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Basic Translational

Influences of proton pump inhibitor on *Helicobacter pylori* adherence to the gastrointestinal cell lines

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Original Article

ABSTRACT

Background/Aims: *Helicobacter pylori* is a carcinogenic bacterium that could induce P-glycoprotein expression in the human gastrointestinal tract. Bacterial adherence to the gastrointestinal cell lines could be influenced by the level of P-glycoprotein. This study aimed to determine the influence of proton pump inhibitors that exhibit an inhibitory effect on P-glycoprotein in gastrointestinal carcinoma cell lines, namely Caco-2 and LS174T, in relation to *H. pylori* adherence.

Materials and Methods: Caco-2 and LS174T cells lines treated with omeprazole and esomeprazole were used in this study to assess the bacterial attachment of *H. pylori* within certain incubation periods.

Results: The presence of proton pump inhibitors increased the *H. pylori* adherence in a time-dependent manner in both Caco-2 and LS174T cell lines. The double inhibition of P-glycoprotein using proton pump inhibitor and P-glycoprotein inhibitor caused low P-glycoprotein expression in the cell lines, resulting in higher *H. pylori* adherence compared to the control cell lines.

Conclusion: Proton pump inhibitors may alter P-glycoprotein expression in the gastrointestinal tract, and subsequently *H. pylori* adherence on the cell lines, and may contribute to resistance to drug therapy.

Keywords: Proton pump inhibitor, protein expression, *H. pylori*

INTRODUCTION

Helicobacter pylori is a Gram-negative microaerophilic spiral bacillus that colonizes the gastric mucosa, and is the leading cause of gastric cancer worldwide (1). The human stomach, particularly the antrum and duodenum, acts as the principal reservoir for *H. pylori* infection. Peptic ulcer disease is characterized by mucosal damage secondary to pepsin and gastric acid secretion (2). Antibiotics and proton pump inhibitors have been used to eradicate *H. pylori* infection in patients with peptic ulcers (3). The proton pump inhibitor-clarithromycin-amoxicillin or metronidazole are currently the first choice treatments; however, the eradication rates were found to be less than 80% (4). There has been an increasing number of reports worldwide observing the incidence of antibiotic resistance that is linked to failed *H. pylori* eradication. Another important reason for eradication failure was reduced permeability of cell membranes to certain drugs, reduced drug binding to intracellular targets, and the role of P-glycoprotein (5). P-glycoprotein is a 170–180 kDa adenosine triphosphate (ATP)-dependent efflux protein that mediates the export of drugs from cells in the gut, providing a protective function for the body against xenobiotics (6). Over or low expression of P-glycoprotein may further decrease or increase the bioavailability of the drug, leading to substrate alteration and toxic effects, respectively (7).

It has been reported that *H. pylori* induces P-glycoprotein expression in the human gastrointestinal tract, and the bacterial adherence to the gastrointestinal cell line is influenced by the level of P-glycoprotein expression, which could be manipulated by the use of P-glycoprotein inducer or inhibitor (5,8). Therefore, increases in P-glycoprotein expression levels in gastric mucosal tissue will influence the extent of *H. pylori* binding, and exploitation of these levels could potentially prevent the de-

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velopment of *H. pylori*-associated gastrointestinal disease, such as gastric cancer.

Caco-2 cells express high levels of P-glycoprotein and have been widely used for the study of this drug reflux protein function and intestinal absorption (9). LS174T cells provide a suitable in-vitro model to test for the effect of Pregnane X receptor (PXR), which mediates the induction of intestinal P-glycoprotein using rifampicin (10). Proton pump inhibitors, such as omeprazole, esomeprazole, and pantoprazole, are P-glycoprotein inhibitors and are commonly used in *H. pylori* eradication regimens; however, little has been known of the influence of proton pump inhibitors on bacterial adherence to the gastrointestinal cell lines. In this study, we aimed to investigate the extent of *H. pylori* adherence to human gastrointestinal cell lines in the presence of P-glycoprotein inhibitors, namely omeprazole and esomeprazole.

