Helicobacter pylori Urease Inhibition by Rabeprazole, a Proton Pump Inhibitor

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We investigated the inhibitory effects of four gastric proton pump inhibitors (PPIs): rabeprazole, a novel benzimidazole PPI, omeprazole, lansoprazole and AG-2000, on the urease activity of Helicobacter pylori (H. pylori). Their 50% inhibitory concentrations (I_{50}) were found to be 0.29, 5.4, 9.3 and 0.3 μM respectively. Rabeprazole and omeprazole were also potent inhibitors of Jack bean and Proteus mirabilis cellular ureases. The thioether derivative of rabeprazole, one of its metabolites, had no inhibitory effect on H. pylori urease, despite being reported as a more potent inhibitor of H. pylori growth than rabeprazole.

The inhibitory effect of rabeprazole was prevented completely and reversed considerably by the addition of sulfhydryl compounds, such as β-mercaptoethanol, glutathione and dithiothreitol. Moreover, the addition of β-mercaptoethanol recovered the urease activity inhibited by rabeprazole. From these results, we expected that rabeprazole inhibited H. pylori urease activity by forming disulfide bonds between it and the active site of the enzyme.

Key words Helicobacter pylori; lansoprazole; omeprazole; proton pump inhibitor; rabeprazole; urease

Helicobacter pylori (H. pylori) has been implicated as an etiological pathogen of gastritis and peptic ulcer disease since it was isolated by Warren and Marshall in 1983. It is a gram-negative, spiral bacterium characterized by strong urease activity. Ammonia generated by the hydrolysis of urea protects this acid-sensitive bacterium from gastric acid. Furthermore, high concentrations of ammonia may have a direct toxic effect on gastric mucosal cells. H. pylori also produces a toxin that induces eukaryotic cell vacuolation, and its toxicity may be potentiated by urease-mediated ammonia production. Therefore, H. pylori urease has been suggested to be associated with gastroduodenal disorders.

The gastric proton pump inhibitors (PPIs), such as rabeprazole (E-3810), omeprazole and lansoprazole, are used as antiulcer agents, which inhibit acid secretion into the stomach by the potent and continuous blockade of (H^+ + K^+)-adenosine triphosphatase (ATPase). The PPIs accumulate in an acidic environment, such as gastric gland lumens or gastric vesicle intravesicular spaces, and are then transformed into active compounds under such acidic conditions. This inhibition is known to be associated with the binding of PPIs to the cysteinyl residues of the proton pump active sites.

Recently, it was reported that PPIs were potent inhibitors of H. pylori growth in vitro at concentrations similar to those that inhibit gastric (H^+ + K^+)-ATPase activity. In order to clarify the mechanism responsible for the growth inhibition by PPIs, we investigated the effects of PPIs on H. pylori urease activity in the present study.

MATERIALS AND METHODS

Materials Rabeprazole and its thioether derivative were donated by Eisai Co., Japan; lansoprazole and its acid-activated analog (AG-2000) were from Takeda Chemical Industries Co., Japan; and omeprazole was from Fujisawa-Astra Co., Japan. Rabeprazole was dissolved in ethanol, except for the study of the preventive effects of sulphydryl (SH) compounds, when it was dissolved in H_2O. AG-2000 was dissolved in methanol and the other PPIs used were dissolved in ethanol. β-Mercaptoethanol (β-ME), glutathione (GSH) and dithiothreitol (DTT) were purchased from Wako Pure Chem. Ind., Japan.

Preparation of Urease H. pylori ATCC 43504 was kindly provided by the Research Institute for Microbial Diseases, Osaka University. It was cultured on brucella HK agar plates (Kyokuto Seiyaku Kogyo, Japan) for 4 d at 37°C in an anaerobic jar with Anaero Pack Campylo (Mitsubishi Gas Chemical Co., Japan), then it was inoculated into 10 ml of brucella broth (BBL, U.S.A.) supplemented with 10% v/v fetal calf serum in a 50 ml flask, which was placed in an anaerobic jar, and cultured micro-aerobically for 4 d at 37°C. Proteus mirabilis was isolated clinically and cultured for 2 d at 37°C in nutrient broth (Difco, U.S.A.). All the bacterial cells were harvested by centrifugation (20000 x g, 20 min, 4°C).

