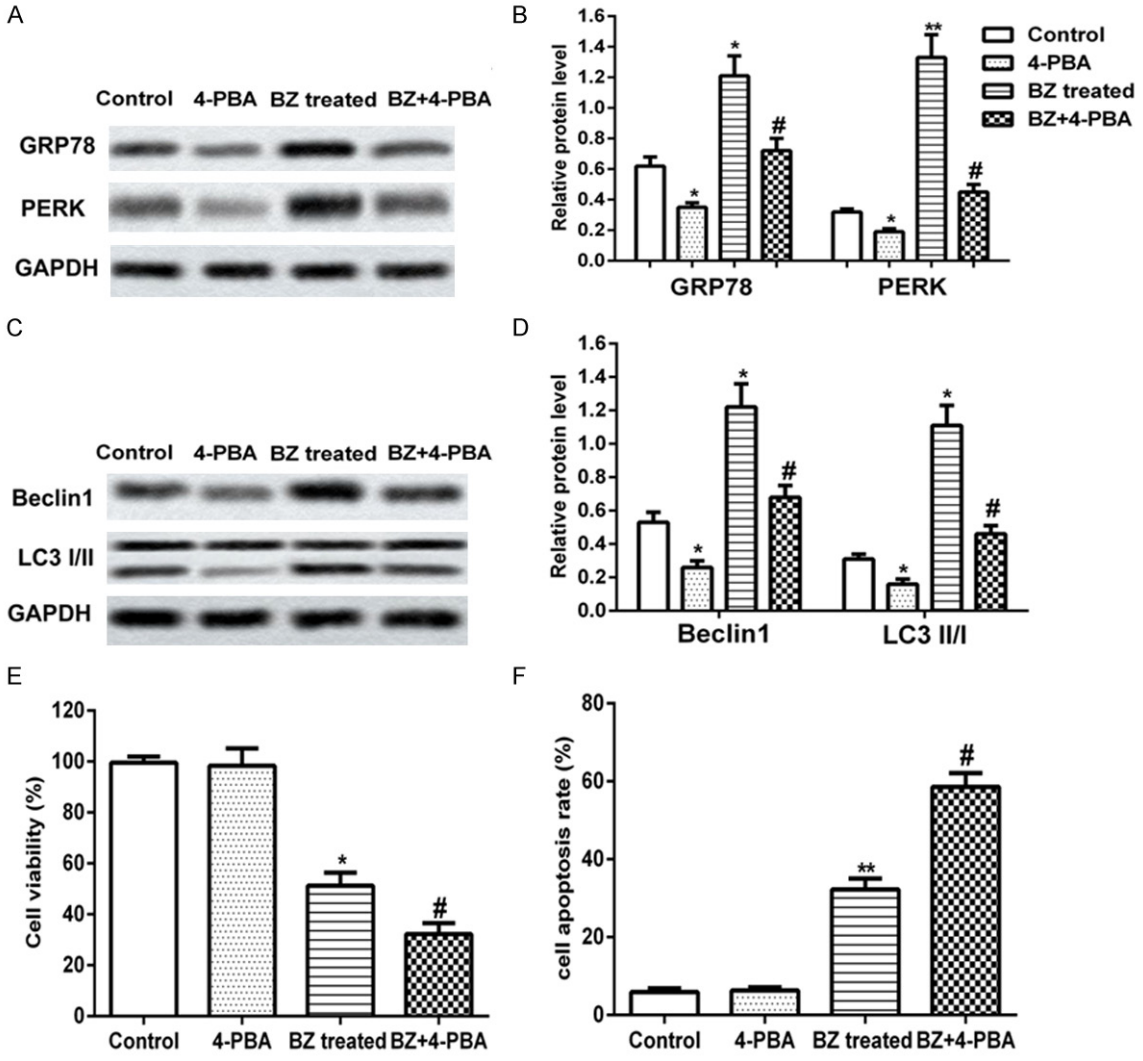

Figure 4. BZ induces autophagy through ER stress-mediated way in EC cells. EC cells were treated with 4-PBA, BZ, BZ + 4-PBA, respectively, with non-treated cells as control. A, B. Relative expression of GRP78 and PERK was measured by Western blot. C, D. Relative expression of Beclin1 and LC3 I/II was measured by Western blot. E. Cell viability was detected by CCK-8 assay. F. Cell apoptosis rates were measured by flow cytometric analysis. All data are represented as the mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group, $^{\#}P < 0.05$ compared with BZ treated group.