MATERIALS AND METHODS

Caco-2 and LS174T Cell Culture

Caco-2 (ATCC®HTB-37) and LS174T (ATCC®CL-188) cell lines were cultured in in Nunc 25 cm^2 tissue culture flasks and fed with Dulbecco Modified Eagle Minimal Essential Medium (DMEM; Sigma-Aldrich, New South Wales) supplemented with 10% fetal calf serum, 2 mM L- glutamine, 1 mM non-essential amino acid and 1% penicillin/streptomycin. The cell cultures were maintained in humidified conditions at 37°C with 5% CO_2, with the culture media being replaced every two days. A hemocytometer was used to determine the number of cells in the suspension and every single well on the 96-well plate was fed with 2,000 cells per well. The 96-well plate was fed every other day for 19–21 days before it was ready for the bacterial adherence experiment.

Bacteria Culture

A strain of *H. pylori* was kindly donated by our local health institution. *H. pylori* was inoculated into blood agar supplemented with laked horse blood and *H. pylori*-selective supplement (Dent, Oxoid, Australia). All the blood agar plates were incubated at 37°C under microaerobic conditions for five–seven days.

Cell Viability

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assays were performed to evaluate the survival of cells in the presence of omeprazole and esomeprazole. Different concentrations of omeprazole and esomeprazole were used to determine the exact drug concentration that could allow 80%–90% cell survival. Omeprazole (20 mg, Pharmaniaga Manufacturing Berhad, Malaysia) and esomeprazole (20 mg, AstraZeneca, UK) were dissolved using its solvents and 0.9% NaCl, respectively. Caco-2 and LS174T cell lines were seeded at a density of 2,000 cells/well in a 96-well plate in 100 µL medium for 24 hours at 37°C. After 24 hours, omeprazole or esomeprazole was added at various concentrations (0 µM–600 µM). MTT solution (0.5 mg/mL, Sigma) was added to each well and the plate was incubated for 4 hours at 37°C. The formazan crystals were dissolved well before the absorbance of dye was measured using a Tecan plate reader (Infinite® 200 PRO, Life Sciences) at 570 nm wavelength. The percentage of inhibition was expressed by comparing the absorbance values of drug-treated cells with those of untreated controls: ((treated-cell absorbance/untreated cell absorbance) × 100) (11).

BacLight Green Preparation

On the day of the experiment, *H. pylori* colonies were scraped from the blood agar plates and suspended in cold sterile phosphate buffered saline (PBS, Sigma-Aldrich, New South Wales). The bacterial suspension was centrifuged before each of the pellets were re-suspended in cold PBS. Nine microliters of a 100 µM BacLight green solution in DMSO Molecular Probes (Eugene, Oregon, USA) was added to the bacterial suspensions to detect and monitor live bacteria. The stained bacteria will then exhibit bright green fluorescence (absorption/emission about 480/516 nm). All the tubes were then incubated for an hour at 37°C in a rotating incubator and given multiple washes with cold PBS.

For the bacterial attachment study in the presence of P-glycoprotein inhibitor, Hepes Balanced Salt Solution (HBSS) was prepared. A tube containing HBSS and 10% fetal calf serum (FCS), and a combination of HBSS, FCS, and 4 µM PSC-833, was also prepared. PSC-833 is a specific potent inhibitor of P-glycoprotein. To make up the bacterial solution, two Eppendorf tubes were prepared. One Eppendorf tube contained HBSS, FCS, and bacterial solution, while the other Eppendorf tube contained HBSS, FCS, bacterial solution, and PSC-833.

Bacterial Adherence Study

Two 96-well plates were seeded with Caco-2 cells and maintained for 21 days. On the day of the experiment, all medium was replaced with HEPES Balanced Salt Solution at pH 7.4. A potent P-glycoprotein inhibitor, PSC-833, was incubated with Caco-2 cells for 30 minutes prior to the first bacterial incubation period to allow P-glycoprotein transport sites to be blocked prior to exposure to bacteria (12). The bacterial solutions were loaded into the 96-well plates accordingly. In this study, 10 µM omeprazole or 10 µM esomeprazole was used to treat the Caco-2 cell lines 24 hours before introducing the BacLight green-labeled *H. pylori* into the cells. The *H. pylori* adherence to Caco-2 cells treated with omeprazole for 24 hours before bacterial introduction was compared to the bacterial adherence on the Caco-2 cells that were incubated with 4 µM of PSC-833 and treated with omeprazole for 24 hours before bacterial introduction after 30, 60, 90, 120, 180, and 240 minute incubation periods. In parallel, *H. pylori* adherence to the Caco-2 cells treated with omeprazole during 4 hours of bacterial introduction was compared to bacterial adherence on Caco-2 cells that were incubated with 4 µM of PSC-833 and treated with omeprazole during 4 hours of bacterial introduction after 30, 60, 90, 120, 180, and 240 minute incubation periods.

