

Inhibition of lysosomal enzyme activities by proton pump inhibitors

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Abstract

Background Proton pump inhibitors (PPIs) are pro-drugs requiring an acidic pH for activation. The specificity of PPI toward the proton pump is mainly due to the extremely low pH at the parietal cell canalicular membrane where the pump is located. Reactivity of PPIs was also observed in moderately acidic environments like the renal collecting duct. But no PPI effect on lysosomal enzymes has been observed possibly because the previous studies were performed with liver tissue, where PPIs are metabolized.

Methods The reactivity of PPIs (omeprazole, lansoprazole and pantoprazole) with a cysteine-containing peptide was analyzed by mass spectrometry, and the impact of PPIs on lysosomal enzymes was evaluated in cultured cells and mice. The effect of PPIs on the immune system was examined with a mouse tumor immunotherapy model.

Results Incubation of a cysteine-containing peptide with PPIs at pH5 led to the conversion of most of the peptide into PPI-peptide adducts. Dose dependent inhibition of lysosomal enzyme activities by PPIs was observed in cultured cells and mouse spleen. Further, PPI counteracted the tumor immunotherapy in a mouse model.

Conclusions Our data support the hypothesis that many of the PPI adverse effects are caused by systematically compromised immunity, a result of PPI inhibition of the lysosomal enzymes. This novel mechanism complements the existing mechanisms in explaining the increased incidence of tumorigenesis and infectious diseases among PPI users and underlie the ongoing concern about the overuse of PPIs in adult and pediatric populations.

Keywords Omeprazole · Lansoprazole · Pantoprazole

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Abbreviations

AP	Acid phosphatase
CID	Collision-induced dissociation
GERD	Gastroesophageal reflux disease
MPO	Myeloperoxidase
NAG	β -N-acetylglucosaminidase
PPIs	Proton pump inhibitors

Introduction

Proton pump inhibitors (PPIs) such as omeprazole, lansoprazole and pantoprazole are substituted benzimidazole compounds used to inhibit gastric acid secretion in the

treatment of peptic ulcer, gastroesophageal reflux disease (GERD) and other acid-related diseases. PPIs are superior to H₂ receptor antagonists for more potent suppression of acid production [1–4] and no tachyphylaxis [5]. In many circumstances, PPIs have been a life-saving medicine and have significantly reduced the incidence of gastroesophageal surgery in the US since the implementation of PPI therapy in 1989 [6, 7].

PPIs are produced in a lipophilic inactive form which freely crosses cell membranes until they arrive at an acidic compartment where these weak bases are protonated and are no longer membrane permeable [8]. In the acidic environment, PPIs undergo a series of rearrangement reactions resulting in the formation of sulphenamides as the major reactive agents toward the thiol groups of proteins [8]. The irreversible covalent binding of PPI molecules to the thiol groups at the active site of the gastric proton pump (the H, K-ATPase) inactivates the pump [8]. The specificity of PPIs is not because PPIs react preferably with the thiol groups of the proton pump, but rather a consequence of the extremely low pH in the stomach during active secretion which serves to locally accumulate as well as to activate the drug.

As such, concerns have been raised regarding possible reactivity of PPIs in acidic compartments other than the stomach. Inhibitory effects of PPIs were observed in the renal collecting duct [9] and in osteoclasts [9–11], but not on lysosomal enzymes [12–14]. However, the studies of PPI effects on lysosomal enzymes were performed only with liver tissue, the very tissue where PPIs are metabolized. Therefore, a possible effect of PPI on lysosomal enzyme activities in other tissues remains unknown. Here, we report that PPIs and cysteine-containing peptides readily formed adducts at pH5. Subsequently, we demonstrated that PPIs are capable of inhibiting lysosomal enzyme activities *in vitro* and *in vivo*.

Methods

Formation of PPI-laminin (925–933) adduct

Each of the PPIs omeprazole, lansoprazole and pantoprazole (Sigma-Aldrich, St. Louis, MO) were prepared as 30 mM stock in ethanol. Each PPI was mixed with laminin peptide (aa925–933, Sigma-Aldrich, cat# C0668) at a molar ratio of PPI:peptide = 10:1 in a solution with a final pH5.0. The constituents of the mixture were: 6 mM PPI, 0.6 mM laminin (925–933), 2 mM HCl, 6 mM acetic acid, 20 % ethanol. HCl is needed to attain pH5.0 as these PPIs are weak bases. The mixture was incubated for 10 min at room temperature to allow formation of PPI-laminin (925–933) adduct.

Mass spectrometry analysis of PPI-laminin (925–933) adduct

Equivalent amounts of each PPI, laminin (925–933) peptide and their reaction product were lyophilized to dryness using a SpeedVac concentrator. These samples were then reconstituted with the loading solution (50 % acetonitrile, 0.1 % formic acid) before nano-LC-ESI-Qq-TOF tandem mass spectrometry analysis. The instrument settings for the mass spectrometry analysis was the same as described previously [15].

