

Selective Induction of Apoptosis with Proton Pump Inhibitor in Gastric Cancer Cells

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ABSTRACT

Purpose: To survive in an ischemic microenvironment with a lower extracellular pH, ability to up-regulate proton extrusion is critical for cancer cell survival. Gastric H⁺/K⁺-ATPase exchanges luminal K⁺ for cytoplasmic H⁺ and is the enzyme primarily responsible for gastric acidification. On the basis of the fact that blocking the clearance of acidic metabolites are known to induce the cell death, we hypothesized that pantoprazole (PPZ), one of gastric H⁺/K⁺-ATPase inhibitors used frequently to treat acid-related diseases, could inhibit growth of tumor cells.

Experimental Design: Genomic DNA fragmentation, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling assay, and annexin V staining were performed to detect PPZ-induced apoptosis. Mitogen-activated protein kinase activation and heat shock proteins expression were determined by immunoblot with specific antibodies. The antitumor effect of PPZ was evaluated *in vivo* by a xenograft model of nude mice.

Results: After PPZ treatment, apoptotic cell death was seen selectively in cancer cells and was accompanied with extracellular signal-regulated kinase deactivation. By contrast, normal gastric mucosal cells showed the resistance to PPZ-induced apoptosis through the overexpression of anti-apoptotic regulators including HSP70 and HSP27. In a xenograft model of nude mice, administration of PPZ significantly inhibited tumorigenesis and induced large-scale apoptosis of tumor cells.

Conclusions: PPZ selectively induced *in vivo* and *in vitro* apoptotic cell death in gastric cancer, suggesting that proton pump inhibitors could be used for selective anticancer effects.

INTRODUCTION

The H⁺/K⁺-ATPase of gastric parietal cell exchanges luminal K⁺ for cytoplasmic H⁺ and is the enzyme primarily responsible for gastric acidification (1–3). The enzyme consists of two subunits, a 114 kDa α -subunit and a 35 kDa β -subunit. The α -subunit containing ATP and cation binding sites carries out the catalytic and transporting function of the proton pump. The heavily glycosylated β -subunit is required for endocytic retrieval of the H⁺/K⁺-ATPase from the canalicular membranes and is also essential for protecting proton pump from the environment of acid milieu. Because abnormally controlled gastric acids secreted by H⁺/K⁺-ATPase caused several gastrointestinal acid-related diseases including gastroesophageal reflux disease, gastric ulcer, duodenal ulcer, and Barrett's esophagus, gastric proton pump inhibitors have been developed as the treatment for these acid-related diseases (4–7). Pantoprazole (PPZ) of these proton pump inhibitors, a substituted 2-pyridyl methyl/sulfinyl benzimidazole derivative, is a prodrug requiring protonation for functional activation at acidic conditions, accumulating selectively in acidic gastric luminal space and ultimately inhibiting acid secretion by the covalent binding with cysteine residues in α -subunit of H⁺/K⁺-ATPase.

Besides of gastric H⁺/K⁺-ATPase, several kinds of vacuolar-type H⁺-ATPase are ubiquitously found on the membrane of various intracellular compartments of eukaryotic cells such as lysosomes, endosomes, the Golgi complex, and secretory granules (8). Essential regulation of pH in cytoplasmic, intraorganelar, and local extracellular spaces through vacuolar-type H⁺-ATPase has been suggested to play an important role in the mechanism of cell survival. These facts can be credited by several studies showing that vacuolar-type H⁺-ATPase inhibitor, bafilomycin A or concanamycin A, strongly induced apoptotic cell death (9–12).

Cell survival and cell death are tightly controlled by numerous signal enzymes and regulators such as mitogen-activated protein kinases (MAPKs) and heat shock proteins (HSPs). MAPK signal enzymes are divided into three major groups, extracellular signal-regulated kinases (ERKs), c-Jun NH₂-terminal kinases (JNKs)/stress-activated kinases, and p38 (13, 14). The ERKs appear to play a crucial role in the process of extracellular signals to the nucleus leading to induction of cellular growth, proliferation, and differentiation (15, 16). Heat shock proteins, which function mainly as molecular chaperones, allow cells to adapt to their environmental changes and to survive in otherwise lethal conditions (17–23). Because HSPs include pro- and antiapoptotic proteins that interact with a variety of cellular proteins, the type of HSP induced and its level of expression can determine the fate of a cell in response to a death stimulus. Generally, HSP60, HSC70, and HSP90 are constitutively expressed in mammalian cells, whereas HSP70 and HSP27 are strongly induced by different stresses, such as heat, oxidative stress, or anticancer drugs. It is well known that

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HSP27 and HSP70 play a cytoprotective role in gastrointestinal damage including apoptosis (24–27).

Maintenance of intra- or extracellular pH is very much important for cell function, and cancer cells *in vivo* often exist in an ischemic microenvironment with a lower extracellular pH than surrounding normal cells (28–33). The acidity in tumors is due to the increased production of acidic metabolites from rapid and large amounts of glycolysis and is provoked by the limited ability of the tumor vasculature to remove these acidic products. To overcome this hypoxic microenvironment and to prevent the accumulation of the increased acidic metabolites, the ability to dispose of intracellular protons is critical for cancer cell survival (34–37). These findings support the rationale of the present study that the inhibition of proton extrusion might be more susceptible or vulnerable to cell death of cancer cells than normal cells.

In this study, we have demonstrated for the first time that PPZ, the H^+/K^+ -ATPase inhibitor, induced apoptosis selectively in gastric cancer cells and significantly inhibited tumorigenesis in a tumor xenograft model. We also documented the mechanism of the selectivity of this proton pump inhibitor on apoptosis of cancer cells. Our novel finding suggests that proton pump inhibitors could be considered as the selective anticancer agents in gastric cancers.

