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Effects and mechanisms of proton pump inhibitors as a novel chemosensitizer on human gastric adenocarcinoma (SGC7901) cells

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Abstract

Upregulation of proton extrusion is critical for tumor cell survival in an ischemic microenvironment with a lower extracellular pH (pHe). Lower pHe and higher intracellular pH (pHi) benefit cancer cells for invasion and growth. Vacuolar H^+-ATPases (V-H^+-ATPases) play a critical role in regulating the transmembrane pH gradient. Proton Pump Inhibitors (PPI), mainly treating acid-related diseases, could inhibit the expression of V-H^+-ATPases. We have investigated whether PPI decreases the pHi of the human gastric adenocarcinoma cell line, SGC7901, by inhibiting V-H^+-ATPases so as to enhance the cytotoxicity of anti-tumor drugs. We have assessed the optimal treatment time, pretreatment dosage of PPI and the possible mechanism of action. PPI exceeding 10 μg/ml inhibited protein expression of V-H^+-ATPases in a dose-dependent manner, decreased the pHi value and reversed the transmembrane pH gradient, whereas PPI at final concentration of 1 μg/ml could not. Changes of the pH gradient were positively correlated with PPI concentration. The inhibitory effects of PPI on V-H^+-ATPases primarily occurs from 12 h to 24 h after PPI pretreatment ($P < 0.05$). The pHi value of SGC7901 was lowest 24 h after PPI pretreatment ($P < 0.05$). Administration of anti-tumor drugs 24 h after PPI pretreatment produced the most cytotoxic effects on SGC7901 ($P < 0.05$) and significantly improved the early and total apoptosis rates ($P < 0.01$). PPI exceeding 20 μg/ml also significantly reduced the ADR-releasing index, thereby enhancing the intracellular ADR concentration ($P < 0.01$). Therefore, PPI could enhance the cytotoxic effects of anti-tumor drugs on the SGC7901 cells.

Keywords: Vacuolar H^+-ATPases; Tumor acidity; Proton pump inhibitors; Transmembrane pH gradient

1. Introduction

A major barrier for the effective treatment of gastric cancer is the phenomenon of Multidrug Resistance (MDR) exhibited by tumor cells (Juranka et al., 1989; Roninson, 1987). A common feature of MDR cells is a net decrease in the intracellular accumulation of drugs (Gottesman and Pastan, 1993). In fact, tumor microenvironment is characterized by reversed pH gradient, with an acidic extracellular pH (pHe) and an alkaline intracellular pH (pHi) (pHe < pHi) compared to the normal tissues (pHe > pHi) (Stubbs, 1998). However, the acid pHe will effectively impair the entry of weakly basic anti-tumor drugs into cancer cells since it could neutralize or sequester drugs into the acidic intracellular vesicles (Mahoney et al., 2003).

Vacuolar H^+-ATPases (V-H^+-ATPases), specific proton pumps of the cell, have an important role in maintaining a relatively neutral pHi, an acidic luminal pH, and an acidic pHe, through pumping protons into extracellular environment or lumen of some membrane-bound organelles (Nishi and Forgac, 2002). V-H^+-ATPases are overexpressed in many types of metastatic cancers and positively correlated to their invasion and

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metastasis (Sennoune et al., 2004). Some human tumor cells, particularly those selected for MDR, also exhibit enhanced V-H^+-ATPases activity (Marquardt and Center, 1991; Martinez-Zaguilan et al., 1999).

Some molecules that inhibit V-H^+-ATPases and may reverse the MDR to cytotoxic drugs have been identified (Martinez-Zaguilan et al., 1993). Although their toxicity and poor results in preclinical tests have limited their development as therapeutic agents, recent insight into the mechanism of tumor acidification may provide new strategies that mainly target V-H^+-ATPases (Fais et al., 2007). And Proton Pump Inhibitors (PPI) could represent a class of drugs suitable to this purpose (Barrison et al., 2001).

Since the introduction of Omeprazole in 1989, PPI have demonstrated gastric acid suppression superior to that of histamine H_2-receptor blockers (Vanderhoff and Tahboub, 2002). Currently some researches have reported that PPI might be considered as chemosensitizer agents. According to Luciani et al. (2004), PPI pretreatment sensitized tumor cell lines to the effects of chemotherapeutic drugs suggesting that proton pump activity may affect cancer cells homeostasis and particularly acidic vesicles trafficking. Moreover, it seems highly conceivable that tumor acidity may induce a selective accumulation of PPI in the tumor tissues. By analogy with the gastric compartment, PPI may be protonated and transformed into the active form in the acidic tumor microenvironment (De Milito and Fais, 2005a,b).

We therefore investigated whether PPI could inhibit the expression of V-H^+-ATPases, reverse the transmembrane pH gradient and chemosensitize SGC7901 cells to anti-tumor agents. We sought the optimal administration time, the optimal dose of PPI, and look at possible mechanisms.

2. Materials and methods

2.1. Reagents and drugs

The human gastric adenocarcinoma cell line, SGC7901, was kindly given by the Department of Oncology, Drum Tower Hospital of Nanjing University, Medical School. Nigericin was obtained from Alexia Biochem, USA. Vacuolar H^+-ATPase $6V_1A$ mouse polyclonal antibody was purchased from Taiwan Abnova, China. MTT Cell Proliferation and Cytotoxicity Assay Kit, BCA Protein Assay Kit and Annexin V-FITC PI double staining Apoptosis Detection Kit were provided by KEYGEN Biotech, China. BCECF-AM pH-sensitive fluorescent probe was bought from Beyotime Biotec, China. Other materials and chemicals were purchased from commercial sources.

Pantoprazole sodium salts (Altana Pharma AG D-78467 Konstanz, Germany) were resuspended in normal saline (0.85%) at 1 mg/ml immediately before use. Cisplatin (Qilu Pharmo Co. Ltd, China. NO.7100361DC) was resuspended in phosphate buffered saline (PBS) at a stock concentration of 1 mg/ml and stored at −20 °C. 5-Fluorouracil (5-FU, Jiangsu Hengrui Pharma. Co. Ltd, China. NO.0710152) was supplied in solution at 25 mg/ml and stored at room temperature.

2.2. Cell line and cell culture

SGC7901 cells were cultured in RPMI-1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials, China) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified air with 5% CO_2 atmosphere at 37 °C (Thermo Direct Heat CO_2, USA).

2.3. Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis of V-H^+-ATPase $6V_1A$ were done as previously described (Ranta et al., 2008). Briefly, cells were lysed in lysate (containing 0.01% PMSF, 150 mM NaCl, 50 mM Tris (pH = 8), 0.1% SDS, 0.2% EDTA, 1% Triton X-100, 1% sodium deoxycholate) supplemented with protease inhibitors (aprotinin, leupeptin, phenylmethylsulfonyl fluoride, sodium orthovanadate; Roche) and incubated for 30 min on ice, and centrifuged at 12000 rpm in 4 °C (Eppendorf centrifuge 5804R, Germany) for 15 min to remove nuclei and cell debris. Protein concentration of the extracts was determined by the BCA Protein Assay Kit, following the manufacturer's instructions. Thirty micrograms of each cell extract was electrophoresed on 10% gels and electroblotted to a nitrocellulose membrane using a semidry transfer system (Bio-Rad, USA). Non-specific binding was blocked by incubating the membrane in 1 × TBST (Tris Buffered Saline containing 0.05% Tween-20) supplemented with 5% nonfat dry milk for 1 h. Blots were incubated with a polyclonal mouse antibody to V-H^+-ATPase subunit V_1A (1:2500) and a monoclonal mouse antibody to β-actin (1:3000, Santa Cruz, USA) as a control for protein loading. Antibody binding was detected by incubating the blot with a horseradish peroxidase-conjugated goat anti-mouse antibody (1:1000, KPL, USA). Antibody staining was visualized by enhanced chemiluminescence (Santa Cruz, USA). The images of Western blot products were collected and analyzed by Quantity One V4.31 (Bio-Rad, USA).

