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Lansoprazole induces apoptosis of breast cancer cells through inhibition of intracellular proton extrusion

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ABSTRACT

The increased glycolysis and proton secretion in tumors is proposed to contribute to the proliferation and invasion of cancer cells during the process of tumorigenesis and metastasis. Here, treatment of human breast cancer cells with proton pump inhibitor (PPI) lansoprazole (LPZ) induces cell apoptosis in a dose-dependent manner. In the implantation of the MDA-MB-231 xenografts in nude mice, administration of LPZ significantly inhibits tumorigenesis and induces large-scale apopotosis of tumor cells. LPZ markedly inhibits intracellular proton extrusion, induces an increase in intracellular ATP level, lysosomal alkalinization and accumulation of reactive oxygen species (ROS) in breast cancer cells. The ROS scavenger N-acetyl-L-cysteine (NAC) and diphenyleneiodonium (DPI), a specific pharmacological inhibitor of NADPH oxidases (NOX), significantly abolish LPZ-induced ROS accumulation in breast cancer cells. Our results suggested that LPZ may be used as a new therapeutic drug for breast tumor.

1. Introduction

Solid tumors often exist in a hypoxic microenvironment [1,2], and possess a high-glycolytic metabolite, which results in proton accumulation in the cytoplasm [3,4]. However, intracellular pH (pHi) must be controlled within a narrow range to maintain basic cell functions such as membrane permeability, enzyme activity, cellular metabolism, ATP maintenance, cell proliferation, and apoptotic mechanisms [5]. Consequently, the tumor cells enhance the ability to dispose of intracellular protons, which results in an acidic extracellular environment [1,6]. Acidic pH, in particular, has pleiotropic effects on the resistance to chemotherapy [7], proliferation [8], and metastatic behavior [9] during the process of tumorigenesis and metastasis. Inhibition of several proton extrusion mechanisms adopted by malignant cancer cells presents one promising therapeutic strategy [1,10,11].

Proton pump inhibitors (PPIs), substituted 2-pyridyl methyl/sulfinyl benzimidazole derivatives, have been developed for the treatment of acid-related disorders including gastroesophageal reflux disease (GERD), gastric ulcer, duodenal ulcer, and Barrett's esophagus [12–14]. Gastric acidification primarily depends on the H^+/K^+-ATPases of gastric parietal cell which can exchange luminal K^+ for cytoplasmic H^+ [15]. PPIs require protonation for functional activation at acidic conditions, accumulate selectively in acidic gastric luminal space, and ultimately inhibit acid secretion by covalently binding to cysteine residues in α-subunit of H^+/K^+-ATPase.

By analogy with gastric compartment, PPIs may be protonated and transformed in active form in the acidic tumor microenvironment [16,17]. According to Luciani et al. [18], PPI pretreatment could sensitize tumor cell lines to the effects of chemotherapeutic drugs and directly induce tumor cell killing. PPIs were shown to selectively induce apoptosis of gastric cancer cells by inhibiting gastric H^+/K^+-ATPases [19]. PPIs could induce apoptosis of human B-cell tumors through severe alteration of pH gradients regulation, including ROS production [20]. PPIs could inhibit the expression of H^+/K^+-ATPase, reverse the transmembrane pH gradient and chemosensitize SGC7901 cells to anti-tumor agents [21]. The PPI esomeprazole kills melanoma cells through a caspase-dependent pathway involving cytosolic acidification and alkalinization of tumor pH, lysosomal membrane permeabilization and ROS generation in human melanoma cells [22,23]. In addition, PPZ pretreatment enhances the cytotoxic effects of anti-tumor drugs on SGC7901 cells and reverses MDR of SGC7901/ADR by down-regulating the V-ATPases/mTOR/HIF-1α/P-gp and MRP1 signaling pathway [24].

In present study, we treated human breast cancer cells with PPIs, omeprazole (OPZ), esomeprazole (EPZ), pantoprazole (PPZ) and lansoprazole (LPZ), to study their effects on cell apoptosis. Our data showed that LPZ is more effective to induce apoptosis

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in breast cancer cells than the others. LPZ significantly inhibited tumorigenesis and induced large-scale apopotosis of tumor cells *in vivo*. LPZ had an antineoplastic effect in human breast cancer cells, targeting tumor acidic pH, intracellular ATP level, lysosomal pH and intracellular ROS generation. Our results suggested that LPZ may be used as a potential new drug against human breast cancer.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-453, MDA-MB-468, SK-BR-3 and T-47D) were cultured in RPMI 1640 (GIBCO) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine in a humidified chamber 5% CO_2 atmosphere at 37 °C.

