Ixazomib induces apoptosis and suppresses proliferation in esophageal squamous cell carcinoma through activation of the c-Myc/NOXA pathway


1Department of Pharmacy, Huashan Hospital, Fudan University, Shanghai, China (T.W., Z.P., L.C., H.Q., H.C., L.Z., M.Z., X.S., Q.L.)

2Department of Cardio-Thoracic Surgery, Huashan Hospital, Fudan University, Shanghai, China (Y.Z.)

#These two authors contributed equally to this work.
Running title: Ixazomib suppresses esophageal squamous cell carcinoma

*Correspondence address:
Quanyi Li, Department of Pharmacy, Huashan Hospital, Fudan University, No.12 Urumqi Middle Road, Shanghai 200040, China. E-mail: qyli1234@163.com.
Xiaojin Shi, Department of Pharmacy, Huashan Hospital, Fudan University, No.12 Urumqi Middle Road, Shanghai 200040, China. E-mail: xiaojin_shi@hotmail.com.

Word count:
Abstract: 243 words;
Introduction: 781 words;
Discussion: 713 words
Figures: 7;
References: 52

Abbreviations: Bcl-2, BCL2 apoptosis regulator; c-Myc, transcriptional regulator Myc-like; EA, esophageal adenocarcinoma ERS, endoplasmic reticulum stress; ESCC, esophageal squamous cell carcinoma; NOXA, phorbol-12-myristate-13-acetate-induced protein 1; UPS, ubiquitin-proteasome system

Recommended section: Chemotherapy, Antibiotics, and Gene Therapy
Abstract

Esophageal squamous cell carcinoma (ESCC) is one of the major subtypes of esophageal cancer. More than half of the ESCC patients in the world are in China, and the 5-year survival rate is less than 10%. As a new oral proteasome inhibitor, ixazomib has shown strong therapeutic effect in many solid tumors. In this study, we aimed to investigate the effects of ixazomib on the proliferation inhibition and apoptosis of ESCC cells. We used four human ESCC cell lines, cell viability assay, cell cycle and apoptosis assay, RT-PCR, Western blot, immunohistochemistry and ESCC xenografts model to clarify the roles of the therapeutic effect and mechanism of ixazomib in ESCC. Ixazomib significantly inhibited the proliferation and induced apoptosis in ESCC cells. RT-PCR results showed that the expression of endoplasmic reticulum stress-related gene NOXA and c-Myc significant increase after treatment with ixazomib in ESCC cell. Then we knockdown the NOXA and c-Myc by siRNA, the therapeutic effect of ixazomib markedly decrease, which confirmed that c-Myc/NOXA pathway played a key role in the treatment of ESCC with ixazomib. In vivo, the xenograft ESCC model mice were given 10 mg/kg of ixazomib every other day for 30 days. The results showed that the tumor size in the treatment group was significantly smaller than the control group. These results suggested that ixazomib is known to suppress proliferation and induce apoptosis in an ESCC cell lines, and this effect was likely mediated by increased activation of the c-Myc/NOXA signaling pathways.

Significance Statement

Esophageal squamous cell carcinoma (ESCC) is the common worldwide malignant tumors, but conventional chemotherapeutics suffer from a number of limitations. In this study, our results suggested that ixazomib is known to suppress proliferation and induce apoptosis in an ESCC cell lines. Therefore, ixazomib may be a potential new strategy for ESCC therapy.
Introduction

Currently, esophageal cancer is the seventh most common worldwide malignant tumors. More than 456,000 new cases of esophageal cancer and 300,000 deaths were recorded globally in 2018, accounting for 3.2% and 5.3% of all cancers, respectively (Bray et al., 2018). According to histopathological classification, esophageal cancer is composed of two main histologic subtypes, oesophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EA).

East Asia is a region with a high incidence of esophageal cancer, and China accounts for approximately half of the world’s esophageal cancer cases (Ferlay et al., 2010). Notably, more than 90% patients are diagnosed with ESCC in China (Zhao et al., 2012; Liang et al., 2017). The major treatment for ESCC patients is surgical resection, and the prognosis is favorable in the early stages. But the early symptoms of ESCC are more hidden, and invasive and aggressive, cause most diagnosed ESCC patients are in moderate and advanced stages (Mariette et al., 2007). Therefore, for these patients, chemotherapy helps to reduce the primary tumor bulk, to increase the possibility of radical resection and to treat micro-metastatic disease and prolong the survival period of patients (Lordick et al., 2016). These chemotherapeutic drugs include combinations of platinum-based drugs, 5-fluorouracil, paclitaxel and doxorubicin. However, conventional chemotherapeutics is often seriously hindered by a number of limitations, including high-systemic toxicity, chemoresistance and short in vivo circulation half-life.

Currently, targeted therapy was recognized as promising strategy for cancer treatment due to its high specificity and minimal side effects. The ubiquitin-proteasome system (UPS) plays a critical role in regulating 80-90% protein degradation and turnover as well as regulation of multiple cellular events, including the cell cycle, signal transduction, response to oxidative stress, cell proliferation and apoptosis (Ciechanover, 1994; Hochstrasser, 1995; Jana, 2012). As the critical endpoint for the UPS, the 26S proteasome is the chief proteolytic effector responsible for recognized and degraded the ubiquitylated protein, which consists of a core particle 20S proteasome, and two 19S regulatory particles (Bard et al., 2018). In the past decade, proteasome as a target for cancer treatment, has gained increasing attention.
them, the proteasome inhibitor (e.g., bortezomib, carfilzomib) has been confirmed to show excellent therapeutic effects in the treatment of multiple myeloma and other tumors (Johnson, 2015; Manasanch and Orlowski, 2017).