2.4. Immunofluorescence staining analysis

Dispersed single cells (2×10^5 cells per well) were grown on $22 \times 22 \times 1$ mm^3 glass coverslips (pretreated with 0.3% gelatin) in 6-well culture plates. After 36–48 h incubation or 24 h PPI pretreatment, cells were fixed in ice-cold acetone for 10 min at 4 °C. The cells were blocked with 10% normal goat serum (Boster Biotech, China) for 30 min and probed with V_1 A subunit of V-H^+-ATPase antibodies (1:100) at 4 °C overnight. Alexa Fluor Dye Conjugated secondary antibodies (1:100, Alexa Fluor 488 goat anti-mouse IgG (H + L) highly cross-adsorbed, 2 mg/ml, Invitrogen, USA) were used to incubate for 1 h to be visualized under a fluorescent microscope (Imager A_1, Axio, Zeiss). DAPI (2 μg/ml, Invitrogen, USA) was used to stain nuclei.

2.5. Cytotoxic assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, MTT assay)

The cytotoxicity of PPI and/or anti-tumor drugs was determined by MTT assay (Wang et al., 2008). Cells (1×10^4/well) were plated in 200 μl of medium/well in 96-well plates. After overnight incubation, PPI was added with the final concentration of 10 μg/ml. After 0, 12, 24 h PPI treatment, 5-Fu and cisplatin were added at 25 and 1 μg/ml, respectively. After further 24 h incubation, 50 μl of 5 mg/ml MTT (pH 4.7) was added and the cells cultured for another 4 h. The supernatant was removed and 150 μl DMSO was added per well. Samples were shaken for 10 min. The absorbance at 570 nm was measured with a microplate reader (Tecan Sunrise, Switzerland), using wells without cells as blanks and using untreated cells as a negative control. The effects of PPI in enhancing cytotoxicity of chemotherapeutic drugs were expressed as relatively cell viability, using the following formula: Percent cell viability (% = OD of drug-treated sample/OD of untreated sample) ×100%.

2.6. Annexin V-FITC apoptosis detection

Apoptosis detection in untreated and pretreated cells was performed by Annexin V-FITC and Propidium Iodide (PI) double staining Apoptosis Detection Kit by flow cytometry (BD Biosciences, USA), as previously described by Pfaffel-Schubart et al. (2008). Briefly, the cells were trypsinised, washed with PBS, centrifuged and resuspended with Annexin-binding buffer (500 μl). The cells were incubated with 5 μl 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 in order to distinguish necrotic cells. The samples were analyzed by FACS with CellQuest version 3.3 software within 1 h.

2.7. Measurements of intracellular and extracellular pH values

pH standard buffer solutions A and B were prepared as described in Table 1, and the pH value of solution A was regulated to 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4 and 7.6. The pH value of solution B was regulated to 7.4. The 2 solutions were stored at 4 °C. Nigericin was diluted with ddH_2O at 5 mM (3.375 mg Nigericin:1 ml ddH_2O) and stored at −20 °C. 1 μl Nigericin solution was added into 1 ml solution A with the final concentration of 5 μM. BCECF-AM pH-sensitive fluorescent probe was diluted into 5 mM with DMSO and stored at −20 °C away from the light. 1 μl BCECF-AM solution was added into 1 ml solution B with the final concentration of 5 μM.

Table 1
The pH standard buffer solution prescription.

Elements (MW)	Solution A mM (g)	Solution B mM (g)
NaCl (58.5)	0	135 (7.897)
KCl (74.5)	133 (9.908)	5 (0.038)
Cholin-Cl (139.6)	7 (0.977)	0
$CaCl_2$ (111)	1 (0.111)	1.8 (0.199)
$MgCl_2$ (95)	1 (0.095)	0
$MgSO_4$ (120)	0	0.8 (0.096)
KH_2PO_4 (136)	2 (0.272)	0
Glucose (180)	5 (0.9)	5 (0.9)
HEPES (238.3)	6 (1.429)	10 (2.383)

The pHi value was measured in the monolayers using the pH-sensitive fluorescent probe 2′, 7′-bis-(2-carboxyethyl)-5-carboxyfluorescein (BCECF) as previously described (Xia et al., 1999). The first step was to establish a standard curve. Cells were cultured for 24 h in six-well plates at a density of 1×10^5 cells per well under conditions as mentioned above. Then the upper medium was removed and the cells were washed with the solution B twice, five min each time. Then the solution B containing BCECF-AM (1 μl/1 ml, 5 μM) was then added and the cells were incubated for 1 h. The supernatant was removed and the cells were washed twice by the solution A at different pH values. Solution A containing Nigericin (1 μl/1 ml, 5 μM) was added into each well and the cells were incubated for 15 min under normal conditions. The cells were trypsinized and resuspended with 1 ml solution A at different pH values. The BCECF fluorescence intensity was recorded by flow cytometry. The wavelengths of excitation light and emission light were 490 nm and 530 nm. Bivariate correlation analysis between fluorescence intensity of 490 nm and the pH value was performed, and then the pHi standard curve was developed. Secondly, the cells were treated as described above after PPI pretreatment with different concentrations of 1, 10 and 100 μg/ml for 12, 24 and 48 h, except that solution A was replaced by solution B at the last thee steps. The fluorescent intensity of 490 nm was recorded and then the pHi value could be calculated according to the pHi standard curve.

The pHe values of culture medium at 0 h and 24 h after PPI pretreatment were measured by pH211 Calibration Check Microprocessor pH Meter (HANNA Instrument, Italy).

2.8. Intracellular Adriamycin (ADR) concentration analysis

Fluorescence intensity of intracellular ADR was determined by flow cytometry. The wavelengths of excitation light and emission light were 488 nm and 575 nm (Hong et al., 2007). Briefly, cells (2×10^5 cells per well) were seeded in 6-well culture plates. After 24–36 h culture, some cells were pretreated with PPI (1, 10, 20, 50 and 100 μg/ml) and others were left untreated. After another 24 h, cells continued to be cultured for 1 h in the RPMI-1640 containing ADR with a final concentration of 10 μM. Cells were trypsinized and harvested (for detection of ADR accumulation) or, alternatively, cultured in drug-free RPMI-1640 for another 1 h followed by trypsinization and harvesting (for detection of ADR retention). The ADR-releasing index of cells was calculated according to the formula: the ADR-releasing index = (accumulation value-retention value)/accumulation value.

2.9. Statistical analysis

The data were expressed as mean ± SD of the 3 independent experiments at least. Statistical analysis was performed with the software package SPSS 13.0. The significant difference between the means of different groups was evaluated by one-way ANOVA, using SNK, LSD and Dunnett's methods. Statistical significance was defined as $P < 0.05$ for all tests.