2.2. Cell survival rate assay

The survival rates of breast cancer cells were measured by MTT assay as described previously [25]. Briefly, cells were plated in 96-well plates at a concentration of 5×10^4 cells/mL at 100 μL per well in RPMI 1640 medium. The next day, the medium in 96-well plates was replaced by the medium containing a variety of PPI concentrations. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed after the cells were kept in culture at 37 °C for 48 h.

2.3. Hoechst 33342 staining for the detection of apoptotic cells

To certificate apoptosis phenomenon, the Hoechst 33342 staining assay was carried out. Hoechst 33342 can permeate cells and stain the nuclei by showing blue fluorescence [26]. Briefly, the cells were incubated with 5 μg/mL Hoechst 33342 (Sigma) for 15 min at 37 °C. The fluorescence was visualized under a fluorescent microscope at 340 nm excitation and 510 nm emission. Apoptotic cells were characterized by condensed and fragmented nuclei, and apoptosis was expressed as ratios of nuclear condensation cells to all cells.

2.4. Tumor xenograft

MDA-MB-231 cells were suspended in serum-free RPMI 1640 at density of 1×10^7 cells/mL. The cells (5×10^6) were s.c. injected into the left upper flank region of each mouse (4–6 weeks of age, female, BalB/c nu+/nu+, from Academy of Military Science, Beijing) on day 0. Tumor dimensions were measured with calipers once every two days, and the volume of each tumor (mm^3) was calculated according to $L \times W^2/2$ (L, length; W, width). The animals were randomly divided into two groups (5 per group). Once the tumors' volume reached ~100 mm^3 (about 1 week), Group A received an intratumoral injection of saline (0.85% NaCl) daily, while Group B received an intratumor injection of 12 mg/kg LPZ daily. After 3 weeks all mice were sacrificed. Isolated tumors were fixed in formalin and embedded in paraffin. Five-micrometer sections were stained with haematoxylin/eosin, and analyzed for Ki-67. These studies were approved by the Animal Use Committee of Institute of Hematology (Chinese Academy of Medical Sciences, Tianjin) approved all protocols for treating animals.

2.5. Measurement of intracellular pH

Intracellular pH was measured in the monolayers using the pH-sensitive fluorescent probe BCECF-AM, as described previously [27].

2.6. Determination of intracellular ATP concentration

ATP level was measured by the luciferinluciferase method as reported elsewhere [28].

2.7. Evaluation of lysosomal acidity

LysoSensor Green DND-189 (LSG) was used to monitor changes in the pH of acidic vesicles. LSG accumulates in lysosomes and acidic organelles [29], which exhibits a pH-dependent increase or decrease in fluorescence intensity upon lysosome and acidic organelle acidification or alkalinization. Cells were incubated with 0.2 μM LSG for 30 min at 37 °C and the fluorescence of LSG was detected at 488 nm excitation and 510 nm emission.

2.8. ROS measurement

Dihydroethidium (DHE), a reduced form of ethidium bromide and membrane-permeable dye, was used to detect the level of intracellular ROS. DHE will be oxidized in the presence of ROS and binds to double-stranded DNA, thus giving a deep red fluorescence. Cells were incubated with 50 μM DHE for 30 min at 37 °C and were washed three times with Hank's balanced salt solution (HBSS; 138 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.35 mM Na_2HPO_4), then observed with a fluorescence microscope at 488 nm excitation and 610 nm emission. The fluorescent intensity represents the level of intracellular ROS.

2.9. Statistical analysis

All statistics were performed using SPSS 16.0 software. Measurement data were represented as mean ± SD. Comparison of the mean between groups was performed by *t* test. *P* values < 0.05 were considered significant. Survival analysis was assessed using Kaplan–Meier method and survival rate was compared by log-rank test.