Similar methods and study arrangements were applied for another proton pump inhibitor, namely esomeprazole. All 96-well plates used were incubated at 37°C during the study time period and a 96-well fluorescence plate reader (Varioskan plate reader, Thermo Scientific) was used to detect fluorescent bacteria in these 96-well plates. A 485 nm excitation filter and 520 nm emission filter were used for detection.

Another two 96-well plates were seeded with LS174T cells and maintained for 9 days. In LS174T cell lines, P-glycoprotein expression was induced with rifampicin (8). After four days of cell growth, 10 µM rifampicin was added to the medium for LST174T cells. The growth medium with rifampicin was then replaced every 2 days. The LST174T cells were considered ready for experiments after 6 days of exposure to rifampicin. For a bacterial adherence study in LS174T cells in the presence of P-glycoprotein inhibitor (PSC-833), the method used was similar to that described for the Caco-2 cell lines.

Western Blot Analysis

The inhibitory effect of omeprazole and esomeprazole on P-glycoprotein expression has been reported elsewhere (11). In our study, the P-glycoprotein levels of the Caco-2 cell lines that had been incubated with proton pump inhibitors for 24 hours were evaluated by Western blot analysis. The P-glycoprotein expression in the treatment groups (21 day old Caco-2 cells treated with omeprazole or esomeprazole for 24 hours) was compared to that in the control group (21 day old Caco-2). Protein was collected from these flasks using protein lysis buffer, and the supernatants were kept at −20°C until the protein determination and Western blot analysis could be done.

In order to determine the influence of P-glycoprotein induction by rifampicin, P-glycoprotein expression in 10 day old LS174T was measured by Western Blot Analysis. The P-glycoprotein expression in the treatment groups (9 day old LS174T cells treated with rifampicin plus omeprazole or esomeprazole for 24 hours) was compared to that in the control group (9 day old LS174T cells treated with rifampicin alone). The Western Blot Analysis was performed based on the method outlined in our previous study (12). A Fusion FX1 instrument (Vilber Lourmat, Marne-La-Vallée, France) was used to determine the P-glycoprotein expression from Western Blotting. Our study does not require ethical approval as it is *in-vitro* research using already derived and established human cell lines.

Statistical Analysis

Analysis of variance (ANOVA) with Dunnett multiple comparisons was performed to determine the omeprazole and esomeprazole concentrations that allowed cell viability of 80%–90%. In a bacterial adherence study using Caco-2 cells grown for 21 days in a 96-well plate, the fluorescence intensity for cells that were incubated with omeprazole or esomeprazole for 24 hours before bacterial introduction was compared with the fluorescence intensities for Caco-2 cells that were incubated with 4 µM of PSC-833 and treated with omeprazole or esomeprazole for 24 hours before bacterial introduction, Caco-2 cells treated with omeprazole or esomeprazole for 4 hours of bacterial introduction, and Caco-2 cells that were incubated with 4 µM of PSC-833 and treated with omeprazole or esomeprazole for 4 hours of bacterial introduction at different time points using the Student's t-test. Results were presented as the mean±standard error of the mean of triplicate wells for each incubation period.

For bacterial attachment studies using LS174T cells, the fluorescence intensities for cells that were incubated with 4 µM PSC-833, 10 µM rifampicin, or 4 µM PSC-833 plus 10 µM rifampicin were compared to that of the control cells using the Student's t-test. Results were also presented as the mean±standard deviation of triplicate wells for each incubation period. SPSS software was used in this study and, in all analyses, p-values less than 0.05 were taken to indicate a statistically significant difference.

RESULTS

Drug Effects on Cell Viability

The inhibitory effect of omeprazole and esomeprazole was observed in both Caco-2 and LS174T cell lines at various drug concentrations (Figure 1). All drugs diminished cell viability in a concentration-dependent manner. Both cell lines showed a decrease in cell viability at a minimum dose of 10 µM of either omeprazole or esomeprazole after 24 hours of treatment. In this study, a 10 µM dose of omeprazole and esomeprazole provided the highest cell viability for Caco-2 (93% with omeprazole and 80% with esomeprazole; $p<0.05$) and LS174T (87% with both proton pump inhibitors; $p<0.05$).