3. Results

3.1. Protein expression of V-H^+-ATPases by western blot analysis

The expression of V-H^+-ATPases in SGC7901 cells was examined by western blot analysis. After 24 h PPI pretreatment at concentrations of 1, 10 and 100 μg/ml, the expression of V-H^+-ATPases was altered when compared to those in the control group (Fig. 1). The expressions of V-H^+-ATPases in the 10 and 100 μg/ml PPI groups were significantly less than in the control group ($P < 0.05$), whereas those in the 1 μg/ml PPI group were significantly higher than in the control group ($P < 0.05$). Similarly, significant difference was found among the 3 different-dosage PPI groups ($P < 0.05$). The inhibitory effects of PPI pretreatment on the expressions of V-H^+-ATPases also gradually became evident with time. From the beginning to 12 h after PPI pretreatment, the expression of V-H^+-ATPase diminished continuously, particularly at 6 h and 12 h ($P < 0.05$). However, at 24 h it reverted to the same level as the control group (Fig. 2). A dose-dependent relationship was found between the PPI concentration and the expression of V-H^+-ATPases. Regression analysis demonstrated that a negative regression relationship existed between the PPI concentration and the expression of V-H^+-ATPases ($R^2 = 0.762$, $P < 0.05$) (Fig. 9).

3.2. Intracellular distribution of V-H^+-ATPases via immunofluorescence analysis

As an inhibitor of V-H^+-ATPases, PPI pretreatment (10 μg/ml) for 24 h caused a different intracellular localization of V-H^+-ATPase-expressing vesicles, which had previously accumulated in the perinuclear spaces (Fig. 3A and B). After PPI pretreatment, the intracellular distribution of V-H^+-ATPase expression was rather sparser (Fig. 3C) than in the control group. Meanwhile the fluorescent intensity of V-H^+-ATPase expression after 24-h PPI pretreatment also significantly decreased compared to the controls.

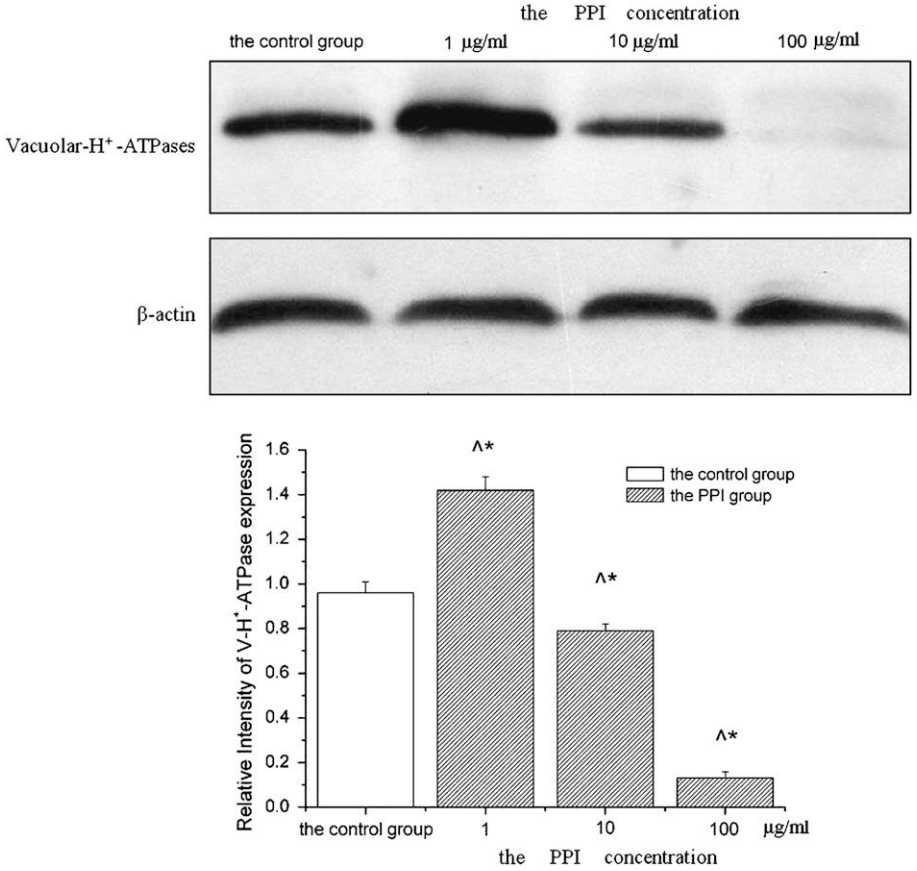

Fig. 1. Effects of PPI pretreatment with different concentrations on Vacuolar-H+-ATPases expression of SGC7901.*$P < 0.05$, significant difference was revealed when compared to that in the control group. ^$P < 0.05$, there were significant differences between any two of the three different-dosage PPI groups.

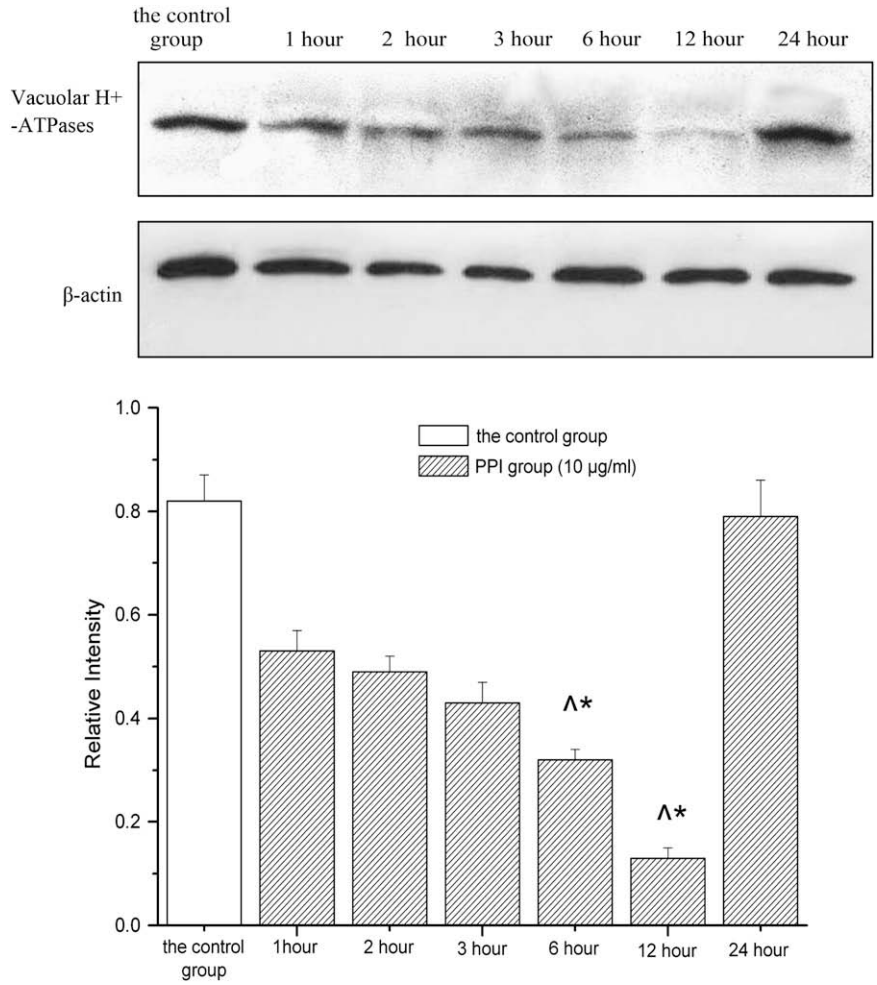

Fig. 2. Effects of PPI pretreatment on Vacuolar-H+-ATPase expression of SGC7901 at different time points. * $P < 0.05$, significant difference was revealed when compared to that in the control group. ^ $P < 0.05$, there were significant differences between one group and other five groups among six groups at various time points.

