Pantoprazole inhibits human gastric adenocarcinoma SGC-7901 cells by downregulating the expression of pyruvate kinase M2

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Abstract. The Warburg effect is important in tumor growth. The human M2 isoform of pyruvate kinase (PKM2) is a key enzyme that regulates aerobic glycolysis in tumor cells. Recent studies have demonstrated that PKM2 is a potential target for cancer therapy. The present study investigated the effects of pantoprazole (PPZ) treatment and PKM2 transfection on human gastric adenocarcinoma SGC-7901 cells *in vitro*. The present study revealed that PPZ inhibited the proliferation of tumor cells, induced apoptosis and downregulated the expression of PKM2, which contributes to the current understanding of the functional association between PPZ and PKM2. In summary, PPZ may suppress tumor growth as a PKM2 protein inhibitor.

Introduction

Aerobic glycolysis, first studied by Otto Warburg, is the process whereby tumor cells use glycolysis with reduced mitochondrial oxidative phosphorylation to metabolize glucose, even under aerobic conditions (1). The metabolic switch may be required to support cell growth more than the defects in mitochondrial respiration. In certain cancer cell lines, the most glycolytic tumor cells were demonstrated to also be the most aggressive cells (2). In another study, aerobic glycolysis was an essential step that ultimately resulted in tumorigenesis, which was characterized by enhanced activity of glycolytic enzymes and distinct changes in the glycolytic isoenzyme (3). There were three rate-limiting enzymes of glycolysis, consisting of hexokinase, phosphofructokinase and pyruvate kinase (4). Among these enzymes, pyruvate kinase catalyzed the final step of glycolysis, which is associated with the production of adenosine triphosphate and pyruvate, and was important during the process of glycolysis (5). Four pyruvate kinase isoenzymes are known in mammals: Liver-type pyruvate kinase (PKL) is expressed in the kidney and liver; protein kinase R (PKR) is expressed in red blood cells; the M1 isoform of pyruvate kinase (PKM1) is expressed in the majority of adult tissues; and the M2 isoform of pyruvate kinase (PKM2) is the embryonic form, and is a splice variant of PKM1 (6). PKM2 was overexpressed in numerous cancer cells. The expression of PKM2 was critical for rapid growth in cancer cells (7,8). However, inhibition of PKM2 may undesirably affect cancer cell metabolism (9-11).

Gastric cancer is one of the most common cancers, and is a leading cause of cancer-associated mortality in the world, accounting for ~8% of novel cancers (12). Numerous trials have demonstrated that PKM2 is overexpressed in gastric cancer and that the inhibition of PKM2 has well-supported anti-tumor effects (13-15). The critical role of PKM2 in gastric cancer suggests that identifying novel inhibitors of PKM2 may provide a promising future in the treatment of gastric cancer.

Proton pump inhibitors (PPIs) are highly effective in the treatment and symptomatic relief of peptic ulcers, by inhibiting H^+,K^+-adenosine triphosphate (ATP)ase in the gastric parietal cells (16). The effects of pantoprazole (PPZ), one type of PPI, on PKM2 were hypothesized to be associated with the inhibitory efficacy of PPZ (17,18). Yeo *et al* reported that PPZ selectively induced apoptotic cell death in *in vivo* and *in vitro* gastric cancer models (19), suggesting that PPIs may exert selective anticancer effects. However, there are few relevant studies, and the mechanisms involved remain unclear.

The present study aimed to determine whether PPZ inhibits proliferation and selectively induces apoptosis in SGC-7901 cells, possibly via a PKM2-mediated mechanism. The hypothesis was examined by altering the expression of PKM2 *in vitro*.

Materials and methods

Cell line and cell culture. The human gastric adenocarcinoma cell line SGC-7901 was kindly provided by the Department of Oncology, Medical School, Drum Tower Hospital of Nanjing

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University (Nanjing, Jiangsu, China). The cells were cultured in RPMI-1640 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials, Hangzhou, Zhejiang, China) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) in a humidified air with 5% CO_2 at 37˚C (Direct Heat CO_2) (Thermo Fisher Scientific, Waltham, MA, USA).

Drugs and experimental design. Pantoprazole sodium salts (Takeda Pharmaceuticals International GmbH, Zurich, Switzerland) were resuspended in normal saline (0.85%) at 5 mg/ml immediately prior to use. When the SGC-7901 cells had reached 60-70% confluence, PPZ was added at a final concentration of 20 μg/ml, and the cells were cultured for additional experiments.