The harvested H. pylori cells were washed with 5 ml of 20 mM citrate buffer (pH 5.0), 20 mM phosphate buffer (pH 7.0) or 20 mM triethanolamine buffer (pH 8.5), then resuspended in the same buffers and used for the assay of cellular urease activity at different pH values. The cells were disrupted by sonication, centrifuged at 20000 x g for 20 min at 4°C, and the supernatants were used for the assay of cell-free urease activity. The P. mirabilis cells were washed with 0.1 M phosphate buffer (pH 7.0), resuspended in this buffer, and this suspension was used as Proteus cellular urease. Jack bean powder (10 g) was stirred for 2 min with 50 ml of 0.1 M phosphate buffer (pH 7.0), followed by centrifugation at 20000 x g for 20 min at 4°C, and the supernatant was used as Jack bean urease solution. The activities of the H. pylori cellular urease solutions were 1 to 3 IU/mg protein and those of the cell-free solutions were 31 IU/mg protein. The specific activities of the P. mirabilis and Jack bean ureases were 5.1 and 0.80 IU/mg protein, respectively.

Urease Assay and Inhibition Reaction mixtures com-

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prising 100 μl of enzyme solution and 300 μl of buffers at different pH values containing 400 mM urea were incubated at 37°C for 30 min, after which 100 μl 1N H2SO4 was added. The buffers used in this assay were 0.1 M citrate (pH 5.0), 0.1 M phosphate (pH 7.0) and 0.1 M triethanolamine buffer (pH 8.5). For ammonia determination using the indophenol method,12) 2.5 ml each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and an alkali reagent (5.5% w/v Na2HPO4·12H2O, 0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each reaction mixture. After incubation at 65°C for 20 min, the absorbance at 630 nm was measured. In order to determine the 50% inhibition ($I_{50}$) values, mixtures of 50 μl of each enzyme solution and test compound solutions at various concentrations were preincubated at 37°C for 15 min, after which the urease activities were measured using the indophenol method described above. The activity was calculated as a percentage of that in the control experiment, in which 50 μl of solvent was added instead of the test compound solution. The final concentration that caused 50% inhibition ($I_{50}$) was determined using the program provided by Dr. Ono (Tokyo University).

For the glutamate dehydrogenase (GDH)-coupling method13) of determining urease activity, 50 μl of reaction mixture, 1 ml of H2O, 500 μl of 2-oxoglutarate/ADP/TEA reagent (35 mM 2-oxoglutarate, 1.82 mM adenosine diphosphate and 0.5 mM triethanolamine, pH 8.0) and 50 μl of reduced nicotinamide adenine dinucleotide (NAIDH) solution (6 mM β-NADH disodium salt (Oriental Yeast Co., Japan) and 12 mM NaHCO3) were incubated for 10 min at room temperature and the absorbance [A] at 340 nm was measured. To this mixture, 10 μl of GDH (Boehringer Mannheim Yamanouchi, Japan, 1200 KU/l) was added, kept for 20 min at room temperature, after which the absorbance [B] at 340 nm was measured and the amounts of NADH consumed were calculated from [A] - [B].

The SH compounds of various concentrations were added to cell-free urease solution (pH 7.0), 50 μl of which was preincubated with 50 μl of rabeprazole aqueous solution at 37°C for 15 min, after which the urease activity was measured by the GDH-coupling method described above.

In order to determine whether the inhibitory effect of rabeprazole could be reversed by β-ME, 50 μl of cell-free urease solution (pH 7.0) was incubated with or without 50 μl of 0.16 mM rabeprazole at 37°C for 60 min. After incubation for 15 min, 10 μl of 2 mM β-ME or H2O was added to the mixtures, and the urease activities were determined at 0, 5, 10, 15, 20, 30, 45 and 60 min during the incubation.

**Protein Determination** The amounts of protein in the soluble fractions were measured by the Folin–Lowry method14) or Bio-Rad protein assay15) with bovine serum albumin (fraction V, Sigma Co., U.S.A.) as the standard.

