cancers

MDPI

Article

Proton Pump Inhibitors Enhance the Antitumor Effect of Chemotherapy for Esophageal Squamous Cell Carcinoma

Shinya Matsumura [1,2], Takeshi Ishikawa [1,*], Juichiro Yoshida [1], Ryuichi Morita [1], Tomoki Sakakida [1], Yuki Endo [1], Toshifumi Doi [1], Ryohei Hirose [1], Ken Inoue [1], Osamu Dohi [1], Naohisa Yoshida [1], Kazuhiko Uchiyama [1], Tomohisa Takagi [1], Hideyuki Konishi [1], Kohichiroh Yasui [3], Yuji Naito [1] and Yoshito Itoh [1]

[1] Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan; matsumu@koto.kpu-m.ac.jp (S.M.); jyoshida@koto.kpu-m.ac.jp (J.Y.); mryuich@koto.kpu-m.ac.jp (R.M.); stomoki@koto.kpu-m.ac.jp (T.S.); endo0622@koto.kpu-m.ac.jp (Y.E.); t-doi@koto.kpu-m.ac.jp (T.D.); ryo-hiro@koto.kpu-m.ac.jp (R.H.); keninoue71@koto.kpu-m.ac.jp (K.I.); osamu-d@koto.kpu-m.ac.jp (O.D.); naohisa@koto.kpu-m.ac.jp (N.Y.); k-uchi@koto.kpu-m.ac.jp (K.U.); takatomo@koto.kpu-m.ac.jp (T.T.); hkonishi@koto.kpu-m.ac.jp (H.K.); ynaito@koto.kpu-m.ac.jp (Y.N.); yitoh@koto.kpu-m.ac.jp (Y.I.)
[2] Department of Gastroenterology, Kyoto Chubu Medical Center, Kyoto 629-0197, Japan
[3] Department of Nursing, Faculty of Health Sciences, Bukkyo University, Kyoto 603-8301, Japan; yasuik@koto.kpu-m.ac.jp
* Correspondence: iskw-t@koto.kpu-m.ac.jp; Tel.: +81-75-251-5519; Fax: +81-75-251-0710

Simple Summary: The use of proton pump inhibitors (PPIs) as V-ATPase inhibitors has been reported to enhance the effectiveness of chemotherapy in some cancers. This study aimed to evaluate the effect of PPIs on 5-Fuorouracil (5-FU)-based therapy for advanced esophageal cancer based on in vitro experiments and a clinical study. In the present study, PPIs showed a dose-dependent antitumor effect on esophageal cancer cells and enhanced the sensitivity of esophageal cancer cells to 5-FU at sublethal concentrations. In the clinical setting, patients treated with oral PPIs showed a superior tumor response to 5-FU and better overall survival in comparison to the non-PPI group. These results indicate that PPIs can enhance chemosensitivity in esophageal cancer patients treated with 5-FU.

Abstract: Background: Vacuolar ATPase (V-ATPase) is involved in cancer development. The use of proton pump inhibitors (PPIs) as V-ATPase inhibitors has been reported to enhance the effectiveness of chemotherapy in certain cancers. This study aimed to evaluate the effect of PPIs on chemotherapy for esophageal cancer. Methods: To investigate the effects of PPIs on esophageal cancer cells, human KYSE50 and 70 cells were plated and 3 PPIs (lansoprazole, esomeprazole, vonoprazan) were added at various concentrations with 5-Fluorouracil (5-FU) to the corresponding cells for a cell viability assay. To investigate the effects of PPI treatment on patients undergoing 5-FU-based therapy in the clinical setting, we retrospectively analyzed the clinical outcomes and chemotherapy-related adverse events in 40 esophageal cancer patients who received 5-FU chemotherapy in our hospital between May 2013 and April 2017. Results: In the viability assays, all PPIs significantly enhanced the cytotoxic effect of 5-FU on the two esophageal cancer cell lines. In the clinical study, PPI-treated patients showed better overall survival (OS) than patients managed without PPI treatment. A multivariate analysis revealed that PPI treatment was independently associated with OS (p = 0.009, HR, 0.33; 95% CI, 0.12–0.76). Conclusions: PPI treatment may safely enhance chemosensitivity in esophageal cancer patients.

Keywords: esophageal cancer; vacuolar ATPase (V-ATPase); proton pump inhibitors (PPIs); 5-Fluorouracil (5-FU); lansoprazole; esomeprazole; vonoprazan

Citation: Matsumura, S.; Ishikawa, T.; Yoshida, J.; Morita, R.; Sakakida, T.; Endo, Y.; Doi, T.; Hirose, R.; Inoue, K.; Dohi, O.; et al. Proton Pump Inhibitors Enhance the Antitumor Effect of Chemotherapy for Esophageal Squamous Cell Carcinoma. *Cancers* **2022**, *14*, 2395. https://doi.org/10.3390/cancers14102395

Academic Editor: Zui Pan

Received: 9 April 2022
Accepted: 11 May 2022
Published: 12 May 2022

1. Introduction

Esophageal cancer causes more than half a million cancer-related deaths worldwide each year, and squamous cell carcinoma is the most prevalent (87.8%), especially in Asia

and Eastern Africa, and alcohol and tobacco are important risk factors for that histological subtype [1,2]. Most patients are unresectable or metastatic disease at diagnosis, and many cases treated with curative intent have a relapse. Although 5-Fuorouracil (5-FU)-platinum-based chemotherapy has been a widely used for these cases, it often results in poor outcomes (median survival, <1 year) [3]. One reason for this result is acquired resistance to anticancer drugs; thus, overcoming acquired resistance is essential for improving the survival of advanced/metastatic cases treated with chemotherapy [4].

The upregulation of glycolysis (the Warburg effect), a universal property of cancers, leads to microenvironmental acidosis, which leads to evolution to phenotypes resistant to acid-induced cell toxicity [5]. Protons produced by glycolysis are transported across the membrane from cancer cells by the overexpressed proton pump, which prevents intracellular acidosis and relieves them from dangerous protons. Among these proton pumps, the most prominent is vacuolar H+-ATPase (V-ATPase) [6]. V-ATPase is an ATP-driven proton pump that functions to both transport protons across the plasma membrane and to acidify intracellular compartments [7]. The action of this pump leads to the selection of more aggressive tumor cell phenotypes that are able to survive in this highly hostile microenvironment. The acidic tumor microenvironment has also been shown to increase the chemoresistance of solid tumors [8]. The expression of V-ATPase has been reported to be associated with pathological grade, TNM stage and tumor metastasis in esophageal squamous cancer cells. The expression of V-ATPase may be strongly associated with drug resistance and tumor metastasis [9]. The extracellular pH of solid tumors is lower than that of normal tissues, which inhibits the uptake of weakly basic chemotherapeutic drugs and, hence, reduces their cytotoxicity [10].

Recently, the inhibition of the acidic microenvironment by blocking the activity of V-ATPases with the use of cytotoxic agents has been shown to synergistically kill chemotherapy-resistant tumors [11–13]. Proton pump inhibitors (PPIs), such as lansoprazole and esomeprazole, inhibit gastric acid secretion by targeting the gastric acid pump, and are used to treat gastrointestinal disorders (e.g., gastroesophageal reflux disease, duodenal ulcers and gastric ulcers [14–16]), and are commonly used concomitant to cancer treatment. PPIs, which were previously thought to be specific inhibitors of H+/K(+)-ATPase, also inhibit V-ATPase [17–19]. Recent studies have demonstrated that the sensitivity of various tumors to chemotherapeutic agents can be enhanced by PPIs [20–22]. Thus, PPIs may be a chemosensitizer that enhances the sensitivity of esophageal cancer cells to chemotherapeutic agents. The aim of this study was to evaluate the effect of PPIs on cytotoxicity of 5-Fuorouracil (5-FU) in vitro and the clinical impact of PPIs on the response to chemotherapy for patients with unresectable or recurrent esophageal cancer.