3.3. Effects of anti-tumor drugs with presence or absence of PPI pretreatment on the cell viability using MTT assay

The design and results of combination strategy including PPI and/or anti-tumor drugs are summarized in Fig. 4. The cell viability in the chemo group and other 3 PPI + chemo groups was clearly lower than in the PPI group ($74.3 \pm 1.8\%$, $71.6 \pm 1.5\%$, $71.9 \pm 0.9\%$ and $58.7 \pm 1.2\%$ versus $95.8 \pm 1.8\%$) ($P < 0.01$). Meanwhile, the cell viabilities in the PPI + chemo group-1 and -2 also were lower than the chemo group, but no significant difference was found between any 2 of the 3 groups. Furthermore, significant differences were found in the cell viability between the PPI + chemo group-3 and other 4 groups ($P < 0.01$).

3.4. Apoptosis detection

A quantitative analysis of the fluorescent signals was performed by Fluorescence Activated Cell Sorting (FACS). The results are summarized in Fig. 5A and B. In PPI + chemo group the total apoptosis rate and early apoptosis rate ($80.8 \pm 1.16\%$ and $77.5 \pm 1.13\%$) were significantly higher than those in the control group ($12.4 \pm 0.81\%$ and $8.3 \pm 0.69\%$), the PPI group ($23.9 \pm 0.55\%$ and $6.9 \pm 0.54\%$) and the chemo group ($26.4 \pm 1.19\%$ and $23.2 \pm 0.92\%$) ($P < 0.01$). However, the late apoptosis rate in the PPI group was significantly higher than that in other 3 groups ($17 \pm 0.9\%$ versus $4.1 \pm 0.43\%$, $3.2 \pm 0.46\%$, $3.3 \pm 0.81\%$) ($P < 0.01$) and also higher than the early apoptosis rate in the PPI group ($17 \pm 0.91\%$ versus $6.9 \pm 0.54\%$) ($P < 0.01$).

3.5. Measurements of the intracellular and extracellular pH values

The standard curve of fluorescence intensity versus the pHi value of SGC7901 is shown in Fig. 6A. According to regression analysis, the mathematic model was established following the formula: $Y = 1610 + 1.53 \times 10^{-5}\ e^{(2.83x)}$, $R^2 = 0.9782$. The pHi values of SGC7901 before and after PPI pretreatment with different concentrations at various time points were calculated based on this formula. Fig. 6B shows that the fluorescent intensity of BCECF in SGC7901 pretreated by PPI at a final concentration of 10 μg/ml for 24 h was obviously weaker than that in the control group. At 12 h after PPI pretreatment, no

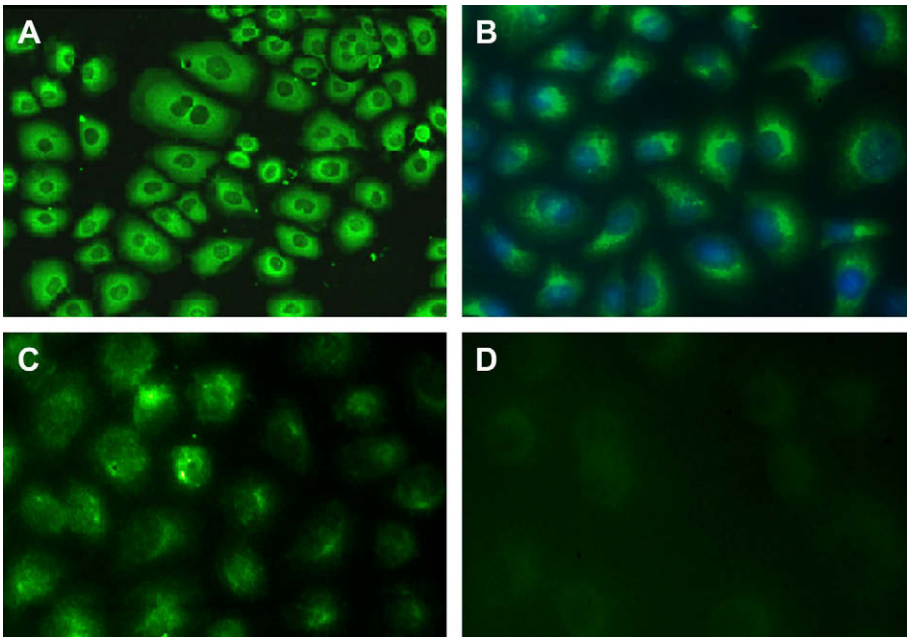

Fig. 3. Effects of PPI pretreatment on the intracellular distributions of Vacuolar-H^+-ATPases in SGC7901. A) Intracellular Distribution of Vacuolar-H^+-ATPases in SGC7901 before PPI pretreatment (×200). B) Intracellular Distribution of Vacuolar-H^+-ATPases in SGC7901 before PPI pretreatment using double staining (×200) DAPI stained for nuclei (DAPI: 0.5 μg/ml, Invitrogen). C) Intracellular Distribution of Vacuolar-H^+-ATPases in SGC7901 after 24-h PPI pretreatment (10 μg/ml) (×200). D) Negative Control (PBS as primary antibody) (×200).

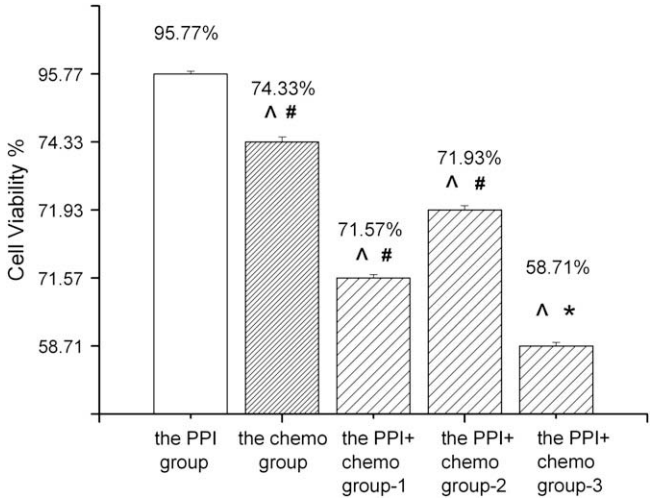

Fig. 4. Comparison of the cell viability of SGC7901 after the treatment of PPI and/or anti-tumor drugs. The PPI group: After 24-h incubation, the PPI solution was added into cells. The chemo group: After 48-hr incubation, 5-Fu and cisplatin were added into cells for 24 h. The PPI+chemo group-1: After 48-h incubation, PPI, 5-Fu and cisplatin were simultaneously added into cells for 24 h. The PPI+chemo group-2: PPI was added into cells after 36-hr culturing and then at 48 h 5-Fu and cisplatin were added for 24 h. The PPI+chemo group-3: PPI was added into cells after 24-h culturing and then at 48 h 5-Fu and cisplatin were added for 24 h. MTT ASSAY was performed to detect the cell viability at 72 h. **Dosage:** PPI (10 μg/ml); 5-Fu (25 μg/ml); cisplatin (1 μg/ml) ^ $P < 0.01$, the cell viabilities in the last four groups were significantly lower than that in the PPI group. # $P > 0.05$, the cell viabilities in the PPI+chemo group-1 and -2 also were lower than that in the chemo group, but no significant difference was found between any two of the three groups. * $P < 0.01$, significant differences were found in the cell viability between the PPI+chemo group-3 and other four groups.

significant difference was found among the 3 PPI groups, and similar result was also found when comparing to the control group (Fig. 6C). The pHi value of SGC7901 at 24 h after PPI pretreatment (10 and 100 μg/ml) was significantly lower than in the control group ($P < 0.01$). Conversely, the pHi value of cells after PPI pretreatment (1 μg/ml) was non-significantly higher than in the control group. However, significant difference also existed among the 3 PPI groups at 24 h ($P < 0.01$). At 48 h a remarkable difference was similarly found in the pHi value after PPI pretreatment (100 μg/ml), not only comparing to that in the control group ($P < 0.01$), but also in the 2 other PPI groups ($P < 0.05$). There was no significant difference in the pHi value between the 2 PPI groups (1 and 10 μg/ml). Meanwhile, a dose-dependent relationship between the pHi value and the PPI concentration showed that the pHi value of cells was negatively correlated with the PPI concentration. Similarly, the pHi value of SGC7901 was negatively correlated with expression of V-H^+-ATPases (Fig. 9).