### RESULTS

Preincubation of H. pylori urease at 37°C for 15 min with rabeprazole, omeprazole, lansoprazole and AG-2000 resulted in concentration-dependent inhibition of this enzyme. Rabeprazole was the most potent inhibitor among these PPIs, the $I_{50}$ values of which were influenced strongly by the pH of the reaction mixture. In an acidic condition (pH 5.0), the PPIs were more effective inhibitors, as shown in Table 1. However, the $I_{50}$ values of AG-2000 were not pH-dependent. Rabeprazole, lansoprazole and AG-2000 inhibited both cellular and cell-free urease equipotently. The inhibitory effects of omeprazole on H. pylori cellular urease, however, were markedly weaker than those on cell-free urease at pH 7 and 8.5. The rabeprazole thioether derivative, one of the metabolites of this drug, did not inhibit urease activity despite being a much stronger inhibitor of H. pylori growth than the mother compound.16)

As shown in Table 2, rabeprazole and omeprazole were potent inhibitors of Jack bean urease and weak inhibitors of P. mirabilis enzyme, whereas lansoprazole did not inhibit these ureases even at the high concentrations.

SH compounds, β-ME, DTT and GSH, prevented the inhibition of H. pylori urease activity by rabeprazole in a concentration-dependent manner when rabeprazole was preincubated with cell-free urease solution containing these compounds (Fig. 1), and β-ME similarly prevented the inhibition of Jack bean urease activity by rabeprazole (Fig. 2).

The addition of β-ME reversed the urease activity inhibition by rabeprazole. When H. pylori cell-free urease solution was incubated with 80 μM rabeprazole at 37°C for 15 min, its activity was decreased to 18.3% of the

<table>
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<th>Inhibitor</th>
<th>Ureaase</th>
<th>$I_{50}$ (μM)</th>
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<tr>
<td></td>
<td>pH 5.0</td>
<td>pH 7.0</td>
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<tr>
<td>Rabeprazole</td>
<td>Cellular 0.24</td>
<td>4.2</td>
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<tr>
<td></td>
<td>Cell-free 0.29</td>
<td>7.6</td>
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<tr>
<td>Thioether</td>
<td>Cellular &gt;1000</td>
<td>&gt;1000</td>
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<td></td>
<td>Cell-free &gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>Cellular 8.0</td>
<td>72</td>
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<td></td>
<td>Cell-free 5.4</td>
<td>5.6</td>
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<tr>
<td>Lansoprazole</td>
<td>Cellular 14</td>
<td>20</td>
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<td></td>
<td>Cell-free 3.3</td>
<td>10</td>
</tr>
<tr>
<td>AG-2000</td>
<td>Cellular 1.2</td>
<td>2.5</td>
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<td></td>
<td>Cell-free 0.3</td>
<td>1.0</td>
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Ureaase activity was measured by the indophenol method.

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<tr>
<td>Rabeprazole</td>
<td>P. mirabilis Cellular 90</td>
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<td>Omeprazole</td>
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<tr>
<td>Lansoprazole</td>
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<tr>
<td>AG-2000</td>
<td>P. mirabilis Jack bean &gt;1000</td>
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Ureaase activity was measured by the indophenol method.
control value. However, the addition of 0.2 mM β-ME to the reaction mixture resulted in the progressive recovery of the urease activity with time and it reached 58.3% after 45 min (Fig. 3).