2. Materials and Methods

2.1. Cell Culture

Human esophageal cancer cells (KYSE50 and KYSE70 cells), obtained from the JCRB Cell Bank (Osaka, Japan), were grown in D-MEM (high glucose) with L-Glutamine and Phenol Red medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), 100 µg/mL streptomycin and 100 U/mL penicillin at 37 °C under 5% CO_2 and 90% humidity. The cells were grown in a single cell layer that was attached to treated plastic surfaces, and were subcultured 1 or 2 times per week. In the experiments, cells during the exponential growth phase were used.

2.2. Western Blotting

Cellular debris was removed by washing with ice-cold PBS. Then, the cells were lysed in Lysis Buffer (CelLytic M; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), then scraped and incubated on ice for 15 min. Supernatants were collected, and total protein was mixed with SDS sample buffer. The protein content was measured using the Bradford method; 20 µg of protein was used per lane. The protein lysates were then separated by electrophoresis on 10% SDS-PAGE, then transblotted to polyvinylidene fluoride mem-

branes (Atto Corporation, Tokyo, Japan). Membranes were blocked using 10% EzBlock (Atto Corporation, Tokyo, Japan) in TBS-T (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20 V/V) for 60 min at room temperature. They were then washed 3 times with TBS-T, and incubated overnight at 4 °C with mouse anti-human-V-ATPase C1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and goat anti-mouse-β-actin (Abcam, Cambridge, UK) antibodies in TBS-T (diluted 1:500–1:1000). Membranes were then washed with TBS-T 3 times, and incubated with secondary anti-mouse (GE Healthcare Japan Corporation, Tokyo, Japan) and anti-goat (GE Healthcare Japan Corporation, Tokyo, Japan) IgG antibodies in TBS-T (diluted 1:5000–1:10,000) for 1 h at room temperature. An ECL-kit (ECL plus, GE Healthcare Bio-Sciences K.K., Tokyo, Japan) was used to detect immunoreactive proteins. Blots were analyzed using ImageJ (version 1.51).

2.3. Cell Viability Assay

A Cell Counting Kit-8 (DOJINDO Laboratories, Kumamoto, Japan) was applied to evaluate cell viability. KYSE50 and KYSE70 cells were plated at 1×10^4 cells per well in 100 µL of D-MEM with 10% FBS in 96-well plates for 24 h. Then, 3 types of PPIs (lansoprazole (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), esomeprazole (Cayman Chemical Company, Ann Arbor, MI, USA), and vonoprazan (Cayman Chemical Company, Ann Arbor, MI, USA) at various concentrations were freshly prepared and added to the corresponding cells. After 72 h, 10 µL of Cell Counting Kit-8 solution was added to each well, and cells were incubated for another 4 h. The absorbance was measured using a multi-well spectrophotometer (SpectraMax M2, Molecular Devices, San Jose, CA, USA) at 450 nm. Cell viability was calculated as follows: cell viability (%) = absorbance of treated wells/absorbance of control wells (without PPIs) × 100.

In the assay for the combined use of PPIs and 5-FU, various concentrations of 5-FU (2, 20 and 200 µM, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were added to cells 24 h after the administration of a PPI. After another 48 h, we evaluated cell viability. In this case, the sublethal dose was defined and set as the PPI concentration at which >85% of cells of each type were viable at 72 h after PPI administration.

The combination index (CI) was calculated by Compusyn software (ComboSyn, Inc., Paramus, NJ, USA). The quantitative definition of drug combinations is CI = 1 for additive effect, CI < 1 for synergism, and CI > 1 for antagonism [23].

2.4. Measurement of Intracellular pH

Intracellular pH (pHi) was measured using the fluorescent pH indicator (2-carboxyethyl)-5-carboxyfluorescein (BCECF)-AM (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. BCECF-AM was diluted to 1 mM with DMSO. KYSE50 and KYSE70 cells were plated at 1×10^4 cells per well in 100 µL of D-MEM with 10% FBS in a 96-well optical bottom plate polymerBase Black (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h. Then, 3 types of PPIs (lansoprazole, esomeprazole and vonoprazan) at the sublethal concentrations were freshly prepared and added to the corresponding cells. After 72 h, BCECF-AM was diluted to 3 µnol/L with HEPES-buffered Ringer solution (DOJINDO Laboratories, Kumamoto, Japan), adjusted to pH 7.4. Next, the culture medium was replaced, and cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2, for 15 min. Then, the culture medium was washed several times with HEPES, and pHi was measured using BCECF fluorescence (excitation wavelength: of 440 nm and 490 nm). Using a multi-well spectrophotometer, at 37 °C, fluorescence intensities were measured every 25 s and monitored at a wavelength of 535 nm. Data acquisition was conducted using SoftMax Pro (v5.4.6, Molecular Devices, San Jose, CA, USA). Calibration of the measurements of each experiment was performed by successively replacing HEPES-buffered Ringer solution with modified Ringer solution at pH 6.4, 6.8, 7.4 and 7.8, with each replacement solution containing the K+/H+ exchanger nigericin at a concentration of 10 µmol/L (Sigma-Aldrich, St. Louis, MO, USA), in order to determine the pHi.

2.5. Clinical Research

We retrospectively investigated the clinical outcomes and chemotherapy-related adverse events of 40 consecutive patients who underwent 5-FU-based chemotherapy for esophageal squamous cell carcinoma (clinical stage IVB according to the 8th edition the Union for International Cancer Control (UICC) staging of cancers of the esophagus) in our hospital between May 2013 and April 2017 (Figure 1) [24]. The 40 cases included patients with postoperative recurrence and patients who received radiotherapy for palliative treatment (e.g., radiation therapy for bone metastases). Patients who used a PPI for at least 30 days from the start of the first chemotherapy treatment were defined as the PPI group (n = 18), other patients were defined as the non-PPI group (n = 22). The efficacy of treatment was assessed by overall survival (OS) and the response to treatment at the end of 2 courses (RECIST criteria ver. 1.1). The common Terminology Criteria for Adverse Events version 5.0 (CTCAE) was used to assess adverse events. Patient data were collected from their electronic medical records. The present study was approved by the Medical Ethics Review Committee of Kyoto Prefectural University of Medicine (approval no. ERB-E-42). All procedures in this study were in accordance with the ethical standards of the Medical Ethics Review Committee of Kyoto Prefectural University of Medicine, as well as the Declaration of Helsinki. The requirement for informed consent from individual study participants was waived by the Medical Ethics Review Committee of Kyoto Prefectural University of Medicine due to the retrospective nature of this study.

