Proton Pump Inhibitor-Treated *H. pylori* Adjust Cell Envelope Fatty Acid and Cholesterol Content to Survive

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**Abstract**

**Background:** Proton pump inhibitors (PPIs) with lipophilic nature may interact with lipid components of *H. pylori* cell membrane, disrupting cell structure and viability. In this study, the effect of PPIs on fatty acid and cholesterol components of *H. pylori* cell membrane was assessed.

**Methods:** One *H. pylori* isolate was treated with 1X and 2X MICs (μg/mL) of lansoprazole (LPZ: 8 and 16) and pantoprazole (PAN: 128 and 256) in brain heart infusion broth plus serum. Treated *H. pylori* was cultured on brucella blood agar (BBA) and tetrazolium egg yolk agar (TEYA). Bacterial cells stained with Live/Dead kit were examined by fluorescent microscopy. Fatty acid and cholesterol contents of treated *H. pylori* were measured by gas chromatography.

**Results:** PPI-treated *H. pylori* did not grow on BBA but grew on TEYA. Fluorescent microscopy showed *H. pylori* stained red. Analyses showed high frequency of saturated fatty acids, C14:0, C16:0 and C18:0. Among unsaturated fatty acids, C18:1 and C18:2c were increased, while five were eliminated and five were synthesized de novo. Cholesteryl-6-O-tetradecanoyl-D-glucopyranoside was detected as the only glycosylated cholesterol in treated *H. pylori*. Growth of PPI-treated *H. pylori* on cholesterol-rich TEYA showed that occurrence of cholesterol can reverse the growth inhibition by PPIs. Red bacilli form of *H. pylori* showed dye entry through damaged cell membrane without lysis.

**Conclusion:** Incorporation of lipophilic PPI into *H. pylori* cell membrane disrupted lipids and inhibited growth. However, *H. pylori* adjusted the defected membrane by replacing the lipid components and resisted lysis.

**Keywords:** Cell membrane, Cholesterol, Fatty Acids, Helicobacter pylori, Proton pump inhibitors

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**Introduction**

Proton pump inhibitors (PPIs), used alone or included in antibacterial regimen against *H. pylori*, proved to be effective in improvement or resolution of gastric acid- and *H. pylori*-associated peptic diseases, gastritis, peptic ulcer, gastric cancer, intestinal metaplasia and mucosa-associated lymphoid tissue lymphoma. PPIs were first identified as acid suppressing agents that helped in ulcer healing. Furthermore, it was revealed that PPIs provide neutral pH appropriate for *H. pylori* growth and better efficacy of antimicrobials. Shortly after, the antimicrobial activity of PPIs against *H. pylori* was reported. It was indicated that in acidic pH, PPIs convert to sulfonamide that inhibit acid pumps of gastric parietal cells and *H. pylori* urease by binding to cysteine residues in proteins. However, the results of further studies suggested that the inhibitory action of PPIs on *H. pylori* growth was not due to inactivation of enzymes such as urease or F- and P-type ATPases or dependent on acidic pH. Several in *vitro* studies have demonstrated that PPIs inhibit *H. pylori* growth and urease activity and cause the spiral forms of bacteria to turn into coccoid. However, except for urease, other target molecules of these drugs in *H. pylori* have not been identified. It has been proposed that PPIs with lipophilic property probably interact with components of *H. pylori* cell membrane.

The cell envelope of *H. pylori*, a Gram-negative bacterium, is composed of peptidoglycan layer, outer and inner membranes and periplasmic space. The membranes are made of phospholipids composed of polar heads of phosphates and fatty acids. Lipid-associated proteins are also involved in membrane structure and function. Membrane fatty acids and their associated proteins play important roles in vital activities of the bacterial cell including solute transport, biosynthesis of components, energy production, enzyme secretion, signal transduction, and regulation of DNA replication and cell division.

The cell membrane of members of the Helicobacter genus is unique in accumulating large amounts of free cholesterol which comprise >70% of neutral lipids.
_H. pylori_ extracts free cholesterol from host gastric epithelial cells, and α-glycosylates it and incorporates the resulting cholesteryl glucosides (CGs) into its membrane. Three CGs have been identified in _H. pylori_, cholesteryl-α-D-glucopyranoside (CGGL), cholesteryl-6-O-tetradecanoyl-α-D-glucopyranoside (CAG), and cholesteryl-6-O-phosphatidyl-α-D-glucopyranoside (CPG). CGGL converts to CPG when phosphorylated and to CAG when acylated. It has been shown that CGs are involved in maintenance of _H. pylori_ spiral shape, normal membrane permeability, resistance to antibacterial influx, bacterial virulence and cell division. Accordingly, damage to _H. pylori_ cell membrane, leading to changes in fatty acid and cholesterol content, can exert a negative impact on bacterial cell structure, viability and growth.