3. Results

3.1. LPZ induces apoptosis in breast cancer cells

To test whether the anti-proliferative effect was dependent on PPI-induced cytotoxicity, proton pump inhibitors (PPIs), omeprazole (OPZ), esomeprazole (EPZ), pantoprazole (PPZ) and lansoprazole (LPZ), were added to the medium one day after plating the cells, and survival rates were measured 48 h latter by MTT assay. As shown in Fig. 1A, PPIs induced a dose-dependent and kind-dependent inhibition of cell proliferation in breast cancer cells, in which LPZ was more effective to induce death in breast cancer cells. The LPZ concentrations of all death in MDA-MB-231, MCF-7, MDA-MB-453, MDA-MB-468, SK-BR-3 and T-47D cells were 80, 140, 140, 120, 180 and 160 μM, respectively. As shown in Fig. 1B (left panel), the cell nuclei occurred to concentrate after treated with 100 μM LPZ for 24 h. However, the ROS scavenger NAC obviously prevented the LPZ-induced nuclear condensation. The data was also showed in histogram (Fig. 1B, right panel). Those results suggested that LPZ induces apoptosis in breast cancer cells.

3.2. Administration with LPZ markedly retards tumor growth in a xenograft model of nude mice in vivo

To assess the anti-neoplastic role of LPZ *in vivo*, we tested whether LPZ administration could affect the growth of human breast cancer in the MDA-MB-231 xenografts. The isolated tumor from mice with intratumor administration of LPZ was remarkably