P-Glycoprotein Inhibitory Effects on the Bacterial Adherence on Caco-2 Cells

The presence of proton pump inhibitors was observed to increase the *H. pylori* adherence in a time-dependent manner. The longer the incubation period, the greater the bacterial adherence observed. The *H. pylori* adherence to the Caco-2 cells was observed to be highest when the cells had been incubated with omeprazole for 24 hours before bacterial introduction, and in the presence of PSC-833 ($p<0.05$) (Figure 2a). The *H. pylori* adherence to the Caco-2 cells remained high when omeprazole was used to incubate the cell lines for 24 hours and no PSC-833 was given to the cell lines, although there was no significant difference between this adherence level and that on the control cell lines. The bacterial adherence was deemed to be lowest when only omeprazole was introduced during 4 hours of the incubation period. In our study, esomeprazole also demonstrated similar behavior with regard to *H. pylori* adherence to the Caco-2 cell lines, with $p<0.05$ (Figure 2b).

Effects of P-Glycoprotein Inhibition on the Bacterial Adherence in LS174T Cells

In LS174T cell lines, the presence of omeprazole 24 hours before bacterial introduction tended to increase the *H. pylori* ad-

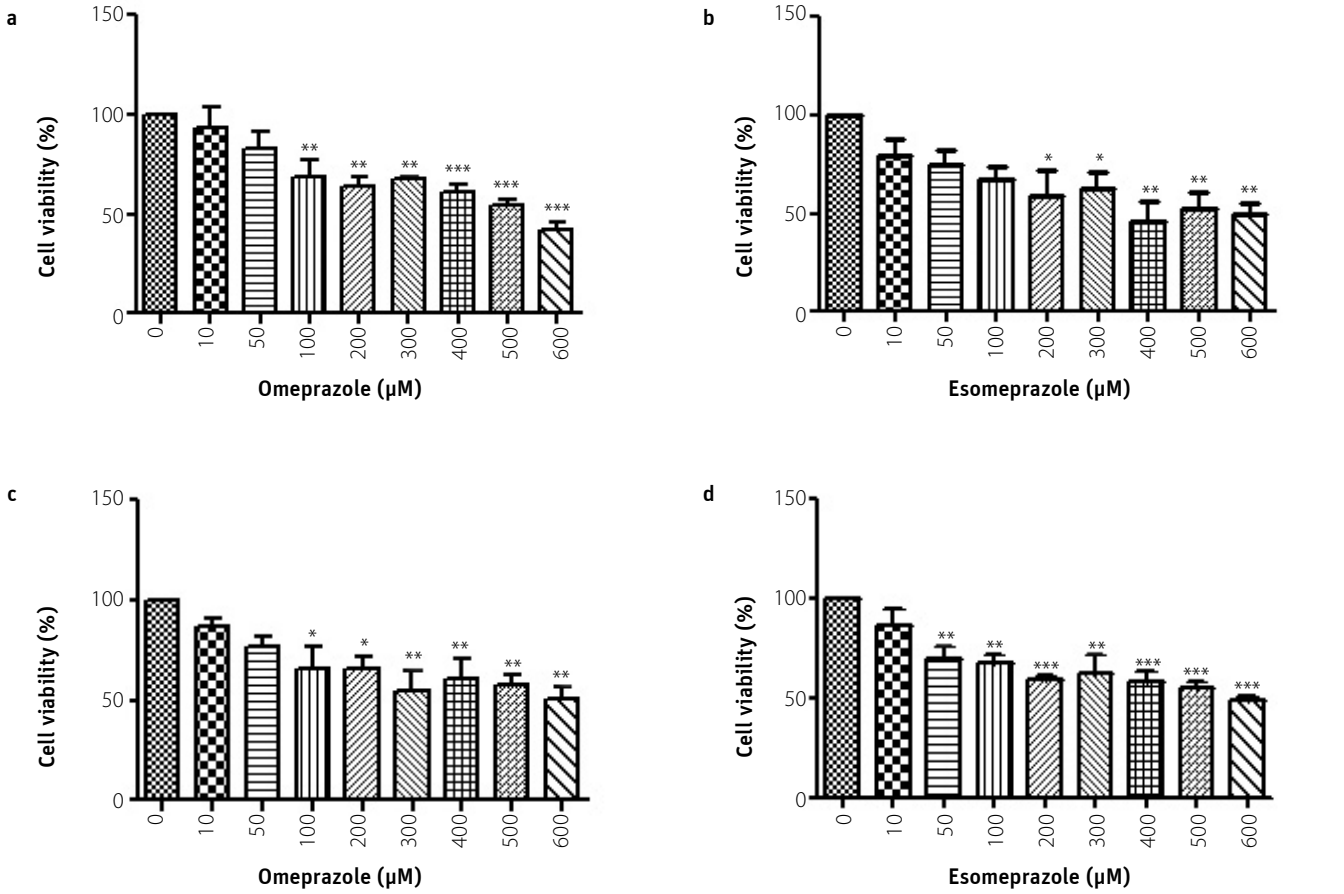

Figure 1. a-d. The viability assay for safest drug concentration used to treat cell lines. Varying concentrations ranging from 0 to 600 μM were used for (a) omeprazole on Caco-2 cells, (b) esomeprazole on Caco-2 cells, (c) omeprazole on LS174T cells and (d) esomeprazole on LS174T cells

herence in a time-dependent manner, and the co-administration of PSC-833, along with omeprazole, indeed increased the bacterial adherence, although the adherence levels between these two experimental conditions did not differ significantly compared to that for the control cell line ($p>0.05$) (Figure 3a). The *H. pylori* adherence was found to increase when PSC-833 was given to the LS174T cells that were treated with omeprazole during 4 hours of bacterial introduction as compared to LS174T cells treated with omeprazole during 4 hours of bacterial introduction.