MATERIALS AND METHODS

Cell Culture and Reagents. Human gastric cancer cell lines (AGS, Kato III, SNU-1, SNU-601, MKN-28, and MKN-45) were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine and 100 units/mL penicillin in a humidified 5% CO_2 atmosphere. As counter cells of these cancer cells, we cultured normal rat gastric mucosal RGM-1 cells and normal rat intestinal epithelial IEC-6 cells, which were maintained with DMEM-F12 and DMEM, high glucose (Life Technologies, Inc.) supplemented with 10% bovine insulin, respectively, and COS-1 cell, normal human fibroblast cells with RPMI 1640.

Solutions of PPZ and omeprazole were obtained from Altana Pharma AG (Konstanz, Germany) and AstraZeneca, respectively, and lansoprazole (Takeda, Japan) was solved in PBS (adjusted pH 2.0) with HCl overnight at room temperature.

Measurements of Intracellular pH. Intracellular pH was measured in the monolayers using the pH-sensitive fluorescent probe 2',7'-bis-(2-carboxyethyl)-5-carboxyfluorescein (BCECF). Cells were loaded with BCECF for 10 minutes at room temperature in solution A containing 2.5 μ mol/L BCECF-AM and mounted in the miniature Ussing chamber described for $[Ca^{2+}]_i$ measurements. BCECF fluorescence was recorded and calibrated using a protocol described previously (38). Briefly, the fluorescence at excitation wavelengths of 490 and 440 nm was recorded using a recording setup (Delta Ram; PTI Inc., St Louis, MO), and the 490:440 ratios were calibrated intracellularly by perfusing the cells with solutions containing 145 mmol/L KCl, 10 mmol/L HEPES, and 5 μ mol/L nigericin with the pH adjusted to 6.2 to 7.6.

Detection of Apoptosis. Induction of apoptosis was determined by assaying a genomic DNA fragmentation. Briefly, cells were lysed for 15 minutes in 10 mmol/L Tris.Cl (pH 7.4), 5 mmol/L EDTA, and 1% Triton X-100 and centrifuged at

12,000 rpm for 15 minutes. The supernatant was incubated with 0.1 mg/mL proteinase K at 37°C for 1 hour and extracted with an equal volume of phenol-chloroform, and the cellular DNA was precipitated with 1:10 volumes of 0.3 mol/L sodium acetate and 2 volumes of absolute EtOH overnight at $-70^\circ C$. The precipitate was dissolved in 20 μ L TE buffer containing 200 μ g/mL RNase and incubated for 1 hour at 37°C. The extracted DNA was resolved on 1.8% agarose gel and stained with ethidium bromide.

Caspase-3 and poly(ADP-ribose) polymerase (PARP) proteolysis were assessed by immunoblotting with specific antibodies (Cell Signaling Technology, Beverly, MA). Terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling (TUNEL) and annexin V/propidium iodide staining were detected by Fluorescent FragEL DNA fragment detection kit (Oncogene, Boston, MA) and annexin V-FITC apoptosis detection kit (BD Biosciences, San Diego, CA), according to the manufacturer's instructions, respectively.

Western Blot Analysis. Total proteins were extracted from PPZ-treated cells electrophoresed on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes using a semidry transfer system (Hoeffer Phamacia Biotech, San Francisco, CA). Membranes were blocked in 5% nonfat dry milk and probed with specific antibodies corresponding to phospho-p38 (Cell Signaling Technology, Beverly, MA), phospho-ERK, phospho-JNK, HSP70, HSP60, or HSP27 (all from Santa Cruz Biotechnology, Santa Cruz, CA), respectively.

Immunofluorescence Staining. Dispersed single cells (2×10^5 cells per well) were grown on $22 \times 22 \times 1$ mm³ glass coverslips in 6-well culture plates. After 24-hour culture, cells were fixed in ice-cold methanol for 5 minutes in a $-20^\circ C$ freezer and permeabilized with 1% Triton X-100/PBS for 10 minutes at room temperature. The cells were blocked with 5% bovine serum albumen for 30 minutes and probed with anti- α subunit of H^+/K^+ -ATPase antibodies (1:100 diluted in 5% bovine serum albumen, Santa Cruz Biotechnology) for 2 hours. Cy3-conjugated secondary antibodies were used to visualize under a confocal microscope (BX50F, Olympus, Japan).

Tumor Xenograft. Subconfluent MKN-45 cells were dissociated with 0.25% trypsin and 1 mmol/L EDTA (Life Technologies, Inc.) and suspended in PBS at density of 5×10^7 cells/mL. Each mouse was s.c. inoculated with MKN-45 cells (5×10^6 per site) on the left and right side of the back on day 0. The animals were randomly divided into two groups (9 per group). Group A received an intratumoral injection of PBS daily from day 14; Group B daily received an intratumor injection of 0.4 mg/kg PPZ daily from day 14. The shortest and longest diameters of the tumor were measured with calipers at 2-day intervals, and the volume of each tumor (mm³) was calculated. Mice were sacrificed at day 22, and isolated tumor tissues were analyzed for microscopic gross finding and TUNEL stain. These studies were approved by the Institutional Animal Care and Use Committee and complied with the highest international criteria for human use of animals in research.

RESULTS

Human Gastric Cancer Cells Were More Tolerant of Acidity in the Culture Media Than Normal Cells. To determine whether cancer cells tolerate acidic conditions better

than noncancer cells, we cultured several gastric cancer cell lines (AGS, KATO III, MKN-28, MKN-45, SNU-1, and SNU-601) and noncancer cell lines (RGM-1, IEC-6, and COS-1) in media maintained at different pHs, including 7.4, 6.9, 6.5, 5.9, and 5.4. The cancer cells adapted well to lower pH, whereas the normal cells were much less tolerant of acidity in the culture media (Fig. 1A). At pH 5.4, most of cancer cell lines tested showed >80% viability, but normal cell line RGM-1 significantly decreased viability by 21.6%.