Mass spectra were analyzed using Analyst QS software (version 2.0). All collision-induced dissociation (CID) spectra were manually examined to verify that most of the y sequence ions were detected. The total ion intensities of each molecule were determined using the Bayesian peptide reconstruction module provided in the BioAnalyst extension of the Analyst QS software.

Cell cultures and PPI treatments

A549, Caco2, HEK293, and HepG2 cell lines were obtained from the American Type Culture Collection. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum at 37 °C, under a humidified atmosphere of 5 % carbon dioxide. After 24 h of plating in 10-cm culture dishes, cells were treated with PPIs (omeprazole, lansoprazole, or pantoprazole) at concentrations of 0, 10, or 30 μM for 5 times with interval of 12 h, and then harvested for enzyme activity assay. To test for the specificity of the PPI effect, a similar procedure was performed with cimetidine at concentrations of 0, 10, and 30 μM.

Measurement of lysosomal enzyme activities

Cell pellets or mouse tissues were resuspended or added in homogenization buffer (0.5 % hexadecyltrimethylammonium bromide (HTAB), 5 mM EDTA, and 50 mM potassium phosphate buffer, pH6). Homogenates were made using a tissue grind pestle (Kontes). After incubation at 55 °C for 1 h, samples were centrifuged at 16,000g for 10 min. The supernatants were used for enzyme assays. Acid phosphatase (AP) and β-N-acetylglucosaminidase (NAG) activities were measured using kits from Sigma, according to the manufacturer's instructions. Myeloperoxidase (MPO) activity was measured by continuously monitoring the H₂O₂ dependent oxidation of o-dianisidine dihydrochloride (ODH) at a wavelength of 460 nm for 90 s with intervals of 2 s. MPO activity was calculated as the absorbance change per minute over the linear portion of the curve. Protein concentrations were measured using the BCA method (Pierce). All enzyme

activities were normalized to the protein contents in the samples. Enzyme activities in PPI treated groups were compared with control groups whose enzyme activities were expressed as 100.

Mice and PPI treatments

All experimental protocols were approved by the Institutional Animal Care and Use Committee of University at Buffalo.

To evaluate the PPI effect on lysosomal enzyme activities, C57BL6 male mice were purchased from Harlan Laboratories and housed in the institutional Laboratory Animal Facility. At 8 weeks of age, animals were injected intraperitoneally (IP) with omeprazole at doses of 0.57 or 2.85 mg/kg, five times consecutively every 12 h. Control animals were injected with the vehicle only. Two hours after the last injection, the animals were sacrificed and tissues were collected for enzyme activity assays.

To examine the PPI effect on tumor immunotherapy, a single tumor was implanted in each 6-week-old BALB/c mouse (Harlan Laboratories) by injection with 1×10^6 4T1 cells (a syngeneic BALB/c mammary carcinoma) in 0.1 ml DMEM behind the neck just above the scapula, as described previously [16]. The mice were then randomized and separated into three treatment groups: (1) the control group was injected with the vehicle, (2) the immunotherapy group was given a single injection of slow release IL-12/GM-CSF on day 0 as specified previously [17], and injections of vehicle, and (3) the omeprazole + immunotherapy group was given a single injection of slow release IL-12/GM-CSF on day 0, and omeprazole injections twice a day starting from day 0. Tumors were monitored until they reached 300–400 mm³ in size.

Statistical analysis

One-way ANOVA and Tukey post hoc tests were used to analyze the differences among control and PPI treated groups with different doses. A *p* value less than 0.05 was considered statistically significant.

Results

Formation of PPI-laminin (925–933) adduct at pH5

To explore the reactivity of PPI with peptide at pH5, laminin (925–933) peptide was selected because it contains a free thiol group from the side chain of cysteine and a C-terminal arginine, which facilitates the analysis of the PPI-peptide adduct by LCMSMS. As shown in Fig. 1, the omeprazole–laminin (925–933) adduct was readily formed

at pH5. Figure 1a demonstrated the isotopic peaks of the intact omeprazole–laminin (925–933) adduct. Isotopic peaks 0.5 Da apart indicated that this is a doubly charged ion, therefore having a molecular mass of 1293.6 Da, an exact match for the calculated molecular mass of the omeprazole-peptide adduct. According to the calculated theoretical molecular masses of the fragmentation ions (Fig. 1b), the identity of the doubly charged ion from Fig. 1a was confirmed to be the omeprazole–laminin (925–933) adduct by the collision-induced dissociation spectrum (Fig. 1c) of the fragmentation products of the adduct.

To assess the completeness of the reaction between omeprazole and the laminin (925–933) peptide, the mass spectra of the starting materials and the product were quantified (Fig. 1d). Since omeprazole was in excess to the peptide in the reaction mixture, abundant omeprazole sulphenamide was detected in the end product. Laminin (925–933) peptide was also detected in the end products, but the intensity was greatly diminished compared to the starting material. Importantly, the omeprazole–laminin (925–933) adduct was a major component in the end product. Apparently the majority of the laminin peptide was converted to the omeprazole-peptide adduct. Similar results were obtained for lansoprazole (Fig. 2 a, b) and pantoprazole (Fig. 2c, d), when incubated with laminin (925–933) at pH5.