Ixazomib, an oral 20S subunit-selective inhibitor being developed used for treatment of a broad range of human malignant tumor. Compared with bortezomib, ixazomib has a shorter proteasome dissociation half-life which is a critical role in improving the distribution of drug in solid tumor tissues (Kupperman et al., 2010). Currently, ixazomib was approved for treatment of multiple myeloma patient combination with lenalidomide and dexamethasone by the US FDA (Shirley, 2016). Besides haematological neoplasms, a series of studies have suggested that ixazomib also have therapeutic anticancer functions in solid tumor, including colorectal cancer, bladder cancer, osteosarcoma and breast cancer (Kupperman et al., 2010; Sato et al., 2017; Yue and Sun, 2019; Harris et al., 2020). However, the detailed mechanisms underlying ixazomib induced cancer cell death remain unclear.

In recent years, endoplasmic reticulum stress (ERS) pathway is discovered regulated cellular processes, including protein folding, sorting, secretion, and play a crucial role in the process of apoptosis (Oakes and Papa, 2015; Minchenko et al., 2016). Early ERS is a response promoting survival, while the unfolded protein response reduces the accumulation of unfolded protein and restores the function to the endoplasmic reticulum (Oakes and Papa, 2015). However, when the unfolded protein response is not sufficient to protect cell survival, the endoplasmic reticulum will act as the trigger point of apoptosis signals to induce apoptosis and promote the expression of apoptosis-inducing factors, such as the Bcl-2 homology 3 (BH3)-only pro-apoptotic protein NOXA, a key mediator for ERS-induced apoptosis (Kelly et al., 2012; Cano-Gonzalez et al., 2018). Proteasome inhibitor MG-132 was found induces apoptosis via activation of NOXA signaling pathways in hepatic stellate and chronic lymphocytic leukemia cell lines (Baou et al., 2010; Sosa Seda et al., 2010). Furthermore, Many studies have found evidence of sustained and high-level activation of ERS in esophagus cancer cells (Rosekrans et al., 2015; Hu et al., 2019). These studies suggest that ixazomib could potentially be used for induce ESCC cell apoptosis via ER stress-mediated NOXA induction.

In the present study, we evaluate the preclinical efficacy of ixazomib in ESCC cell lines and
animal xenograft models by a series of in vitro and in vivo experiments. Our results demonstrate that ixazomib is strongly apoptotic and blocks ESCC cell growth and survival. We further show that ixazomib exerts its pro-apoptotic action via a mechanism involving the activation of the NOXA-c-Myc pathway. These results indicate that the ixazomib may be an additional useful strategy to improve the treatment outcome for ESCC patient.

Materials and Methods

Cell culture
The human esophageal squamous carcinoma cell lines KYSE 150, KYSE 510, KYSE520, TE1 and human esophageal epithelial cells lines HECC were purchased from American Type Culture Collection. KYSE 150, KYSE520 and TE1 were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640, Hyclone). KYSE 510 were cultured in 50% F12 medium (Hyclone) and 50% RPMI-1640 mixed medium. HECC cultured in high glucose Minimum Essential Medium (MEM, Hyclone). Both media were supplemented with 10% fetal bovine serum (FBS, Yeasen Biological Teacknology Co., Ltd), and 100 U/ml penicillin/streptomycin (Wisent) at 37°C in a humidified incubator with 5% CO2 and 95% air.

Reagents
Ixazomib purchased from Med Chem Express (HY-10453, USA) were dissolved in DMSO. These reagents were stored at -20°C until use.

Cell viability assay
The cell viability assay was performed using the Cell Counting Kit-8 (Proteintech Group, Inc, USA). Briefly, the ESCC cells were seeded into 96-well plates at 6×10^3 cells/well and incubated under standard culture conditions for 24 h, then the cells were treated with gradient concentrations of ixazomib for 24-72 h. After treatment, 10 µl of CCK-8 solution was added into each well of the 96-well plate and incubated for 1 h at 37°C. The optical density (OD)
values were detected at 450 nm using a microplate reader (Biotek, Winooski, VT, USA). Each experiment was independently repeated three times with five replicates.

**Colony formation assay**

For colony formation assay, KYSE150 and KYSE510 cells were plated in 6-well plates (1×10³ cells/well) and cultured under standard culture conditions overnight. Then, the medium was replaced with gradient concentrations of ixazomib and vehicle for 2 weeks until the colonies were visible in the culture plate. The supernatants were discarded and washed with PBS twice, 4% paraformaldehyde used to fix the colonies, and stained with 0.1% crystal violet solution for 30 minutes. Finally, the excess staining solution was washed away with distilled water and photographed on a microscope and used Image J software to calculate the number of clones.

**Cell cycle analysis**

For cell cycle analysis, we performed using the Cell Cycle Kit (Beyotime, China). KYSE150 and KYSE510 cells were treated with vehicle (0.1% DMSO) or ixazomib (0.25, 0.5, 1 μM) for 24 h. Then these cells were washed by PBS (0.1M, pH 7.4) three times, and fixed in 70% ethanol solution at -20°C overnight. The fixed cells were washed with cold PBS to remove excess ethanol solution and stained with propidium iodide (PI) staining buffer. Samples were incubated at 37°C for half an hour and using BD Accuri™ C6 (BD Bioscience, USA) for assay. The experiments were repeated three times, and data were analyzed with ModFit LT software (Verity Software House, Topsham, ME).