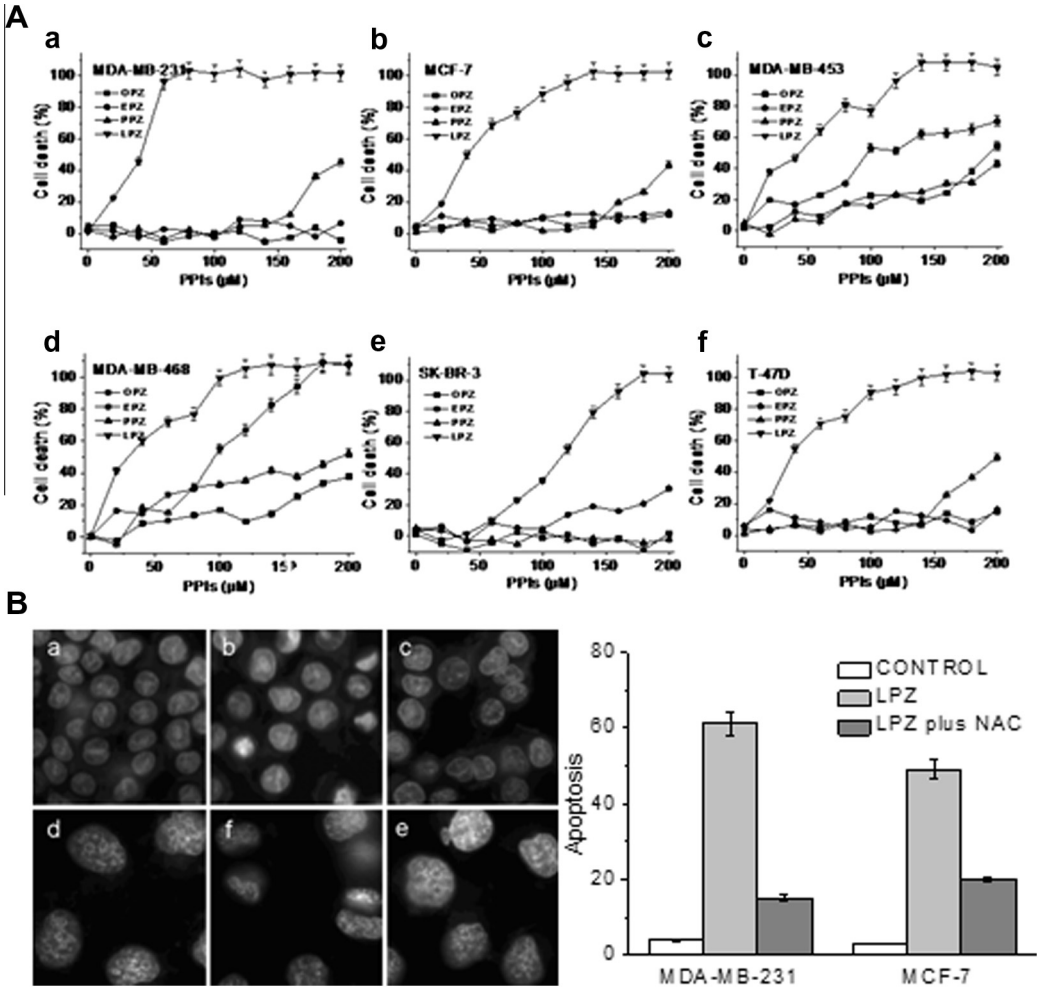

Fig. 1. PPIs inhibit proliferation and induce apoptosis in breast cancer cells *in vitro*. (A) PPIs induce death in human breast cancer cells. a, MDA-MB-231; b, MCF-7; c, MDA-MB-453; d, MDA-MB-468; e, SK-BR-3; f, T-47D. ■, omeprazole (OPZ); ●, esomeprazole (EPZ); ▲, pantoprazole (PPZ); ▼, lansoprazole (LPZ). In these PPIs, LPZ is more effective to induce the cell death. Values are means ± SD (n = 3). (B) MDA-MB-231 and MCF-7 were stained with Hoechst 33342. Left panel: imaged after different treatments for 24 h. (a) MDA-MB-231 control, (b) MDA-MB-231 LPZ (100 μM), (c) MDA-MB-231 LPZ (100 μM) plus NAC (5 mM), (d) MCF-7 control, (e) MCF-7 LPZ (100 μM), (f) MCF-7 LPZ (100 μM) plus NAC (5 mM). The data also showed in histogram (right panel). Data are presented as mean ± SD (n = 3).

smaller than that of mice with intratumor administration of saline (Fig. 2A and B). Haematoxylin/eosin stain of tumor sections from mice revealed that LPZ administration induces considerable apoptosis, and most tumor cells were replaced by apoptotic cells comparing with saline administration. Immunohistochemical analysis of tumor sections from mice with intratumor administration of LPZ showed diminished levels of Ki-67, suggesting a reduced proliferative potential of residual tumor cells (Fig. 2C). These results suggested that LPZ cytotoxicity on tumor cells is paralleled by concomitant inhibition of the proliferative rate within the tumor mass *in vivo*.

3.3. Acidification of cytosolic pH occurs in LPZ-induced cell death

We analyzed the changes in the intracellular pH (pHi) following treatment with LPZ in MDA-MB-231 and MCF-7 cells for 12 h in order to assess the role of LPZ in intracellular proton extrusion. After 12 h of 100 μM LPZ treatment, the pHi in MDA-MB-231 cells was significantly decreased from 7.33 to 6.63, while the pHi in MCF-7 cells was changed from 7.35 to 6.83 (Fig. 3A). These findings showed that LPZ treatment suppresses proton extrusion in breast cancer cells.

3.4. LPZ induces an increase in intracellular ATP level in breast cancer cells

Intracellular ATP levels determine cell death fate of cancer cells [30] and influence V-ATPase activity, so we measured the changes of the intracellular ATP levels following treatment with LPZ (100 μM) in MDA-MB-231 and MCF-7 cells for 1 h to assess the role of LPZ in cell death and intracellular proton extrusion. LPZ induced higher levels of intracellular ATP than untreated cells in MDA-MB-231 cells, and NAC inhibited the change induced by LPZ. However, LPZ treatment had a minor change in intracellular ATP level in MCF-7 cells (Fig. 3B).

3.5. LPZ induces alkalinization of lysosomal pH in breast cancer cells

We analyzed the effect of LPZ on lysosomal pH in MDA-MB-231 cells by detecting the fluorescence intensity of LysoSensor Green DND-189 (LSG). The fluorescence intensity was decreased after loading a concentration of 0.5 mM LPZ (Fig. 3C), suggesting an increase in lysosomal pH occurred. This finding clearly indicated that LPZ treatment induces lysosomal alkalinization.