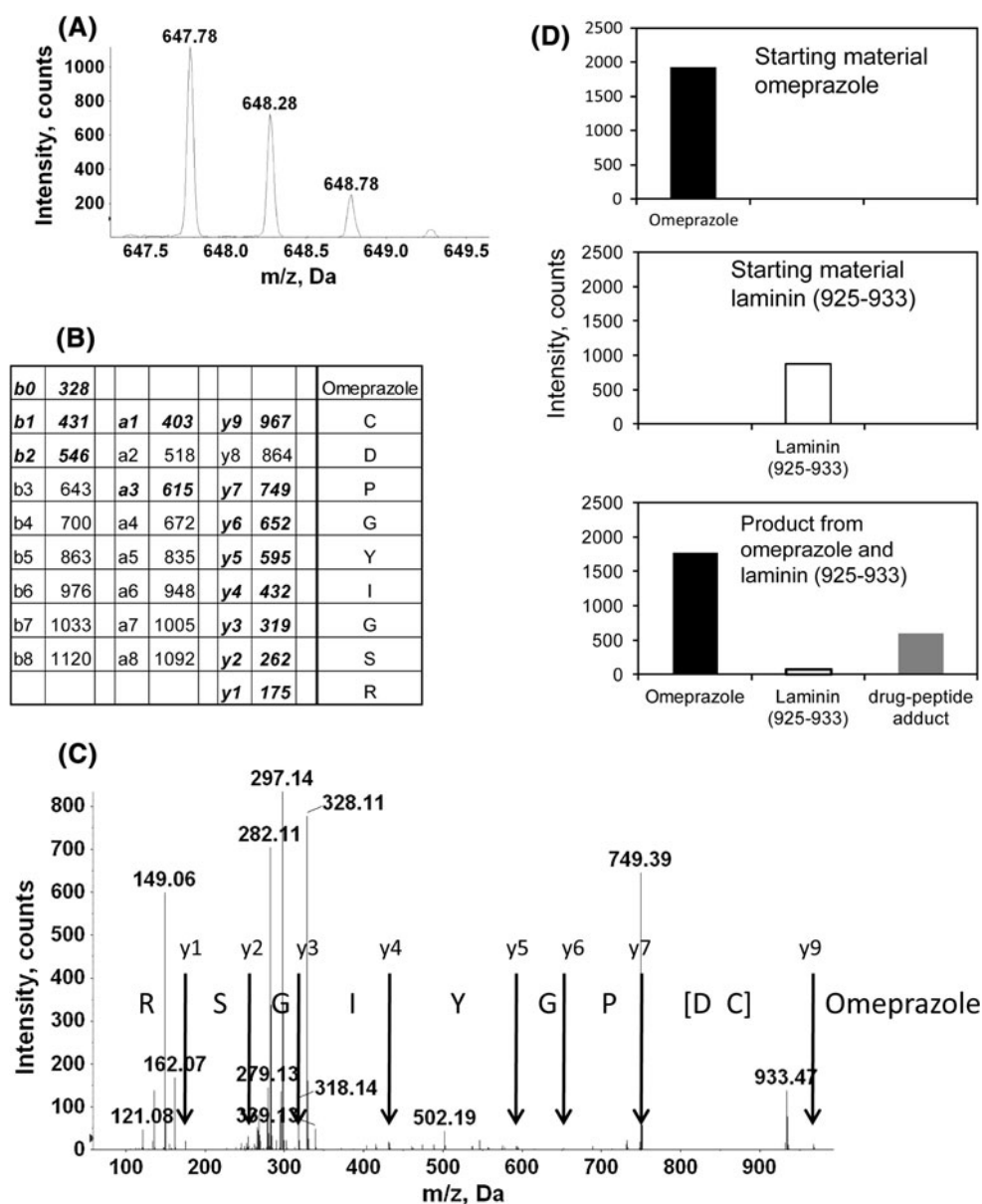
PPIs inhibited lysosomal enzyme activities in cultured cells

The results described above suggested activation of PPIs at pH5 and their reactivity with cysteine-containing peptide at pH5. Since pH5 is commonly found in the lysosome, a universal organelle important for most cells in the body, the possible inhibition of lysosomal enzymes by PPIs was evaluated with four cell lines derived from different human tissues/cells: A549 (lung), Caco2 (colon), HEK293 (kidney) and HepG2 (hepatocyte). Compared to cells treated with vehicle (controls), dose-dependent inhibition of both AP and NAG by omeprazole was observed with all four cell lines (Fig. 3a, b). Both low dose (10 μM) and high dose (30 μM) omeprazole treatment significantly inhibited the lysosomal activities in all four cells tested.