**DISCUSSION**

In the present study, we found that the PPIs, rabeprazole, omeprazole and lansoprazole, potently inhibited *H. pylori* urease activity, probably by binding to the active site of the enzyme, and rabeprazole was about 10 times more potent than the other PPIs. Nagata *et al.*\(^{17}\) reported that the \(I_{50}\) values of lansoprazole and omeprazole against *H. pylori* cell-free urease at pH 7.0 were 9.3 and 9.5 µM respectively, which are similar to our results. They also reported that the \(I_{50}\) values of lansoprazole and omeprazole against cellular urease were 10 times higher than those against cell-free urease.\(^{17}\) We
observed similar results only with omeprazole; the $I_{50}$ values of rabeprazole and lansoprazole against cellular urease were virtually the same as those against cell-free urease. Urease inhibition by these PPIs depended on the pH of the preincubation mixture and their effects became more potent under acidic conditions. The PPIs are known to be transformed into their active forms within the acidic compartment of the parietal cells and these active compounds inhibit (H$^+$ + K$^+$)-ATPase activity by reacting with the SH groups of this enzyme. As urease is known to belong to the SH enzymes, we presumed that the acid-induced forms exhibited inhibitory effects against urease in the same manner as against the proton pump. The active analog of lansoprazole, AG-2000, inhibited *H. pylori* urease more potently than lansoprazole itself, and it was pH-independent. These two observations concerning the pH-dependent effects of PPIs on the proton pump and urease activity lend support to the above presumption.

Rabeprazole and omeprazole also inhibited Jack bean urease potently and *P. mirabilis* cellular urease weakly, whereas lansoprazole inhibited neither, but AG-2000 inhibited *P. mirabilis* urease. Their inhibitory actions on *P. mirabilis* cellular urease may have been hindered more by the cell membranes of this organism than by those of *H. pylori* cells, probably because *H. pylori* urease is distributed on or near the cell membrane surface.

The SH compounds, β-ME, GSH and DTT, markedly prevented the inhibitory effect of rabeprazole on urease activity and reversed the inhibition of urease activity by rabeprazole progressively with time. Similar effects of SH compounds against the inhibitory actions of PPIs on (H$^+$ + K$^+$)-ATPase have been reported. 18,19 Nagata et al. found that GSH and DTT prevented lansoprazole-induced urease inhibition. 170 Our results also showed that the inhibitory effects of PPIs on urease activity were very similar to those on (H$^+$ + K$^+$)-ATPase.

In addition to these observations, we were interested in another finding in this study that the rabeprazole thioether derivative was a more potent inhibitor of *H. pylori* growth [minimum inhibitory concentration (MIC)$_{50}$: 0.20 μg/ml] than the other PPIs, but did not inhibit its urease activity at all. The MIC$_{50}$ against the *H. pylori* growth of lansoprazole, 10 omeprazole 10 and rabeprazole 16 have been reported to be 6.25, 25.0 and 1.56 μg/ml, respectively. As these MIC$_{50}$ (on molar basis) were similar to the $I_{50}$ values against urease activity, the inhibitory effects of PPIs on *H. pylori* urease activity were considered to be an explanation of their ability to inhibit the growth. In view of these results and other reports that acetylhydroxamic acid, another urease inhibitor, 20 did not inhibit *H. pylori* growth in *vivo*, 21 we concluded that there was no direct relationship, at least in *vivo*, between the growth and urease inhibitory actions of PPIs.

In this study we have demonstrated that PPIs inhibited not only the gastric (H$^+$ + K$^+$)-ATPase, but also the ureases from *H. pylori* and other sources. These enzymes belong to the SH enzyme family and PPIs are believed to inhibit them by binding to cysteiny1 residues in their active sites. This suggests that PPIs may inhibit other SH enzymes, for example, alcohol dehydrogenase, amylase and others. In fact, Morii et al. 22 demonstrated omeprazole binding to pepsin, and Roine et al. 23 observed that omeprazole inhibited the alcohol dehydrogenase activity of *H. pylori*. Further study into the effects of PPIs on other SH enzymes is needed.

A characteristic of *H. pylori* is the production of a potent urease, which catalyzes the hydrolysis of urea to form ammonia that protects this acid-sensitive bacterium from gastric acid and enables it to colonize in human gastric mucosa. Urease-deficient *H. pylori* was unable to colonize in piglet stomachs, 24 suggesting that *H. pylori* urease plays an essential role in its infection of the gastric mucosa. The inhibition of *H. pylori* urease by PPI or hydroxamic acid may prove to be a beneficial therapy for gastritis and peptic ulcer disease. Further studies are required to prove or refute this hypothesis in *vivo*.

REFERENCES