Oxygen Concentration Influences Proton Pump Inhibitor Activity against Helicobacter pylori In Vitro

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Omeprazole and lansoprazole are proton pump inhibitors that have shown activity against Helicobacter pylori and other Helicobacter species when tested by agar dilution. Lansoprazole was more active against H. pylori than was omeprazole, and the activity was independent of urease production. Disk susceptibility tests and agar dilution MIC determinations were performed to investigate the effects of incubation under different sets of atmospheric conditions on H. pylori inhibition. Oxygen concentration was found to influence proton pump inhibitor activity in vitro, with higher concentrations leading to greater susceptibility. The method of testing is important in determining the anti-Helicobacter activity of proton pump inhibitors.

The proton pump inhibitors (PPIs) omeprazole and lansoprazole are substituted benzimidazoles that inhibit acid secretion by gastric parietal cells through alteration of the H+/K+-ATPase (2). Each of these drugs is not active against the enzyme in its natural state but is transformed within the acid compartments of the parietal cell into the active inhibitor, a sulfenamide (7). Both drugs are currently used to treat patients with gastroesophageal reflux disease, peptic ulcer disease, and Zollinger-Ellison syndrome. Both omeprazole and lansoprazole have been used in combination with an antibiotic (amoxicillin or clarithromycin) to successfully eradicate Helicobacter pylori from the gastric mucosa of infected individuals (14).

It has been suggested that the increased gastric pH due to the PPI enables the antibiotic to be more active against the organism by inhibiting gastric juice volume and thereby increasing the concentration of antibiotic. Another mechanism of action that has been suggested for PPIs is the protection of amoxicillin activity through acid suppression. Our studies on human gastric mucosal levels of amoxicillin show that levels of amoxicillin as measured by bioassay are decreased in the presence of gastric acid suppression by H2 receptor antagonists (1). Omeprazole has recently been shown to increase the bioavailability of clarithromycin in the human gastric mucosa when the two drugs are coadministered (4). There are preliminary data indicating that the H. pylori cell membrane contains a phosphorylated or p-type ATPase that is not common in prokaryotic cells (8). It is not known whether this enzyme is an H+ or a K+ ATPase, but it has been suggested that benzimidazoles inhibit the ATPase activity of H. pylori by covalently binding to sulphydryl groups of cysteine residues within the α subunit of the enzyme.

Initial in vitro studies with PPIs and H. pylori suggested that these drugs had no activity on this organism (3). Subsequently, specific anti-H. pylori activity was demonstrated, with no activity being shown against a range of aerobic and anaerobic gram-positive and gram-negative bacteria at neutral pH (5). At pH 4.0, omeprazole has been shown to be active against gram-negative bacteria other than H. pylori (8).

Although the hypothesis that PPIs are in fact directly antimicrobial is still controversial, we undertook studies to evaluate whether methodological factors may explain the previously seen variation in activity.

MATERIALS AND METHODS

Strains. To investigate the specificity of the anti-Helicobacter activity of PPIs, the following type strains were used: H. pylori NCTC 11637, H. pylori ATCC 51110 (a urease-deficient mutant strain), H. mustelae ATCC 43775, H. cinaedi ATCC 11611, and H. fennellae ATCC 11612. The susceptibilities of two type strains of H. pylori and two recent clinical isolates (one metronidazole susceptible and the other metronidazole resistant) to omeprazole, lansoprazole, and metronidazole were tested under three sets of atmospheric conditions by agar dilution and disk diffusion methods.

MIC determinations. MICs were determined by agar dilution following the standardized National Committee for Clinical Laboratory Standards methodology (11) in Mueller-Hinton agar (pH 7.5) with 5% horse blood. With brain heart infusion broth, a suspension of each organism was made to a turbidity corresponding a McFarland standard of 1 (approximately 7 × 108 CFU per spot). Each drug was initially dissolved in dimethyl sulfoxide and then diluted in distilled water to final concentrations ranging from 0.125 to 256 μg/ml.

Incubation conditions. The MIC plates were incubated at 35°C in a microaerophilic environment (Camp GasPak; Oxoid, Basingstoke, United Kingdom) for 72 h. Two other sets of atmospheric conditions for incubation were investigated further by agar dilution and disk diffusion. The compositions of the three gas mixtures examined were as follows: (i) 7% CO2–20% O2–0.01% H2–72.99% N2 (5% CO2 incubator), (ii) 10% CO2–6% O2–0% H2–84% N2 (Oxoid Camp GasPak [microaerophilic]), and (iii) 7% CO2–1% O2–8% H2–84% balance N2 (evacuation of the jar to ~70 Pa and replacement with a gas mixture consisting of 10% CO2–10% H2–80% N2). Fifty cubic milliliters of each gas mixture was collected into a stoppered syringe at the start of incubation, and the oxygen and carbon dioxide concentrations were measured with a Radiometer ABL 505 blood gas analyzer.