**Cell apoptosis and caspase-3 activity determination**

For simultaneous measurement of cell apoptosis, we performed using the Annexin V-FITC/PI apoptosis kit (Beyotime, China) according to the manufacturer's instruction. KYSE150 and KYSE510 cells were treated with vehicle (0.1% DMSO) or ixazomib (0.25, 0.5, 1 μM) for 24 h. Then the cells were harvested and washed twice with cold PBS, and Annexin binding buffer (500 μl) was added to each sample. Thereafter, cells were stained by Annexin V–FITC
and PI solution in the dark for 15 min. The stained cells were analyzed by flow cytometry (Novocyte, ACEA Biosciences) at the fluorescence emission of 530 nm. Because the activation of caspase3 represents an essential step in the apoptotic process. Thus, GreenNuc™ Caspase-3 Assay Kit (Beyotime, China) was used to count caspase-3 activity in cells according to the manufacturer’s introductions. Esophageal cancer cells treated with vehicle (0.1% DMSO) or ixazomib (0.5, 1 μM) for 24 h, caspase 3 positive cells were observed and counted by fluorescence microscope.

**Western blot analysis**
Western blotting was done as described previously (Zhang et al., 2019). Bcl-2, NOXA, c-Myc, cleaved-caspase8, cleaved-caspase9 (Cell Signaling Technology), were used as primary antibodies. These primary antibodies were followed by appropriate secondary antibodies conjugated to horseradish peroxidase. Antibody-protein complexes were detected using enhanced chemiluminescence (ECL) immunoblotting detection reagent. The signals were analyzed using a LAS-3000 image analyzer and MultiGauge software (Fuji Film). Densitometric analysis of WB results was performed with ImageJ software.

**Quantitative real-time polymerase chain reaction**
KYSE150 cells were treated with DMSO (0.1%) or ixazomib (10 μM) for 24h. Total RNA was isolated using the Trizol reagent and Ultrapure RNA kit (CW Biotech, China) according to the manufacturer’s instructions. 2 mg of total mRNA was reverse-transcribed with the Superscript™ reverse transcription system (Takara, Dalian, Japan). Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) reaction was carried out on ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR master mix reagents (Takara, Dalian, Japan). Relative quantification of target gene mRNA level was calculated after normalization to GAPDH mRNA using the 2-ΔΔCt method. The primer sequences were selected based on the published literature (Du et al., 2017).

**Gene silencing using small interfering RNA (siRNA)**
Kyse150 and Kyse510 cells were transfected using Lipofectamine 2000 (Invitrogen, life technologies) with siRNA mixture targeting NOXA or c-Myc for 48 h and further treated with ixazomib (1 μM) for another 48 h. Then cells were collected for cell viability assay. The siRNA (siNOXA, sic-Myc) and control siRNA (siCtrl) were purchase from HanBio Technology Co. Ltd. (HanBio, Shanghai, China).

The sequences of the siRNA are as follows:

siNOXA#1: GUAAUUAGACACAAUUC
siNOXA#2: GGUGCACGUUUCAUCAAUUUG
sic-Myc#1: AACGUUAGCUUCACCAACA
sic-Myc#2: CGAGCUAAACGGAGCUUU

**Esophageal squamous carcinoma xenografts**

Human esophageal squamous carcinoma cell xenografts were generated by subcutaneously injecting 2×10⁶ KYSE150 cells suspended with PBS/Matrigel into the right flank region of 6-week-old BALB/c nude mice (Zhang et al., 2016). Tumor growth was monitored once per 5-days after implantation. When subcutaneous tumors reached an average size of >100 mm³, mice were treatments intragastrically with ixazomib or vehicle (5% DMSO+45% PEG300+ddH2O) were then initiated. During the study, Mice were weighed per 10-days during and after the drug-treatments. Tumor volumes were calculated using the formula \( V = \text{length} \times \text{width}^2 \times 0.5 \), where length and width represent the longer and shorter tumor diameters, respectively. All procedures were performed in accordance to approved protocols by the Fudan University Institutional Animal Care and Use Committee. Every effort was made to minimize potential distress, pain, or discomfort to the animals throughout all experiments.

**Histopathology and immunohistochemistry**

Tumour tissues obtained from the mice bearing KYSE150 tumors were subjected to immunohistological analysis. Briefly, at the end of the treatment, mice were killed and tumor tissues were obtained. Immunohistological detection of anti-c-Myc (Cell Signaling Technology, Cat No.#9402)and NOXA (Cell Signaling Technology, Cat No.#14766) were performed according to the manufacture's instructions using paraffin-embedded sections of
tumor. Apoptotic cells in the tumor tissue were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining using an apoptotic cell detection kit (Beyotime, China). In addition, the major organs were removed for histological examination by hematoxylin/eosin (HE) staining.