Transfection. The human gene encoding PKM2 was amplified using reverse transcription-polymerase chain reaction (RT-PCR) and the total-RNA extracted from the SGC-7901 cells. The PKM2 gene was ligated to an internal ribosome entry site (pIRES2)-enhanced green fluorescent protein (EGFP) plasmid (Invitrogen; Thermo Fisher Scientific) using T4 DNA ligase following digestion by the restricted endonucleases *Eco*RI and *Bam*HI. SGC-7901 cells were transfected with the pIRES2-EGFP-PKM2 expression vector using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific), according to the manufacturer's protocol for 24 h prior to PPZ treatment.

Cell proliferation assay. The SGC-7901 cells were seeded in 100 μl of medium per well, at a density of $1x10^4$/well, in 96-well plates and treated with 5 mg/ml PPZ for 24 h. The cytotoxicity of PPZ was assessed by a cell counting kit-8 assay (KeyGen Biotech Co., Ltd., Nanjing, Jiangsu, China), according to the manufacturer's protocol. The cytotoxicity was expressed as the relative cell viability, using the following formula: Cell viability (%) = (OD of drug-treated sample / OD of untreated sample) x 100. The experiment was repeated 3 times.

Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection. The SGC-7901 cells were seeded into 6-well plates. Following treatment with 5 mg/ml PPZ, the cells were trypsinized, washed twice with phosphate-buffered saline (PBS), resuspended and then stained with an Annexin V-FITC apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd.). The apoptosis of cells was analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The experiment was repeated 3 times.

Western blot analysis. The expression of PKM2 was evaluated using western blot analysis. The cells were kept on ice for 30 min in lysate containing 0.01% phenylmethanesulfonylfluoride, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% sodium dodecyl sulfate (SDS), 0.2% ethylenediaminetetraacetic acid, 1% Triton X-100 and 1% sodium deoxycholate, supplemented with the protease inhibitors leupeptin, aprotinin, phenylmethylsulfonyl fluoride and sodium orthovanadate (Roche Diagnostics, Basel, Switzerland). The lysed cells were then centrifuged at 9,660 x g at 4˚C (Centrifuge 5804R; Eppendorf, Hamburg, Germany) for 15 min, leaving a supernatant

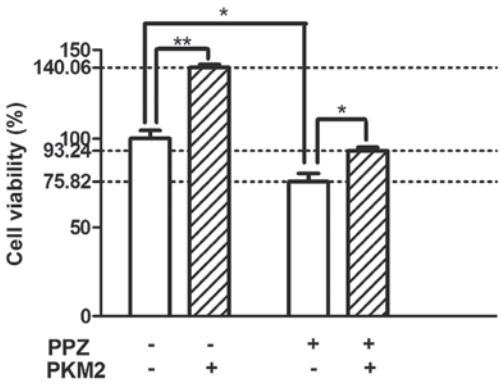

Figure 1. Comparison of the cell viability of SGC-7901 subsequent to treatment with 5 mg/ml PPZ and PKM2 transfection for 24 h. The experiment was repeated three times. *P<0.05; **P<0.01. PPZ, pantoprazole; PKM2, M2 isoform of pyruvate kinase; PPZ^+, treatment with PPZ; PPZ^-, without PPZ treatment; $PKM2^+$, transfection with PKM2; $PKM2^-$, without PKM2 transfection.

of extracted proteins. The protein content was estimated according to the bicinchoninic acid protein assay kit (Nanjing KeyGen Biotech Co., Ltd.). In total, 50 μm of each protein sample was subjected to western blotting on a denaturing 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Immobilon-P; EMD Millipore, Billerica, MA, USA) using a semidry transfer system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The non-specific binding was blocked by the incubation of membranes in 1X Tris-buffered saline containing 0.05% Tween-20 with 5% nonfat dry milk for 1 h. Subsequently, the membranes were incubated overnight at 4˚C with monoclonal rabbit antibodies against PKM2 (dilution, 1:1,000; no. 4053; Cell Signaling Technology Inc., Danvers, MA, USA, USA) and glyceraldehyde 3-phosphate dehydrogenase (dilution, 1:3,000; no. sc-51905; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), which was used as an internal control for protein loading. Antibody binding was detected by incubating the blot with a horseradish peroxidase-conjugated goat anti-rabbit antibody (dilution, 1:1,000; KPL, Gaithersburg, MD, USA). The specific signals were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology, Inc.). Digital images were obtained and analyzed by Quantity One V4.31 (Bio-Rad Laboratories, Inc.).