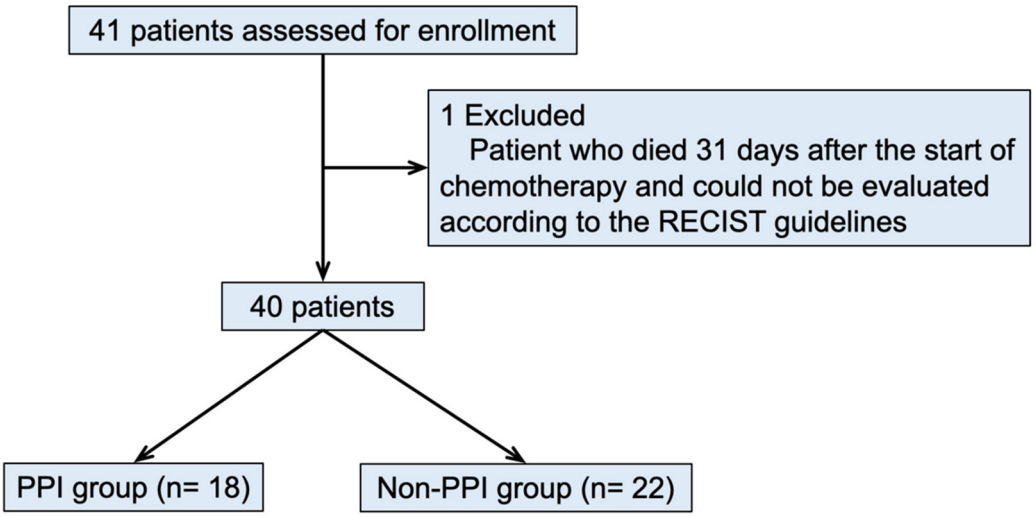

Figure 1. Flow chart of patient enrollment.

2.6. Statistical Analysis

Quantitative data were evaluated using Student's t-test. A one-way ANOVA followed by post hoc Steel's multiple comparison test was used to compare multiple groups. Fisher's exact test was used to compare categorical variables. Overall survival was compared according to the Kaplan–Meier method using a log-rank test. p values of <0.05 were considered to indicate statistical significance. JMP Pro (version 14.0, SAS International Inc., Cary, NC, USA) was used to perform the statistical analyses.

3. Results

3.1. In Vitro Experiment

We evaluated the efficiency of PPIs in increasing the chemosensitivity of the KYSE50 and KYSE70 cell lines to 5-FU. First, we examined the expression of V-ATPase on KYSE50 and KYSE70 cells by western blotting (Figure 2, the whole western blots can be found at Figure S1). We confirmed that V-ATPase was constitutively expressed at the protein level in both cell lines, and was more highly expressed in KYSE70 cells.

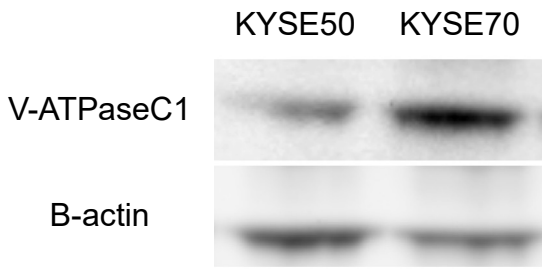

Figure 2. Western blotting to determine for protein expression of V-ATPase in esophageal cancer cell lines.

Next, we assessed whether PPIs impacted the survival of esophageal cancer cell lines. Figure 3 shows an overview of the dose–response bar graph of PPI treatment at various concentrations. In both cell lines, PPIs reduced cell viability in dose-dependent manner, thus providing evidence to support that PPI treatment had a negative impact on the survival of these cells. In this context, the sublethal doses (defined as a survival rate of at least 85%) were 25 µM for lansoprazole, 10 µM for esomeprazole and 10 µM for vonoprazan in KYSE50 cells, and 5 µM for lansoprazole, 5 µM for esomeprazole and 50 µM for vonoprazan in KYSE70 cells.

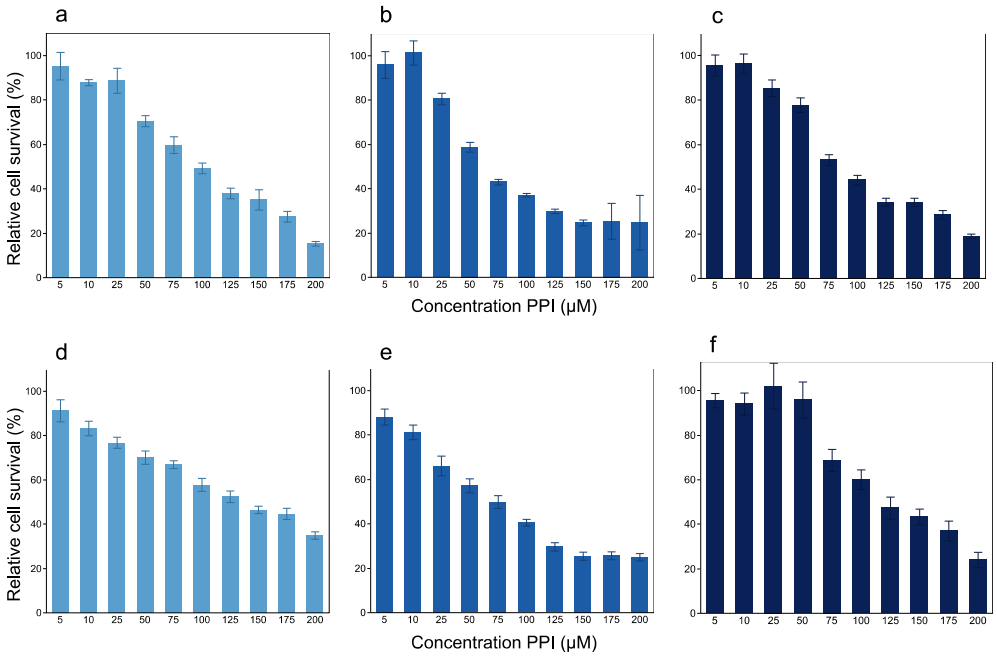

Figure 3. The dose–response bar graph for PPI treatment at various concentrations in the KYSE50 (**a**–**c**) and KYSE70 (**d**–**f**) cell lines. KYSE50 cells treated with (**a**) lansoprazole, (**b**) esomeprazole and (**c**) vonoprazan. KYSE70 cells treated with (**d**) lansoprazole, (**e**) esomeprazole and (**f**) vonoprazan.

We investigated whether PPIs at sublethal concentrations increased the sensitivity of both cell lines to 5-FU in vitro. The combination of sublethal concentrations of PPIs with 2, 20 or 200 µM 5-FU reduced the viability of both cell lines at all 5FU concentrations in comparison to cells without PPI treatment (Figure 4). These results suggest that PPIs may increase sensitivity of esophageal cancer to 5-FU.