Disk diffusion. For disk diffusion studies, each paper disk was moistened with 20 μl of a 500-μg/ml solution of either omeprazole or lansoprazole, dried for 1 h, and stored desiccated at 4°C. To investigate the influence of incubation atmosphere on organism growth rates, a viable count of each strain was performed by diluting an agar plug (6-mm diameter) in 10 ml of phosphate-buffered saline and then performing serial dilutions to 10−10. This was done for each strain, after incubation under each set of test atmospheric conditions, from Mueller-Hinton blood agar plates lacking PPIs.

Statistics. Two-tailed paired t tests were used to compare the differences in MICs and zones of inhibition for the three sets of atmospheric conditions investigated. Statistics were performed by using Microsoft Excel, version 5.0. A P value of <0.05 was considered significant.

RESULTS

The specificity of PPI antibacterial activity was investigated by agar dilution. Table 1 shows the MICs and MIC ranges of both PPIs for several type strains and clinical isolates of H. pylori and other Helicobacter species.
Both omeprazole and lansoprazole had activity against the *Helicobacter* species tested, with lansoprazole having higher potency than omeprazole and *H. pylori* being the most susceptible species.

To investigate the influence of incubation conditions on the anti-*Helicobacter* activity of PPIs, agar dilution MIC determinations were performed. The incubation results and medium pHs for the three different sets of atmospheric conditions are summarized in Table 2.

The MICs of the PPIs were statistically significantly lower when the plates were placed in the CO₂ incubator (atmosphere i) than after microaerophilic incubation (atmosphere ii, $P = 0.013$; atmosphere iii, $P = 0.001$). The MIC range was between four and six doubling dilutions.

Metronidazole susceptibility was not significantly affected by the differences in atmospheric conditions in this study and did not vary by more than one dilution. The effect of atmosphere on PPIs that was seen in the agar dilution experiments was confirmed by disk diffusion studies in which zone sizes around omeprazole and lansoprazole disks (both 10 μg) and metronidazole disks (5 μg) were measured. The differences in viable count of each strain under each set of atmospheric conditions were minor (less than $2 \log_{10}$ dilutions), with $\log_{10}$ CFU per milliliter determinations ranging from 5.88 to 7.51.

**DISCUSSION**

The antibacterial activity of PPIs against *H. pylori* and, to a lesser extent, other *Helicobacter* species has been confirmed in this study. Lansoprazole was more active against *H. pylori* and *H. fennelliae* than was omeprazole by agar dilution. The susceptibility of the urease-deficient mutant *H. pylori* ATCC 51110 was identical to that of urease-competent *H. pylori* NCTC 11637. This indicates a urease-independent mechanism of action for PPIs, which is in agreement with the results of previous studies (9, 10). Methodological factors can greatly influence the anti-*Helicobacter* activity of PPIs in vitro. For both the agar dilution and disk diffusion methods of testing PPI activity, incubation under microaerophilic conditions resulted in little inhibition by either PPI (i.e., resistance). Conversely, incubation in the CO₂ incubator resulted in large zones of inhibition and low PPI MICs (i.e., susceptibility). The densities of growth under all three sets of atmospheric conditions were similar for all four strains of *H. pylori*. The difference in susceptibility after incubation in a CO₂ incubator compared with that after incubation in either microaerophilic atmosphere was statistically significant ($P < 0.005$ by paired t test) for both test methods and for both PPIs but not for metronidazole. Previous studies have shown that CO₂ can alter in vitro susceptibility by changing the pH of the medium through the action of carbonic acid (12). Acid activation of PPIs has also been shown to be important for anti-*Helicobacter* activity (13). The pH ranges of uninoculated Mueller-Hinton blood agar plates over 72 h of incubation under the different sets of atmospheric conditions were similar. Thus, pH changes due to different incubation conditions do not fully account for the variation in inhibition by PPIs seen. The concentration of carbon dioxide in the gas mixture does not seem to influence the antibacterial effect of the PPIs, since it is similar for all three atmospheres. Nitrogen gas is relatively inert and unlikely to influence the PPIs. Hydrogen gas was found to be important for the isolation of some *Campylobacter* spp., e.g., *Campylobacter concisus* (6). The concentration of hydrogen varied from 0% in the Camp GasPak method (atmosphere ii) to 8% in the gas evacuation method (atmosphere iii), with only a minor change in PPI activity against *H. pylori*. The oxygen concentration varied from 20% for CO₂ incubator tests to 10% for the Camp GasPak, with a fourfold increase in PPI MIC. When the oxygen concentration was further reduced to 1% (atmosphere iii), the PPI MIC increased a further fourfold. This MIC increase was not seen with metronidazole. The concentration of oxygen is likely to have a major influence on PPI activity against *H. pylori* in vitro. Whether this in vitro phenomenon has clinical implications concerning treatment of *H. pylori* with PPIs remains to be elucidated.

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