**Statistical analysis**

Graph Pad Prism 7.0 and SPSS 16.0 software were used for statistical evaluation. Group differences in vitro experiments were compared perform one-way analysis of variance (ANOVA) with Dunnett’s post hoc test, and we used the Chou-Tallay isobologram by compusyn software to evaluate the synergistic effect of ixazomib combined with cisplatin and 5-fluorouracil. The animal experiments were compared using independent-samples Student $t$ tests. Prior to analysis, all data underwent the Kolmogorov-Smirnov normality test and Levene’s test; results showed that all data from different group of data satisfied the normality and homogeneity of variance. Data are expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

**Results**

**Ixazomib inhibited the proliferation of human esophageal squamous carcinoma cells**

Ixazomib is an orally available proteasome inhibitor that potently, reversibly, and selectively targets the proteasome. Here, we aimed to evaluate its therapeutic potential toward esophageal squamous carcinoma. Cell viability assay and colony formation assay were used to explore the anti-cancer effect of the ixazomib in four commonly used ESCC cell lines. Results showed that ixazomib displays excellent anti-proliferation activity as measured by the CCK-8 assay in ESCC cell lines. Treatment with 1 μM ixazomib inhibited cell viability by 40.3%, 27.4% and 13.5% at 24h and 72.5%, 50.1% and 37.4% at 48h for KYSE150, KYSE510, KYSE520, respectively ($P < 0.05$ for both), but the inhibition of cell viability was slightly weaker in TE1 cell lines than in other cell lines. (Figure 1A). And with an IC50 value at 0.85, 2.63, 10.61 and 42.27 μM for KYSE150, KYSE510, KYSE520 and TE1 cell line, respectively (Figure 1B). We also found that ixazomib had no effect on proliferation in the
esophageal epithelial cell line (HECC). The measurement of cell viability demonstrated that Kyse150 and Kyse510 cells were more sensitive to ixazomib treatment, therefore we selected these ESCC cell lines in the following study.

When evaluated by the colony formation assay, high-dose ixazomib (0.5 μM) significantly reduces in the number of colonies by 58.9% and 69.4% for KYSE150 and KYSE510, respectively (P < 0.01 for both) (Figure 1C, D). All these findings demonstrate that ixazomib has anticancer activity toward ESCC.

**Ixazomib induced G2/M cell cycle arrest**

To understand the underlying mechanisms of antiproliferative activity of ixazomib, we first examined the effect of ixazomib-treated ESCC cells. The Kyse150 and Kyse510 cells were treated with a gradient concentration of 0.25, 0.5 and 1μM of ixazomib for 24 h. The results show that a dose-dependent increase of cells in the G2/M phase indicated the ixazomib induction of a G2/M phase arrest (Figure 2A, B). The results revealed that ixazomib treatment increased the expression of G2/M phase related proteins p21 (Figure 2C). These results suggest that ixazomib has been found to induce cell cycle arrest in G2/M phase in ESCC cells.

**Ixazomib promotes apoptosis in ESCC cells**

ESCC cells treated with ixazomib shrank and turned round was observed microscopically after 48 h, suggestive that ixazomib treatment could trigger apoptosis. Therefore, we used the Annexin V-FITC/PI assay to confirm this speculation. In the apoptotic assays, Kyse150 and Kyse510 cells treated with ixazomib at 0.25-1 μM increased the Annexin V-positive cell populations in a dose-dependent manner (Figure 3A-D). In addition, we used the GreenNuc™ Caspase-3 assay kit to observe the expression of caspase 3 in live esophageal cancer cells after treatment with ixazomib. The results showed that the number of caspase 3 positive cells was significantly increased in the ixazomib (0.5, 1μM) treatment group compared with the control group in kyse 150 and kyse 510 cells (Figure 3E, F). In addition, ixazomib treatment also the activation of caspase 3, cleaved-caspase 9 and inhibition of Bcl-2 was confirmed by western blot analysis in ESCC cells (Figure 3G, H), suggesting that
ixazomib-induced apoptosis was involved in mitochondrial/intrinsic apoptosis pathway. These results confirmed that ixazomib could trigger apoptosis in ESCC cells.

c-Myc/NOXA pathway participates in ixazomib induced proliferation inhibition in ESCC cells.

Based on these previous studies, the proteasome inhibitors increased the accumulation of polyubiquitinated proteins and trigger the endoplasmic reticulum stress-induced apoptosis in several cancer cells (Fribley et al., 2004; Mozos et al., 2011; Niewerth et al., 2015). To assess the role of the ixazomib in apoptosis by endoplasmic reticulum stress in ESCC cells, we determined by RT-PCR the expression levels of endoplasmic reticulum stress-related genes in Kyse150 cell treated with ixazomib. Results of these experiments showed significant increase in the mRNA expression levels of endoplasmic reticulum stress-related genes in response to ixazomib. The increased in NOXA expression was the most prominent compared with other ERS related gene (Figure 4A). We knocked down the NOXA by siRNA, the results show that siNOXA significantly decreased the expression level of NOXA protein in ESCC cells (Figure 4B), and silencing NOXA rescued ixazomib-induced apoptosis, proliferation inhibition (Figure 4C, D) and cell viability inhibition (Supp. Figure 1A, C) in ESCC cells.