For esomeprazole, the observation was quite varied, although a similar pattern of bacterial adherence to that with omeprazole was established (Figure 3b). The *H. pylori* adherence to the cells was observed to be highest when the cells had been incubated with esomeprazole for 24 hours before bacterial introduction and in the presence of PSC-833 ($p<0.05$). There was a steady increase in bacterial adherence when PSC-833 was also used in LS174T cells that were treated with esomeprazole during 4 hours of bacterial introduction. Nevertheless, there was no significant difference observed with regard to the bacterial adherence between the other two experimental conditions and the control cell lines.

Effects of P-Glycoprotein Induction and Inhibition on Bacterial Adherence on LS174T Cells

In this study, the P-glycoprotein expression in another set of LS174T cell lines was induced by rifampicin. The co-administration of PSC-833 to the cell lines that had been incubated with omeprazole 24 hours before bacterial introduction tended to increase the *H. pylori* adherence (Figure 4a). There was an increasing trend of bacterial adherence when the respective P-glycoprotein inhibitor was used for the LS174T cell lines that were treated with omeprazole during 4 hours of bacterial introduction. The lowest adherence rate of *H. pylori* in the P-glycoprotein-induced LS174T cell lines was observed when only omeprazole was given during 4 hours of bacterial introduction ($p>0.05$).

For esomeprazole, the observation was quite different to that with omeprazole (Figure 4b). In the LS174T cell lines that were treated with esomeprazole 24 hours before bacterial introduction, the bacterial adherence rate remained low. The presence of PSC-833 in the LS174T cell lines that were incubated with esomeprazole 24 hours before bacterial introduction increased the *H. pylori* adherence; however, this finding was similar to those with the other two experimental conditions ($p>0.05$).