For single-celled bacteria directly and continuously exposed to environmental stresses, adjustment of fatty acid composition to maintain biophysical properties of membranes is pivotal for survival and adaptation. It has been indicated that cationic antimicrobials with lipophilic property can damage membranes by: 1) preferentially binding to anionic lipids, causing separation and rearrangement of membrane lipids, leading to pore formation and leakage; 2) interacting with anionic lipids in bacterial cell membrane, leading to their clustering, separation from natural domain and loss of function; and 3) targeting intracellular molecules as the primary or enhancing mechanism for effective killing.

It has been demonstrated that at pH 7 lansoprazole (LPZ), the benzimidazole PPI degrades to sulfide compounds that are lipophilic and interact with components of cell membrane. Sulfoxides exert inhibitory effect on both resting and growing _H. pylori_ cells at pH 5 and 7. Furthermore, compared with antibiotics, sulfoxides kill bacteria in a shorter time with no emergence of resistance. It has been suggested that sulfoxides’ targets in bacterial cell must be structural components rather than proteins that need active bacteria for expression. Growth of PPI-treated _H. pylori_ has been shown to be inhibited within 20 hours; however, this inhibitory effect is dose- and time-dependent, being reversible at low drug dose and short exposure time.

The aim of this study is to examine whether the cell membrane components of _H. pylori_ are the target molecules of PPIs. We investigated the impact of two PPIs, LPZ and pantoprazole (PAN) on lipid components of _H. pylori_ cell membrane. _H. pylori_ was exposed to PPIs at 1X and 2X of minimum inhibitory concentrations (MICs) and changes in morphology, viability, culturability as well as fatty acid and glycosylated cholesterol contents of bacterial cells were assessed. After 20 h exposure of _H. pylori_ cells to PPIs, their culturability was assessed on Brucella blood agar (BBA) and tetrazolium-egg yolk agar (TEYA). Changes in bacterial morphology and viability were examined by light and fluorescent microscopy. Furthermore, whole cells of PPI-treated _H. pylori_ were used for extraction of fatty acids and cholesterol. Gas chromatography-flame ionization detector (GC-FID) was used for measurement of lipid profiles and the results obtained in PPI-treated _H. pylori_ were compared with those found in untreated controls. The relationship between changes in cell membrane components of PPI-treated _H. pylori_ and bacterial morphology, viability and culturability was assessed.

**Materials and Methods**

Selection of one _H. pylori_ isolate with High Susceptibility to LPZ and PAN

One _H. pylori_ isolate was used in this study. This isolate was selected out of 25 _H. pylori_ isolates which were tested by agar dilution and disc diffusion methods for susceptibility to PPIs (LPZ and PAN) with MICs determined in our previous study at 8 μg/mL for LPZ and 128 μg/mL for PAN. Dimethyl sulfoxide (DMSO) with no antibacterial activity on _H. pylori_ was used for preparation of PPI solutions. Fresh cultures of 25 _H. pylori_ isolates were used for preparation of bacterial suspensions with the turbidity of McFarland unit No. 2 (6×10⁸ cell/mL). BBA (Pronadisa, Spain) plates containing final concentrations of LPZ (1X: 8 μg/mL) and PAN (1X: 128 μg/mL) were spot inoculated with a 10-μL volume of each bacterial suspension. BBA plates without PPIs were used as controls. After 5-7 days of incubation under microaerobic atmosphere at 37°C, the plates were inspected for bacterial growth. Disc diffusion method was used for detection of one _H. pylori_ out of 25 with highest susceptibility to LPZ and PAN. Blank paper discs (Padtanteb Co, Iran) were superimposed on BBA plates that were surface-inoculated with 100 μL of fresh _H. pylori_ suspensions with the turbidity of McFarland unit No. 2. A 10-μL volume of each PPI (MIC: 1X) was inoculated into the blank discs and the plates were incubated as mentioned above. One _H. pylori_ isolate that produced equal inhibition zone diameters upon exposure to both PPIs was selected for examining the impact of PPIs (LPZ [1X:8 and 2X:16 μg/mL] and PAN [1X:128 and 2X:256 μg/mL]) on growth, morphology and fatty acids and cholesterol contents of _H. pylori_.