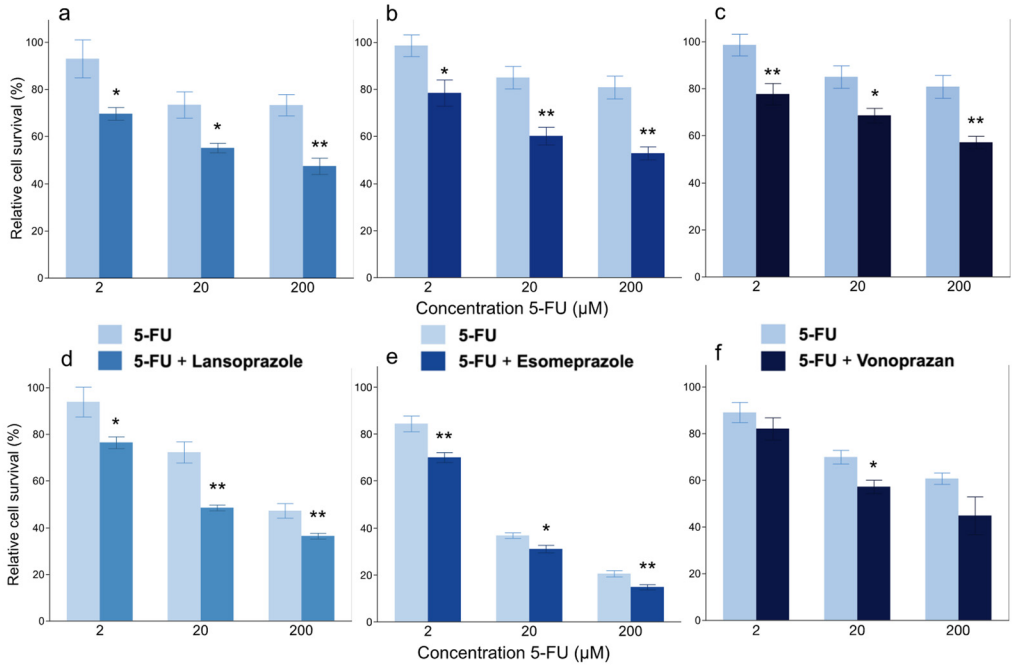

Figure 4. Effect of PPIs on the sensitivity of KYSE50 (**a**–**c**) and KYSE70 (**d**–**f**) cells to 5-FU. KYSE50 cells with (**a**) lansoprazole, (**b**) esomeprazole and (**c**) vonoprazan. KYSE70 cells with (**d**) lansoprazole, (**e**) esomeprazole and (**f**) vonoprazan. *: $p < 0.05$, **: $p < 0.01$.

To test the potential synergistic effects of combination therapy, drug combination index (CI) were analyzed by CompuSyn software (Table S1). All combinations except combination of 2 μM of 5-FU and 50 μM vonoprazane against KYSE70 cells showed a combined synergistic effect (CI < 1).

Since, in addition to an acidic extracellular microenvironment, alkaline cytosols have been shown to play a role in resistance to chemotherapy [25], it was considered that a decrease in cytoplasmic pH due to PPI treatment is one of the mechanisms through which PPIs increased the sensitivity of esophageal cancer cell lines to 5-FU. Therefore, intracellular pH (pHi) after PPI treatment was measured using BCECF-AM (a fluorescent pH indicator). The pHi in KYSE50 cells treated with each sublethal concentration of lansoprazole, esomeprazole and vonoprazan for 72 h was significantly lower in comparison to untreated controls ($p \leq 0.001$, $p = 0.042$ and $p = 0.017$ respectively, Figure 5a). In KYSE70 cells, the pHi was significantly lower at each sublethal concentration of esomeprazole and vonoprazan. ($p < 0.001$ and $p = 0.004$, respectively). The pHi of lansoprazole-treated KYSE70 cells was not significantly different from that of untreated control cells (Figure 5b).

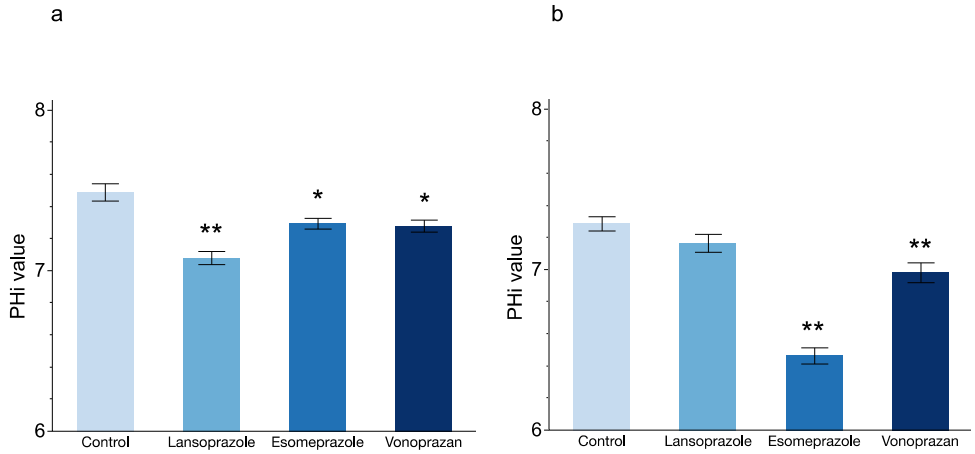

Figure 5. Effect of PPI treatment on intracellular pH. The figure presents the results of intracellular pH measurement of KYSE50 (**a**) and KYSE70 (**b**) cells at 72 h after treatment with sublethal concentrations of PPIs. *: $p < 0.05$, **: $p < 0.01$.

3.2. Clinical Research

The baseline characteristics in a retrospective study that compared two groups (PPI group (n = 18) and non-PPI group (n = 22)), are shown in Table 1. There were no significant difference in the clinical background factors (e.g., age, sex, body mass index, performance status, the Charlson comorbidity index and selected chemotherapy regimen). The Glasgow prognostic score (GPS), prognostic nutrition index (PNI), and neutrophil/lymphocyte ratio (NLR) at the start of treatment, which are reported to be prognostic factors, were compared; however, there was no significant difference between the two groups.

Table 1. Baseline characteristics of esophageal cancer patients taking PPIs vs. esophageal cancer patients not taking PPIs.

Characteristics		PPI (n = 18)	Non-PPI (n = 22)	p Value
Age, mean (SD), years		67.9 (7.83)	65.6 (7.90)	0.364
Sex, n				
	Male	16	17	0.328
	Female	2	5	
PS, n				
	0	6	11	
	1	12	10	0.264
	2	0	1	
BMI, mean (SD), kg/m^2		21.2 (3.90)	19.4 (3.12)	0.112
Location, n				
	U	2	4	
	M	7	11	0.490
	L	9	7	
Disease status, n				
	Metastatic	17	18	0.212
	Recurrent	1	4	
Prognostic factors				
CCI, n				
	0	9	14	
	1–2	7	7	0.596
	≥3	2	1	

Table 1. *Cont.*

Characteristics	PPI (n = 18)	Non-PPI (n = 22)	p Value
GPS, n			
0	9	11	
1	7	6	0.548
2	2	5	
PNI, mean (SD)	44.8 (4.23)	43.6 (7.53)	0.529
NLR, mean (SD)	3.6 (2.32)	5.2 (7.84)	0.409
Regimen (1st course), n			
FP	11	16	
DCF	5	4	0.500
Nedaplatin FU	1	2	
FOLFOX	1	0	
Radiation therapy, n			
Yes	13	11	0.150
No	5	11	
PPI subtypes, daily dose, n			
Lansoprazole			
15 mg	2		
30 mg	6		
Rabeprazole			
10 mg	3	-	-
Esomeprazole			
20 mg	4		
Vonoprazan			
10 mg	1		
20 mg	2		

PS, performance status; BMI, body mass index; CCI, Charlson comorbidity index; GPS, Glasgow prognostic score; PNI, prognostic nutritional index; NLR, neutrophil lymphocyte ratio; FP, fluorinated pyrimidines; DCF, docetaxel plus 5-fluorouracil and cisplatin; Nedaplatin FU, Nedaplatin and 5-fluorouracil; FOLFOX, oxaliplatin plus fluorouracil and leucovorin.

