Effect of pantoprazole on human gastric adenocarcinoma SGC7901 cells through regulation of phospho-LRP6 expression in Wnt/β-catenin signaling

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Abstract. Recent studies have found that an acidic tumor microenvironment is the key to managing cancer progression and metastasis. Our previous study found that proton pump inhibitors (PPIs) inhibit the expression of vacuolar-ATPases (V-ATPases) and reverse the transmembrane pH gradient. The present study was conducted to explore the effect of pantoprazole on gastric adenocarcinoma through the regulation of Wnt/β-catenin signaling. We used SGC7901 human gastric cancer cells as an *in vitro* model to study the effect of pantoprazole. The antiproliferative, pro-apoptotic and anti-invasive effects of pantoprazole were examined. The effects of pantoprazole on the expression of the Wnt/β-catenin signaling pathway were also studied by western blotting. Our study found that pantoprazole inhibited the proliferation and induced the apoptosis of SGC7901 human gastric cancer cells. The expression of V-ATPases was decreased following treatment with pantoprazole. Further study found that pantoprazole treatment caused a decrease in phospho-LRP6, but not in LRP6. β-catenin in Wnt/β-catenin signaling and its target genes c-Myc and cyclin D1 were also decreased upon the inhibition of V-ATPases. Therefore, pantoprazole could be characterized as a V-ATPase inhibitor for treating gastric cancer by inhibiting the phosphorylation of LRP6 in Wnt/β-catenin signaling.

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Introduction

Gastric cancer is the leading cause of cancer-related mortality in China and is the third leading cause of cancer-related mortality in North America and Western Europe (1). Previous studies have found that an acidic tumor microenvironment is key to managing cancer progression and metastasis (2-4). Vacuolar-ATPases (V-ATPases), specific proton pumps of the cell, have an important role in maintaining a relatively neutral intracellular pH (pHi), an acidic luminal pH, and an acidic extracellular pH (pHe). They are overexpressed in many types of metastatic cancers and are positively correlated with invasive and metastatic tumor potential (5). Furthermore, blocking the expression of V-ATPases can inhibit the growth and metastasis of human cancer (6).

Some molecules and drugs that inhibit V-ATPases have been identified (7), such as bafilomycin, concanamycin and NiK-12192, but their toxic effect and poor results in preclinical tests have limited their development as therapeutic agents. Recent insight into the mechanism of tumor acidification has provided new strategies for targeting V-ATPases (8). Proton pump inhibitors (PPIs) could represent a class of drugs suitable to this purpose (9). PPIs have demonstrated gastric acid suppression and have been applied in acid-related diseases generally with good safety and few side effects. A specific target of PPIs is K^+/H^+-ATPase which is located in gastric parietal cells. Moreover, our previous study found that PPIs can inhibit the expression of V-ATPases, and reverse the transmembrane pH gradient (10).

Among the cancer-related signaling pathways, the canonical Wnt pathway, also known as the Wnt/β-catenin pathway, is involved in gastrointestinal carcinogenesis. Wnt ligands engage their receptor complex, stabilize intracellular levels of β-catenin, and allow the nuclear accumulation of β-catenin, together with the transcription factor lymphoid enhancer-binding factor 1/T cell-specific factor, followed by transcriptional activation of Wnt/β-catenin target genes such as c-Myc and cyclin D1 (11). In this study, we explored the effect of a PPI on gastric carcinoma by regulating Wnt/β-catenin signaling.

Materials and methods

Drugs. Pantoprazole was purchased from Altana Pharma (Konstanz, Germany; AGD-78467). According to our previous study, a concentration >20 mg/ml of pantoprazole inhibited the proliferation of SGC7901 cells. Pantoprazole was resuspended in normal saline at 20 mg/ml immediately before use.

Cell line and cell culture. The human gastric adenocarcinoma cell line, SGC7901, was kindly provided by the Department of Oncology, Drum Tower Hospital of the Nanjing University Medical School. Cells were cultured in RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hangzhou Sijiqing Biological Engineering Materials, China) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in humidified air with a 5% CO_2 atmosphere at 37˚C (Direct Heat CO_2 incubator; Thermo Scientific, Rockford, IL, USA).

Transfection. Cells plated in 100-mm dishes were transfected at 50-80% confluence with V-ATPase expression vectors or with control vectors, using the liposome-mediated transfection method. SGC7901 cells were transfected with an shRNA-V-ATPase or negative control vector (GAPDH) for 2 days, then trypsinized and plated at low density. Stable clones were selected by maintaining cells in medium containing G418 antibiotic.