Dose-dependent inhibition of acid phosphatase and β-N-acetylglucosaminidase were also observed with lansoprazole (Fig. 3c, d). Compared to omeprazole, lansoprazole seemed to have more potent inhibitory effects on both enzymes. It is worth noting that the inhibitory effects of lansoprazole in HepG2 cells were less pronounced compared to other cells. No significant inhibition of acid phosphatase by lansoprazole at 10 μM in HepG2 cells was observed. Dose-dependent inhibition of acid phosphatase

Fig. 1 Formation of omeprazole–laminin (925–933) adduct at pH5. Omeprazole and laminin (925–933) peptide were incubated at pH5 and analyzed by LC-MS/MS.

a Detection of the intact omeprazole–laminin (925–933) adduct by mass spectrum. Isotopic peaks 0.5 Da apart indicated that the detected ion was doubly charged. **b** Expected and detected fragments of omeprazole–laminin (925–933) adduct after CID. All expected *b* ions, *a* ions and *y* ions were listed, with the detected ions bolded and italicized. **c** Mass spectrum of the CID fragmentation products of omeprazole–laminin (925–933) adduct. C terminal Arg facilitated the detection of *y* ions, which were labeled by arrows. **d** Quantitation of the mass spectra of the starting materials (omeprazole and laminin peptide, respectively) and the product (omeprazole–laminin peptide adduct). Counts are the summary of all isotopic peaks of differently charged ions of the same molecule in the whole LCMSMS run. Similar PPI-peptide adducts were obtained with lansoprazole and pantoprazole at pH5



and β -*N*-acetylglucosaminidase by pantoprazole were observed in Caco2 and HEK293 cells, but not in A549 or HepG2 cells (Fig. 3e, f).

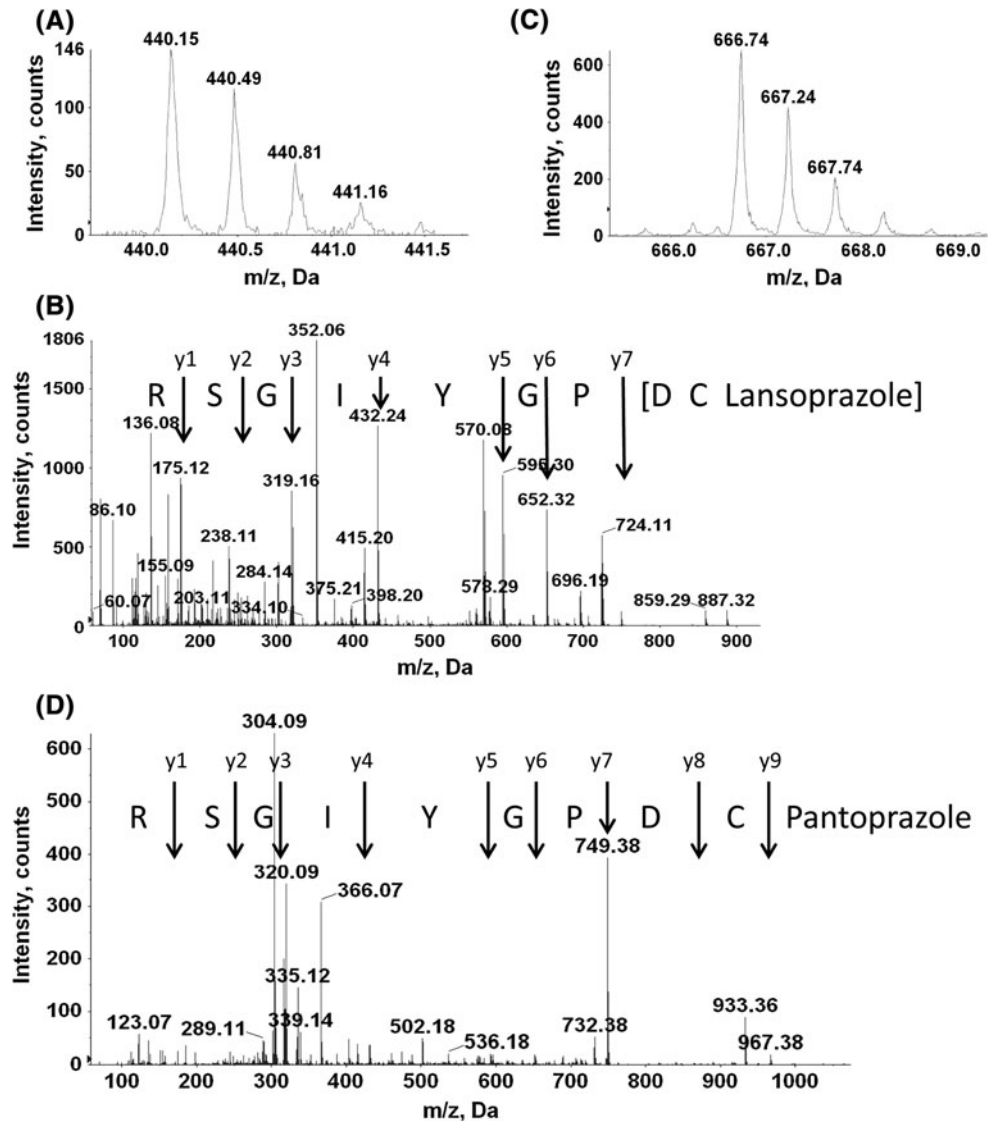
No inhibitory effect on AP or NAG by cimetidine was observed with both low dose (3 μ M) and high dose (30 μ M) treatment (Fig. 3g, h).