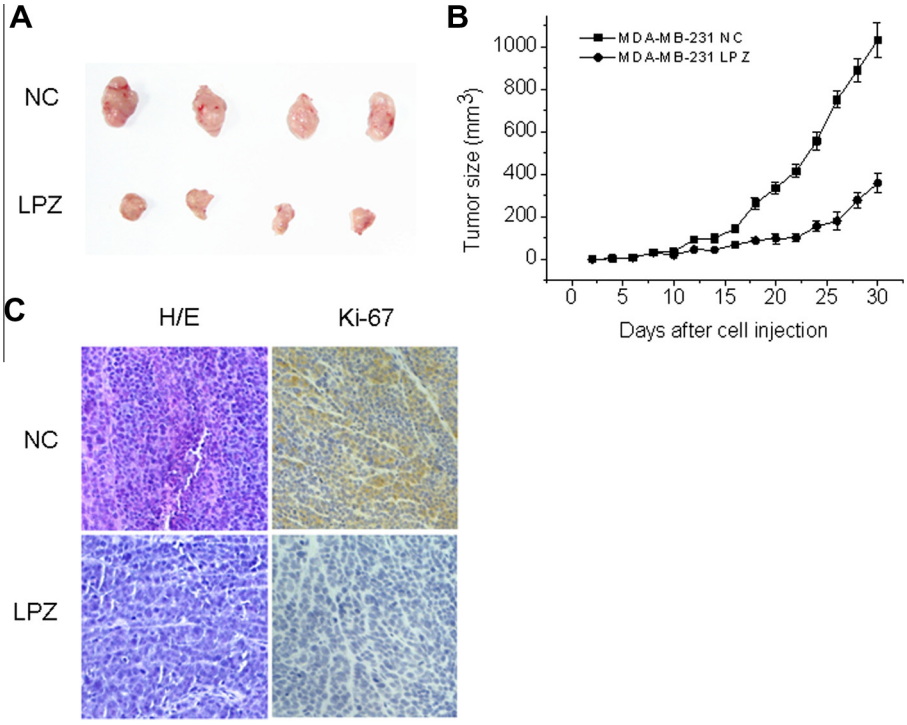

Fig. 2. LPZ inhibits tumor growth in a xenograft model of nude mice. (A, B) Administration with LPZ suppresses the rate of xenograft tumor growth. (C) Immunohistochemical analysis of tumor sections from mice injected with saline and LPZ by haematoxylin/eosin stain and Ki-67. The presence of large necrotic areas is observed in LPZ treated MDA-MB-231 *in vivo*.

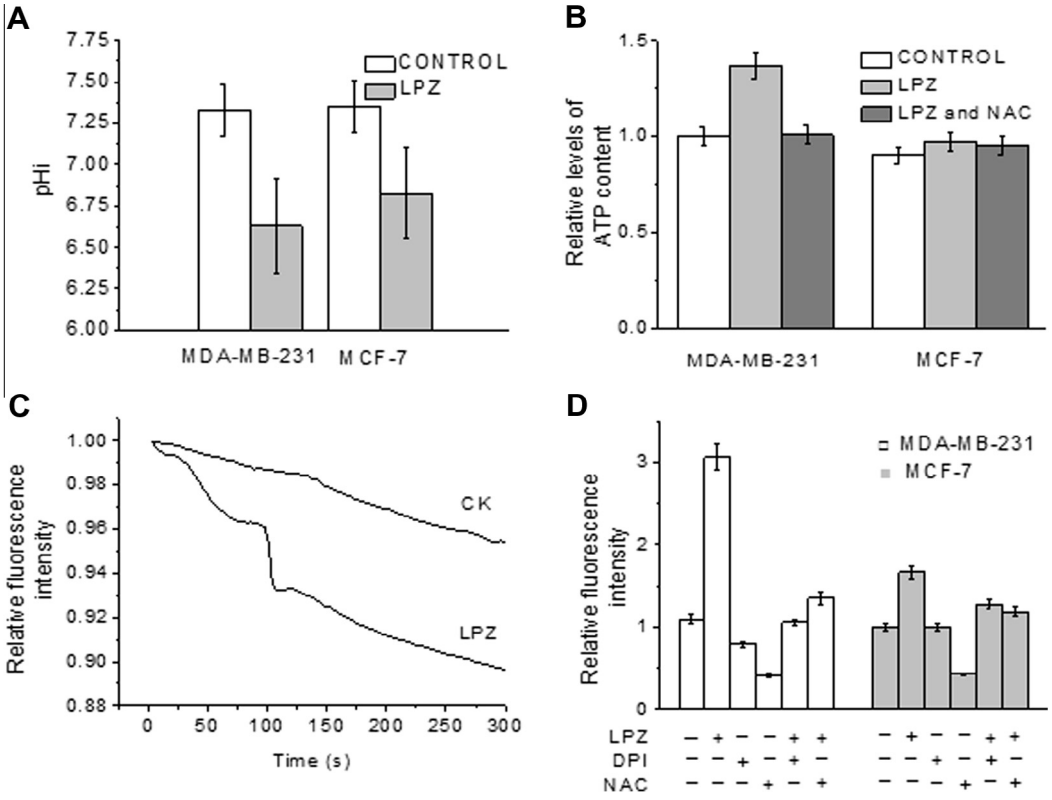

Fig. 3. LPZ induces breast cancer cell death through acidification of cytosolic pH, higher level of intracellular ATP, alkalinization of lysosomal pH and ROS accumulation. LPZ induces a decrease in intracellular pH in MDA-MB-231 and MCF-7 cells (A), an increase in intracellular ATP level (B), an increase in lysosomal pH in MDA-MB-231 cells (C), and ROS accumulation in MDA-MB-231 and MCF-7 cells (D).