To investigate whether this difference in cell survival at lower pH was due to the ability to dispose or disperse H^+ , we immunocytochemically stained two cancer cells (AGS and MKN-45) and two noncancer cells (RGM-1 and IEC-6) with antibodies against the gastric proton pump, α subunit of H^+/K^+ -ATPase. We found that there was a much larger amount of proton pump in cancer than noncancer cells (Fig. 1B). Immunofluorescence images showed a predominant expression of the α subunit of H^+/K^+ -ATPase in the membrane and cytoplasm of cancer cells. These results indicated the enhanced ability to dispose of H^+ due to overexpression of H^+/K^+ -ATPase, which might contribute to cancer cell survival in an acidic microenvironment.

Treatment of PPZ Significantly Inhibits Cancer Cell Viability in a pH-Dependent Manner. Because PPZ is a protonatable weak base, which can convert to active form in an

acidic environment with a low pH, we tested the effects of PPZ on cell growth in culture media maintained at various pHs (Fig. 2). Nevertheless, cancer cells showed tolerance of acidity in culture media as shown in Fig. 1A, and administration of the proton pump inhibitor PPZ led to significant reduction of cancer cell survival (Fig. 2A). At pH 5.0, PPZ treatment reduced cell viability by 6.9% on average in MKN-45 cancer cells (Fig. 2A). On the contrary, normal cell line RGM-1 did not show a significant difference in cell viability between the PPZ-treated and nontreated groups (data not shown). Despite resistance of cancer cells to the acidic environment, cancer cells were much more susceptible to growth inhibition of PPZ at a lower pH.

We evaluated whether this attenuation of cancer cell viability is induced by a decrease of intracellular pH due to the blocking of disposal of intracellular H^+ by specific protonation of this drug. As shown in Fig. 2B, PPZ treatment significantly increased acidity of intracellular pH in AGS, MKN-28, and MKN-45, but SNU-601 did not show a remarkable change (Fig. 2B). Of note, AGS decreased intracellular pH from 7.6 to 7.2. These findings showed that PPZ-induced cell death was caused by the specific protonation of this proton pump inhibitor.

We also investigated whether other proton pump inhibitors such as omeprazole and lansoprazole has a similar effect to pantoprazole on cancer cell viability. Human gastric MKN-45 cells were cultured with each proton pump inhibitor in pH 6.0 of

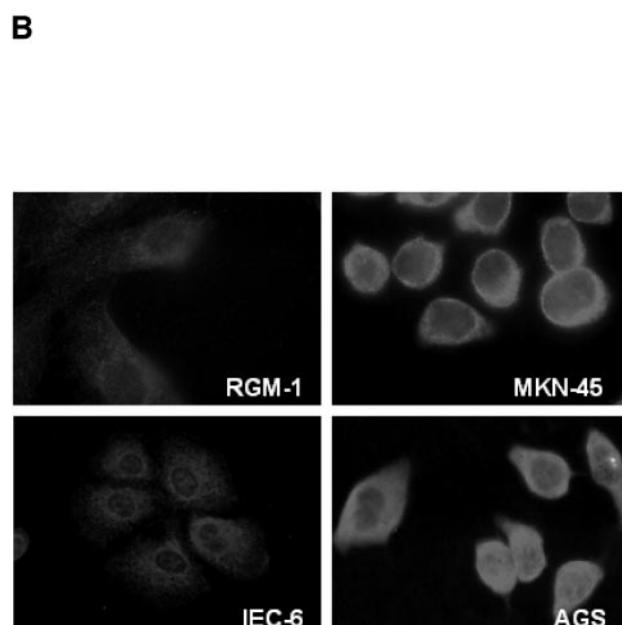
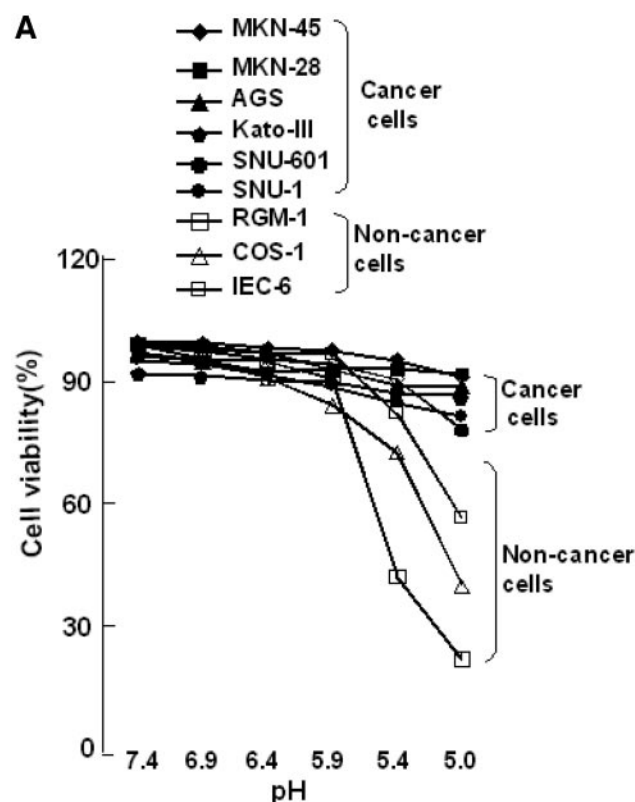


Fig. 1 Effects of pH of culture medium on cell viability and cellular expression of H^+/K^+ -ATPase. Human gastric cancer cell lines (MKN-45, MKN-28, AGS, Kato III, SNU-601, and SNU-1) and noncancer cell lines (RGM-1, Cos-1, and IEC-6) were cultured in media at different pH for 24 hours, and cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (A). Expression of H^+/K^+ -ATPase, α -subunit was detected with specific antibodies and visualized with Cy3-conjugated secondary antibodies. Note that the α -subunit of H^+/K^+ -ATPase was more highly expressed in the plasma membrane and cytoplasm of cancer than noncancer cells (B).