According to the RECIST guidelines, the response rate after 2 courses of chemotherapy was 66.7% in the PPI group and 40.9% in the non-PPI group (p = 0.102), while the disease control rate was 94.4% and 77.3% (p = 0.113), respectively (Table 2), both were better in the PPI group; however, the difference did not reach the statistical significance.

Table 2. Clinical outcomes of esophageal cancer patients in the PPI and non-PPI groups.

Response to Treatment	PPI (n = 18)	Non-PPI (n = 22)	Treatment Difference (95% CI)	p Value
CR, n (%)	0	1 (4.5)		
PR, n (%)	12 (66.7)	8 (36.4)		0.269
SD, n (%)	5 (27.8)	8 (36.4)		
PD, n (%)	1 (5.6)	5 (22.7)		
Response rate, n (%)	12 (66.7)	9 (40.9)	0.35 (0.09–1.27)	0.102
Disease control rate, n (%)	17 (94.4)	17 (77.3)	0.2 (0.02–1.90)	0.113

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

The Kaplan–Meier analysis demonstrated superior survival in the PPI group in comparison to patients who did not receive PPIs (log-rank test p = 0.032) (Figure 6).

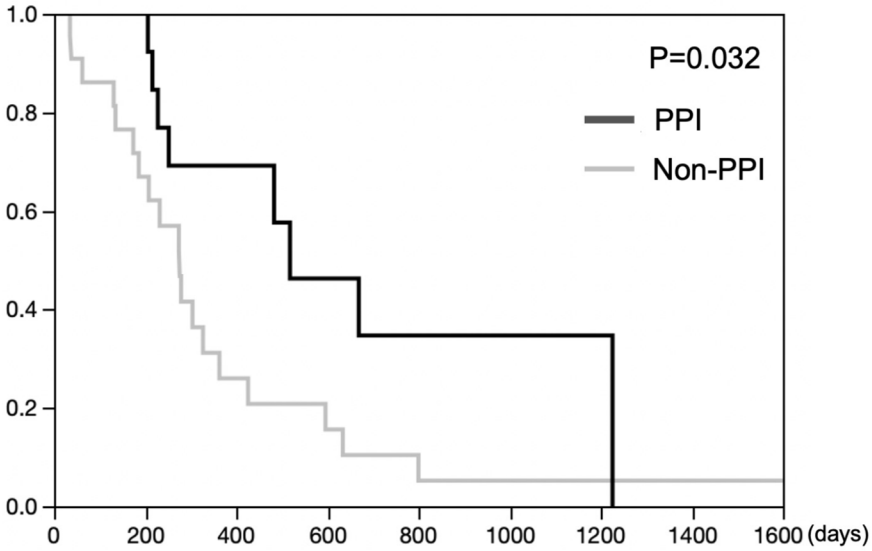

Figure 6. Overall survival (OS) according to PPI use or non-use in esophageal cancer patients receiving 5-FU-based chemotherapy.

The univariate and multivariate analyses for OS in esophageal cancer patients who received 5-FU-based chemotherapy revealed that PPI use was independently associated with OS (p = 0.009, HR, 0.33; 95% CI, 0.12–0.76) (Table 3).

Table 3. Univariate and multivariate analyses of risk factors for overall survival in esophageal cancer patients treated with 5-FU-based chemotherapy.

	Univariate Analysis			Multivariate Analysis		
	HR	**95% CI**	***p* Value**	**HR**	**95% CI**	***p* Value**
Age						
<75 years	1					
≥75 years	0.70	0.20–1.84	0.496			
Sex						
Female	1					
Male	2.05	0.78–7.07	0.161			
PS						
=0	1			1		
≥1	1.20	0.56–2.59	0.642	1.32	0.53–3.30	0.549
Regimen (1st course)						
DCF	1					
Other	0.91	0.42–2.15	0.825			
CCI						
0	1					
≥1	1.38	0.65–2.95	0.408			
GPS						
=0–1	1			1		
=2	2.32	0.76–5.90	0.130	2.59	0.68–8.88	0.154
PNI						
≥45	1					
<45	1.18	0.55–2.59	0.670			
NLR						
<5	1			1		
≥5	1.93	0.74–4.50	0.169	1.88	0.64–5.08	0.236
PPI						
No	1			1		
Yes	0.41	0.17–0.92	0.029	0.35	0.13–0.80	0.012

HR, hazard ratio; CI, confidence interval; PS, performance status; CCI, Charlson comorbidity index; GPS, Glasgow prognostic score; PNI, prognostic nutritional index; NLR, neutrophil lymphocyte ratio.

There were no significant differences in the occurrence of grade ≥ 3 chemotherapy-related adverse events (Table 4). In the PPI group, two patients treated with docetaxel, cisplatin and 5-FU (DCF) developed grade 4 neutropenia, one of these patients developed febrile neutropenia. The other 5 patients (5-FU and cisplatin or 5-FU and nedaplatin) developed grade 3 neutropenia. In addition, grade 3 anorexia, grade 3 creatinine increase and grade 3 sinus bradycardia were observed in one case each. On the other hand, among the non-PPI group, 3 patients (2 5-FU and nedaplatin and 1 5-FU and cisplatin) had grade 4 neutropenia, and 5 patients had neutropenia. Three of these patients had febrile neutropenia. In addition, grade 3 anorexia and grade 3 creatinine increase were observed in one case each. Of the patients who experienced grade ≥ 3 chemotherapy-related adverse events, 8 were subsequently treated at a reduced intensity. In one case of grade 3 bradycardia and in one case of grade 3 creatinine increase, 5-FU and cisplatin was changed to 5-FU and nedaplatin. No cases were terminated due to side effects.

Table 4. Chemotherapy-related adverse events (grade ≥ 3) for esophageal cancer patients taking PPIs vs. patients without PPI treatment.

	PPI (n = 18)	Non-PPI (n = 22)	Odds Ratio (95% CI)	p Value
Myelosuppression, n	7	9	0.92 (0.26–3.28)	0.897
Gastrointestinal toxicity, n	1	1	1.24 (0.07–21.2)	0.884
Heart failure, n	1	0	-	0.202
Renal toxicity, n	1	2	0.59 (0.05–7.07)	0.669

4. Discussion

In the present study, PPIs showed a dose-dependent antitumor effect on esophageal cancer cells and enhanced sensitivity to 5-FU at sublethal concentrations. In actual clinical practice, the oral PPI group showed a superior tumor response to 5-FU and better OS in comparison to the non-PPI group. These results indicate that PPI medication could enhance the chemosensitivity of esophageal cancer patients treated with 5-FU. To the best of our knowledge, this study is the first to demonstrate that PPIs could enhance the chemosensitivity of esophageal cancer both in vitro and in the clinical setting. Furthermore, the results in this study revealed, for the first time, that vonoprazan, a novel potassium-competitive acid blocker, has the effect of enhancing chemosensitivity.