Immunofluorescence staining analysis. Dispersed cells were grown on glass slides within 6-well plates and were then treated with 5 mg/ml PPZ for 24 h. Subsequent to washing with PBS 3 times, the cells were fixed with cold acetone for 10 min at 4˚C. Next, the cells were blocked with 10% normal goat serum (Wuhan Boster Biological Technology, Wuhan, China) for 30 min and probed with the antibody against PKM2 (dilution, 1:100) at 4˚C overnight. Alexa Fluor dye-conjugated secondary antibodies (dilution, 1:100; Alexa Fluor 594) and 2 mg/ml goat anti-rabbit IgG (H + L) highly cross-adsorbed (Molecular Probes; Thermo Fisher Scientific) were incubated with the cells for 1 h to enable the samples to be visualized under a fluorescent microscope (Axio Imager A1; Carl Zeiss AG, Oberkochen, Germany). The nuclei were stained using Invitrogen 4',6-diamidino-2-phenylindole (2 μg/ml; Thermo Fisher Scientific).

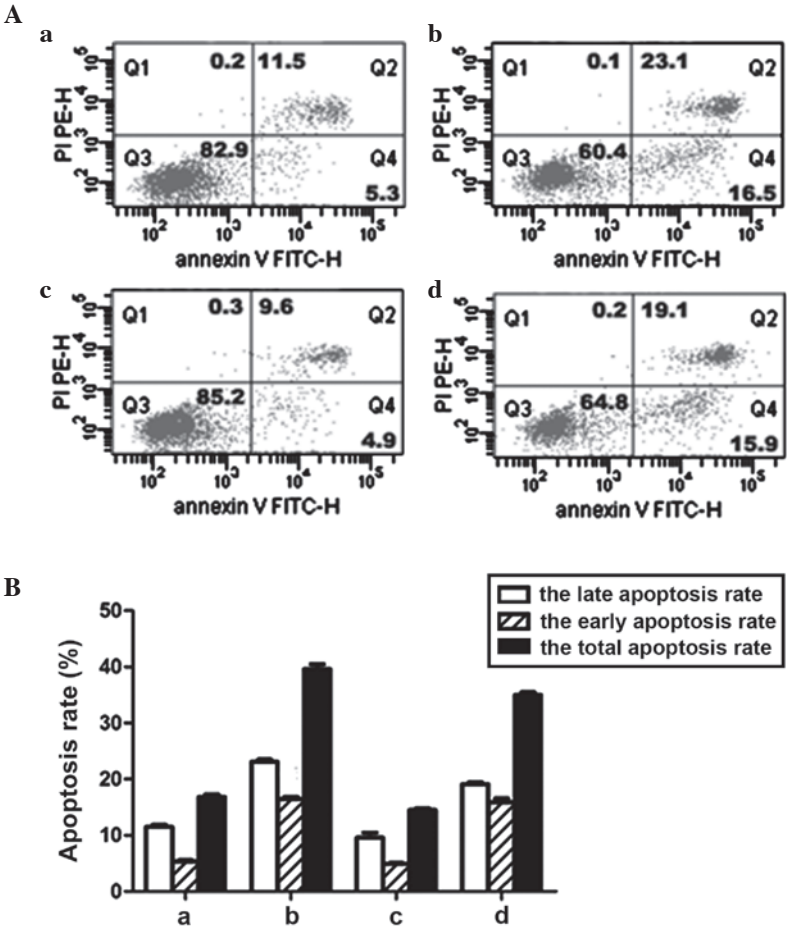

Figure 2. (A) The apoptosis rate of SGC-7901 cells following 5 mg/ml PPZ administration and PKM2 transfection with Annexin V-fluorescein isothiocyanate apoptosis detection. (Aa) $PPZ^-/PKM2^-$ group, without PPZ and PKM2 intervention. (Ab) $PPZ^+/PKM2^-$ group, treatment with PPZ without PKM2 transfection. (Ac) $PPZ^-/PKM2^+$ group, transfection with PKM2 without PPZ treatment. (Ad) $PPZ^+/PKM2^+$ group, intervention with PPZ and PKM2. (B) Comparison of the apoptosis rate of SGC-7901 cells subsequent to intervention with PPZ and PKM2. The experiment was repeated three times. PPZ, pantoprazole; PKM2, M2 isoform of pyruvate kinase.