PPI inhibited lysosomal enzyme activities in vivo

For in vivo examination of the possible inhibition of lysosomal enzyme activities by PPI, omeprazole was used since it is the most popular PPI and its inhibitory effect on lysosomal enzyme activities in cultured cells appeared to

be a good representation of all PPIs tested. Mice were injected (IP) with omeprazole before analysis of lysosomal enzyme activities in the liver, the stomach and the spleen. Analysis of the acid phosphatase activities in the liver and the stomach indicated that animals injected with omeprazole (both 0.57 and 2.85 mg/kg dose) were no different from animals injected with vehicle (data not shown). However, dose-dependent, significant inhibition of acid phosphatase in the spleen was observed in omeprazole-injected mice, compared to animals injected with vehicle (Fig. 4a). Similarly, dose-dependent, significant inhibition of MPO in the spleen was observed in omeprazole-injected mice (Fig. 4b).

Fig. 2 Formation of PPI-peptide adduct by lansoprazole and pantoprazole at pH5. **a** Detection of the intact lansoprazole–laminin (925–933) adduct by mass spectrum. **b** Mass spectrum of the CID fragmentation products of lansoprazole–laminin (925–933) adduct. **c** Detection of the intact pantoprazole–laminin (925–933) adduct by mass spectrum. **d** Mass spectrum of the CID fragmentation products of pantoprazole–laminin (925–933) adduct