spectively. Rapa increased the number of $LC3^{+}$ puncta, Beclin1 expression, and LC3 II/I ratio, significantly, compared with the BZ treated group, while CQ treatment had opposite effects (**Figure 3A-D**, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$). Activation of autophagy increased cell viability and suppressed cell apoptosis, compared with the BZ treated group, while CQ treatment had opposite effects (**Figure 3E**, **3F**, $^{**}P < 0.01$, $^{\#}P < 0.05$). Present findings demonstrate that activation of autophagy counteracted the anti-tumor effects of BZ while inhibition of autophagy enhanced the anti-tumor effects of BZ.

BZ induces autophagy through ER stress-mediated way in EC cells

It has been reported that ER stress can trigger autophagy. Multiple ER stress associated signaling cascades also take part in the autophagy process [9]. The present study found that BZ treatment upregulated expression of ER stress related proteins GRP78 and PERK, compared with the control group. Treatment with ER stress inhibitor 4-PBA downregulated GRP78 and PERK expression, compared with the BZ treated group (**Figure 4A**, **4B**, $^{*}P < 0.05$, $^{**}P <$

0.01, $^{\#}P < 0.05$). In addition, inhibition of ER stress by 4-PBA suppressed Beclin1 expression, LC3 II/I ratio, and cell viability while promoting cell apoptosis rates, compared with the BZ treated group, indicating that inhibition of ER stress suppressed autophagy and enhanced anti-tumor effects of BZ in EC cells (**Figure 4C-F**, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$). Present data demonstrates that BZ induced autophagy through ER stress mediated way in EC cells.

Discussion

BZ is a proteasome inhibitor which can induce apoptosis via many cellular mechanisms associated with proteasome inhibition [13]. BZ is often used as an effective anti-tumor agent for treatment of multiple myeloma and several solid tumors. The present study found that BZ can also induce protective autophagy. This can inhibit the anti-tumor effects of BZ through ER stress mediated way. Therefore, co-treatment with BZ and autophagy inhibitor may enhance the anti-tumor effects of BZ, making it an effective reagent against EC.

BZ was the first proteasome inhibitor used in clinical practice. It is now approved for treatment of multiple myeloma [14]. Many studies have shown its efficacy as an active antitumor agent against various solid tumors, including breast cancer, ovarian cancer, and colon cancer [15-17]. BZ could directly induce death of tumor cells by preventing degradation of pro-apoptotic proteins and promoting degradation of antiapoptotic proteins [12]. Bao reported that BZ induced apoptosis and suppressed cell growth and metastasis of chondrosarcom a cells through inactivation of Stat3 signaling [18]. In agreement with previous studies, the present study observed that BZ treatment suppressed cell viability while promoting cell apoptosis in EC cells in a dose-dependent manner, suggesting that BZ acts as an anti-tumor agent for EC therapy.

Autophagy is an evolutionarily conserved process that degrades proteins and organelles to maintain homeostasis under stress [19]. Autophagy is a double-edged sword which can resist or promote cancer cell death under different stress conditions [20]. Tang's study indicated that protective autophagy could be induced by BRCA1 deficiency to mitigate stress, providing a mechanism for BRCA1 haploinsufficiency in tumorigenesis [21]. Yan also demonstrated that protective autophagy induced by resveratrol could mitigate ER stress in neuronal HT22 cells [22]. These studies have demonstrated that during stress, protective autophagy can be induced to protect cells against stress and promote cell survival. During autophagy, Beclin1 is a crucial regulator of autophagy. It interacts with Class III PI3K VPS34, VPS15 to regulate the autophagy process [23]. Conversion of LC3 I to LC3 II is important in the elongation of the isolation membrane and eventual closure of the autophagosomal membrane. It is often used as a marker to monitor the autophagic process [24]. The present study found that BZ treatment increased the number of $LC3^{+}$ puncta, Beclin1 expression, and LC3 II/I ratio dose-dependently, indicating that BZ induced autophagy when treating EC cells. To investigate the effects of BZ-induced autophagy on EC cells, autophagy activator Rapa and autophagy inhibitor CQ were used to co-treat EC cells with BZ. Present results showed that activation of autophagy upregulated cell viability while suppressing cell apoptosis, compared with the BZ treated group, while inhibition of autophagy had opposite effects. Taken together, present data elucidates that BZ could induce protective autophagy, counteracting the anti-tumor effects of BZ in EC cells.