Several studies have evidenced that c-Myc was a prime transcription factor to transactivate NOXA (Nikiforov et al., 2007; Knorr et al., 2015). We determined the protein levels of NOXA and c-Myc in both Kyse150 and Kyse510 cell lines in response to ixazomib. As shown in Figure 5A, ESCC cells express undetectable NOXA protein levels, which were clearly upregulated following ixazomib treatment. Furthermore, the c-Myc protein expression followed a trend similar to that observed for NOXA protein expression. We knocked down the c-Myc by siRNA, the expression of NOXA significantly decreased in Kyse150 under ixazomib treatment or basic state, suggesting that c-Myc plays an important role in the activation of NOXA by ixazomib (Figure 5C). In addition, same phenomenon can also be found that reduced c-Myc function rescued ixazomib-induced apoptosis, proliferation inhibition (Figure 5D, E) and cell viability inhibition (Supp. Figure 1B, D) in ESCC cells. These observations indicated that c-Myc/NOXA signaling pathways play a major role in ixazomib induced proliferation inhibition in ESCC cells.
Cell viability inhibition effect of combination treatments of ixazomib and chemotherapy agents

Next, we assessed whether the combination of ixazomib and commonly used chemotherapy agents (cisplatin and 5-fluorouracil) exhibits additive or synergistic cell viability inhibition of ESCC cells. We applied a universal reference experimental scheme (Chou–Talalay isobologram analysis) for evaluating the effects of possible synergistic interaction (Ooko et al., 2017). The ESCC cell lines were treated with varying concentrations of ixazomib at indicated concentrations of cisplatin and fluorouracil for 24 h. A combination index (CI) less than 1 indicates the drug combination has synergism. The results showed that the combination of low concentrations of ixazomib and cisplatin triggered synergistic anti-ESCC activity, with a CI < 1.0 (Fig. 6A, B). However, we observed no similar synergistic effects between ixazomib and 5-fluorouracil in ESCC cells (Supp. Figure 2). This phenomenon suggests that the combination of ixazomib and cisplatin has therapeutic potential in the treatment of ESCC.

Ixazomib inhibits the growth of ESCC tumors in mice xenograft models

Based on the results of in vitro studies, we hypothesized that ixazomib might have an anti-tumor effect in vivo. To address this hypothesis, ESCC tumor xenografts were established in nude mice. Figure 7A shows that ixazomib had a strong inhibition on the growth of human ESCC xenografts. At the final measurement, the volumes of tumors from the mice treated with Ixazomib dose of 10 mg/kg every other day was 745 mm$^3$, while the size of the tumors in control mice has already reached 1340 mm$^3$. The tumor growth was inhibited by 44.4% after treatment with ixazomib (Figure 7F). Then the tumor specimens from mouse xenografts were subjected to immunohistochemical staining for NOXA and c-Myc expression. The results show that elevated NOXA expression was accompanied by an increase in c-Myc expression level in the tumor tissue of ixazomib-treated group in comparison with vehicle-treated control (Figure 7B). Additionally, DAPI and TUNEL staining results confirmed that compared to the control group, the apoptosis rate was markedly enhanced in the ixazomib-treated group. In order to observe the delayed organ toxicity on the mice within a certain treatment period, major organs including heart, liver,
kidney and spleen from experimental mice administered with free ixazomib and ixazomib-treatment were sectioned and stained with HE. No obvious histopathological change was observed in heart, lung and spleen of ixazomib treated mice, as shown in Figure 7D. No significant drug-related effects on body weight or any other signs of overt toxicity were shown in the group (Figure 7F). Collectively, these results suggested that ixazomib was an effective and safe anti-tumor drug in ESCC.

Discussion

Moderate and advanced stages of ESCC patients lack effective systemic treatment to eradicating tumor cells, and new treatment alternatives are urgently needed to improve patient prognosis. Currently, a series of studies have supported the accumulation of ubiquitinated proteins causing tumor cell death (Soldatenkov et al., 1998; Mimnaugh et al., 2004; Itoh et al., 2019). Therefore, inducing ubiquitinated protein accumulation has become an increasingly important treatment strategy in cancer therapy, proteasome inhibitors have been reported to have significant antitumor activity against various cancer cells through this mechanism (Sato et al., 2012; Sato et al., 2014; Sato et al., 2017). The novel orally proteasome inhibitor ixazomib is currently used for treatment of multiple myeloma patients, and it has been confirmed to have killing efficiency to tumor cells (Sato et al., 2017; Yue and Sun, 2019; Harris et al., 2020). In this study, we explore whether proteasome inhibitor ixazomib have therapeutic effects on ESCC. Our results proved that ixazomib effectively inhibits cell viability in ESCC cell lines and xenograft tumor model mice. In addition, we elucidate that ixazomib induces the apoptosis and suppresses proliferation in esophageal squamous cell carcinoma through activation of the c-Myc/NOXA pathway. Moreover, our results showed that combined treatment with ixazomib and cisplatin induces synergistic anti-ESCC activity. These results clearly suggest that ixazomib have potential to be used as anti-ESCC therapeutics.