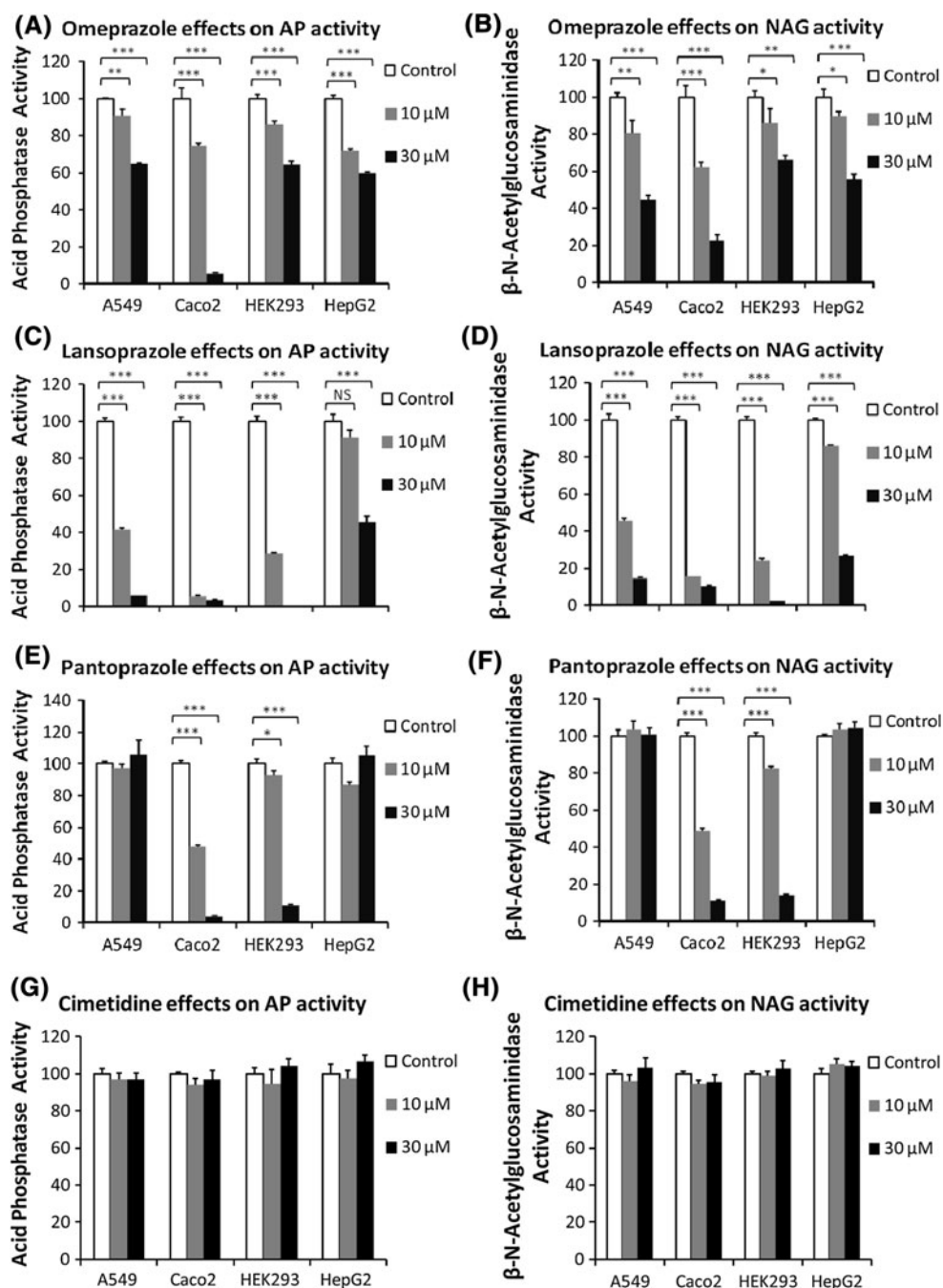


PPI inhibited tumor immunotherapy in a mouse model

The inhibitory effect of PPI on lysosomal enzymes, together with the fact that lysosomal enzymes are required in both humoral and cellular immunity, raised the possibility that some of the adverse effects of PPIs are due to the PPI inhibition of the immune system. To test this hypothesis, we took the advantage of a well-characterized mouse tumor immunotherapy model in which the immunotherapy regimen induces potent immune responses which effectively control and even eradicate the tumor [17]. BALB/C mice with a single tumor implant were subjected to three treatments: (1) mice in the control group were injected with vehicle, (2) mice in immunotherapy group were injected with IL-12/GM-CSF, and (3) mice in immunotherapy + PPI group were given IL-12/GM-CSF and ome-

prazole. The sizes of the tumors were monitored to evaluate PPI impact on immunotherapy. The tumor growth curve of the immunotherapy group is indicative of a two-phase event (Fig. 5a). Days 0–2 were the inflammation phase in which the tumor growth of the immunotherapy group was boosted by the IL-12/GM-CSF treatment. During this phase, the tumor growth of the immunotherapy + PPI group was significantly slower than the immunotherapy group (Fig. 5b). Days 2–10 were the next phase in which the tumor growth of the immunotherapy group was tightly controlled. In contrast, the tumor size of the control group increased exponentially over time. The tumors of the immunotherapy + PPI group also exhibited an exponential growth curve. The tumor growth of the immunotherapy + PPI group was significantly faster than that of the immunotherapy group (Fig. 5c).

Fig. 3 Inhibition of lysosomal enzyme activities in cultured cells by PPIs. Cell lines of various origins A549 (lung), Caco2 (colon), HEK293 (kidney) and HepG2 (hepatocyte) were treated with the pro-drugs omeprazole (a, b), lansoprazole (c, d) or pantoprazole (e, f), respectively. The final concentrations of the PPIs were 10 and 30 μ M, with cells treated with vehicle as controls. To test the specificity of the PPI effects, a histamine H₂-receptor antagonist, cimetidine, was used to treat cells at same concentrations (g, h). The activities of lysosomal enzymes acid phosphatase (a, c, e, g) and β -N-acetylglucosaminidase (b, d, f, h) were analyzed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS not significant



Discussion

We demonstrated that PPIs are readily activated at pH5 and reacted with cysteine-containing peptide. We then showed potent inhibition of lysosomal enzyme activities by PPIs in vitro and in vivo. Further, we showed that PPI negatively affected tumor immunotherapy in a mouse model.

Reaction of PPIs with peptides other than the proton pump is expected, as the activated sulphenamide form of PPIs can react with any free thiol group abundantly available in many proteins. Brandstrom et al. [8] reasoned

that unprotected oral PPI intake would cause a significant loss of PPI activity because PPIs activated in the stomach will react with the content of the GI tract before reaching the proton pumps at the canalicular membrane of the parietal cells. Reaction of PPIs with thiol groups in other peptides was suggested by the observation that PPIs inhibit the V-ATPase in the mildly acidic environment of kidney and osteoclast [9]. Our observation that PPIs readily reacted with the thiol group of a peptide at pH5 provides direct support to those studies performed with the kidney and the osteoclast.