Fig. 7 shows the pHi and pHe values at 0 and 24 h after PPI pretreatment. The pHi value at 0 h after PPI pretreatment was 7.51 ± 0.05 and the pHe values at 0 h after PPI pretreatment with different concentrations were significantly higher than in the control group ($P < 0.05$). After 24 h, PPI pretreatment at 10, 20, 50 and 100 μg/ml, the pHi values were significantly lower than in the control group ($P < 0.05$), whereas the pHi value in 1 μg/ml PPI group was not. Conversely, the pHe values at 24 h after different-dosage PPI pretreatment were significantly higher than in the control group ($P < 0.05$). Fig. 8A demonstrates that pretreatment with PPI exceeding 10 μg/ml could reverse the pH gradient (pHe > pHi), whereas PPI at the dose of 1 μg/ml could not. Meanwhile, changes of pH gradient after 24-h PPI pretreatment were also positively

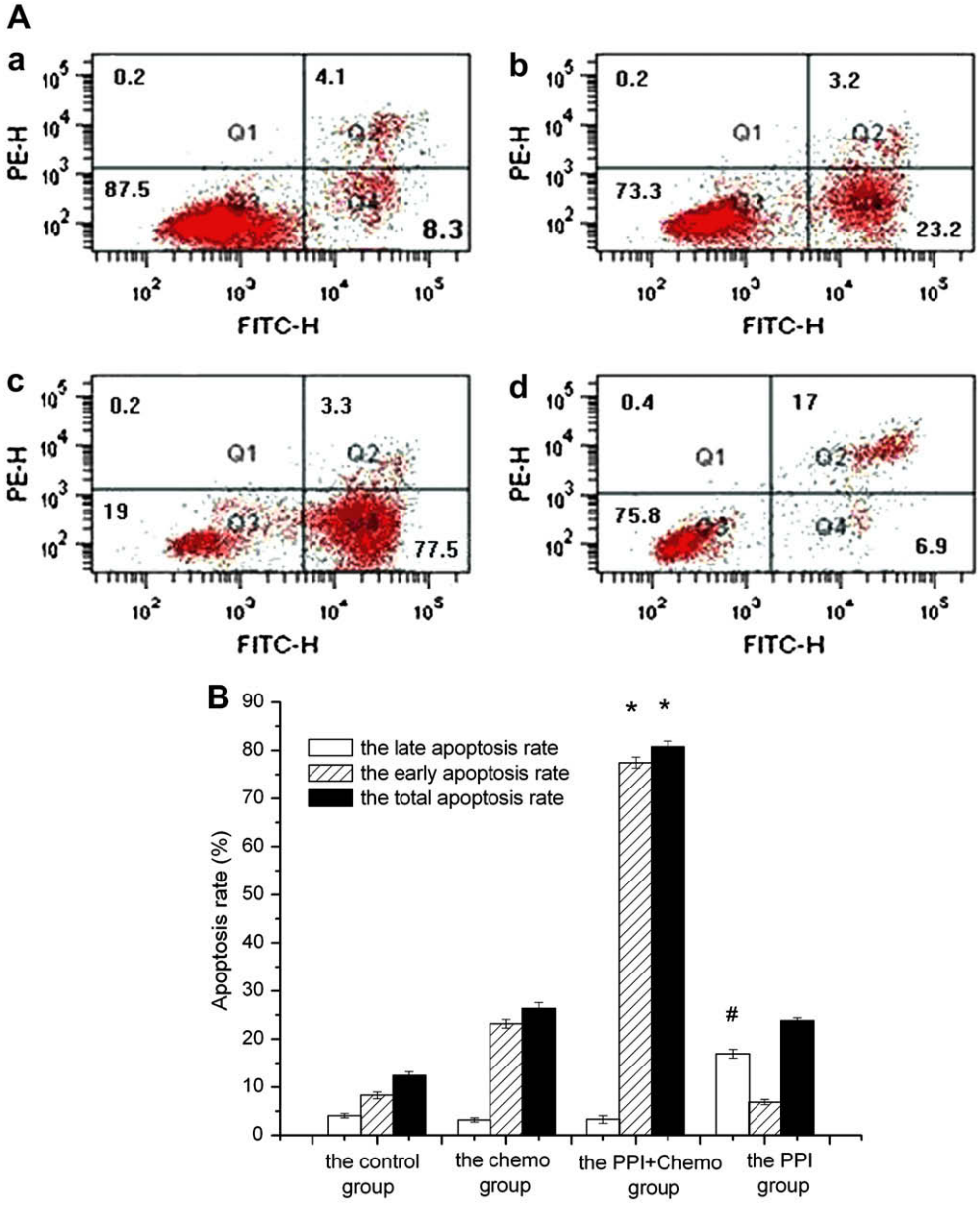

Fig. 5. A) Comparison of the apoptosis rate of SGC7901 cells after the treatment of PPI and/or anti-tumor drugs, a) The control group: No treatment, b) The chemo group: 5-Fu + Cisplatin were added into cells for 24 h after 48-h incubation, c) The PPI + chemo group: PPI was added into cells after 24-h incubation and then at 48 h 5-Fu and cisplatin were added for 24 h. d) The PPI group: PPI was added into cells for after 24-h incubation. **Dosage:** PPI (10 μg/ml); 5-Fu (25 μg/ml); cisplatin (1 μg/ml). B) Comparison of the apoptosis rate of SGC7901 after the treatment of PPI and/or anti-tumor drugs. * $P < 0.01$, the total apoptosis rate and the early apoptosis rate in the PPI+chemo group were significantly higher than those in the control group, the PPI group and the chemo group. # $P < 0.01$, the late apoptosis rate in the PPI group was significantly higher than those in the other three groups and also was higher than the early apoptosis rate in the same group.

correlated with the PPI concentration. The exponential relationship between them is given by the formula $Y = -0.332*e^{(-x/3.2486)} + 0.0671$, $R^2 = 0.95022$ (Fig. 8B).

3.6. Effects of PPI with various concentrations on intracellular ADR concentration

PPI pretreatment could reduce the ADR-releasing index in SGC7901 (Fig. 10). The ADR-releasing index decreased as the PPI concentration increased. PPI exceeding 20 μg/ml could significantly reduce the ADR-releasing index ($P < 0.01$). However, there was no significant difference between any 2 of the 20, 50 and 100 μg/ml PPI groups. After 24 h PPI pretreatment (20 μg/ml), the intracellular fluorescent intensity of ADR was clearly higher than in the control group (Fig. 11).