**Determination of Time Required for Inhibition of _H. pylori_ Growth by PPIs**

A 50-mL flask containing 10 mL brain-heart infusion (BHI) broth (Pronadisa, Spain) (pH 7.0) supplemented with 2.5% horse serum was used for growing the control _H. pylori_. Test 50-mL flasks containing 10 mL BHI broth and serum were supplemented with LPZ and PAN to reach the final concentration of MIC (1X). A 10-mL volume of bacterial suspension with the turbidity of McFarland unit No. 4 was added to 10-mL volume of BHI-serum-PPI flasks to reach the final turbidity of
The incubator at 37°C while 50 μL volumes of bacterial and the absence of secondary bacterial contamination. The from each treated flask on BBA was negative, showing and harvested (8000 rpm, 4°C). Culture of a 50 μL volume CO₂ and reached the turbidity of McFarland unit No. 2. The were inoculated with fresh culture of H. pylori after 5-7 days of incubation under microaerobic conditions at 37°C. The second 50 μL of bacterial suspension was used for Gram staining and light microscopy.

Preparation of PPI-Treated H. pylori Cells for Analysis of Lipid Contents by GC-FID
Each PPI was added to 20 one-liter flasks containing 500 mL of BHI broth with 2.5% horse serum to reach the final concentrations of 1X and 2X of MIC. The flasks were inoculated with fresh culture of H. pylori and the turbidities were adjusted to McFarland unit No. 2. The flasks were incubated in CO₂ incubator at 37°C while shaking. After 20 h, bacteria were washed twice with PBS and harvested (8000 rpm, 4°C). Culture of a 50 μL volume from each treated flask on BBA was negative, showing the absence of secondary bacterial contamination. The 20 flasks were divided into three groups: eight flasks for fatty acid analysis, eight flasks for analysis of glycosylated cholesterols, and four flasks as control.

Microscopy
Examination of PPI-Treated H. pylori Cells for Culturability, Viability and Morphological Changes - Fluorescent Microscopy
A fresh culture of H. pylori was used for inoculating a 150-mL flask containing 50 mL BHI broth with 2.5% serum to reach the turbidity of McFarland unit No. 2. After 20 h exposure to 1X and 2X concentrations of PPIs, bacterial cells were washed twice with PBS buffer (× 5000 rpm) and resuspend in 1 mL of PBS. For examining the culturability of PPI-treated H. pylori, 50 μL volumes of bacterial suspensions were surface-inoculated on BBA and TEYA plates. For examination of culturability of PPI-treated H. pylori, 50 μL volumes of bacterial suspensions were surface-inoculated on BBA and TEYA plates. One milliliter of test and control suspensions were surface-inoculated on BBA for examining the culturability of PPI-treated H. pylori after 5-7 days of incubation under microaerobic conditions at 37°C. The plates were incubated and inspected as mentioned above. To examine the viability and changes in morphology of PPI-treated H. pylori cells, 500-μL volumes of washed cells were stained with LIVE/DEAD BacLight Bacterial Viability Kit L-7012. According to the manufacturer’s instructions and examined by fluorescent microscope (Olympus, Tokyo, Japan). A suspension of 128 μg/mL of PAN in DMSO was stained with LIVE/DEAD kit and used as control.

Results
Selection of One H. pylori Isolate with High Susceptibility to PPIs
Compared with untreated control H. pylori isolates that showed confluent growth on BBA, all the 25 isolates were susceptible to LPZ and PAN and did not show visible growth on PPI-containing BBA plates. One H. pylori isolate that showed high susceptibility to LPZ and PAN by producing IZD of 45 mm was selected for examining the inhibitory effect of PPIs.