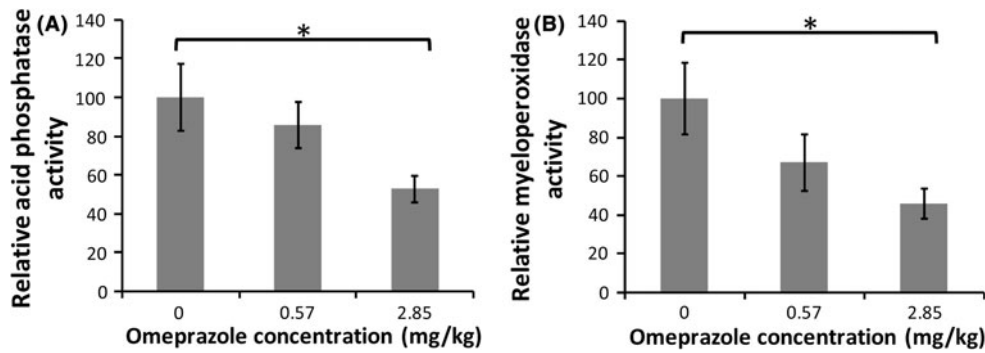
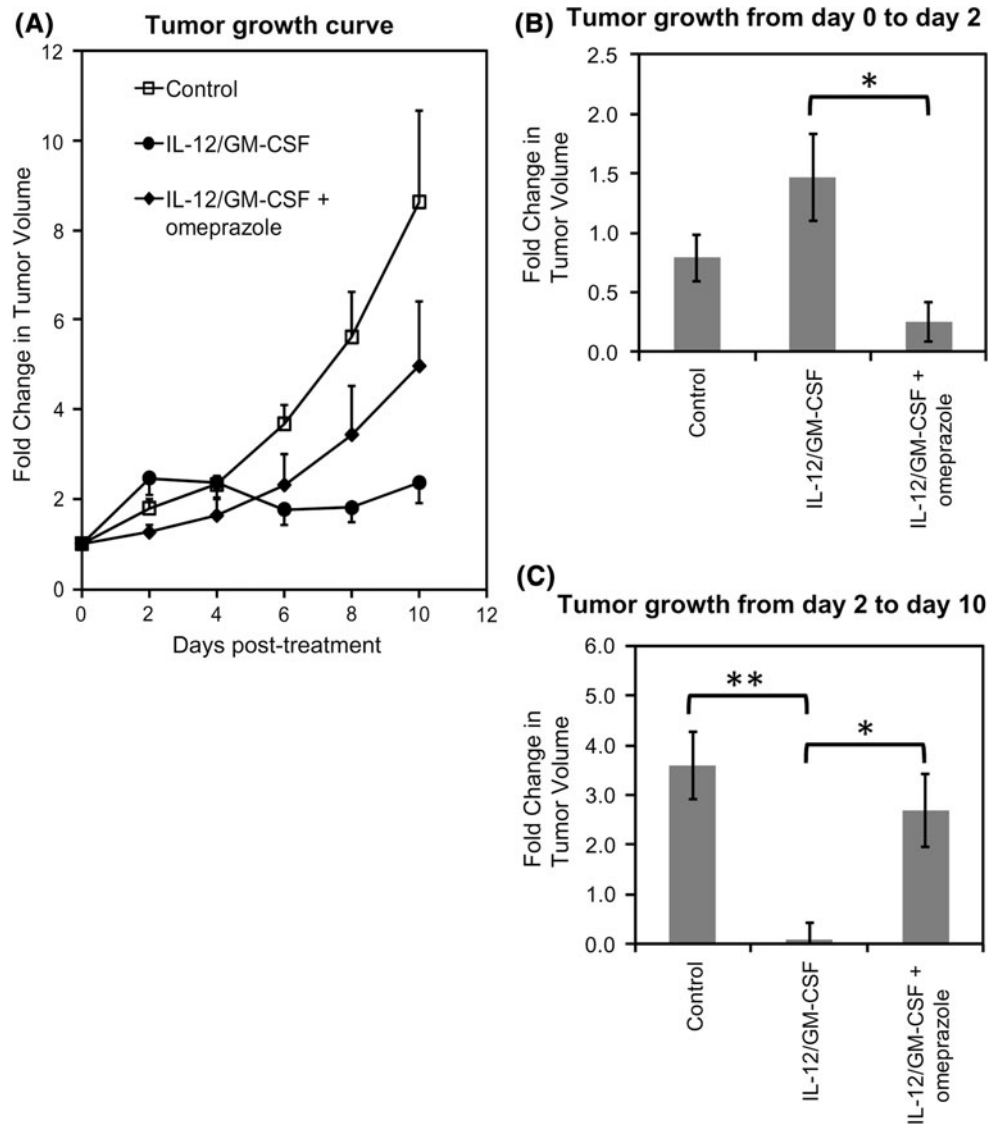


Fig. 4 Omeprazole inhibition of lysosomal enzyme activities in mice. Mice treated with 0.57 mg omeprazole per kg ($n = 6$), 2.85 mg omeprazole per kg ($n = 6$), or vehicle ($n = 6$) were sacrificed and

analyzed for lysosomal enzyme activities in the spleen. For both acid phosphatase (a) and myeloperoxidase (b) activities, a dose-dependent inhibition by omeprazole were observed. $*p < 0.05$

Fig. 5 Omeprazole effect on tumor immunotherapy. A single tumor was planted subcutaneously in each mouse. Mice were then randomized and separated into three treatment groups: the control group, the immunotherapy group and the immunotherapy + PPI group. **a** Tumor growth curve. The average tumor volume for each group was plotted. **b** Tumor growth from day 0 to 2. Drastic increase in tumor size was observed for immunotherapy group due to the inflammation induced by the therapy. Inflammation was not observed with the immunotherapy + omeprazole group. **c** Tumor growth from day 2 to 10. Tumor growth was significantly slower in the immunotherapy group. Suppressed immunotherapy was observed with the immunotherapy + PPI group. $*p < 0.05$, $**p < 0.01$