4. Discussion

Evidence is accumulating that hypoxia and acidity are involved in cancer progression and in the sensitivity of tumors to chemotherapy (Raghunand and Gillies, 2000). Besides, hypoxia and acidity may also contribute to the progression from benign to malignant growth (De Milito and Fais, 2005a). Tumor acidity, in particular, has a role in resistance to

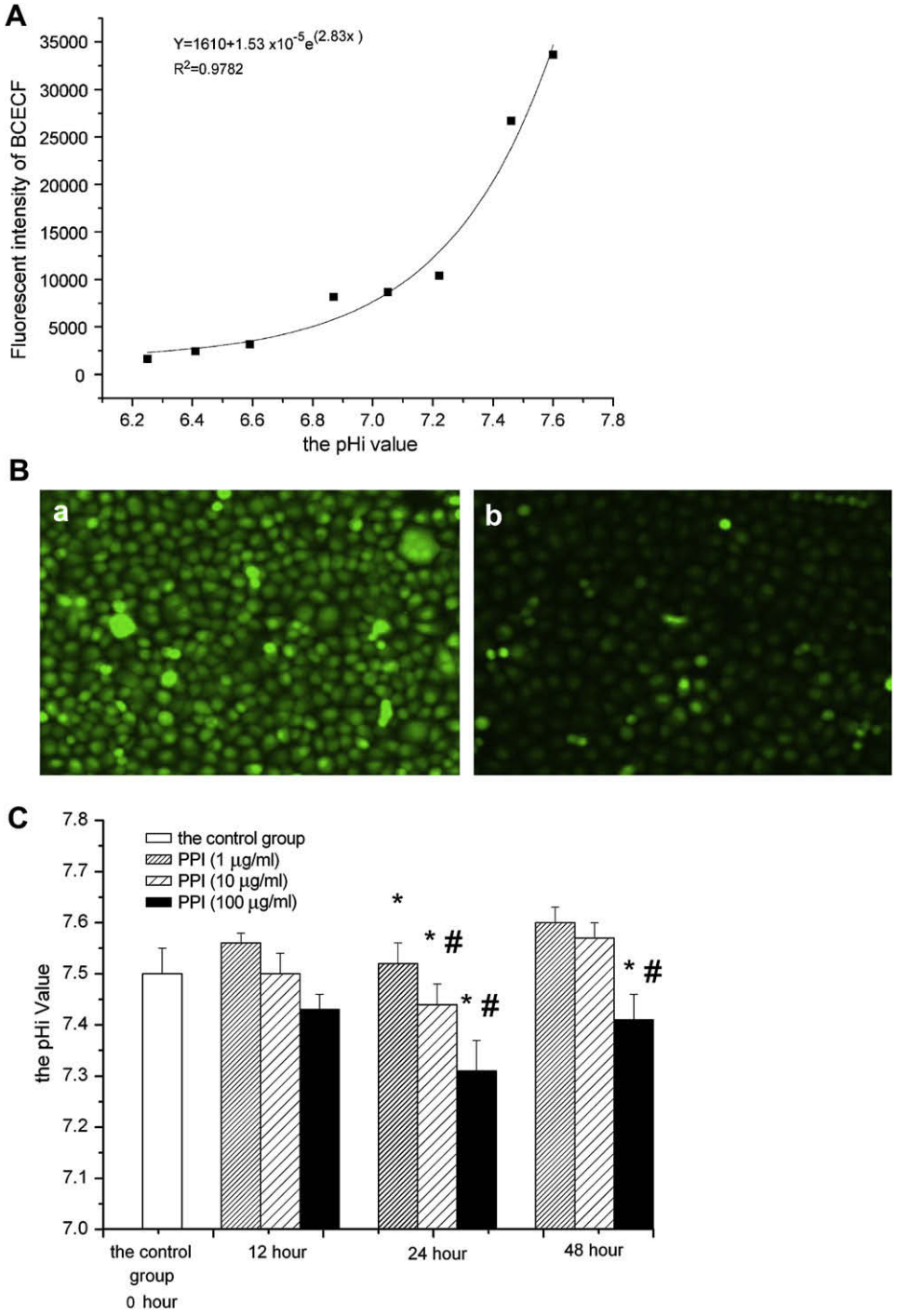

Fig. 6. A) Standard curve of fluorescence intensity of BCECF versus the pHi value of SGC7901. B) Comparison of the fluorescent intensity of BCECF in SGC7901 before and after 24-h PPI pretreatment A. the control group (×200) B. the PPI group (×200). The cells with presence or absence of 24−h PPI pretreatment (10 μg/ml) were incubated with the solution B containing BCECF (5 μM) for 1 h to visualize under a fluorescent microscope. C) Changes of the pHi value of SGC7901 after PPI pretreatment with different concentrations at different time points. # $P < 0.01$, significant difference could be found in different-dosage PPI groups when comparing to the control group. * $P < 0.05$, significant difference could be found between any two of the three groups or between one group and the other two groups at the same time point.

chemotherapy (Raghunand et al., 2001), proliferation (Morita et al., 1992) and metastatic behavior (Martinez-Zaguilan et al., 1996).

As to chemoresistance, many anticancer drugs are classified as either weak basic or molecules whose binding to cellular structures is pH dependent. Accumulation of these drugs within tumor cells should be affected by the transmembrane pH gradient (Raghunand et al., 1999). Thus lower pHe and higher pHi perhaps contribute to promoting MDR when drugs which partition across the membrane would be protonated and ironically trapped in the cytosol or acid vesicles in their biologically active form (Simon et al., 1994). Thus agents that

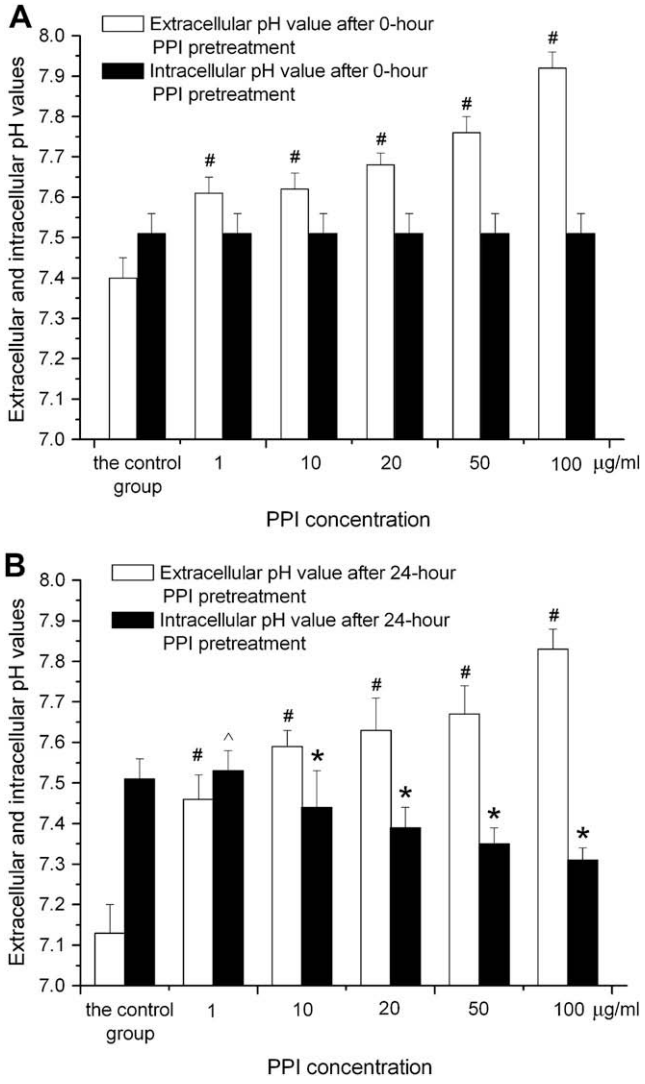

Fig. 7. A) Comparison of extracellular and intracellular pH values of SGC7901 after 0-h PPI pretreatment with different concentrations. # $P < 0.05$, the pHe values of the PPI groups at 0 h after PPI pretreatment were all significantly higher than in the control group. The pHi values after 0-h PPI pretreatment with different concentrations in six groups remains at the same level. B) Comparison of extracellular and intracellular pH values of SGC7901 at 24 h after PPI pretreatment with different concentrations. # $P > 0.05$, the pHe values of the PPI groups at 24 h after PPI pretreatment were all significantly higher than in the control group. ^ $P < 0.05$, no significant difference was found in the pHi value between the PPI group (1 μg/ml) and the control group. * $P < 0.05$, significant differences were found in the pHi value between the PPI group (10, 20, 50 and 100 μg/ml) and the control group. There also were significant differences in the pHi value between any two of the 1, 10, 20, 50 and 100 μg/ml PPI groups.