The pro-apoptotic NOXA is a Bcl-2 family protein, and it is a crucial role that interacts with several apoptosis-related proteins such as Bad, Mcl-1, Bim and Puma (Morsi et al., 2018). In addition, endoplasmic reticulum stress plays an important role in mediating tumor cell apoptosis by inducing the upregulation of NOXA (Cano-Gonzalez et al., 2018). The c-Myc
oncogene is well known and acts as a driving force to activate a variety of oncogenic signaling pathways (Yoshida, 2018). c-Myc is frequently dysregulated or overexpressed in multiple tumor cells, and its abnormal expression contributes to reprogramming cell metabolism and to maintaining the high rate of proliferation rate in cancer cells (Dejure and Eilers, 2017). Therefore, apoptosis in response to increased expression of c-Myc is an important endogenous protection mechanism against failure that inhibits the oncogenic properties and tumorigenesis of c-Myc (Pelengaris et al., 2002; Nilsson and Cleveland, 2003). Wirth M et al. using quantitative promoter-scanning chromatin immunoprecipitation revealed the binding of c-Myc to the promoters of NOXA upon proteasome inhibition (Wirth et al., 2014). Other scholars also reported that c-Myc was a prime transcription factor to transactivate NOXA (Nikiforov et al., 2007). A series of studies have shown that proteasome inhibitor bortezomib can induce c-Myc-dependent up-regulation of NOXA, leading to apoptosis in colorectal and pancreatic cancer cells (Yue and Sun, 2019; Lankes et al., 2020). Here, this study suggested that ixazomib-mediated upregulation the expression of NOXA and c-Myc in a time- and concentration-dependent manner, on the other hand, NOXA and c-Myc silencing using siRNA partially rescued the proliferation inhibitory effect of ixazomib in ESCC cells. These results confirm that the ixazomib inhibits the proliferation of ESCC cells by target on the c-Myc-NOXA pathway.

Bortezomib is the first proteasome inhibitor for used as a monotherapy for the treatment of multiple myeloma and mantle cell lymphoma approved by the FDA (Richardson, 2003). A series of clinical studies found that bortezomib combined with other drugs, including corticosteroids, lenalidomide and thalidomide can significantly improve the therapeutic effect (Kapoor et al., 2012). In recent years, research has explored the potential value of bortezomib in combination with cytotoxic drugs in in solid malignancies. Bortezomib combined with platinum-based agents has achieved encouraging results in Phase I/II study in ovarian cancer and non-small-cell lung carcinomas (Ramirez et al., 2008; Piperdi et al., 2012; Zhao et al., 2015). There is a pervasive belief that the synergism between two drugs was due to platinum agents can block bortezomib-induced STAT1 activation which suppresses apoptosis.(Fribley et al., 2006; Kao et al., 2013) Given that ixazomib, like bortezomib, is a boronate-based
molecules, we explored whether ixazomib similarly enhances the anti-ESCC activity of the nonspecific cytotoxic agent (cisplatin and fluorouracil).

In summary, our studies have identified a novel mechanism of the proteasome inhibitor ixazomib induced proliferation inhibition and apoptosis in ESCC. It is possible that this effect be mediated by c-Myc/NOXA pathway, which might give a new insight into the anti-tumor effect and mechanism of ixazomib in ESCC.

Acknowledgments
The work was supported by the National Natural Science Foundation of China (81901399, 81973399), the Shanghai ‘Rising Stars of Medical Talent’ Youth Development Program (Youth Medical Talents-Clinical Pharmacist Program), Shanghai Key Clinical Specialty Projects-Clinical Pharmacy (shslczdzk06502), and Scientific Research Project of Shanghai Health and Family Planning Commission to (20184Y0194, 20204Y0445).

Conflict of interest
The authors declare no conflicts of interest

Authorship Contributions
Participated in research design: Tianxiao Wang, Pengying Zhang, Qunyi Li.
Performed data analysis: Tianxiao Wang, Xiaojin Shi, Qunyi Li.
Wrote or contributed to the writing of the manuscript: Tianxiao Wang, Mingkang Zhong, Xiaojin Shi, Qunyi Li.
References


Figure and legend

Figure 1
Ixazomib inhibits growth and colony formation of esophageal squamous carcinoma cells. (A), Effect of ixazomib on the viability of breast cancer cells Kyse150, Kyse510, Kyse520, TE1 and HECC. Cells were treated with vehicle or ixazomib (0.1, 1, 10 μM) for 24 h and 48h, the viability was detected with the CCK-8 kit. (B), ESCC cells were treated with various concentrations of ixazomib for 24 hours. Cell viability was determined by the CCK-8 assay. IC50 value of ixazomib in ESCC cell lines was then determined. (C, D), Efficacy of ixazomib on colony formation of ESCC cells. ESCC cells were treated with ixazomib (0.25, 0.5 μM) for 7days, and then fixed, stained and counted. Values are the means ± SD (n = 3). *P < 0.05 or **P < 0.01 indicates significant differences from the vehicle group as assessed by one-way ANOVA with post hoc Dunnett’s test.

Figure 2
Ixazomib triggers G2/M cell cycle arrest in esophageal cancer cells. (A, B) Ixazomib induced G2/M cell cycle arrest in Kyse150 and Kyse510 cells were treated with ixazomib at different concentration for 24 h, followed by PI staining and FACS analysis for cell cycle profile. (C), Distribution was analyzed by Modift and Graphpad software (lower panel). Values are the means ± SD (n = 5-6). *P < 0.05 or **P < 0.01 indicates significant differences from the vehicle group as assessed by one-way ANOVA with post hoc Dunnett’s test.

Figure 3
Ixazomib induces apoptosis in ESCC. Kyse150 and Kyse510 cells were treated with ixazomib for 48 h. (A-D) Apoptosis was determined by FACS analysis using Annexin V-FITC/PI double-staining kit and Annexin V+ cell populations were defined as apoptosis. (E-F) Using GreenNuc™ Caspase-3 assay kit to observe the expression of caspase 3 in live esophageal cancer cells after treatment with ixazomib. (G-H), Treatment with ixazomib increased the expression of caspase3, cleaved caspase 9 and decrease the expression of Bcl-2. ESCC cells were treated with ixazomib for 48 h and cell lysates were assessed by western blot. GAPDH was used as loading control. Values are the means ± SD (n = 5-6). *P < 0.05 or **P < 0.01 indicates significant differences from the vehicle group as assessed by one-way ANOVA with post hoc Dunnett’s test.