The extracellular pH of solid tumors is more acidic in comparison to normal tissue as a consequence of high glycolysis and poor vascular perfusion [26]. Tumor cells have, thus, evolved the ability to function in a more acidic environment than normal cells. The activity of V-ATPase, a key pH regulator in tumor cells, is important for the excretion of excess acid into the extracellular environment, which results in a "reversed" pH gradient with a constitutively increased intracellular pH that is higher than the extracellular pH in tumor cells [27]. This reversal not only allows tumor cells to evade apoptosis, but is also involved in inducing drug resistance. At extracellular low pH, many drugs are protonated and lose their ability to enter cells, thus protecting the DNA of tumor cells from the effects of drugs [28,29]. In addition, the pH gradient between the cytoplasm and lysosomal compartment is also involved in resistance to the weakly basic chemotherapeutic drugs [30]. Therefore, the inhibition of V-ATPase by PPIs could alter pH regulation in tumor cells and overcome drug resistance. Consistent with previous reports [31], we confirmed that PPIs decreased the intracellular pH of esophageal cancer cell lines, suggesting that the increased sensitivity to 5FU induced by PPIs is mainly mediated by the changes in tumor cell pH regulation. However, in this study, the decrease in intracellular pH in lansoprazole-treated KYSE70 cells was weak, but the sensitivity to 5-FU was strongly enhanced in these cells, so there may be other mechanisms for enhancing the chemosensitivity of PPIs besides the change of intracellular pH. Further research is needed to elucidate the molecular mechanism by which PPIs exert enhanced chemosensitivity.

It has been reported that the rate of V-ATPase expression increases with the pathological grade and TNM stage in esophageal squamous cancer cells collected from patients [9]. It has also been reported that epithelial ovarian cancer patients whose mRNA expression of V-ATPase was in the upper 75th percentile showed significantly poorer overall survival in comparison to patients whose mRNA expression of V-ATPase was in the lower 25th percentile [22]. A number of these previous studies suggested that V-ATPase is a crucial factor that is involved in drug resistance, and tumor progression, and may represent a suitable and specific target for novel anticancer strategies. Pharmacologic inhibitors of V-ATPase activity have been used in the past with a high level of efficacy in vitro; however, their potential application in the clinical setting is hampered by predicted toxicity on normal cells [25,32]. Since the safety of PPIs is clinically well-established, the use of PPI as a V-ATPase inhibitor during chemotherapy is promising strategy for enhancing chemosensitivity. Recently, PPIs have been shown to enhance the clinical effect of chemotherapy in several cancer types [20,33]. For 5-FU-based chemotherapy, Wang et al. have shown to be better for OS (p = 0.04, RR, 0.72; 95% CI, 1.02–1.90) and PFS (p = 0.01, RR = 0.67; 95% CI, 1.10–2.05) in patients with colorectal cancer who were treated with a FOLFOX regimen along with PPIs than without PPIs [34]. In a cohort of 596 patients with previously untreated head and neck squamous cell carcinomas, PPI use was associated with improved OS ($p < 0.0001$, HR = 0.55; 95% CI, 0.42–0.73) [35]. Consistent with these reports, the analysis of our clinical data showed that OS in the oral PPI group was superior to that in the non-PPI group, and a multivariate analysis revealed that PPI use was independently associated with OS. Importantly, no additional toxicity was observed with PPI administration in the present study. Our results and recently published observations indicate a new path to anticancer therapy for drug-resistant tumors.

Although vonoprazan may be considered to be a PPI in a broad sense, it is a potassium-competitive acid blocker (P-CAB) with a different mechanism of action from lansoprazole and esomeprazole, which are PPIs in a narrow sense. A P-CAB was used as a PPI in this study because they are commonly and widely used in actual clinical practice as an alternative to PPIs due to their additional clinical advantages over conventional PPIs [36,37]. In the present study, vonoprazan showed a dose-dependent antitumor effect on esophageal cancer cell lines and enhanced sensitivity to 5-FU. This is the first study to evaluate the effect of vonoprazan on cancer cells, and these results suggest that vonoprazan is likely to function as a chemosensitizer in cancer treatment, similar to conventional PPIs.

The present study was associated with several limitations. First, this was a single-center, retrospective study with a relatively small sample size. Second, in clinical practice, the criteria for administering PPIs were based on the judgment of the attending physician.

5. Conclusions

PPIs showed a dose-dependent antitumor effect on esophageal cancer, and sublethal concentrations of PPIs showed synergistic cytotoxicity with 5-FU in vitro. In actual clinical practice for patients treated with 5-FU for advanced esophageal cancer, the OS of the oral PPI group was superior to that of the non-PPI group, and the occurrence of chemotherapy-related adverse events in the two groups did not differ to a statistically significant extent. Although this was a small-sized retrospective study, these results suggest that the use of PPIs could safely enhance chemosensitivity in patients with esophageal cancer. The effect of PPIs as a chemosensitizer in patients with esophageal cancer needs to be investigated in a large prospective controlled study and the underlying molecular mechanisms also need to be further studied in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14102395/s1, Figure S1: Whole western blot and densitometry readings/intensity ratio of V-ATPase C1 in the KYSE50 and KYSE70 cell lines; Table S1: The combination index (CI) was calculated by Compusyn software, and a CI value < 1 represents synergism.

Author Contributions: Conceptualization, S.M. and T.I.; methodology. S.M., J.Y., R.M., T.S., Y.E., T.D. and T.I.; data curation, S.M., J.Y., R.M., R.H., K.I. and T.I.; resources, T.I., K.U., K.Y. and Y.N.; visualization, O.D., N.Y. and H.K.; writing—original draft preparation, S.M.; writing—review and editing, T.I.; supervision, T.I., T.T., K.Y., Y.N. and Y.I. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by JSPS KAKENHI Grant Number JP 20K07787 and by a research fund of Kyoto Prefectural University of Medicine.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Kyoto Prefectural University of Medicine (approval no. ERB-E-42, 15 September 2021).

Informed Consent Statement: Given the retrospective nature of this work, informed consent was waived for the individual participants included in the study in accordance with the standards of Kyoto Prefectural University of Medicine Institutional Medical Ethics Review Committee.

Data Availability Statement: Data will be available from the corresponding author upon reasonable request.

Conflicts of Interest: Y.I. received scholarship funds from Takeda Pharmaceutical Co., Ltd., Tokyo, Japan; Daiichi Sankyo Co., Ltd., Tokyo, Japan; Otsuka Pharma Co., Ltd., Tokyo, Japan; Eisai Co., Ltd., Tokyo, Japan; EA Pharma Co., Ltd., Tokyo, Japan; ASKA Pharmaceutical Co. Ltd., Tokyo, Japan and Pfizer Inc., New York, NY, USA and has received lecture fees from Takeda Pharmaceutical Co., Ltd., Tokyo, Japan; Daiichi Sankyo Co., Ltd., Tokyo, Japan; Otsuka Pharma Co., Ltd., Tokyo, Japan; Eisai Co., Ltd., Tokyo, Japan and ASKA Pharmaceutical Co., Ltd., Tokyo, Japan. Y.N. received lecture fees and joint research funds from Takeda Pharmaceutical Co., Ltd., Tokyo, Japan. T.I. has received scholarship funds from Daiichi Sankyo Co., Ltd., Tokyo, Japan. K.U. has received lecture fees from Mitsubishi Tanabe Pharma Co., Osaka, Japan. T.I., J.Y. and T.D. have received lecture fees from Daiichi Sankyo Co., Ltd., Tokyo, Japan. The authors declare that all these conflict of interest are not connected with the issue of this paper. The other authors have no conflict of interest to declare.