It has been established that accumulation of misfolded and unfolded proteins in ER under pathophysiological stress will lead to ER stress. Cells react to ER stress by activating a complex intracellular signal transduction pathway named UPR, which aims to re-establish ER homeostasis in cells [25]. Accumulating evidence has supported that ER stress could activate autophagy, which determines survival of cells under ER stress [26]. Recent studies have emphasized the importance of ER stress and autophagy induced by anticancer therapies. UPR is governed by three transmembrane ER stress sensors, PKR-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) [27]. GRP78 is an initiator of early ER stress. It can activate and elevate along with aggravated ER stress [28]. To understand the relationship between ER stress and autophagy under BZ treatment, ER stress inhibitor 4-PBA was used to treat EC cells along with BZ. Inhibition of ER stress was found to suppress autophagy in EC cells. Moreover, inhi-

bition of ER stress enhanced the anti-tumor effects of BZ through inhibiting cell viability while promoting cell apoptosis, compared with the BZ treated group. In summary, present results demonstrate that inhibition of ER stress could enhance the anti-tumor effects of BZ via suppressing protective autophagy in EC cells.

Present data indicates that BZ exerted anti-tumor effects and induced protective autophagy through ER stress-mediated way in EC cells. Combination of BZ with autophagy inhibitors or ER stress inhibitors could enhance the anti-tumor effects of BZ for EC therapy.

Disclosure of conflict of interest

None.

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References

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127: 2893-917.

[2] Dikken JL, Lemmens VE, Wouters MW, Wijnhoven BP, Siersema PD, Nieuwenhuijzen GA, van Sandick JW, Cats A, Verheij M, Coebergh JW, van de Velde CJ. Increased incidence and survival for oesophageal cancer but not for gastric cardia cancer in the Netherlands. Eur J Cancer 2012; 48: 1624-32

[3] Hawes C, Kiviniemi P and Kriechbaumer V. The endoplasmic reticulum: a dynamic and well-connected organelle. J Integr Plant Biol 2015; 57: 50-62.

[4] Stefani IC, Wright D, Polizzi KM and Kontoravdi C. The role of ER stress-induced apoptosis in neurodegeneration. Curr Alzheimer Res 2012; 9: 373-87.

[5] Balduini W, Carloni S and Buonocore G. Autophagy in hypoxia-ischemia induced brain injury: evidence and speculations. Autophagy 2009; 5: 221-3.

[6] Thorburn A. Autophagy and its effects: making sense of double-edged swords. PLoS Biol 2014; 12: e1001967.

[7] Hu X, Mei S, Meng W, Xue S, Jiang L, Yang Y, Hui L, Chen Y, Guan MX. CXCR4-mediated signaling regulates autophagy and influences acute myeloid leukemia cell survival and drug resistance. Cancer Lett 2018; 425: 1-12.

[8] Zhang K, Chen J, Zhou H, Chen Y, Zhi Y, Zhang B, Chen L, Chu X, Wang R, Zhang C. PU.1/microRNA-142-3p targets ATG5/ATG16L1 to inactivate autophagy and sensitize hepatocellular carcinoma cells to sorafenib. Cell Death Dis 2018; 9: 312.

[9] Yin Y, Sun G, Li E, Kiselyov K and Sun D. ER stress and impaired autophagy flux in neuronal degeneration and brain injury. Ageing Res Rev 2017; 34: 3-14.

[10] Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J, Anderson KC. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res 2001; 61: 3071-6.

[11] Tucker D and Rule S. Novel agents in mantle cell lymphoma. Expert Rev Anticancer Ther 2017; 17: 491-506.