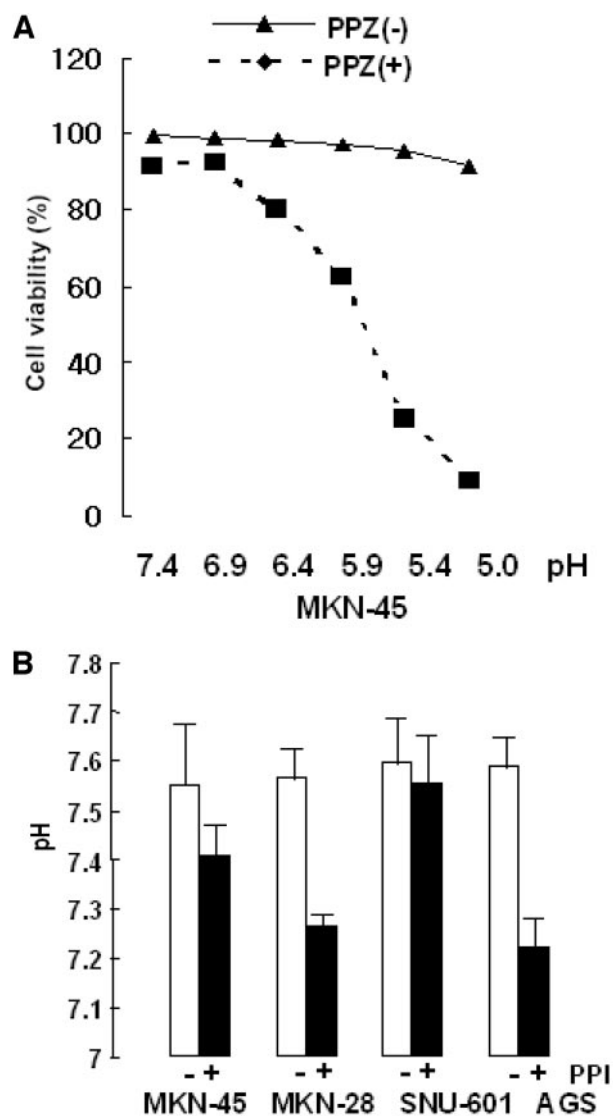


Fig. 2 Inhibition of cancer cell viability by PPZ treatment. In different pH media (7.4, 6.9, 6.4, 5.9, or 5.0), gastric MKN-45 cancer cells were incubated in the presence or absence of 0.5 mmol/L PPZ, and cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (A). Although MKN-45 showed resistance to acidity of culture media, PPZ significantly decreased this cancer cell viability in a pH-dependent manner. To evaluate effect of PPZ on intracellular pH (*pHi*) of cancer cell, gastric cancer cells were plated in a 6-well culture dish, treated with 0.5 mmol/L PPZ for 16 hours, and measured according to Materials and Methods as described previously (B).

media for 24 hours, and cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The results showed that whereas the PPZ or omeprazole-treated cells remarkably reduced cell viability in a dose-dependent manner, lansoprazole did not show any changes in cancer cell viability (Fig. 3). This difference suggested that the decreased cell viability comes from the specific effect of proton pump inhibition. Omeprazole and PPZ are available for parenteral injection, but lansoprazole is only available for oral administra-

tion, suggesting that lansoprazole lost its proton pump inhibiting ability in aqueous form

PPZ Selectively Induced Apoptotic Cell Death in Human Gastric Cancer Cells. To determine whether PPZ preferentially induces apoptosis in cancer cells, we administered this agent to two cell lines, a normal gastric mucosal cell line, RGM-1, and a gastric cancer cell line, MKN-45 (Fig. 4). Although normal gastric mucosal RGM-1 cells significantly reduced cell viability in a low pH media as shown in Fig. 1A, an apoptotic genomic DNA fragmentation was detected by neither pH change nor PPZ treatment (Fig. 4A). However, human gastric cancer cell MKN-45 showed a significant apoptotic genomic DNA fragmentation by PPZ treatment, but acidity of culture media could not induce DNA fragmentation. As shown by a genomic DNA fragmentation, caspase-3 and its substrate, PARP, were cleaved by PPZ treatment in a dose-dependent manner in gastric cancer cells but not detected in normal gastric mucosal cell (Fig. 4B). PPZ also induced alteration of phosphatidylserine distribution and permeability of plasma membrane only in cancer that which were detected by annexin V/propidium iodide staining (Fig. 4C). These results indicated much higher vulnerability and selective sensitivity to apoptosis in gastric cancer MKN-45 cells than normal mucosal RGM-1 cells.

PPZ Suppressed ERK Phosphorylation in Human Gastric Cancer Cells. To assay the involvement of MAPKs in PPZ-induced apoptosis, phosphorylation of ERKs, JNKs/stress-activated kinases, and p38 after PPZ treatment was detected

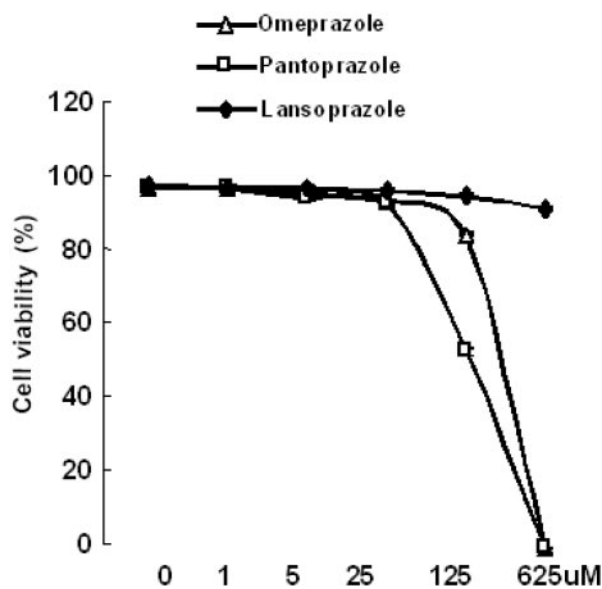


Fig. 3 Effect of proton pump inhibitors on gastric cancer cell viability. To compare effect of other proton pump inhibitors (omeprazole and lansoprazole) with PPZ on cancer cell viability, MKN-45 cells were cultured in the presence of each proton pump inhibitor indicated at pH 6.0 for 24 hours and were measured cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability (%) was calculated by formula as follows: Cell viability (%) = (sample absorbance - total absorbance)/(spontaneous absorbance - total absorbance) \times 100. Total absorbance was obtained from absorbance of 0.1% Triton X-100-treated cells as negative control, and spontaneous absorbance indicated absorbance of PPZ-untreated control cells.