Figure 4
NOXA participates in ixazomib induced proliferation inhibition in ESCC cells. (A) Kyse150 cell treated with or without 1μM ixazomib for 48h. The expression of ER stress related gene was examined by RT-PCR. (B) Western blots assay was used to evaluate the knockdown effect of single siRNA and pooled siRNA on NOXA protein. (C, D) After transfected with the NOXA siRNA for 48 h, Kyse150 and Kyse510 cells were further treated with ixazomib (1μmol) for 48 h and the cell apoptosis and colony formation were examined. Values are the means ± SD (n = 4-5). *P < 0.05 or **P < 0.01 indicates significant differences from the vehicle group, ###P < 0.01 indicates significant differences from the siRNA control group as assessed by one-way ANOVA with post hoc Dunnett’s test.

Figure 5
c-Myc/NOXA pathway plays an important role in ixazomib induced proliferation inhibition in ESCC cells. (A) Kyse150 and Kyse 510 were treated with or without ixazomib 1μM for 48h, Cell lysate was prepared and c-Myc and NOXA protein level was determined by Western blotting analysis. (B) Western blots assay was used to evaluate the knockdown effect of single siRNA and pooled siRNA on c-Myc protein. (C) The protein expression of NOXA in Kyse150 after knockdown of c-Myc. (D, E) After transfected with the c-Myc siRNA for 48 h, Kyse150 and Kyse510 cells were further treated with ixazomib (1μmol) for 48 h and the cell apoptosis and colony formation were examined. Values are the means ± SD (n = 4-5). *P < 0.05 or **P < 0.01 indicates significant differences from the vehicle group, ###P < 0.01 indicates significant differences from the siRNA control group as assessed by one-way ANOVA with post hoc Dunnett’s test.

Figure 6
Combination of low doses of ixazomib and cisplatin triggers synergistic anti-ESCC activity. KYSE150 (A) and KYSE510 (B) cells were treated for 24 hours with indicated concentrations of ixazomib, cisplatin, or ixazomib plus cisplatin and then assessed for viability by CCK8 assays. Isobologram analysis shows the synergistic cytotoxic effect of ixazomib plus cisplatin. The graph (top) is derived from the values given in the table (bottom). Numbers 1 to 15 in graph represent combinations shown in the table. Fa, fraction of cells showing decrease in viability with ixazomib plus cisplatin treatment. CI < 1 indicates synergy. All experiments were carried out in triplicate, and the mean value is shown.

Figure 7
Ixazomib inhibits the growth of ESCC tumors in mice xenograft models. (A) The images of xenograft tumors at the end of the experiment. (B) Representative images of NOXA and
c-Myc staining of ESCC-xenografted tumor sections in different treatment groups (200X magnification). (C) The results of DAPI and TUNEL staining in different treatment groups. (D) The organs including heart, spleen, liver and kidney from the mice treated with ixazomib 10 mg/kg were shown in the first row. The organs from the mice treated with vehicle control were shown in the second row. (E) Volume measurements of Kyse150 xenograft tumors treated with vehicle or ixazomib at the indicated dosages for 30 days. Ixazomib was administered by intragastric administration every other day at 10 mg/kg. (F) The body weight of mice in different treatment groups. Values are the means ± SD (n = 6). *P < 0.05 or **P < 0.01 indicates significant differences from the vehicle group as assessed by a two-tailed unpaired Student’s t test.
Figure 1

A. Relative cell growth (%) at 24h and 48h for KYSE150, KYSE510, KYSE520, TE1, and HECC treated with ixazomib (0.1μM, 1μM, 10μM) and vehicle.

B. Logarithmic graph showing the relative cell growth (%) for KYSE150, KYSE510, KYSE520, TE1, and HECC treated with different concentrations of ixazomib.

C. Images depicting cell morphology for KYSE150 and KYSE510 treated with vehicle and ixazomib (0.5μM).

D. Graph showing relative colony forming ability (%) for KYSE150 and KYSE510 treated with vehicle, ixazomib 0.25μM, and ixazomib 0.5μM.
**Figure 2**

A) **Kyse 150**
- Vehicle
- 0.25 μM
- 0.5 μM
- 1 μM

B) **Kyse 510**
- Vehicle
- 0.25 μM
- 0.5 μM
- 1 μM

C) **Kyse 150**
- G0/G1
- S
- G2/M

**Kyse 510**
- G0/G1
- S
- G2/M

**Cell cycle distribution**

- Vehicle
- IXA 0.25 μM
- IXA 0.5 μM
- IXA 1 μM

* denotes p < 0.05
** denotes p < 0.01
Figure 4

A. KYSE150

mRNA expression (relative levels)

B. KYSE150

siNOXA

Con  |  #1  |  #2  |  #1+#2
---  | ---  | ---  | ---
NOXA | 0.66 | 0.64 | 0.37

C. KYSE 150

% of Apoptotic cells

D. KYSE 150

Relative colony forming ability (%)

vehicle  |  siControl+Ixazomib 0.5 μM  |  siNOXA+Ixazomib 0.5 μM
---  | ---  | ---
KYSE 510  |  **  |  **