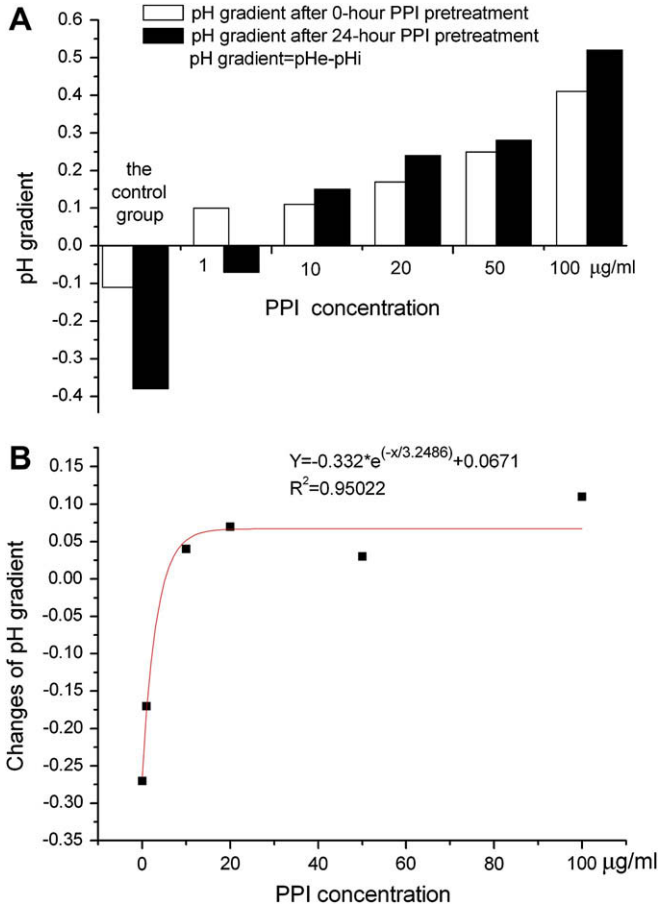

Fig. 8. A) Comparisons of pH gradients in SGC7901 at 0 h and at 24 h after PPI pretreatment with different concentrations. pH gradient=the pHe value-the pHi value. B) Scatterplot of changes of pH gradient versus the PPI concentration. Changes of pH gradient=pH gradient at 24 h after PPI pretreatment-pH gradient at 0 h after PPI pretreatment.

disrupt or normalize the pH gradient in tumors may reverse MDR and/or directly inhibit growth (De Milito and Fais, 2005b). In the case of proliferation, the unfavorable environment may favor the selection of tumor cells able to survive in acidic and hypoxic conditions. However, the normal cells are almost unable to survive in the same microenvironment (Morita et al., 1992; Cosse and Michiels, 2008). As to the metastatic behavior, many tumor cells secrete lysosomal enzymes that participate in degradation of the extracellular matrix, which is necessary for metastatic invasion. Because these enzymes have a lower pH optimum, their activity is greatly enhanced by an acidic extracellular environment (Otero-Rey et al., 2008). Therefore, how to inhibit tumor acidity might be the new strategy in future chemotherapy.

V-H^+-ATPases is a large, complex enzyme, composed of a cytosolic V_1 domain and a transmembrane V_0 domain, which are encoded by multiple genes (the ATP6V gene family) (Bowman et al., 2003; Murakami et al., 2001; Torigoe et al., 2002). Plasma membrane V-H^+-ATPases function in both normal and disease processes, which have been identified at the plasma membrane of certain tumor cells where they are thought to participate in the invasive properties of these cells (Sennoune et al., 2004). Therefore, V-H^+-ATPases are being investigated as a potential target in tumor treatment and PPI have been demonstrated to inhibit the activity of V-H^+-ATPases (Fais et al., 2007).

According to our results, PPI exceeding 10 μg/ml not only could inhibit the expression of V-H^+-ATPases and reverse the pH gradient, but could also affect its intracellular distribution and activity using immunofluorescent staining, strongly indicating the inhibitory effects of PPI on V-H^+-ATPases.

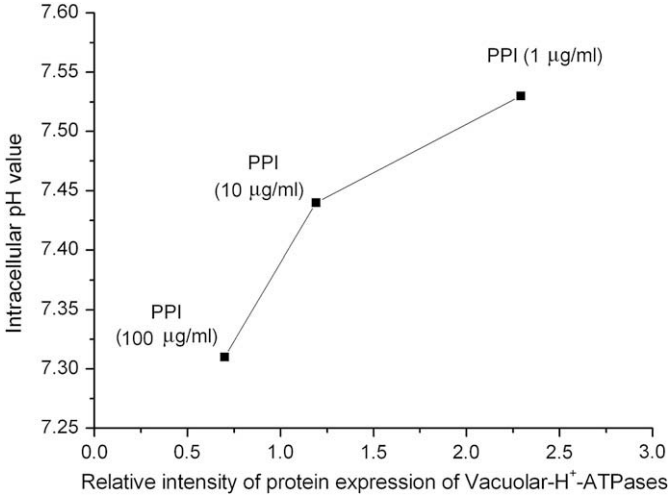

Fig. 9. Scatterplot of intracellular pH value versus Vacuolar H^+-ATPase expression in SGC7901.

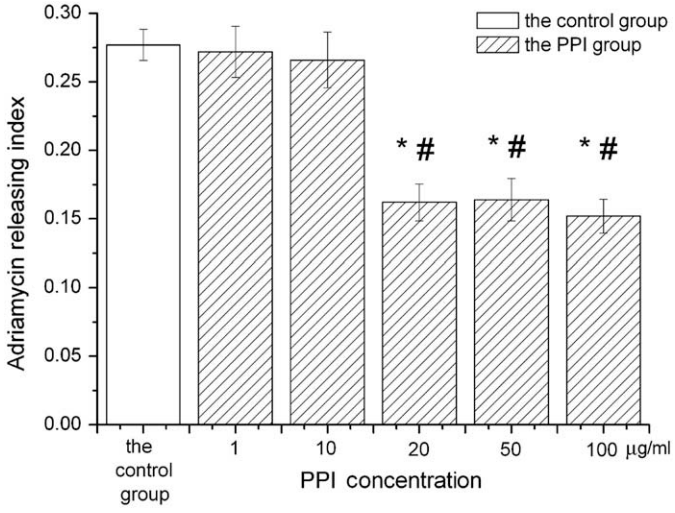

Fig. 10. Changes of ADR-releasing index after 24-h PPI pretreatment with different concentrations in SGC7901. * $P < 0.01$, significant differences in ADR releasing index were found in the 20, 50 and 100 μg/ml PPI groups when respectively comparing to that in the control group. # $P > 0.05$, no significant difference was found in ADR releasing index between any two of the three PPI groups.