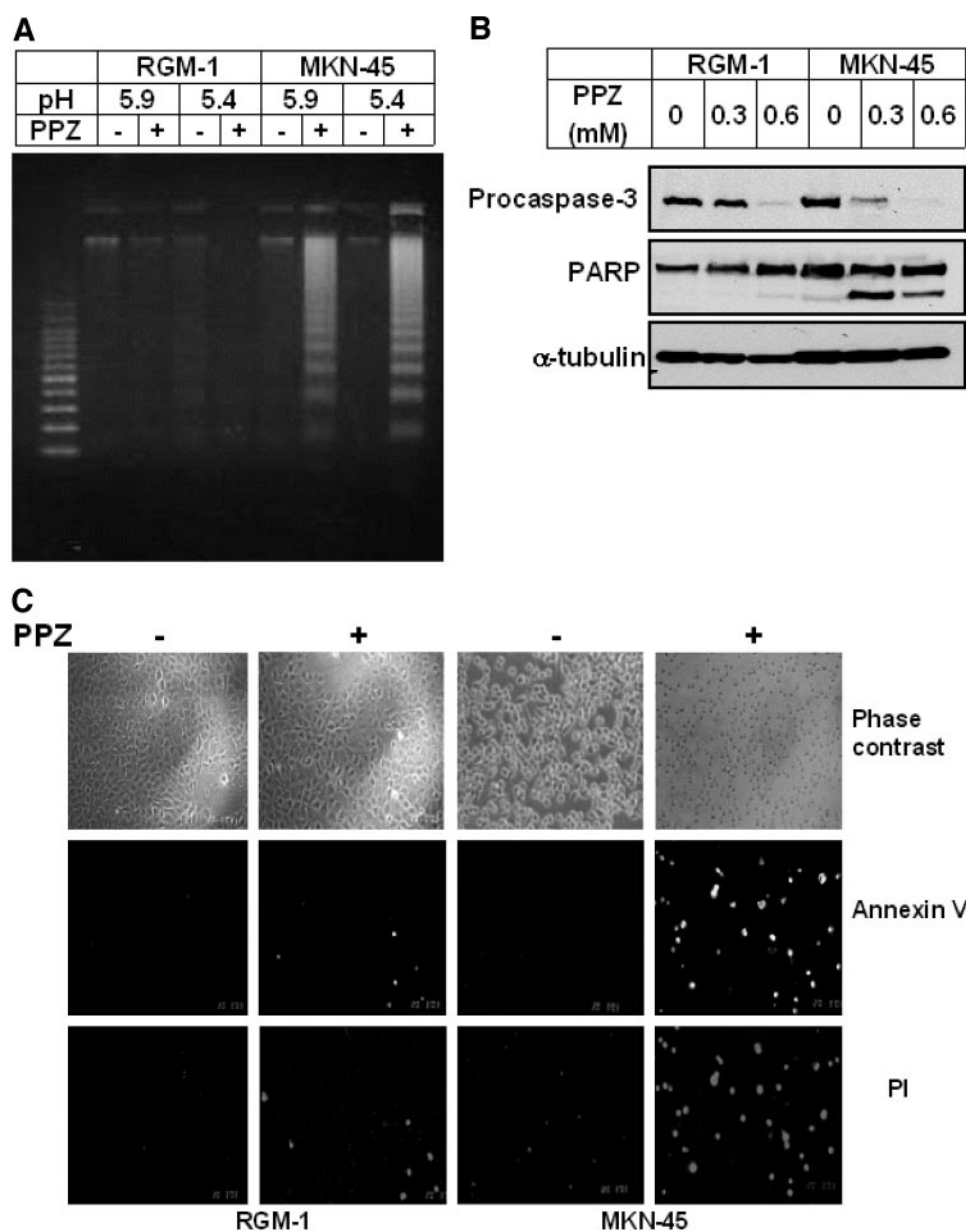


Fig. 4 Selective induction of apoptosis with PPZ. Cells were cultured in the presence or absence of PPZ for 24 hours, and induction of apoptosis was detected by genomic DNA fragmentation (A), caspase-3 activation and PARP cleavage (B), and localization of annexin V and propidium iodide (C). Annexin V-positive cells were stained *green*, and *red* represented propidium iodide-positive cells.

(Fig. 5A and B). In MKN-45 cells, PPZ significantly and selectively inhibited the phosphorylation of ERK and increased the phosphorylation of p38 but had no apparent effects on the phosphorylation of JNK. After 8-hour treatment of 0.5 mmol/L PPZ, ERK phosphorylation was completely inhibited in MKN-45 cells despite an equal amount of total proteins controlled by α -tubulin (Fig. 5A). In addition, inhibition of p38 by SB203580 blocked PPZ-induced apoptosis in MKN-45 cells (data not shown). As shown in Fig. 5B, RGM-1 cells did not change the degree of phosphorylation of ERK and p38, but phosphorylation of JNK was significantly decreased in a time-dependent manner. Taken together, these findings suggest that inhibition of ERK phosphorylation may be responsible for the attenuation of cancer cell survival, whereas activation of p38 contributes to proton pump inhibitor-induced apoptosis.