References

1. Arnold, M.; Ferlay, J.; van Berge Henegouwen, M.I.; Soerjomataram, I. Global Burden of Oesophageal and Gastric Cancer by Histology and Subsite in 2018. *Gut* **2020**, *69*, 1564–1571. Available online: https://gut.bmj.com/content/gutjnl/69/9/1564.full.pdf (accessed on 30 April 2022). [CrossRef]
2. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424. Available online: https://acsjournals.onlinelibrary.wiley.com/doi/pdfdirect/10.3322/caac.21492?download=true (accessed on 10 November 2021). [CrossRef] [PubMed]
3. Miyata, H.; Yamasaki, M.; Kurokawa, Y.; Takiguchi, S.; Nakajima, K.; Fujiwara, Y.; Konishi, K.; Mori, M.; Doki, Y. Survival Factors in Patients with Recurrence after Curative Resection of Esophageal Squamous Cell Carcinomas. *Ann. Surg. Oncol.* **2011**, *18*, 3353–3361. Available online: https://link.springer.com/article/10.1245/s10434-011-1747-7 (accessed on 1 May 2022). [CrossRef] [PubMed]
4. Yoshida, T.; Miyoshi, T.; Seike, J.; Yamai, H.; Takechi, H.; Yuasa, Y.; Tangoku, A. Gene Expression Changes in a Chemoresistant Model with Human Esophageal Cancer Xenografts Using cDNA Microarray. *Anticancer Res.* **2009**, *29*, 1163–1168. Available online: https://ar.iiarjournals.org/content/anticanres/29/4/1163.full.pdf (accessed on 24 May 2021). [PubMed]
5. Gatenby, R.A.; Gillies, R.J. Why Do Cancers Have High Aerobic Glycolysis? *Nat. Rev. Cancer* **2004**, *4*, 891–899. Available online: https://www.nature.com/articles/nrc1478 (accessed on 21 May 2021). [CrossRef] [PubMed]
6. Spugnini, E.P.; Baldi, A.; Buglioni, S.; Carocci, F.; de Bazzichini, G.M.; Betti, G.; Pantaleo, I.; Menicagli, F.; Citro, G.; Fais, S. Lansoprazole as a Rescue Agent in Chemoresistant Tumors: A Phase I/II Study in Companion Animals with Spontaneously Occurring Tumors. *J. Transl. Med.* **2011**, *9*, 221. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3264547/pdf/1479-5876-9-221.pdf (accessed on 20 May 2021). [CrossRef] [PubMed]
7. Cipriano, D.J.; Wang, Y.; Bond, S.; Hinton, A.; Jefferies, K.C.; Qi, J.; Forgac, M. Structure and Regulation of the Vacuolar ATPases. *Biochim. Biophys. Acta* **2008**, *1777*, 599–604. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2467516/pdf/nihms57639.pdf (accessed on 12 November 2021). [CrossRef]
8. Trédan, O.; Galmarini, C.M.; Patel, K.; Tannock, I.F. Drug resistance and the solid tumor microenvironment. *J. Natl. Cancer Inst.* **2007**, *99*, 1441–1454. [CrossRef]
9. Huang, L.; Lu, Q.; Han, Y.; Li, Z.; Zhang, Z.; Li, X. ABCG2/V-ATPase was Associated with the Drug Resistance and Tumor Metastasis of Esophageal Squamous Cancer Cells. *Diagn. Pathol.* **2012**, *7*, 180. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3542252/pdf/1746-1596-7-180.pdf (accessed on 23 May 2021). [CrossRef]

10. Raghunand, N.; He, X.; van Sluis, R.; Mahoney, B.; Baggett, B.; Taylor, C.W.; Paine-Murrieta, G.; Roe, D.; Bhujwalla, Z.M.; Gillies, R.J. Enhancement of Chemotherapy by Manipulation of Tumour pH. *Br. J. Cancer* **1999**, *80*, 1005–1011. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2363059/pdf/80-6690455a.pdf (accessed on 23 May 2021). [CrossRef]
11. Eaton, A.F.; Merkulova, M.; Brown, D. The H(+)-ATPase (V-ATPase): From proton pump to signaling complex in health and disease. *Am. J. Physiol. Cell Physiol.* **2021**, *320*, C392–C414. [CrossRef] [PubMed]
12. Kolosenko, I.; Avnet, S.; Baldini, N.; Viklund, J.; De Milito, A. Therapeutic implications of tumor interstitial acidification. *Semin. Cancer Biol.* **2017**, *43*, 119–133. [CrossRef]
13. You, H.; Jin, J.; Shu, H.; Yu, B.; De Milito, A.; Lozupone, F.; Deng, Y.; Tang, N.; Yao, G.; Fais, S.; et al. Small interfering RNA targeting the subunit ATP6L of proton pump V-ATPase overcomes chemoresistance of breast cancer cells. *Cancer Lett.* **2009**, *280*, 110–119. [CrossRef] [PubMed]
14. Wallmark, B.; Larsson, H.; Humble, L. The relationship between gastric acid secretion and gastric H+, K+-ATPase activity. *J. Biol. Chem.* **1985**, *260*, 13681–13684. [CrossRef]
15. Puscas, I.; Coltau, M.; Baican, M.; Domuta, G. Omeprazole Has a Dual Mechanism of Action: It Inhibits Both H(+)K(+)ATPase and Gastric Mucosa Carbonic Anhydrase Enzyme in Humans (In Vitro and In Vivo Experiments). *J. Pharmacol. Exp. Ther.* **1999**, *290*, 530–534. Available online: https://jpet.aspetjournals.org/content/290/2/530.long (accessed on 23 May 2021).
16. Horn, J. The Proton-Pump Inhibitors: Similarities and Differences. *Clin. Ther.* **2000**, *22*, 266–280. Available online: https://www.clinicaltherapeutics.com/article/S0149-2918(00)80032-6/pdf (accessed on 23 May 2021). [CrossRef]
17. Mizunashi, K.; Furukawa, Y.; Katano, K.; Abe, K. Effect of Omeprazole, an Inhibitor of H+,K(+)-ATPase, on Bone Resorption in Humans. *Calcif. Tissue Int.* **1993**, *53*, 21–25. Available online: https://link.springer.com/article/10.1007/BF01352010 (accessed on 23 May 2021). [CrossRef]
18. Graber, M.L.; Devine, P. Omeprazole and SCH 28080 Inhibit Acid Secretion by the Turtle Urinary Bladder. *Ren. Physiol. Biochem.* **1993**, *16*, 257–267. Available online: https://www.karger.com/Article/Abstract/173771 (accessed on 23 May 2021). [CrossRef]
19. Sabolić, I.; Brown, D.; Verbavatz, J.M.; Kleinman, J. H(+)-ATPases of Renal Cortical and Medullary Endosomes are Differentially Sensitive to Sch-28080 and Omeprazole. *Am. J. Physiol.* **1994**, *266*, F868–F877. Available online: https://journals.physiology.org/doi/abs/10.1152/ajprenal.1994.266.6.F868 (accessed on 23 May 2021). [CrossRef]
20. Lu, Z.N.; Shi, Z.Y.; Dang, Y.F.; Cheng, Y.N.; Guan, Y.H.; Hao, Z.J.; Tian, B.; He, H.W.; Guo, X.L. Pantoprazole pretreatment elevates sensitivity to vincristine in drug-resistant oral epidermoid carcinoma in vitro and in vivo. *Biomed. Pharmacother.* **2019**, *120*, 109478. [CrossRef]
21. Avnet, S.; Lemma, S.; Cortini, M.; Pellegrini, P.; Perut, F.; Zini, N.; Kusuzaki, K.; Chano, T.; Grisendi, G.; Dominici, M.; et al. Altered pH Gradient at the Plasma Membrane of Osteosarcoma Cells is a Key Mechanism of Drug Resistance. *Oncotarget* **2016**, *7*, 63408–63423. Available online: https://www.oncotarget.com/article/11503/pdf/ (accessed on 1 May 2022). [CrossRef] [PubMed]
22. Lee, Y.Y.; Jeon, H.K.; Hong, J.E.; Cho, Y.J.; Ryu, J.Y.; Choi, J.J.; Lee, S.H.; Yoon, G.; Kim, W.Y.; Do, I.G.; et al. Proton pump Inhibitors Enhance the Effects of Cytotoxic Agents in Chemoresistant Epithelial Ovarian Carcinoma. *Oncotarget* **2015**, *6*, 35040–35050. Available online: https://www.oncotarget.com/article/5319/pdf/ (accessed on 29 December 2020). [CrossRef] [PubMed]
23. Chou, T.C. Drug combination studies and their synergy quantification using the chou-talalay method. *Cancer Res.* **2020**, *70*, 440–446. [CrossRef] [PubMed]
24. Rice, T.W.; Patil, D.T.; Blackstone, E.H. 8th Edition AJCC/UICC Staging of Cancers of the Esophagus and Esophagogastric Junction: Application to Clinical Practice. *Ann. Cardiothorac. Surg.* **2017**, *6*, 119–130. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5387145/pdf/acs-06-02-119.pdf (accessed on 30 April 2022). [CrossRef] [PubMed]
25. De Milito, A.; Fais, S. Tumor Acidity, Chemoresistance and proton Pump Inhibitors. *Future Oncol.* **2005**, *1*, 779–786. Available online: https://www.futuremedicine.com/doi/10.2217/14796694.1.6.779?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub%3Dpubmed (accessed on 7 November 2021). [CrossRef]
26. Hashim, A.I.; Zhang, X.; Wojtkowiak, J.W.; Martinez, G.V.; Gillies, R.J. Imaging pH and Metastasis. *NMR Biomed.* **2011**, *24*, 582–591. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3740268/pdf/nihms-385819.pdf (accessed on 12 November 2021). [CrossRef]
27. Webb, B.A.; Chimenti, M.; Jacobson, M.P.; Barber, D.L. Dysregulated pH: A Perfect Storm for Cancer Progression. *Nat. Rev. Cancer* **2011**, *11*, 671–677. Available online: https://www.nature.com/articles/nrc3110 (accessed on 11 November 2021). [CrossRef]
28. Hindenburg, A.A.; Gervasoni, J.E., Jr.; Krishna, S.; Stewart, V.J.; Rosado, M.; Lutzky, J.; Bhalla, K.; Baker, M.A.; Taub, R.N. Intracellular Distribution and Pharmacokinetics of Daunorubicin in Anthracycline-Sensitive and -Resistant HL-60 cells. *Cancer Res.* **1989**, *49*, 4607–4614. Available online: https://cancerres.aacrjournals.org/content/canres/49/16/4607.full.pdf (accessed on 2 November 2021).
29. Vukovic, V.; Tannock, I.F. Influence of Low pH on Cytotoxicity of Paclitaxel, Mitoxantrone and Topotecan. *Br. J. Cancer* **1997**, *75*, 1167–1172. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2222779/pdf/brjcancer00185-0081.pdf (accessed on 2 November 2021). [CrossRef]
30. Bour-Dill, C.; Gramain, M.P.; Merlin, J.L.; Marchal, S.; Guillemin, F. Determination of Intracellular Organelles Implicated in Daunorubicin Cytoplasmic Sequestration in Multidrug-Resistant MCF-7 Cells Using Fluorescence Microscopy Image Analysis. *Cytometry* **2000**, *39*, 16–25. Available online: https://onlinelibrary.wiley.com/doi/pdfdirect/10.1002/%28SICI%291097-0320%2820000101%2939%3A1%3C16%3A%3AAID-CYTO4%3E3.0.CO%3B2-I?download=true (accessed on 30 April 2022). [CrossRef]