Determination of Time Required for Inhibition of H. pylori Growth by PPIs
Compared with control, the treated H. pylori was inhibited by the two selected PPIs but after different exposure times; 18 h for LPZ and 20 h for PAN. Accordingly, the impact of PPIs on H. pylori viability, morphology and profiles of fatty acids and cholesterol was examined after 20 h. Light microscopy observations showed that compared with control H. pylori that appeared as gram-negative curved bacilli, PPI-treated H. pylori cells appeared as distorted bacilli (Figure 1).
Examination of PPI-treated *H. pylori* Cells for Culturability, Viability and Morphological Changes - Fluorescent Microscopy

Culture of PPI-treated *H. pylori* on BBA showed negative results. However, appearance of red bacterial colonies on TEYA indicated bacterial growth in the presence of egg-yolk cholesterol (Figure 2). Fluorescent microscopy observations showed green spiral cells in control *H. pylori* cultures (Figure 3A) and a mixture of red and green bacilli in PPI-treated cultures (Figure 3B). According to the manufacturer’s comments, live bacterial cells with intact membrane stain green due to the entry of SYTO 9 dye. In contrast, bacteria with damaged cell appear red as a result of the free diffusion of propidium iodide through disrupted membrane.

Analysis of Total Saturated Fatty Acids (SFAs) and Unsaturated Fatty Acids (UFAs) in PPI-treated *H. pylori*

Analysis of fatty acid contents of untreated *H. pylori* showed the frequency of SFAs at 64% and that of UFAs at 36%. However, in PPI-treated *H. pylori*, the frequency of SFAs and UFAs was measured in respective order as 1X LPZ (49% and 51%), 2X (56% and 44%), 1X PAN (27% and 67%) and 2X (54% and 46%). Comparing the frequency of C14- C26 SFAs in 2X LPZ and PAN-treated *H. pylori* with those of control *H. pylori* showed increases in C14 (from 7 to 20 and 31%), C16 (7 to 10 and 12%) and C18 (10 to 15 and 21%) and elimination of C17 (from 7 to 0%), C19 cyc (15 to 0%), C20 (13 to 1 and 0%), C24 (4 to 0%), and C26 (1 to 0%). However, among the C14:1- C24:1 UFAs, those that showed a decrease in frequency were C14:1, C16:1, C17:1, C20:1, C20:5, C22:1, and C22:2 (from 1-9% to 0% or remained at 1%). Those that showed an increase in frequency included C18:1 (from 3 to 9 and 10%), C18:2 (9 to 24 and 15%). Interestingly, C16:2c was synthesized de novo in 2X LPZ (10%), 1X PAN (5%) and 2X PAN (19%), C18:2r in 1X PAN (2%), C18:3 in 2X PAN (1%), C22:6 in 1X LPZ (1%) and 1X PAN (2%) and finally, C24:1 in 1X LPZ (4%), and 1X PAN (10%) (Table 1).

Analysis of Glycosylated Cholesterol Contents in Cell Membrane of PPI-treated *H. pylori*

Measurements of total glycosylated cholesterols (CGL, CAG and CPG) in control and treated *H. pylori* with 1X and 2X concentrations of PPIs showed almost similar results ranging from 18000-19000 ppm taken as 100%. However, compared with control *H. pylori* that contained CGL (80%), CAG (17%) and CPG (3%), 1X LPZ-treated *H. pylori* contained CGL (95%), CAG (3%) and CPG (2%) while 1X PAN-treated *H. pylori* contained CGL (92%), CAG (8%) and CPG (0%). However, when *H. pylori* was treated with 2X concentrations of both PPIs, CAG was detected as total cholesterol components and CGL and CPG were not detected (Table 2).

Discussion

Lipids in bacterial cell membrane are important components for the maintenance of bacterial cell structure and vital activities. However, the anionic nature of lipids makes them highly susceptible to cationic compounds...
In *H. pylori*, like other bacteria, the frequencies of SFAs and UFAs should be maintained in a proper balance to adjust membrane viscosity in response to environmental stresses.24