Statistical analysis. The data were analyzed using SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA) and the independent-samples *t*-test method. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Effects of PPZ and PKM2 on cell proliferation. As demonstrated in Fig. 1, PPZ may inhibit the proliferation of the SGC-7901 cells. The cell viability in the PPZ^+ group was significantly decreased compared with the PPZ^- group (-24.18% vs. the PPZ^- group; P<0.05). In addition, PKM2 transfection may notably increase cell viability (+40.06% vs. the $PKM2^-$ group; P<0.01). As expected, the cell viability in the $PKM2^+$ group was increased compared with the viability in the $PKM2^-$ group subsequent to PPZ intervention (+17.42% vs. the $PKM2^-$ group; P<0.05).

Effect of PPZ and PKM2 on apoptosis in SGC-7901 cells. A quantitative analysis of the fluorescent signals was performed by fluorescence-activated cell sorting (FACS). The results were summarized in Fig. 2A and B. As exhibited in Fig. 2B, the early apoptosis, late apoptosis and total apoptosis rates in the PPZ^+ group were notably increased compared with the PPZ^- group (+1.2, +11.6 and +22.8%, respectively, vs. the PPZ^- group; P<0.001). Following PKM2 transfection, the early and late apoptosis rates were not affected (P>0.05), while the total apoptosis rate was statistically decreased compared with the $PKM2^-$ group (-2.3% vs. the $PKM2^-$ group; P<0.05). Under PPZ intervention, there was no difference between the early apoptosis rates of the $PKM2^+$ and $PKM2^-$ groups (P>0.05), while the apoptosis rate was decreased compared with the $PKM2^-$ group on the late apoptosis rate (-4.0% vs. the $PKM2^-$ group; P<0.01) and total apoptosis rate (-4.6% vs. the $PKM2^-$ group; P<0.05).

Effects of PPZ treatment and PKM2 transfection on protein expression of PKM2 in SGC-7901 cells. The expression of PKM2 in SGC-7901 cells was examined using western blot analysis. Following PPZ treatment without PKM2 transfection, the expression of PKM2 decreased when compared to that in the control group (P<0.01) (Fig. 3A). As Fig. 3B showed, the protein expression of PKM2 notably increased (P<0.001), which confirmed the success of PKM2 transfection. Furthermore, the PKM2 protein expression significantly increased following PKM2 transfection under PPZ treatment (P<0.05) (Fig. 3C).

PPZ intervention and PKM2 transfection affects the intracellular expression of PKM2. As an inhibitor of PKM2, PPZ treatment for 24 h caused decreased intracellular expression