[12] Wang D, Qin Q, Jiang QJ and Wang DF. Bortezomib sensitizes esophageal squamous cancer cells to radiotherapy by suppressing the expression of HIF-1alpha and apoptosis proteins. J Xray Sci Technol 2016; 24: 639-46.

[13] Orlowski RZ and Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res 2008; 14: 1649-57.

[14] Frankland-Searby S and Bhaumik SR. The 26S proteasome complex: an attractive target for cancer therapy. Biochim Biophys Acta 2012; 1825: 64-76.

[15] Ludwig H, Khayat D, Giaccone G and Facon T. Proteasome inhibition and its clinical prospects in the treatment of hematologic and solid malignancies. Cancer 2005; 104: 1794-1807.

[16] Munoz-Galvan S, Gutierrez G, Perez M and Carnero A. MAP17 (PDZKIP1) expression determines sensitivity to the proteasomal inhibitor bortezomib by preventing cytoprotective autophagy and NFkappaB activation in breast cancer. Mol Cancer Ther 2015; 14: 1454-65.

[17] Kao C, Chao A, Tsai CL, Chuang WC, Huang WP, Chen GC, Lin CY, Wang TH, Wang HS, Lai CH. Bortezomib enhances cancer cell death by blocking the autophagic flux through stimulating ERK phosphorylation. Cell Death Dis 2014; 5: e1510.

[18] Bao X, Ren T, Huang Y, Ren C, Yang K, Zhang H, Guo W. Bortezomib induces apoptosis and suppresses cell growth and metastasis by inactivation of Stat3 signaling in chondrosarcoma. Int J Oncol 2017; 50: 477-86.

[19] Klionsky DJ and Emr SD. Autophagy as a regulated pathway of cellular degradation. Science (New York, N.Y.) 2000; 290: 1717-21.

[20] Codogno P and Meijer AJ. Autophagy and signaling: their role in cell survival and cell death. Cell Death Differ 2005; 12 Suppl 2: 1509-18.
[21] Tang MK, Kwong A, Tam KF, Cheung AN, Ngan HY, Xia W, Wong AS. BRCA1 deficiency induces protective autophagy to mitigate stress and provides a mechanism for BRCA1 haploinsufficiency in tumorigenesis. Cancer Lett 2014; 346: 139-47.
[22] Yan WJ, Liu RB, Wang LK, Ma YB, Ding SL, Deng F, Hu ZY, Wang DB. Sirt3-Mediated autophagy contributes to resveratrol-induced protection against ER stress in HT22 cells. Front Neurosci 2018; 12: 116.
[23] Chen Z, Li Y, Zhang C, Yi H, Wu C, Wang J, Liu Y, Tan J, Wen J. Downregulation of Beclin 1 and impairment of autophagy in a small population of colorectal cancer. Dig Dis Sci 2013; 58: 2887-94.
[24] Satoh J, Motohashi N, Kino Y, Ishida T, Yagishita S, Jinnai K, Arai N, Nakamagoe K, Tamaoka A, Saito Y, Arima K. LC3, an autophagosome marker, is expressed on oligodendrocytes in Nasu-Hakola disease brains. Orphanet J Rare Dis 2014; 9: 68.
[25] Gorlach A, Klappa P and Kietzmann T. The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. Antioxid Redox Signal 2006; 8: 1391-418.
[26] Patergnani S, Missiroli S, Marchi S and Giorgi C. Mitochondria-associated endoplasmic reticulum membranes microenvironment: targeting autophagic and apoptotic pathways in cancer therapy. Front Oncol 2015; 5: 173.
[27] Zhu J, Tian S, Li KT, Chen Q, Jiang Y, Lin HD, Yu LH, Bai DQ. Inhibition of breast cancer cell growth by methyl pyropheophenylchlorin photodynamic therapy is mediated though endoplasmic reticulum stress-induced autophagy in vitro and vivo. Cancer Med 2018; 7: 1908-20.
[28] Kaira K, Toyoda M, Shimizu A, Shino M, Sakakura K, Takayasu Y, Takahashi K, Asao T, Chikamatsu K. Expression of ER stress markers (GRP78/BiP and PERK) in adenoid cystic carcinoma. Acta Otolaryngol 2016; 136: 1-7.