Overexpression of HSP27 and HSP70 Played a Cytoprotective Role in PPZ-Induced Apoptosis of Normal Gastric Mucosal Cells. Interestingly, we found that HSP70 and HSP27 were overexpressed in RGM-1 after PPZ treatment but not in MKN-45 cells (Fig. 6A and B). Notably, treatment of 0.6 mmol/L PPZ in RGM-1 cells showed a 3.3- and 36.3-fold increase of HSP27 and HSP70 compared with that of nontreated cells, respectively (Fig. 6C). However, HSP27 and HSP70, as well as HSP60, were not grossly changed in PPZ-treated gastric cancer MKN-45 cells (Fig. 6A). To compare the HSP induction, we also treated geranylgeranylacetone (GGA), which is well known as a HSP inducer in gastric mucosal cells. GGA slightly increased HSP60 and HSP27 in MKN-45 cell, but decreased HSP60 and HSP27 in RGM-1. In comparison with GGA, the ability of PPZ to induce HSP70 is much higher at 5.6-fold than

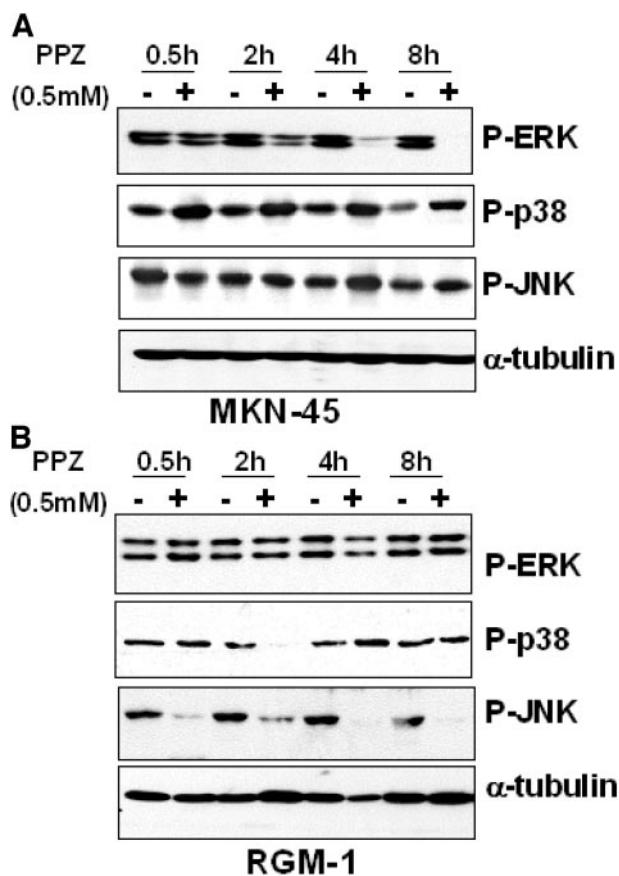


Fig. 5 Distinct MAPK signaling in cancer cells (A) and noncancer cells (B) by PPZ. Cells were treated in the presence or absence of 0.5 mmol/L PPZ for various times, and total proteins were extracted from the cells, electrophoresed on 12% SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes. The membranes were probed with specific antibodies for p-ERK, p-p38, p-JNK, and α -tubulin, respectively.

GGA. These data suggest that overexpression of HSP27 and HSP70 might play a cytoprotective role in PPZ-induced apoptosis of normal gastric mucosal cells.

In a Xenograft Model of Nude Mice, Administration of PPZ Significantly Inhibited Tumorigenesis and Induced Large-Scale Apoptosis of Tumor Cells. We also evaluated the antitumorigenic effects of PPZ in a human gastric cancer xenograft model (Fig. 7). The animals were randomly divided into two groups: an intratumoral injection of PBS and an intratumoral injection of 0.4 mg/kg PPZ. Intratumor administration of PPZ significantly suppressed tumor growth in athymic nude mice, with decreases in tumor volume at day 22 of 44.69% compared with mice injected with PBS (Fig. 7A and B). The isolated tumor from mice with intratumor administration of PPZ was remarkably smaller than that of mice intratumorally injected with PBS (Fig. 7C). Histopathological examination revealed that PPZ treatment provoked considerable apoptosis. Only a few tumor cells survived, and most tumor cells were replaced by apoptotic cells (Fig. 7D). TUNEL staining also showed considerably higher apoptotic cell death in the PPZ-treated group compared with the control group (Fig. 7E).

DISCUSSION

Because the ultimate aim of anticancer treatment is to kill only the cancer cells, the therapeutic efficiency of anticancer agents could be increased by specificity and selectivity to cancer cells. Current understanding of anticancer strategy has led to attempts to screen for agents that selectively increase apoptosis in cancer cells or to use apoptosis pathways specific for tumor cells. Therefore, our current findings suggest that the gastric proton pump inhibitor PPZ has very considerable advantages as anticancer treatment based on the following novel findings. First, PPZ selectively induced apoptosis in cancer cells, and this PPZ-induced apoptosis may be caused by suppressing ERK phosphorylation. This H^+/K^+ -ATPase inhibitor simultaneously stimulated phosphorylation of p38 as well as deactivation of ERK in a dose- and time-dependent manner only in cancer cells. The different regulation of the individual MAPK subfamily by the proton pump inhibitor seems to be better considered as anticancer agents with high selectivity to cancer, because generally p38 is involved in apoptosis and stress signaling pathway, whereas ERK is activated by stimuli of cell growth and differentiation. Second, noncancer cells have mechanisms to counteract the PPZ-induced apoptosis by the induction of antiapoptotic molecules HSP70 and HSP27. We observed that PPZ induced significant amounts of HSP 70 much more than GGA, an agent generally known to induce HSP70 as a key action. Thus, the H^+/K^+ -ATPase inhibitor may act to selectively induce apoptotic cell death in cancer cells without having any significantly adverse effects on the surrounding noncancer cells, and the feature of this agent contributes to safety in an aspect of clinical application. Third, PPZ would be selectively converted into its active form in cancer cells due to the conversion of the active form under the hypoxic and acidic condition, resulting in induction of significant apoptosis. Just like in the canaliculi of parietal cell, pH of inner space of the tumor was reported to be below 6.8 (32, 33). The finding shown in Fig. 2 showed that with lower pH of media, higher activities of apoptosis were observed. In all, these results suggest that PPZ can be used clinically as an anticancer agent.