However, pretreatment with PPI exceeding 1 μg/ml could not, which might be ascribed to negative feedback. In fact, the reverse pH gradient (pHi < pHe) could effectively contribute to the uptake of weakly basic anti-tumor drugs, enabling them to reach their intracellular target, thereby increasing cytotoxicity (De Milito and Fais, 2005a,b). Similarly, selective tumor alkalinisation of tumor pHe *in vivo* to fulfill these pH modifications are associated with regression or delay of tumor growth and also with enhanced chemotherapy (Raghunand and Gillies, 2000). Moreover, PPI are substituted 2-pyridyl-methylsulfinyl benzimidazoles that share a similar core structure (Vanderhoff and Tahboub, 2002) which are protonatable weak basic compounds with pKa values of ∼4 (Rabeprazole pKa = 5). Hence the active protonated form of PPI will necessarily accumulate into acidic compartments. Therefore, it seems conceivable that PPI may specifically get to the tumor site, being an additional acidic compartment in cancer patients (De Milito and Fais, 2005a,b). Besides, weak basic PPI might neutralize acid effluxing from tumor cells maintaining a neutral or weak basic extracellular microenvironment.

In addition, PPI could induce apoptosis in tumor cells. As indicated by Yeo et al. (2004), PPI selectively induces *in vivo and in vitro* apoptotic cell death in gastric cancer, suggesting that PPI could be used for selective anticancer effects which may be caused by suppressing ERK phosphorylation. Other research has indicated the potential use of PPI as antineoplastic agents towards human B-cell tumors (De Milito et al., 2007). However, in our research we investigated the role of PPI as a chemosensitizer so that relatively lower dosage (10 μg/ml) was considered as the optimal dosage, to avoid its apoptosis-inducing effects as well as excessive alkaline pH of the PPI solution.

We also studied the optimal time of administration of PPI on SGC7901. Firstly, the chemo drugs combined with PPI pretreatment were more effective than administration of chemo drugs or PPI alone, indicating their synergistic effects. Then among the PPI + chemo groups, we found that administration of chemo drugs after 24-h PPI pretreatment could exhibit the strongest inhibitory effects on the cell viability. We observed that the pHi value at 24 h after PPI pretreatment was the lowest, which may explain why the moment at 24 h after PPI pretreatment was the optimal time point to add the chemo drug since the extent of partitioning of chemotherapeutic drug molecules across the plasma membrane of a tumor cell is therefore dependent upon the pKa pf the drug as well as the pHi and pHe. In the case of weak bases, the charged form of the drug is the protonated form, and it concentrates on the more acid side of the membrane, leading to greater total drug on the acidic side of the membrane, fulfilling cytotoxicity (Raghunand and Gillies, 2000). In addition, we also found that expression of V-H^+-ATPases diminished after PPI pretreatment but was restored from 12 h to 24 h. It could be inferred that the lowest level of V-H^+-ATPases primarily occurs from 12 h to 24 h after PPI pretreatment, explaining why the lowest pHi could be detected at 24 h after PPI pretreatment.

Another interesting finding was that simultaneous administration of PPI and the chemo drugs was obviously less effective than administration of anti-tumor drugs after 24 h PPI pretreatment. A possible explanation for this phenomenon may lie in the documented ability of some cytotoxic drugs, such as cisplatin, to increased activity of V-H^+-ATPases in treated tumor cells (Murakami et al., 2001). It is also conceivable that the simultaneous administration of PPI and the weakly basic cytotoxic drug may lead to a competition between the 2 drugs for the tumor acidic environment. This competition may in turn lead, on one hand, to the inactivation of the cytotoxic drugs or, on the other hand, to the non-activation of PPI, which needs to be protonated to work. In addition, PPI also need a certain time to exhibit the significantly inhibitory effects on the expression of V-H^+-ATPases and then to reverse the pH gradient, which deserves further investigation.

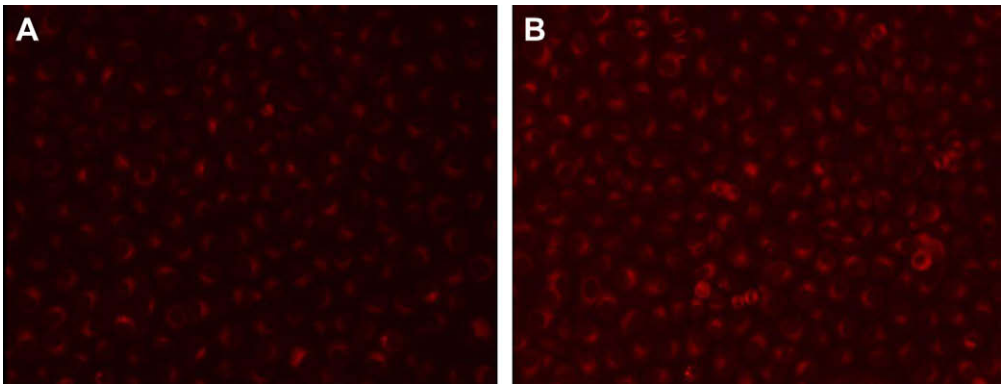

Fig. 11. Comparison of intracellular ADR fluorescent intensity of SGC7901 before and after 24-h PPI pretreatment. A. the control group (×200) B. the PPI group (×200). The cells with or without 24-h PPI pretreatment (20 μg/ml) were incubated with RPMI-1640 containing ADR (10 μM) for 1 h to visualize under a fluorescent microscope.

In detecting apoptosis, PPI (10 μg/ml) alone could not effectively induce apoptosis of tumor cells compared to the chemo drugs, which was possibly related to its lower dosage. But the chemo drugs after 24 h PPI pretreatment could increase the early apoptosis rate more significantly than the chemo drugs alone. Perhaps it was closely linked with PPI pretreatment on reversing the pH gradient and promoting the intracellular accumulation of the chemo drugs. It might be predicted that PPI, as weak basic drugs, could also help to enhance lipotropy of anti-tumor drugs by maintaining a rather weak basic extracellular microenvironment, accelerating them to reach their target. Interestingly, changes of pH gradient also were positively correlated with the PPI concentration, which appeared to enhance the intracellular accumulation of chemotherapeutic drugs. Similarly, Goossens et al. (2000) have reported that intracellular acidification, an early event in apoptosis, increased the susceptibility of cells to killing by chemotherapeutic agents, and was found in HL-60 cells undergoing apoptosis in response to etoposide and camptothecin. These results indicate that PPI, as one of the V-ATPase inhibitors, in combination with low-dose anticancer agents, may provide a new therapeutic approach.

We also confirmed that the ADR-releasing index obviously decreased when the PPI concentration was more than 20 μg/ml. It should be noted that ADR was the common substrates for P-glycoprotein (P-gp) and Multidrug Resistance Protein (MRP) (Gottesman and Pastan, 1993). Hence it can be predicted that PPI pretreatment inhibits the expression of ATP-Binding Cassette transporter superfamily (ABC transporter superfamily) which needs further investigation (Reed, 1999). In particular, ABC transporters, such as P-gp or MRP etc, mediate energy-dependent drug efflux and are closely involved in MDR of tumor cells (Gottesman et al., 2002). Moreover, PPI could inhibit the function of various ATPases and then might influence the intracellular synthesis of ATP, providing the possibility for inhibiting the expression of ABC transporter superfamily.

In conclusion, PPI inhibits the expression of V-H^+-ATPases, and reverses the transmembrane pH gradient so as to sensitize the SGC7901 cells to the anti-tumor drugs. Overexpression of V-H^+-ATPases might play a crucial role in MDR of gastric cancer so that downregulation of V-H^+-ATPases expression by PPI could facilitate the chemosensitivity of SGC7901 to the anti-tumor drugs. However, the possible relevant signaling pathway of PPI involved in enhancing the chemosensitivity needs further research. Further detailed investigation should also be performed to investigate the relationship between inhibition of V-H^+-ATPases and reversal of MDR.

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