31. Liao, C.; Hu, B.; Arno, M.J.; Panaretou, B. Genomic Screening In Vivo Reveals the Role Played by Vacuolar H+ ATPase and Cytosolic Acidification in Sensitivity to DNA-Damaging Agents such as Cisplatin. *Mol. Pharmacol.* **2007**, *71*, 416–425. Available online: https://molpharm.aspetjournals.org/content/71/2/416.long (accessed on 2 November 2021). [CrossRef] [PubMed]
32. Ishisaki, A.; Hashimoto, S.; Amagasa, T.; Nishihara, T. Caspase-3 Activation during the Process of Apoptosis Induced by a Vacuolar Type H(+)-ATPase inhibitor. *Biol. Cell* **1999**, *91*, 507–513. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0248490000882076?via%3Dihub (accessed on 7 November 2021). [CrossRef]
33. Wang, B.Y.; Zhang, J.; Wang, J.L.; Sun, S.; Wang, Z.H.; Wang, L.P.; Zhang, Q.L.; Lv, F.F.; Cao, E.Y.; Shao, Z.M.; et al. Intermittent High Dose Proton Pump Inhibitor Enhances the Antitumor Effects of Chemotherapy in Metastatic Breast Cancer. *J. Exp. Clin. Cancer Res.* **2015**, *34*, 85. Available online: https://jeccr.biomedcentral.com/track/pdf/10.1186/s13046-015-0194-x.pdf (accessed on 6 November 2021). [CrossRef] [PubMed]
34. Wang, X.; Liu, C.; Wang, J.; Fan, Y.; Wang, Z.; Wang, Y. Proton Pump Inhibitors Increase the Chemosensitivity of Patients with Advanced Colorectal Cancer. *Oncotarget* **2017**, *8*, 58801–58808. Available online: https://www.oncotarget.com/article/18522/pdf/ (accessed on 28 December 2020). [CrossRef]
35. Papagerakis, S.; Bellile, E.; Peterson, L.A.; Pliakas, M.; Balaskas, K.; Selman, S.; Hanauer, D.; Taylor, J.M.; Duffy, S.; Wolf, G. Proton Pump Inhibitors and Histamine 2 Blockers Are Associated with Improved Overall Survival in Patients with Head and Neck Squamous Carcinoma. *Cancer Prev. Res.* **2014**, *7*, 1258–1269. Available online: https://cancerpreventionresearch.aacrjournals.org/content/canprevres/7/12/1258.full.pdf (accessed on 4 January 2021). [CrossRef]
36. Miyazaki, H.; Igarashi, A.; Takeuchi, T.; Teng, L.; Uda, A.; Deguchi, H.; Higuchi, K.; Tango, T. Vonoprazan Versus Proton-Pump Inhibitors for Healing Gastroesophageal Reflux Disease: A Systematic Review. *J. Gastroenterol. Hepatol.* **2019**, *34*, 1316–1328. Available online: https://onlinelibrary.wiley.com/doi/10.1111/jgh.14664 (accessed on 10 November 2021). [CrossRef]
37. Murakami, K.; Sakurai, Y.; Shiino, M.; Funao, N.; Nishimura, A.; Asaka, M. Vonoprazan, a Novel Potassium-Competitive acid Blocker, as a Component of First-Line and Second-Line Triple Therapy for Helicobacter Pylori Eradication: A Phase III, Randomised, Double-Blind Study. *Gut* **2016**, *65*, 1439–1446. Available online: https://gut.bmj.com/content/gutjnl/65/9/1439.full.pdf (accessed on 10 November 2021). [CrossRef]