3.6. LPZ induces ROS-dependent cell death in breast cancer cells

To determine whether LPZ-induced apoptosis in breast cancer cells correlates with ROS generation, the levels of intracellular ROS were evaluated after 100 μM LPZ treatment for 1 h by measuring dihydroethidium fluorescence. As shown in Fig. 3D, treatment with LPZ rapidly induced accumulation of ROS in MDA-MB-231 and MCF-7 cells. 5 mM NAC totally abolished the LPZ-mediated ROS production in MDA-MB-231 and MCF-7 cells. In addition, 10 μM DPI remarkably inhibited LPZ-induced ROS generation in MDA-MB-231 and MCF-7 cells. These results suggested that LPZ induces breast cancer cell death through ROS accumulation.

4. Discussion

In the present study, PPIs attenuated viability in breast cancer cell lines tested, but the efficacy showed different manners among them. Our results provided the first preclinical evidence that LPZ induces remarkable cytotoxicity in breast cancer cell lines *in vitro* and inhibits tumor growth in the implantation of the MDA-MB-231 xenografts in nude mice *in vivo*, without evidence of systemic toxicity.

During the last decade, it has become evident that the pH regulation plays an important role in the development and progression of malignant tumors [7–9]. Several types of intracellular pH regulatory mechanisms have been identified in tumor cells: Na^+/H^+ exchangers, bicarbonate (HCO_3^-) transporters, proton-lactate symporters, proton pumps, the vacuolar H^+-ATPase (V-ATPase), and the voltage-gated proton channel Hv1 [25,27,28–30,31–33]. Such selective advantage is conferred to tumor cells by the capacity of surviving in hypoxic-acidic environment, mediated by the up-regulated proton extrusion activity and lysosomal trafficking [17,34]. These findings provide rationale for the present study that cancer cells might be susceptible or vulnerable to the inhibition of proton extrusion. The acidic pH of solid tumor tissues has been proposed as a therapeutic target and a drug delivery system for selective anticancer treatments [35]. It has been reported that the acidification of cytosolic pH precedes apoptosis of leukemia/lymphoma cells [36,37] and that inhibition of V-ATPase activity by RNA interference significantly delays hepatocellular carcinoma growth by decreased proton extrusion [38]. In fact, human melanoma cells treated with omeprazole or esomeprazole had higher levels of intracellular ATP than untreated cells, suggesting potent inhibition of the V-ATPase activity [18]. The fact that metastatic cells express more lysosomal proteins on the cell surface, suggested that an aberrant compartmentalization of lysosomal-like activity operates in malignant tumor cells [39]. Several reports showed that lysosomes lose their proton gradient and occur alkalinization [40] early during apoptosis. In our case, we found that LPZ induces acidification of the intracellular pH, an increase in intracellular ATP level, and lysosomal alkalinization in breast cancer cells, suggesting potent inhibition of intracellular pH regulatory mechanisms.

ROS are generated by the respiratory chain in the mitochondria and by the NOX enzymes in the cytoplasm. In fact, NOX proteins are now considered to be oncogenic proteins, and mitochondrial dysfunction is associated with tumorigenesis [41]. Previous studies have shown that some chemotherapeutic agents can induce apoptosis through ROS generation in certain cancer cells [20,22,23,42–44]. ROS promote apoptosis by stimulating pro-apoptotic signaling molecules, such as ASK1, JNK and p38 [45–47]. ROS also play a pivotal role in p53-induced apoptosis [48]. In addition, ROS can act directly on the apoptotic machinery by accelerating mitochondrial depolarization and dysfunction. LPZ induced a large amount of ROS accumulation, proposing that ROS play a critical role in LPZ-induced cell death in breast cancer cells.

PPI treatment has been proposed as a valid and feasible approach for tumor therapeutics because of the relatively low toxicity and potential selectivity of these drugs. The pharmacological and chemical properties of PPIs make these acid-activated prodrugs as ideal drugs for selective delivery at the acidic tumor site [16,17]. LPZ induces remarkable apoptisis in breast cancer cells through their ability to induce cytosolic acidification, an increase in intracellular ATP level, lysosomal alkalinization, and ROS accumulation. In conclusion, LPZ might be a new potential therapeutic drug for breast cancer.

Acknowledgments

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