**  |  #  |  **

vehicle  |  siControl+Ixazomib 1 μM  |  siNOXA+Ixazomib 1 μM
---  | ---  | ---
KYSE 510  |  **  |  **

**  |  #  |  **
Figure 5

A

<table>
<thead>
<tr>
<th>Ixazomib (µM)</th>
<th>KYSE 150</th>
<th>KYSE 510</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>1.03</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>2.66</td>
<td>2.41</td>
</tr>
<tr>
<td></td>
<td>2.54</td>
<td>1.51</td>
</tr>
<tr>
<td>c-Myc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOXA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>KYSE 150</th>
<th>siMyc</th>
<th>1µM Ixazomib</th>
<th>#1</th>
<th>#2</th>
<th>#1+2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>#1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>#2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>#1+2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>c-Myc</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NOXA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GAPDH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

D

% of Apoptotic cells

- Vehicle
- siControl + Ixazomib 1 µM
- siMyc + Ixazomib 1 µM

E

Relative colony forming ability (%)

- Vehicle
- siControl + Ixazomib 0.5 µM
- siMyc + Ixazomib 0.5 µM
Figure 6

A) KYSE150

B) KYSE510

<table>
<thead>
<tr>
<th>combination</th>
<th>ixazomib (μM)</th>
<th>cis-platinum (μM)</th>
<th>Fa</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>0.5</td>
<td>0.18</td>
<td>0.60</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>1</td>
<td>0.32</td>
<td>0.37</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
<td>2</td>
<td>0.59</td>
<td>0.14</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>4</td>
<td>0.66</td>
<td>0.17</td>
</tr>
<tr>
<td>5</td>
<td>1.6</td>
<td>8</td>
<td>0.77</td>
<td>0.15</td>
</tr>
<tr>
<td>6</td>
<td>0.1</td>
<td>1</td>
<td>0.19</td>
<td>0.99</td>
</tr>
<tr>
<td>7</td>
<td>0.2</td>
<td>2</td>
<td>0.32</td>
<td>0.69</td>
</tr>
<tr>
<td>8</td>
<td>0.4</td>
<td>4</td>
<td>0.63</td>
<td>0.20</td>
</tr>
<tr>
<td>9</td>
<td>0.8</td>
<td>8</td>
<td>0.66</td>
<td>0.32</td>
</tr>
<tr>
<td>10</td>
<td>1.6</td>
<td>16</td>
<td>0.76</td>
<td>0.31</td>
</tr>
<tr>
<td>11</td>
<td>0.1</td>
<td>1.5</td>
<td>0.29</td>
<td>0.62</td>
</tr>
<tr>
<td>12</td>
<td>0.2</td>
<td>3</td>
<td>0.36</td>
<td>0.76</td>
</tr>
<tr>
<td>13</td>
<td>0.4</td>
<td>6</td>
<td>0.54</td>
<td>0.50</td>
</tr>
<tr>
<td>14</td>
<td>0.8</td>
<td>12</td>
<td>0.7</td>
<td>0.35</td>
</tr>
<tr>
<td>15</td>
<td>1.6</td>
<td>24</td>
<td>0.79</td>
<td>0.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>combination</th>
<th>ixazomib (μM)</th>
<th>cis-platinum (μM)</th>
<th>Fa</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>0.5</td>
<td>0.33</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>1</td>
<td>0.35</td>
<td>0.28</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
<td>2</td>
<td>0.45</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>4</td>
<td>0.54</td>
<td>0.24</td>
</tr>
<tr>
<td>5</td>
<td>1.6</td>
<td>8</td>
<td>0.53</td>
<td>0.22</td>
</tr>
<tr>
<td>6</td>
<td>0.1</td>
<td>1</td>
<td>0.27</td>
<td>0.60</td>
</tr>
<tr>
<td>7</td>
<td>0.2</td>
<td>2</td>
<td>0.33</td>
<td>0.63</td>
</tr>
<tr>
<td>8</td>
<td>0.4</td>
<td>4</td>
<td>0.56</td>
<td>0.39</td>
</tr>
<tr>
<td>9</td>
<td>0.8</td>
<td>8</td>
<td>0.74</td>
<td>0.23</td>
</tr>
<tr>
<td>10</td>
<td>1.6</td>
<td>16</td>
<td>0.41</td>
<td>0.58</td>
</tr>
<tr>
<td>11</td>
<td>0.1</td>
<td>1.5</td>
<td>0.31</td>
<td>0.57</td>
</tr>
<tr>
<td>12</td>
<td>0.2</td>
<td>3</td>
<td>0.4</td>
<td>0.46</td>
</tr>
<tr>
<td>13</td>
<td>0.4</td>
<td>6</td>
<td>0.48</td>
<td>0.49</td>
</tr>
<tr>
<td>14</td>
<td>0.8</td>
<td>12</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>15</td>
<td>1.6</td>
<td>24</td>
<td>0.58</td>
<td>0.99</td>
</tr>
</tbody>
</table>
Figure 7

A

Ixazomib 10 mg/kg
vehicle

B

Ixazomib 10 mg/kg
vehicle

Noxa

cMyc

C

DAPI
TUNEL
Merge

Ixazomib 10 mg/kg
vehicle

D

Heart
Liver
Kidney
Spleen

Ixazomib 10 mg/kg
vehicle

E

F

Tumor volume (mm³)
Body weight (g)

Days after treatment

Days after treatment