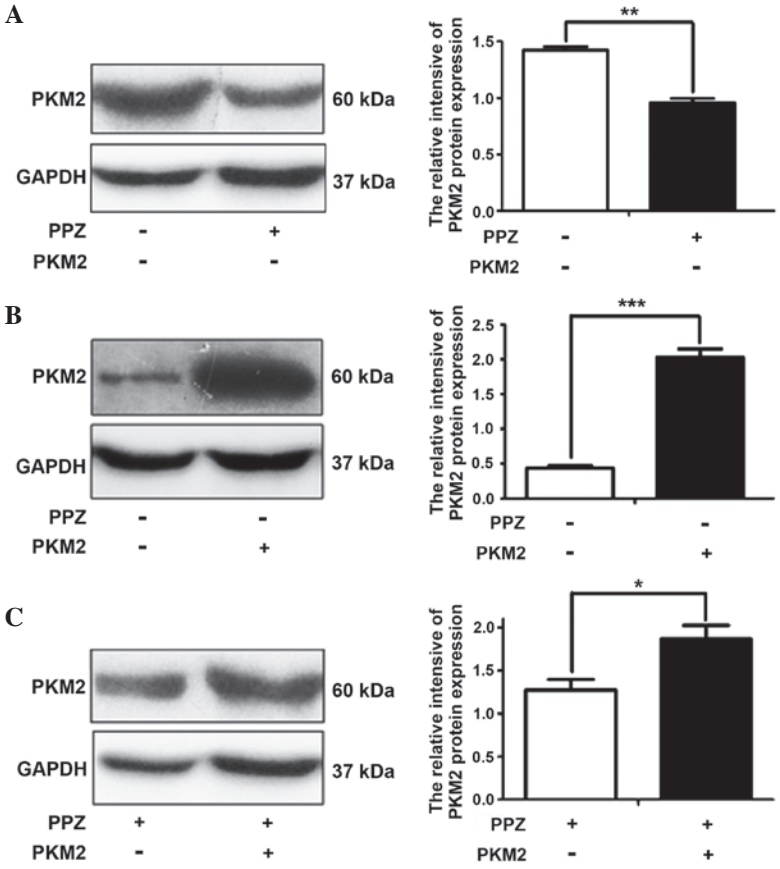

Figure 3. Effects of 5 mg/ml PPZ treatment and PKM2 transfection on PKM2 expression in SGC-7901 cells. (A) Comparison of the PKM2 expression subsequent to PPZ treatment without PKM2 transfection. (B) Comparison of the PKM2 expression subsequent to PKM2 transfection without PPZ treatment. (C) Comparison of the PKM2 expression with PPZ treatment following PKM2 transfection. (*P<0.05; **P<0.01; ***P<0.001). PPZ, pantoprazole; PKM2, M2 isoform of pyruvate kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

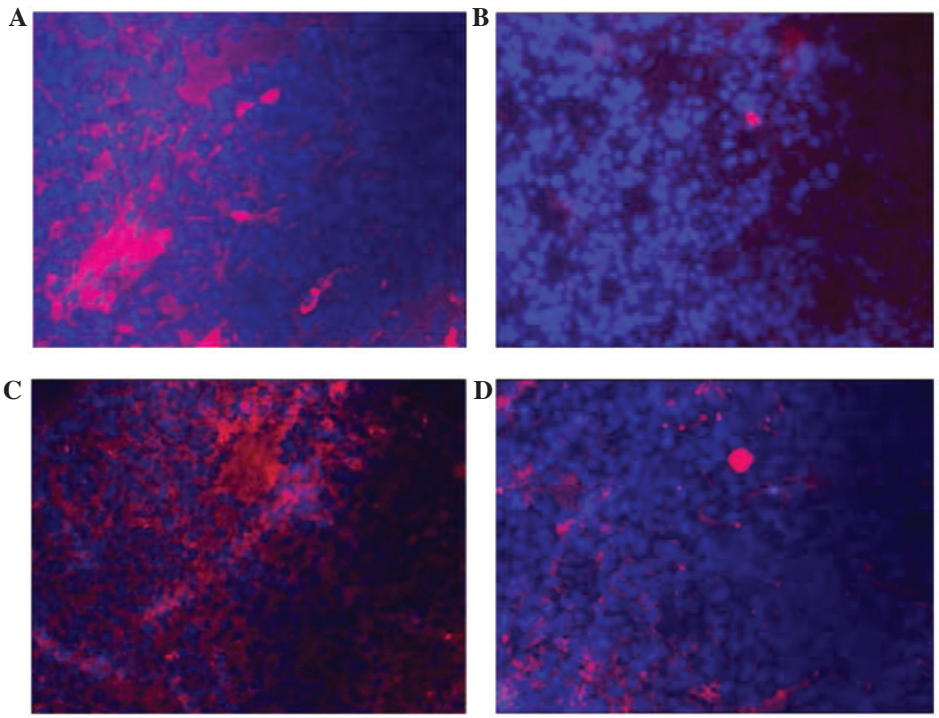

Figure 4. Effects of 5 mg/ml PPZ treatment and PKM2 transfection on the intracellular distribution of PKM2 in SGC-7901 cells. (A) Intracellular distribution of PKM2 in SGC-7901 cells without PPZ treatment and PKM2 transfection (magnification, x200). (B) Intracellular distribution of PKM2 in SGC-7901 cells subsequent to PPZ treatment without PKM2 transfection (magnification, x200). (C) Intracellular distribution of PKM2 in SGC-7901 cells subsequent to PKM2 transfection without PPZ treatment (magnification, x200). (D) Intracellular distribution of PKM2 in SGC-7901 cells subsequent to PPZ treatment and PKM2 transfection (magnification, x200). PPZ, pantoprazole; PKM2, M2 isoform of pyruvate kinase.

of PKM2 (Fig. 4A and B). Subsequent to PKM2 transfection, the fluorescent intensity of PKM2 expression significantly increased compared to the control group (Fig. 4A and C). In addition, the fluorescent intensity of the PKM2 expression in $PKM2^+$ group with PPZ treatment was approximately the median (Fig. 4D).