Cell viability assay. The cytotoxicity of pantoprazole was determined using the MTT (KeyGen Biotech Co., Ltd., China) assay. Cells (1x10^4/well) were plated in 96-well plates in 200 μl of medium/well and then treated with pantoprazole at 20 mg/ml. Control cells were treated with Dulbecco's modified Eagle's medium (DMEM). At different time points, 50 μl of 5 mg/ml MTT was added, and the cells were cultured for another 4 h. The supernatant was removed, and 150 μl of dimethyl sulfoxide (DMSO) was added per well. The absorbance at 570 nm was measured with a microplate reader (Tecan Sunrise, Switzerland), using wells without cells as blanks and untreated cells as a negative control. The viability of the drug-treated cells was expressed as a percentage of the population growth with standard error of the mean relative to that of the untreated control cells.

Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection. Apoptosis detection in cells was performed by the Annexin V-FITC and propidium iodide (PI) double staining apoptosis detection kit (KeyGen Biotech, Co. Ltd.) using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, the cells were trypsinized, washed with phosphate-buffered saline (PBS), centrifuged and resuspended with Annexin V binding buffer (500 μl). The cells were incubated with 5 ml Annexin V-FITC solution for 5 min at room temperature. In the same step, PI was added at 5 μg/ml (5 μl) for another 5 min to distinguish necrotic cells. The samples were analyzed within 1 h by fluorescence-activated cell sorter (FACS) with CellQuest software (version 3.3).

Colony formation assay. Cells (6x10^4/well) were plated in Petri dishes with a 6-cm diameter with 10 ml of DMEM (Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) (HyClone) in the presence or absence of various concentrations of pantoprazole at 37˚C. After culture for 10 days, colonies consisting of >50 cells were counted under the microscope.

Matrigel invasion assay. For the invasion assay, a modified Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) was used. The pore size of the polycarbonate filters was 8.0 μm. The bottom chamber of the Transwell chamber was filled with 30 μl RPMI-1640 containing 10% FBS. The cells were then suspended at a density of 1x10^5 cells/ml in 500 μl of RPMI supplemented with 0.5% FBS and pantoprazole, and added to 8-μm porous BioCoat Matrigel chamber inserts (BD Biosciences) and placed in wells filled with 0.7 ml of medium supplemented with 10% FCS as a chemoattractant. After 2 days of incubation, the upper side of the filter was scraped with a cotton tip to eliminate cells that had not migrated through it. To obtain the total apoptosis rate and inhibition rate by pantoprazole for 48 h, we calculated the number of cells which did not undergo apoptosis (Live cells = plated cells + proliferated cells - apoptotic cells). The invasive ability of the cells was determined by counting the cells that had migrated to the lower side of the filter with a microscope. The relative invasion rate was obtained using the following formula: Relative invasion rate = migrated cells/live cells. Experiments were performed in triplicate, and at least 10 fields were counted for each experiment.

Western blot analysis. Proteins were extracted in lysis buffer (30 mmol/l Tris, pH 7.5, 150 mmol/l sodium chloride, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l sodium orthovanadate, 1% Nonidet P-40, 10% glycerol and phosphatase and protease inhibitors). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4˚C, and then incubated with the secondary antibody. Images of the western blotting products were captured and analyzed by Quantity One v4.31 (Bio-Rad, Hercules, CA, USA).

Data analysis. Statistical comparisons were performed with the software package SPSS 13.0 using the Student's t-test for paired observations or one-way ANOVA with SNK, LSD and Dunnett's methods. All data are presented as means ± standard deviation (SD). $P<0.05$ was considered statistically significant. Mean values and SD were calculated for experiments carried out in triplicate.