The results of this study showed that exposure of *H. pylori* to PPIs, LPZ and PAN at 1X and 2X of MIC, led to dramatic changes in fatty acid and glycosylated cholesterol contents of *H. pylori* cell envelope. These changes manifested as alteration in morphology, viability and culturability of *H. pylori* cells. After 20 h exposure to 1X and 2X concentrations of PPIs, *H. pylori* did not show growth on BBA but appearance of red colonies on TEYA showed that 1X and 2X PPI-treated cells were viable and able to grow on a medium enriched with egg yolk that probably provided cholesterol for bacterial membrane repair and growth.25 Observation with light microscopy showed that compared with the control *H. pylori* that appeared as curved bacilli and stained well when Gram-stained, the PPI-treated *H. pylori* showed as structurally distorted bacilli which were weakly stained with the Gram stain. However, fluorescent microscopy observations showed that compared with the control *H. pylori* that appeared as green bacilli, the PPI-treated cells appeared as a mixture of red and green bacilli which were slightly swollen. It appears that red dye could diffuse into the damaged cell envelope due to changes in fatty acids. Incorporation of PPIs into the bacterial cell envelope may have led to local damage with the consequences of disruption of cell wall integrity and inhibition of bacterial growth and replication. However, photographs showed that bacteria retained their cell structure without lysis.

Observations by transmission electron microscopy on cells treated with LPZ at concentrations several times higher than MIC have demonstrated a considerable change in the cell envelope of *H. pylori* as constriction of bacterial cell, appearance of focal cell wall blebs and release of membrane vesicles. Although the PPI-treated *H. pylori* showed a remarkable reduction in viability, bacterial lysis did not occur, indicating that PPIs inhibit bacterial growth without cell lysis.26 Scanning electron microscopy on LPZ-treated *H. pylori* has also shown shrinkage of *H. pylori* bacilliforms with numerous blebs on the surface, indicating disruption of bacterial cell surface components and increased permeability.27 Another transmission electron microscopy study on *H. pylori* cells which were treated with lipophilic long-chain unsaturated fatty acids (LC-UFAs) demonstrated disruption of bacterial morphology that was associated with growth inhibition, cell membrane fragmentation and lysis. It was suggested that these changes were the result of alterations in the physicochemical properties of bacterial cell membrane by LC-UFA. These changes were reversible at low, but irreversible at higher concentrations of fatty acids. However, calcium, magnesium, ergocalciferol and cholesterol were effective in reversing the inhibitory action that incorporate into them,27 change their structure and disrupt their biological functions.16 When bacteria are exposed to lipophilic compounds, they maintain their cell membrane fluidity mainly through altering the fatty acid composition of phospholipids2 by *de novo* synthesis of fatty acids or remodeling the existing ones.28 For this purpose and to save energy, bacteria may use degraded phospholipids produced as a result of membrane damage.29

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**Table 1.** Frequency of Saturated (SFAs) and Unsaturated Fatty Acids (UFAs) in Cell Membrane of *H. pylori* Treated with lansoprazole (LPZ, 1X:8 and 2X: 16 µg/mL) and Pantoprazole (PAN, 1X:128 and 2X: 256 µg/mL)

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<tr>
<td>Total</td>
<td>36</td>
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<td>44</td>
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*de novo synthesized.*

Cyc, cyclopropane; c, cis isomerization; t, trans isomerization.

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**Table 2.** Frequency of cholesteryl glucosides (CGs) in cell membrane of *H. pylori* treated with lansoprazole (LPZ, 1X:8 and 2X: 16 µg/mL) and pantoprazole (PAN, 1X:128 and 2X: 256 µg/mL)

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<tr>
<td>Total</td>
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CGL, Cholesteryl-α-D-glucopyranoside; CAG, Cholesteryl-6-O-tetradecanoyl-α-D-glucopyranoside; CPG, Cholesteryl-6-O-phosphatidyl-α-D-glucopyranoside.
of fatty acids. Analyses of fatty acid contents of the control *H. pylori* showed the frequency of SFAs and UFAs at 64% and 36%, respectively. These measurements were in accordance with 2/3 SFAs and 1/3 UFAs in a physiologically active bacterial cell. However, SFAs and UFAs in *H. pylori* showed equal frequencies when treated with 2X LPZ (SFAs: 56%, UFAs: 44%) and 2X PAN (SFAs: 54%, UFAs: 46%), indicating a considerable increase in the frequency of UFAs. Increase in UFA contents of bacterial cell membrane in response to environmental changes may lead to increase in membrane fluidity which could play an important role in maintenance of physical structure of bacterial cell against stresses. According to recent reports, LC-UFAs could be involved in antioxidant activity, antibiotic efflux, and formation of microdomains for cell division.