Discussion

Although the Warburg effect was previously proposed, this phenomenon received sufficient attention after the successful application of positron emission tomography (PET) in the clinical diagnosis of tumors (20,21). In recent years, the therapeutic strategies aimed at the glycolytic pathway have been explored extensively; and as one of the key enzymes regulating aerobic glycolysis of tumor cells, PKM2 may be a potential target of cancer therapy. Christofk *et al* reported that PKM2 promoted the rapid growth of cancer cells by diverting glucose metabolites from energy production to anabolic processes, which was attributed to phosphotyrosine signaling regulation (8). Based on those results, targeting PKM2 may provide a novel method of reversing chemotherapy resistance to cancer therapy, by inhibiting tumor growth and promoting increased cancer cell apoptosis *in vitro* and *in vivo* (22). However, the role of PKM2 in tumor growth remains controversial. One study suggested that PKM2 was dispensable for tumor maintenance and growth *in vivo*, and considered that other metabolic pathways occurred in this complicated process (23). Therefore, to clarify the issue, the present study used the gastric adenocarcinoma SGC-7901 cell line with PPZ treatment and PKM2 transfection to observe cell proliferation and apoptosis, and simultaneously detect the protein expression of PKM2.

PPZ may suppress proliferation and induce apoptosis in SGC-7901 cells, which was also confirmed in a previous study (24). In the present study, it was observed that the expression of PKM2 decreased concurrently during this process. It is possible that decreased PKM2 expression impeded cancer cell growth, or the impeded cell growth reduced the protein expression of PKM2. In order to resolve this issue, the expression of PKM2 was increased using transfection, which demonstrated that cancer cell proliferation was elevated and apoptosis was restrained. Thus, it was concluded that PKM2 is crucial for cancer cell growth, to promote proliferation and to inhibit apoptosis. Based on this conclusion, the inhibition of PKM2 may elicit anticancer effects, which has been reported to involve various mechanisms, including the impairment of tumor growth, induction of apoptotic cell death and increased sensitivity to chemotherapy (7,11,25,26). In the present study, increased PKM2 expression by transfection may partially reverse the effects of PPZ in inhibiting cancer cell proliferation and inducing apoptosis. The cancer-specific metabolic transformation associated with therapeutic resistance is a promising target for cancer therapy (27,28). Response of the SGC-7901 cells was not observed subsequent to the inhibition of PKM2 by RNA interference or small-molecule inhibitors. An additional study may therefore be required.

The mechanisms underlying the effect of PPZ on the expression of PKM2 remain unclear. Tumor microenvironments are characterized by acidification and hypoxia. PPIs may inhibit the expression of vacuolar H^+-ATPases (V-ATPases), which are important for maintaining an acidic extracellular pH (pHe) as specific proton pumps of the cell (24). A previous study demonstrated that PPZ treatment significantly inhibited protein expression of V-ATPases, mechanistic target of rapamycin (mTOR) and hypoxia-inducible factor 1α (HIF-1α), accompanied by increased pHe (29). Another study demonstrated that mTOR activation simulated hypoxic effects by inducing HIF-1α expression, which in turn enhanced PKM2 expression through collaboration with c-Myc-hnRNPs splicing regulators, leading to aerobic glycolysis in tumor cells (30). Therefore, PPZ may suppress SGC-7901 cancer cells by downregulating the V-ATPases/mTOR/HIF-1α/PKM2 signaling pathway. Alternatively, as benzimidazole compounds, which were found to regulate the stability of HIF-1α through the Hsp90-Akt pathway (31), PPZ may indirectly reduce the protein expression of PKM2 by inhibiting HIF-1α expression. Therefore, the inhibitory effects of PPZ on the PKM2 protein may involve a variety of factors.

In conclusion, PPZ was demonstrated to effectively inhibit PKM2 expression in human gastric adenocarcinoma SGC-7901 cells, which was associated with cancer cell proliferation and apoptosis. The present study has indicated a novel anticancer mechanism, thus extending the clinical utilization of PPIs as an anticancer drug by inhibiting the expression of PKM2 in gastric cancer cells.

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