One of the well-known properties of cancer cells is aerobic glycolysis, which may cause tumor acidification (39–41). The cancer cell will take in glucose and form lactic acid, which will largely dissociate into the lactate ion and proton (H^+). The lactate ion and H^+ produced from the glycolysis are effluxed into the extracellular fluid leading to low extracellular pH and high intracellular pH of cancer. There are some mechanisms involved in the regulation of tumor pH; the main mechanism by which proton (H^+) is exported is by the sodium-hydrogen antiport, using the energy of the Na^+ gradient (42, 43). Tumor cells may possess an additional mechanism for H^+ export via a vacuolar H^+ -ATPase (V-ATPase) of plasma membrane, the bicarbonate transporter, and the proton-lactate symporter (36, 44). In the present study, we used the H^+/K^+ -ATPase inhibitor for blocking the H^+ export of tumor cells. The H^+/K^+ -ATPase inhibitor successfully suppressed tumor cell viability by inducing apoptotic cell death. Thus, these findings implicate that blockage of another

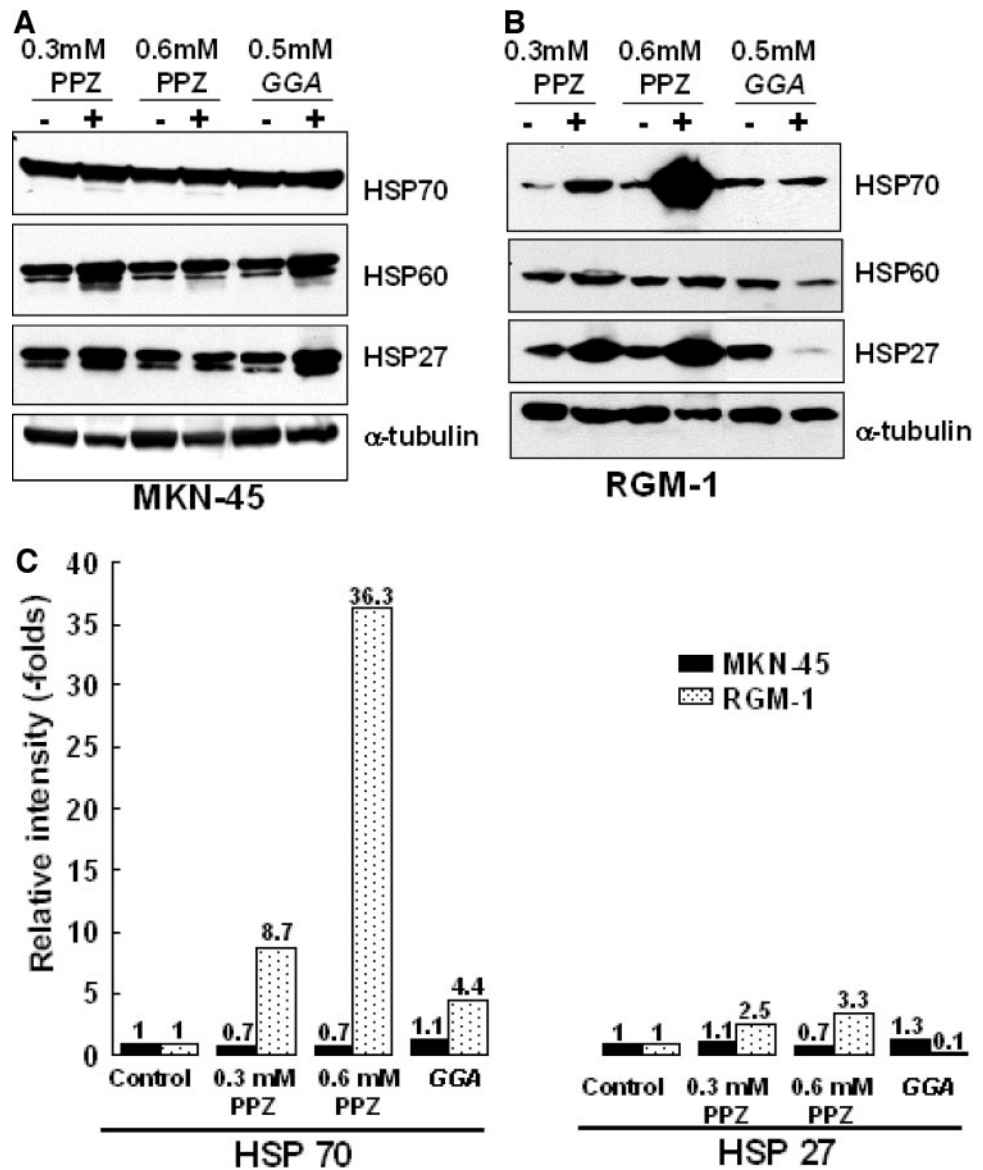


Fig. 6 Effect of PPZ on expression of HSP70 and HSP27 in gastric cancer and noncancer cells. RGM-1 (A) and MKN-45 cells (B) were treated with PPZ or generylgenerylacetone (GGA) for 24 hours, and expression of HSP70, HSP60, and HSP27 was assayed by immunoblotting with specific antibodies. Note that PPZ induced expression of HSP70 and HSP27 in RGM-1 normal gastric mucosal cells but not in MKN-45 gastric cancer cells. Relative intensities of HSP70 and HSP27 compared with nontreated control cells were represented in C.

kind of proton pump predominantly expressed in tumor cells could be used as a promising anticancer drug.

Several inhibitors have been found to interact with vacuolar-type H^+ -ATPase, interfering with both ATP hydrolysis and proton translocation activities (45–47). These inhibitors can be divided largely into two classes: inhibitors acting at a soluble cytoplasmic domain (N-ethylmaleimide and 4-nitrobenzo-2-oxa-1,3-diazole chloride) and inhibitors acting at transmembrane sites (dicyclohexyl-carbodiimide, Bafilomycin A1, and Concanamycin A). Several research groups have already reported that these inhibitors of vacuolar-type H^+ -ATPase can induce apoptotic cell death in several human cancer cell lines including pancreatic cancer, hepatocellular carcinoma, and B-cell hybridoma cells (9–12, 48). They also proved the involvement of cytochrome C release and caspase activation in vacuolar-type H^+ -ATPase inhibitor-induced ap-

optosis. The specific inhibitors of mammalian vacuolar-type H^+ -ATPase belonging to the benzolactone enamide class, such as salicylhalamide, lobatamides, and oximidines, were developed and appear promising as anticancer agents (49, 50). However, some reports are contradicting the proapoptotic effect of the proton pump inhibitor, suggesting that the inhibitor of mitochondria F_0F_1 -ATPase proton pump, oligomycin, prevented apoptotic cell death (51–53).