Since PPIs can be activated at pH5, one would expect that PPIs should have a large inhibitory effect on the enzymes in lysosomes, an important organelle in every cell

of human body. Indeed, the inhibitory effects on lysosomal enzyme activities were observed in all cultured cells tested and in the spleen of intact mice. With the mouse

experiments, we used two doses of omeprazole: 0.57 and 2.85 mg/kg. The 0.57 mg/kg dose is comparable to a regular omeprazole dose for a patient (40 mg for ~70 kg adult, or 0.7 mg/kg for pediatric patient). The higher dose 2.85 mg/kg (5 times that of 0.57 mg/kg) was based on the fact that the basal metabolic rate of mouse is ~7 times that of human [18]. Our PPI dose is also validated by the fact that other PPI studies with rodents used similar doses (e.g. [12]). The spleen is a major organ of the immune system, and lysosomal enzymes are required for antigen presentation (e.g. by T cells) and the removal of tumor cells, infected cells and dysfunctional cells by cytotoxic T lymphocytes (CTL). Therefore, inhibition of lysosomal enzymes in the spleen by PPIs suggests that continuous long-term use of PPIs would cause a systemically compromised immunity. Indeed, with a tumor immunotherapy mouse model, we observed that PPI suppressed the inflammation reaction induced by IL-12/GM-CSF and counteracted the tumor suppression immunotherapy. Many studies have associated PPI use with increased incidence of gastric polyps [19–21], carcinoid tumors [22–27] and gastric cancer [27–29]. Our data reported here suggested that PPIs may promote tumor growth through a compromised immune surveillance.

The often discussed mechanism for increased tumorigenesis among PPI users is that suppression of gastric acid production causes hypergastrinemia. An increased level of gastrin, which has a trophic effect on gastric mucosa and other epithelial tissue, may promote tumorigenesis. This hypothesis cannot fully explain several observations in which hypergastrinemia was not associated with increased risk of gastric cancer in patients with Zollinger-Ellison syndrome (ZES), post-vagotomy, or in other hypergastrinemic states [30–32]. As such, our hypothesis that compromised immunity leads to increased tumorigenesis could be a major mechanism for the increased tumorigenesis among PPI users. In addition, compared to the hypergastrinemia hypothesis, the hypothesis of compromised immunity better explains the observation that PPI alone do not cause cancer but promote the incidence of gastric cancer caused by *H. Pylori* [29].

Our hypothesized mechanism would also predict a similar increased risk for other types of cancer and infectious diseases in long-term PPI users. Long-term PPI use has been associated with increased risk of infections with *Salmonella* [33–37], *Campylobacter* [33, 34, 38] and *Clostridium difficile* [39–42]. The increased risk of bacterial infection in PPI users is commonly attributed to an increased pH in the stomach. However, this explanation is questioned for *C. difficile* infection [43] as this bacterium uses acid-resistant spores as the primary vehicle for transmission. A suppressed immune surveillance could be a

better explanation for increased *C. difficile* infection among long-term PPI users.

The extent of the inhibition of lysosomal enzymes by PPIs was different for each PPI and different in each cell type. With intact mice, no significant effect of PPIs on lysosomal enzymes was observed in stomach or liver, but lysosomal enzymes in the spleen were apparently inhibited by PPI in a dose-dependent manner. Several factors could contribute to these variations. Firstly, the plasma elimination half-lives for omeprazole, pantoprazole and lansoprazole are 0.7, 1.3 and 1.5 h, respectively [44]. This would predict that lansoprazole has the highest bioavailability, followed sequentially by pantoprazole and omeprazole, when the animals were given a similar dose. The differential inhibitory effects of each PPI on lysosomal enzymes in Caco2 and HEK293 cells seemed to be consistent with this pattern as lansoprazole exerted the highest inhibition, followed by pantoprazole and then omeprazole. However, in A549 and HepG2 cells, pantoprazole showed the least inhibition on lysosomal enzymes, suggesting an unidentified mechanism underlying the different effect of PPIs in A549 and HepG2 cells. Secondly, the liver was shown to be the major site for the oxidative metabolism of PPIs [45]. The relatively lower bioavailability of PPI in the liver due to active removal explains our observations that no inhibitory effect of PPI on lysosomal enzymes was observed in the liver. It is also consistent with the relatively diminished inhibitory effects of PPIs on lysosomal enzymes in cultured HepG2 cells, in comparison to other cells. It is noteworthy that the effects of PPI on liver lysosomal enzyme activities were studied previously by several groups [12–14] and none of these previous studies observed significant effects of PPIs on liver lysosomal enzyme activities. It is not known whether the stomach also metabolizes PPIs. However, the parietal cell canalicular membrane is known as a potent sink for PPIs because of its extremely low pH, which could cause a decreased PPI availability in the lysosomes of the stomach cells/tissues. Thirdly, the expression levels and the turn-over rates of each lysosomal enzyme vary in different cells and tissues. For example, in our study, MPO activity was readily detected in mouse spleen, but not in mouse liver or stomach.

Over-prescription of PPIs has been noted in adult [46–50] and pediatric populations [51–53]. Many of these studies suggest that PPIs are often prescribed without diagnosis of acid-related symptoms. Over-prescribing PPIs unnecessarily removes the benefits of gastric acid and brings about myriads of infrequent but serious side effects to PPI users [43]. Here, we demonstrated in vitro and in vivo that PPIs inhibited lysosomal enzyme activities. We also provided evidence indicating that a systematically suppressed immune surveillance could be a major

mechanism for increased incidence of tumorigenesis and infectious diseases among PPI users.

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Conflict of interest The authors declare that they have no conflict of interest.

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