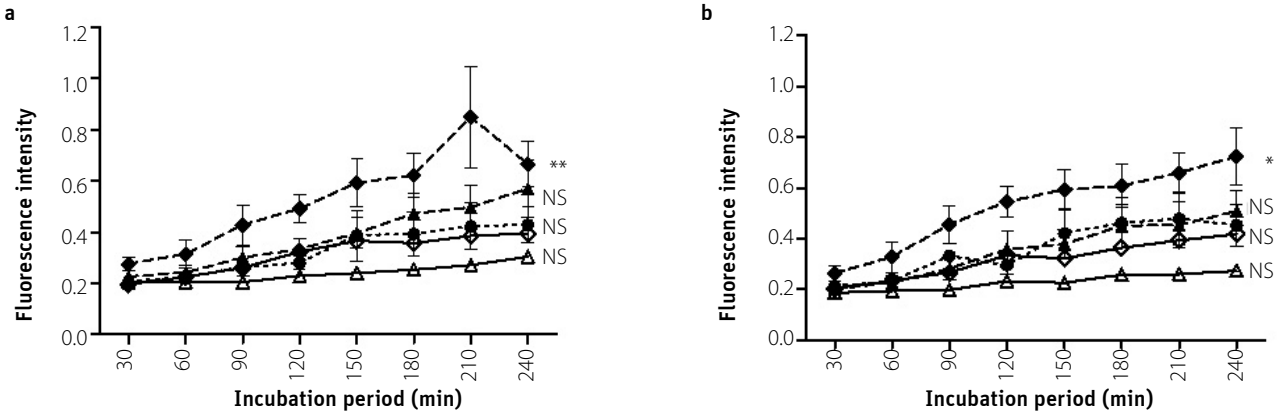

Figure 2. a, b. Four-hour study of BacLight green-labeled *H. pylori* adherence to Caco-2 cells grown for 21 days in a 96-well plate. The fluorescence intensity of Caco-2 cells treated with PPI, omeprazole (a) or esomeprazole (b) for 24 hours before bacterial introduction (filled triangle), Caco-2 cells that were incubated with 4 μM of PSC-833 and treated with PPI for 24 hours before bacterial introduction (filled diamond), Caco-2 cells treated with PPI during 4 hours of bacterial introduction (triangle), and Caco-2 cells that were incubated with 4 μM of PSC-833 and treated with PPI during 4 hours of bacterial introduction (diamond) were compared to the control cell lines (filled round) at different incubation periods

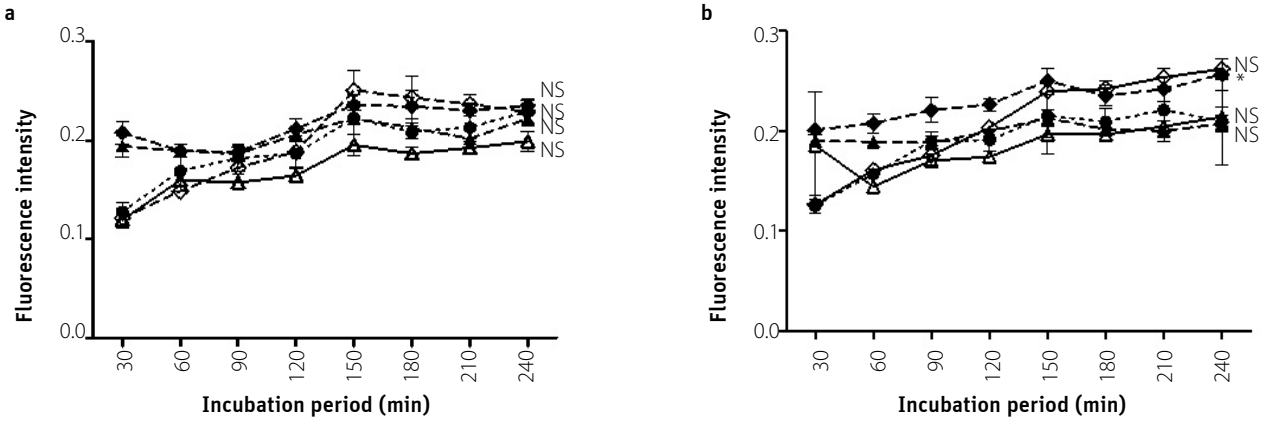

Figure 3. a, b. Four-hour study of BacLight green-labeled *H. pylori* adherence to LS174T cells grown for 9 days in a 96-well plate. The fluorescence intensity of LS174T cells treated with omeprazole (a) or esomeprazole (b) for 24 hours before bacterial introduction (filled triangle), LS174T cells that were incubated with 4 μM of PSC-833 and treated with PPI for 24 hours before bacterial introduction (filled diamond), LS174T cells treated with PPI during 4 hours of bacterial introduction (triangle), and LS174T cells that were incubated with 4 μM of PSC-833 and treated with PPI during 4 hours of bacterial introduction (diamond) were compared to that of the control cell lines (filled circle) for different incubation periods