The results of this study showed that among eight SFAs detected, three showed an increase in frequency (C14:0, C16:0 and C18:0) and five were eliminated (C17:0, C19cyc, C20:0, C24:0 and C26:0). Increase in C14:0 as the main constituent of phosphatidylethanolamine could be related to uptake of cholesterol and increased resistance to antimicrobials. SFAs such as C16:0 pack tightly together and produce a lipid bilayer with high rigidity and low permeability. Increase in SFA also occurred in *Escherichia coli* when exposed to toxic compounds such as benzene and octanol. The membrane cyclopropane SFA C19 (C19cyc) was eliminated in the PPI-treated *H. pylori* but was detected in the control *H. pylori*. Reports indicate that C19cyc is found in actively growing bacteria but absent in stressful conditions. A considerable decrease was detected in C17cyc and C19cyc when members of the genus *Pseudomonas* were treated with toxic compounds such as catechol and phenol and toluene. The reason could be the need for a considerable amount of energy for their synthesis.

The results of this study showed that among 14 UFAs identified, two showed an increase in frequency (C18:1 and C18:2c), two were decreased (C14:1, C16:1), and five were eliminated (C17:1, C20:1, C20:5, C22:1 and C22:2). However, five UFAs were synthesized de novo (C16:2c, C18:2t, C18:3, C22:6 and C24:1). It has been indicated that by introducing an efficient kink in the chain, cis UFAs disrupt the order of the lipid bilayer and increase membrane permeability. UFAs in *Pseudomonas putida* were converted to trans configuration when exposed to toxic compounds. LC-UFAs C20:1-C24:1 observed in the 1X PPI-treated *H. pylori* were not detected in the 2X PPI-treated *H. pylori*. It appears that higher concentrations of PPIs caused their elimination. Occurrence of higher frequency of LC-UFAs in the 1X PPI-treated *H. pylori* could be due to the lower inhibitory activity of PAN that allowed the bacteria to synthesize high amount of LC-UFAs such as C20:1 (18%).

Analysis of CGs of *H. pylori* showed occurrence of CGL, CAG and CPG in the control and 1X PPI-treated *H. pylori*. However, when *H. pylori* was treated with 2X PPIs, CGL and CPG were not detected and CAG was the only CG present. Absence of CPG phospholipid in bacterial cell membrane causes an increase in membrane fluidity, change of FtsZ rings and inhibition of cell division and inactivation of membrane-associated enzymes. Occurrence of CAG as a neutral lipid in the membrane reduces the interactions between molecules, leading to increased fluidity and permeability as well as inhibition of cell division. It has been suggested that an increase in CAG leads to maintenance of *H. pylori* cell integrity and spiral morphology under stressful conditions. Furthermore, occurrence of cholesterol in *H. pylori* cell membrane decreases cell surface negative charge, reducing antibiotic uptake. In the present study, growth of the PPI-treated *H. pylori* on cholesterol-rich TEYA showed that occurrence of cholesterol can reverse the growth inhibition by PPIs at 1X and 2X of MICs.

Pharmacokinetics of PAN was investigated when taken by patients as a single daily dose of 40 mg, intravenously or orally. PAN reached maximum concentration of 7.05 µg/mL in plasma of the former group and 3.12 µg/mL in serum of the latter. These concentrations did not eradicate *H. pylori* even if taken for years. The results of this in vitro study showed that even at concentrations much higher than physiological concentrations of PPIs, *H. pylori* did not lose its viability and the inhibitory effect of PPIs was reversible.

Exposure of *H. pylori* to PPIs led to changes in bacterial cell structure, morphology, viability and culturability. These changes occurred due to degradation of PPIs, at neutral pH, to sulfide compounds that incorporated into *H. pylori* cell membrane. The results were increased lipid clustering, fluidity and permeability and thus pore formation and leakage. However, *H. pylori* adjusted its fatty acid and cholesterol content to stabilize the damaged membrane. Since PPI destabilization occurred in a short time, the *H. pylori* response to this stress also needed to be as fast as possible. For this purpose, *H. pylori* minimized energy expenditure by stopping growth and cell division while retaining structural rigidity to resist lysis and deterioration.

**Authors’ Contribution**
SK and FS designed the experiment and wrote the paper. SK did the research work. AF provided the chemicals and information of their mechanism of action. AS did the data analysis of fluorescent microscopy. SK and ShK did the literature review.

**Conflict of Interest Disclosures**
The authors declare that there are no conflicts of interest.

**Ethical Statement**
All patients signed and informed consent and the study was approved by the research ethics committee of Tehran University of Medical Sciences.
References