Results

Growth inhibition of human SGC7901 cells by pantoprazole. The inhibitory activity of pantoprazole on the proliferation of human gastric cancer SGC7901 cells was investigated. Cells were grown in the absence or presence of pantoprazole (20 mg/ml) for 12, 24 and 48 h. MTT assays were then performed. The growth inhibition occurred in a time-dependent manner. The cell viability of SGC7901 cells in the PPI group (43.1±3.9%) was lower than that in the shRNA-GAPDH group (89±4.9%) at 48 h post treatment (Fig. 1A). The effect
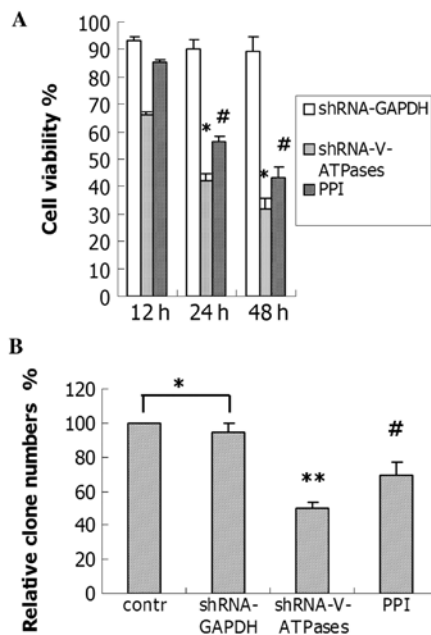
Figure 1. Pantoprazole suppresses the growth of SGC7901 cells. (A) Comparison of the cell viability of SGC7901 cells after being transfected with shRNA-GAPDH or shRNA-V-ATPases after 12, 24 and 48 h. SGC7901 cells were treated with pantoprazole (20 mg/ml) for 12, 24 and 48 h. Growth inhibition was determined by the MTT assay. (B) Colony formation assay. Cell colonies were counted after staining with crystal violet and are shown in the graph. The bars in the graph represent the means ± SD. Experiments were performed in triplicate.
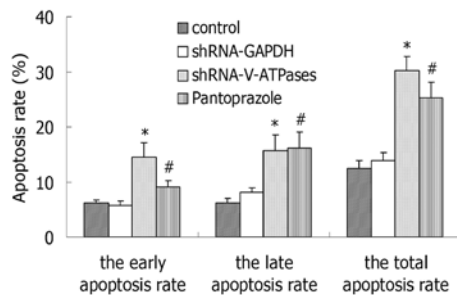

Figure 2. Comparison of the apoptosis rate of SGC7901 cells after treatment with pantoprazole. $^{*,#}P<0.05$, indicating that the apoptosis rate was significantly higher than that in the control group.
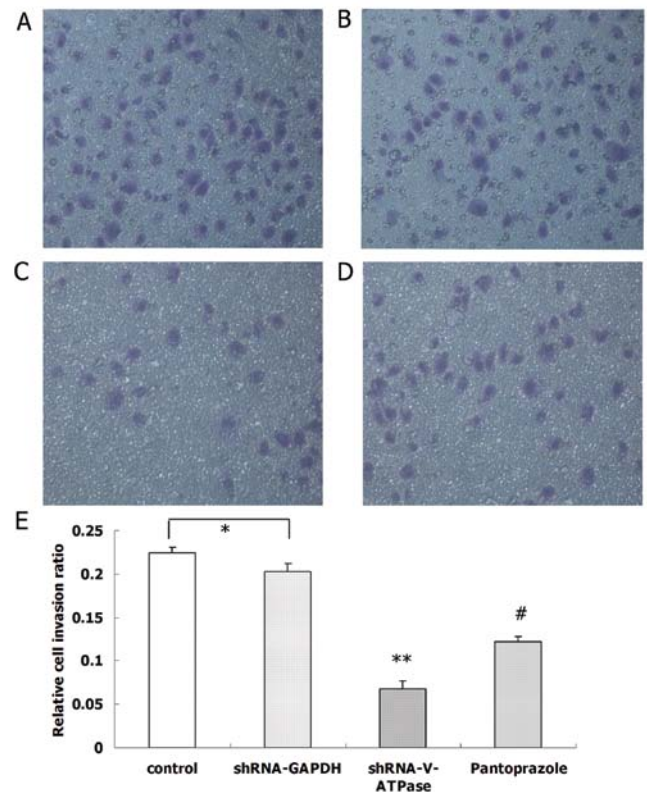

Figure 3. Effects of pantoprazole on the invasion of gastric cancer cells. Cell invasion 48 h after treatment with pantoprazole was evaluated in the Matrigel invasion assay as described in 'Materials and methods'. (A-D) Representative microscope images after pantoprazole treatment (magnification, x200). (A) Control without pantoprazole; (B) shRNA-GAPDH group; (C) shRNA-V-ATPase group; (D) 20 mg/ml pantoprazole group. (E) Comparison of the rate of SGC7901 cell invasion. Data are expressed as percent change (means ± SD) and represent three independent experiments. ($^{**,#}P<0.05$ vs. control; $^{*}P>0.05$ vs. control; one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison).

of pantoprazole on colony formation of the cells was also assessed. On day 10 post-treatment, pantoprazole suppressed the colony formation of the cells (Fig. 1B). These results suggest that pantoprazole preferentially inhibits the growth of SGC7901 cells.