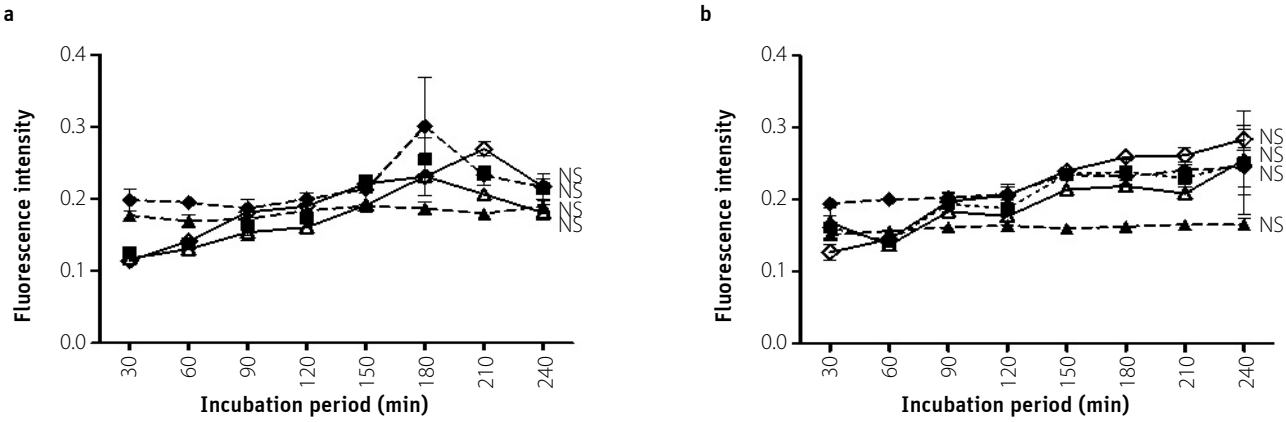

Figure 4. a, b. Four-hour study of BacLight green-labeled *H. pylori* adherence to LS174T cells grown for up to 10 days in a 96-well plate. The fluorescence intensity of LS174T cells treated with 10 μM rifampicin and omeprazole (a) or esomeprazole (b) for 24 hours before bacterial introduction (filled triangle), LS174T cells that were incubated with 10 μM rifampicin, 4 μM of PSC-833, and PPI for 24 hours before bacterial introduction (filled diamond), LS174T cells treated with 10 μM rifampicin and PPI during 4 hours of bacterial introduction (triangle), and LS174T cells that were incubated with 10 μM rifampicin, 4 μM of PSC-833, and PPI during 4 hours of bacterial introduction (diamond) were compared to the control cell lines (filled square) at different incubation periods

P-glycoprotein Expression in Caco-2 Cell Lines and P-Glycoprotein Induction in LS174T Cell Lines

In the bacterial adherence study, it was observed that bacterial adherence to the Caco-2 cells is much higher than to LS174T cells, although the LS174T cell line has been incubated with P-glycoprotein inducer. Based on Western Blot Analysis, the P-glycoprotein expression was found to be higher when either omeprazole or esomeprazole was used to treat the Caco-2 cell lines 24 hours before incubation, as compared to the control cells ($p<0.05$). The use of rifampicin was found to increase the P-glycoprotein level in LS174T, but the protein level remained lower than that observed in the Caco-2 cell lines ($p<0.05$).

DISCUSSION

Bacterial adhesion to the intestinal epithelium is a critical initial step in the pathogenesis of many enteric diseases (13). Adherence to the gastric epithelium is important with respect to the ability of *H. pylori* to initiate the colonization, as bacteria with better adherence properties colonize the host at higher densities. Triple eradication therapy based on a combination of proton pump inhibitors and antibiotics is the routine method used to treat *H. pylori* infection (14). However, the exact role of proton pump inhibitors in eradicating *H. pylori* has not been fully explained (15). Esomeprazole has been reported to be more effective than omeprazole to eradicate *H. pylori* infection due to its single enantiomer, which allows this drug to have a better tolerance rate (16), yet the anti-secretory effects of these two proton pump inhibitors are similar (17).

In this present study, omeprazole and esomeprazole were used to determine the influence of P-glycoprotein inhibitors towards *H. pylori* adherence in human cell lines. The extent of *H. pylori* adherence to Caco-2 cell lines was represented by the intensity of bacterial fluorescence, which corresponded to the amount of bacteria that adhered to human cell cultures coating the wells (12). *H. pylori* adherence to Caco-2 cells was found to increase when P-glycoprotein was inhibited by omeprazole 24 hours before bacterial introduction and in the presence of PSC-833. The double inhibition may cause low P-glycoprotein expression in the cell lines, resulting in the epithelial layer being arrested with a high amount of bacteria. As the inhibition of P-glycoprotein in Caco-2 cells did lead to significant changes in bacterial adherence, the role of P-glycoprotein cannot be dismissed. The P-glycoprotein indeed acts as an important protein at the epithelial level to protect the host. Other recent studies also determined that an alteration in gastrointestinal P-glycoprotein was linked to changes in the adherence level of gut pathogens, such as *Listeria monocytogenes and Escherichia coli* (12,18).