However, there remain two limitations for anticancer application of a vacuolar-type H^+ -ATPase inhibitor like concanamycin A or bafilomycin A. The first one is nonselectivity of apoptosis of vacuolar-type H^+ -ATPase inhibitor, and the second problem is that the vacuolar-type H^+ -ATPase gene is considered a “housekeeping gene” expressed indiscriminately on every cell (54). Hence, there might be similar cytotoxicity provoked by current cytotoxic anticancer drugs. The feasibility of clinical application of these factors

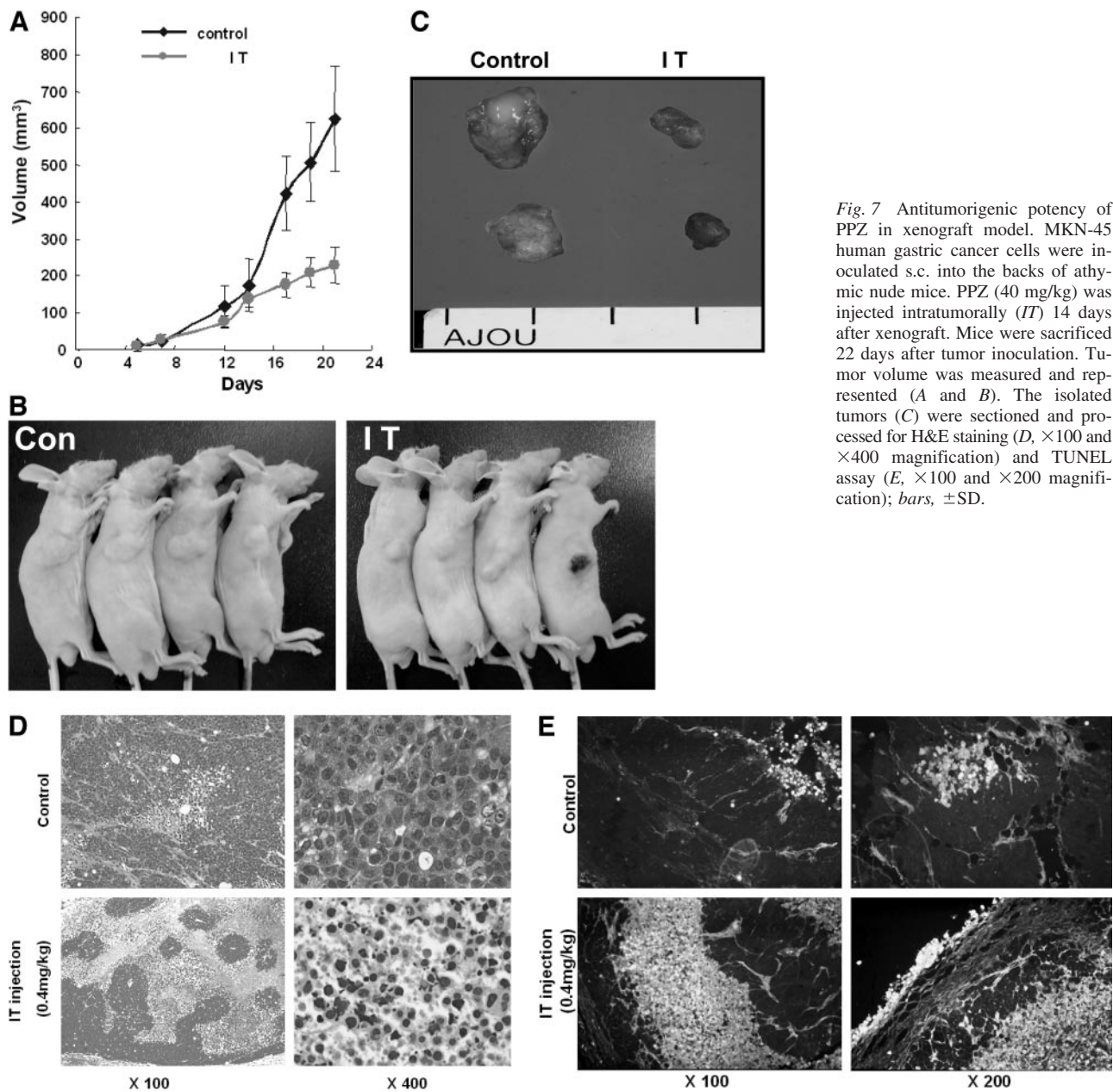


Fig. 7 Antitumorogenic potency of PPZ in xenograft model. MKN-45 human gastric cancer cells were inoculated s.c. into the backs of athymic nude mice. PPZ (40 mg/kg) was injected intratumorally (IT) 14 days after xenograft. Mice were sacrificed 22 days after tumor inoculation. Tumor volume was measured and represented (A and B). The isolated tumors (C) were sectioned and processed for H&E staining (D, $\times 100$ and $\times 400$ magnification) and TUNEL assay (E, $\times 100$ and $\times 200$ magnification); bars, \pm SD.

is difficult despite strong apoptotic-inducing capability. On the other hand, PPZ showed selective induction of apoptosis in cancer cells. It rendered noncancerous cells escaping from apoptotic activities through the induction of antiapoptotic signaling molecules. Moreover, the fact that the proton pump inhibitor is a prodrug requiring protonation after administration brings more hope that protonation is more easily performed within tumor tissues.

In conclusion, our findings provide novel mechanistic insight into the anticancer target of gastric proton pump, H^+/K^+ -ATPase, and expand the repertoire of clinical use of gastric proton pump inhibitor as an anticancer drug by implicating the selective induction of apoptosis in cancer cells.

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