Apoptosis detection. A quantitative analysis of the fluorescent signals was performed by FACS. As noted in Fig. 2, the total apoptosis was increased from 10.5% in the control cells and reached 25.3% following treatment with 20 mg/ml pantoprazole at 48 h. Treatment of SGC7901 cells showed a similar dose-dependent response pattern for the early and late apoptosis rates (Fig. 2).

Matrigel cell invasion assay. We observed a significant difference between SGC7901 cells with and without pantoprazole

treatment in the migration assay. Moreover, SGC7901 cell invasion was reduced by pantoprazole at 20 mg/ml (P<0.05) in the Matrigel invasion assay (Fig. 3).

Effects of pantoprazole on V-ATPases. To determine whether pantoprazole inhibits the expression of V-ATPases, SGC7901 cells were treated with 20 mg/ml pantoprazole and after 48 h were assessed for the presence of V-ATPase expression by immunofluorescence and western blotting.

After 48 h of pantoprazole treatment, the expression of V-ATPases was altered when compared with that in the control group (Fig. 4). The expression of V-ATPases in the pantoprazole group was significantly less than that in the control group (P<0.05), whereas no significant difference was found between the control group and the shRNA-GAPDH group.

Effects on LRP6 and downstream genes by pantoprazole. Research has demonstrated that phosphorylation of LRP6 (which correlates with LRP6 activation) requires V-ATPase activity, suggesting that the receptor may need to enter an acidic intracellular compartment to become phosphorylated. We tested whether pantoprazole inhibits the expression of phospho-LRP6 and its downstream gene, β-catenin. The expression levels of these proteins in SGC7901 cells treated
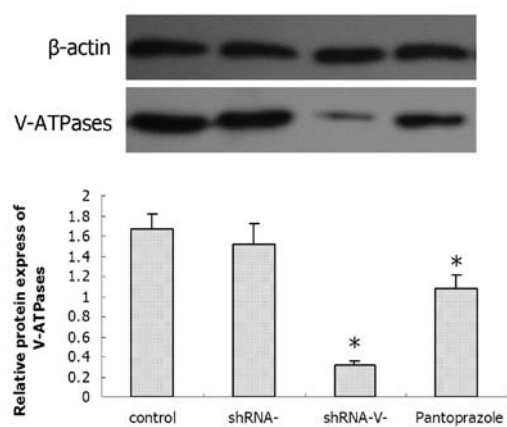
Figure 4. V-ATPase protein detection by western blot analysis. Effects of pantoprazole treatment on V-ATPase expression in SGC7901 cells at 48 h. *P<0.05, significant difference when compared with the control group.

Figure 6. The protein expression of β-catenin, c-Myc and cyclin D1 in SGC7901 cells treated with 20 mg/ml pantoprazole was significantly down-regulated. *P<0.05, compared with that in the control group.
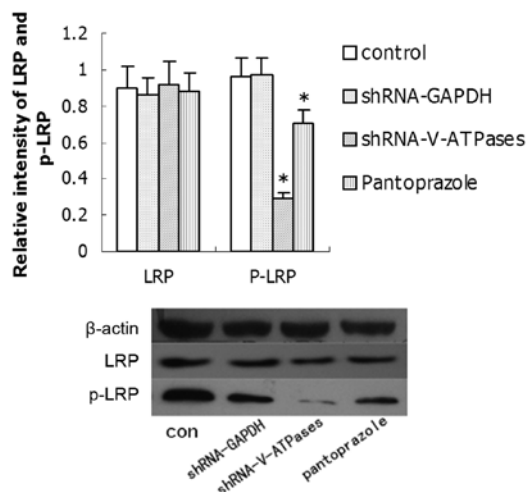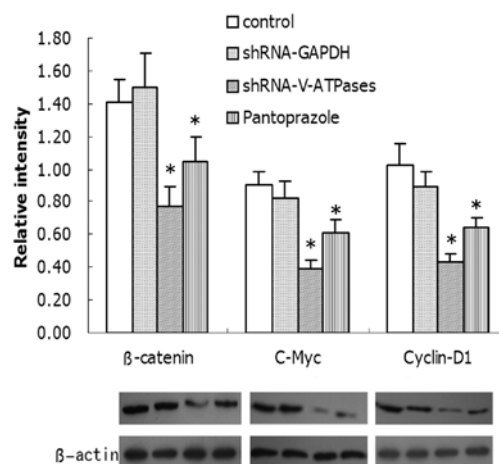

Figure 5. Expression of LRP6 and p-LRP6 following pantoprazole treatment for 48 h. Cytosolic and nuclear proteins were prepared and used in western blotting experiments; β-actin was used as a loading control. Results are presented as the means ± SD. Experiments were performed in triplicate.

with 20 mg/ml pantoprazole after 48 h were detected by western blotting (Fig. 5). The expression of phospho-LRP6 and β-catenin was weak in the SGC7901 cells after pantoprazole treatment compared with that in the control group, but LRP6 showed no difference.