In our study, omeprazole and esomeprazole demonstrated similar behavior with respect to *H. pylori* adherence to Caco-2 cell lines. Although proton pump inhibitors have been known as inhibitors to the ATP-dependent efflux transporter, P-glycoprotein, the inhibitory potency of omeprazole and esomeprazole with regard to P-glycoprotein has yet to be established. In clinical settings, the *H. pylori* eradication rates did not differ very much among different proton pump inhibitors, indicating similar efficacy, with different doses reflecting differences in potency (19). All of these proton pump inhibitors have a common mechanism of action involving chemical rearrangement to a reactive sulfenamide that inhibits the ability of H^+,K^+-ATPase to participate in gastric acid formation (20). *H. pylori* would augment the acid inhibitory potency of omeprazole and enhances the omeprazole-induced proton efflux rate from gastric membrane vesicles (21).

Caco-2 and LS174T cell lines were both derived from colon adenocarcinomas but they differ in characteristics. These two cell lines are suitable *in-vitro* models to study P-glycoprotein induction, localization, and function by xenobiotic drugs (22). Caco-2 was widely used in a study related to P-glycoprotein expression due to it inheriting higher levels of P-glycoprotein. The polarized expression and activity of P-glycoprotein in Caco-2 cells justified its role as a gastrointestinal epithelial barrier (23). Unlike Caco-2 cell lines, LS174T was reported to express less or no P-glycoprotein, but it has PXR, which is considered as a key transcriptional regulator of P-glycoprotein, which allows cells to be induced with rifampicin to express P-glycoprotein. PXR is a nuclear receptor that is able to bind to structurally diverse compounds, including drugs and toxic metabolites (24). As the PXR controls the expression of key genes involved in anticancer drug disposition, its potential role in drug resistance has been studied. The induction of P-glycoprotein in LS174T cell lines by using rifampicin demonstrated an increase in protein expression, although this was not as high as that expressed in Caco-2 cell lines, as observed in our study and elsewhere (10). The level of bacterial adherence was observed to be higher in the Caco-2 cell lines than in the LS174T cell lines. An increase in bacterial adherence was also observed in this study when induced P-glycoprotein expression in LS174T cell lines was reversed with P-glycoprotein inhibitor, PSC-833. Thus, it is convincible that the presence of proton pump inhibitors, along with P-glycoprotein inhibitor, could increase the *H. pylori* adherence to the gastrointestinal cell lines due to inhibition of the expression of P-glycoprotein.

Emerging evidence suggests that P-glycoprotein may restrict bacterial pathogenesis in the gut mucosa. The importance of quantitative evaluation of P-glycoprotein expression during *H. pylori* eradication has been highlighted previously (25). An increase in P-glycoprotein levels could indicate an attempt by the gastrointestinal mucosa layer to protect itself against bacterial invasion (26). Consequently, it was suggested that the potential protective mechanism of P-glycoprotein in the human gastrointestinal tract may be exploited through the use of P-glycoprotein inducers, such as rifabutin, as part of the eradication regimen. Tailored combination therapy with novel rifabutin and ciprofloxacin has been shown to increase the *H. pylori* eradication rates by more than 70%. Therefore, manipu-

lating P-glycoprotein expression using P-glycoprotein inhibitor or inducer could influence treatment outcomes.

The presence of the proton pump inhibitors, omeprazole and esomeprazole, could increase *H. pylori* adherence to gastrointestinal cell lines due to inhibition of the expression of P-glycoprotein. Future study is warranted to determine the potency levels of proton pump inhibitors towards P-glycoprotein expression, to facilitate better understanding of their role in *H. pylori* eradication.

Ethics Committee Approval: N/A.

Informed Consent: N/A.

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Author Contributions: Concept – M.S.O.; Design – M.S.O., E.K.; Supervision – M.S.O., E.K.; Resources – M.S.O., E.K.; Materials – M.S.O., E.K.; Data Collection and/or Processing – N.S.D.; Analysis and/or Interpretation – M.S.O., N.S.D.; Literature Search – M.S.O.; Writing Manuscript – M.S.O.; Critical Review – M.S.O., E.K.; Other – M.S.O., N.S.D., E.K.

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