To examine the relationship between inhibition of V-ATPases and Wnt/β-catenin signaling in gastric cancers, we also detected expression of c-Myc and cyclin D1, which are well-known target genes of the Wnt/β-catenin canonical pathway. c-Myc and cyclin D1 were markedly downregulated in SGC7901 cells after treatment with 20 mg/ml pantoprazole (Fig. 6). Therefore, we confirmed that the inhibition of V-ATPase by pantoprazole reduced the expression of c-Myc and cyclin D1.

Discussion

The V-ATPase is a multiprotein complex localized in intracellular organelles and at the plasma membrane. It is involved

in diverse processes such as phagocytosis, virus entry, metastasis, and embryonic left-right patterning. Its main mechanism is to pump protons and acidify vesicles, thereby promoting vesicular traffic, notably endocytosis (12,13). V-ATPases exist in various cell types, including those of many solid tumors, and are involved in progression and metastasis. These enzymes contribute to the acidic pH of solid tumors, and have been proposed as a therapeutic target for selective anticancer treatments (8,14,15) In recent years, several studies have shown that PPIs such as omeprazole, esomeprazole and pantoprazole have an antineoplastic activity against human hematopoietic and solid tumors (16-18). Our previous study also found that pantoprazole reversed the transmembrane pH gradient and chemosensitized SGC7901 cells to antitumor agents (10). These results suggest that PPIs may be useful as an anticancer agent. However, to date, no precise molecular mode of action in cancer cells has been presented. Thus, we further studied its possible cell targets.

Our previous study (10) found that pantoprazole inhibits the expression of V-ATPases at concentrations of 20 mg/ml, and inhibits the concentration of V-ATPases around the cell membrane, affecting its role in transporting H^+ out of the cell, and thereby decreasing the intracellular pH and increasing the extracellular pH value. Thus, in the present study, the concentration we applied was 20 mg/ml. We found that pantoprazole inhibited the proliferation, induced apoptosis, and decreased the invasive ability of cells. Thus, we confirmed that V-ATPase is a target of pantoprazole in SGC7901 cells and that pantoprazole is a V-ATPase inhibitor. To date, a few V-ATPase inhibitors have been identified, but none have been used clinically because of their toxic effect on normal cells (19-23). PPIs can suppress gastric acid and treat diseases related with gastric acid with few side effects. Therefore, we believe that PPIs, as anticancer agents, may potentially benefit many patient groups.

The Wnt pathway is known to be involved in the tumorigenesis of many human cancers, including colon cancer, breast cancer, lung cancer, melanomas and hepatocellular carcinoma (24-26). Dysregulation of this pathway can be caused by mutations in many molecular components (e.g., CTNNB1,

AXIN or FZD7) in colon cancers, hepatocellular carcinomas and other cancers (27-29). Recently, Cruciat *et al* (30) found that V-ATPase is involved in Wnt/β-catenin signaling, which is related to tumor development and metastasis. LRP6 phosphorylation is accompanied by receptor internalization in caveolin-containing vesicles and endocytosis is essential for Wnt/β-catenin signaling (31,32). This raised the possibility that V-ATPases may influence LRP6 endocytosis, phosphorylation and β-catenin activation. Our study found that, consistent with the inhibition of V-ATPases by pantoprazole, phospho-LRP6 and β-catenin simultaneously decreased, but LRP6 was unchanged. To confirm that pantoprazole inhibits Wnt/β-catenin signaling, we also detected c-Myc and cyclin D1, which are well-known target genes of the Wnt canonical pathway (33). c-Myc and cyclin D1 were markedly downregulated after treatment with pantoprazole at concentrations of 20 mg/ml in SGC7901 cells.

In conclusion, we report that pantoprazole as an inhibitor of V-ATPases can induce cell death in human gastric cancer using the Wnt/β-catenin pathway as a mechanism. Although more careful analyses of the effects of pantoprazole on various organs remain to be carried out, the results of this study showed that V-ATPase is a potential cell target of pantoprazole for the chemotherapy of gastric cancers.

Acknowledgements

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