

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

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Received 9 January 1996/Returned for modification 5 March 1996/Accepted 12 April 1996

**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<sup>+</sup>/K<sup>+</sup> 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 mucosae 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 H<sub>2</sub> 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<sup>+</sup> or a K<sup>+</sup> ATPase, but it has been suggested that benzimidazoles inhibit the ATPase activity of *H. pylori* by covalently binding to sulfhydryl groups of cysteine residues within the  $\alpha$  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 anti-

microbial 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 43773, *H. cinaedi* ATCC 11611, and *H. jennelliae* 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  $7 \times 10^4$  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  $\mu$ g/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% CO<sub>2</sub>-20% O<sub>2</sub>-0.01% H<sub>2</sub>-72.99% N<sub>2</sub> (5% CO<sub>2</sub> incubator), (ii) 10% CO<sub>2</sub>-6% O<sub>2</sub>-0% H<sub>2</sub>-84% N<sub>2</sub> (Oxoid Camp GasPak [microaerophilic]), and (iii) 7% CO<sub>2</sub>-1% O<sub>2</sub>-8% H<sub>2</sub>-84% balance N<sub>2</sub> (evacuation of the jar to -70 Pa and replacement with a gas mixture consisting of 10% CO<sub>2</sub>-10% H<sub>2</sub>-80% N<sub>2</sub>). 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  $\mu$ l of a 500- $\mu$ g/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<sup>-10</sup>. This was done for each strain, after incubation under each set of test atmospheric conditions, from Mueller-Hinton blood agar plates lacking PPI.

**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.

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TABLE 1. Omeprazole and lansoprazole MICs for various *Helicobacter* species, obtained by agar dilution with microaerophilic incubation (Oxoid Camp GasPak)

Organism tested	MIC ( $\mu\text{g/ml}$ ) of:	
	Omeprazole	Lansoprazole
Type strains		
<i>H. pylori</i> NCTC 11637	32	8
<i>H. pylori</i> ATCC 51110	32	8
<i>H. mustelae</i> ATCC 43773	>128	128
<i>H. mustelae</i> Hm180	128	128
<i>H. cinaedi</i> ATCC 11611	64	64
<i>H. fennelliae</i> ATCC 11612	128	64
Clinical isolate		
<i>H. pylori</i>	8–32 <sup>a</sup>	4–8 <sup>b</sup>

<sup>a</sup>  $n = 100$ .

<sup>b</sup>  $n = 31$ .

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 CO<sub>2</sub> 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

TABLE 2. Agar dilution MICs of omeprazole, lansoprazole, and metronidazole for four *H. pylori* strains, obtained under three sets of atmospheric conditions, and pH ranges of uninoculated media

Atmosphere and <i>H. pylori</i> strain tested	MIC ( $\mu\text{g/ml}$ ) of:			pH range <sup>d</sup>
	Omeprazole <sup>a</sup>	Lansoprazole <sup>b</sup>	Metronidazole <sup>c</sup>	
(i) 7% CO <sub>2</sub> –20% O <sub>2</sub> –0.01% H <sub>2</sub>				6.15–7.59
NCTC 11637	8	1	128	
ATCC 51110	8	1	2	
M930312171 <sup>e</sup>	16	2	8	
M940702098 <sup>e</sup>	16	2	8	
(ii) 8% CO <sub>2</sub> –10% O <sub>2</sub> –0% H <sub>2</sub>				6.27–7.44
NCTC 11637	32	16	128	
ATCC 51110	32	16	2	
M930312171	32	16	8	
M940702098	32	16	8	
(iii) 7% CO <sub>2</sub> –1% O <sub>2</sub> –8% H <sub>2</sub>				5.92–7.56
NCTC 11637	128	64	128	
ATCC 51110	64	64	2	
M930312171	128	64	16	
M940702098	128	64	8	

<sup>a</sup>  $P = 0.013$ , i versus ii;  $P = 0.001$ , i versus iii.

<sup>b</sup>  $P = 0.001$ , i versus ii;  $P = 0.0003$ , i versus iii.

<sup>c</sup>  $P = 1$ , i versus ii;  $P = 0.39$ , i versus iii.

<sup>d</sup> pH range in uninoculated medium over a 72-h incubation period.

<sup>e</sup> Clinical isolate.

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  $\mu\text{g}$ ) and metronidazole disks (5  $\mu\text{g}$ ) were measured. The differences in viable count of each strain under each set of atmospheric conditions were minor (less than 2 log<sub>10</sub> dilutions), with log<sub>10</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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.

## ACKNOWLEDGMENTS

Many thanks go to Janni O'Rourke, University of New South Wales, for supplying the non-*H. pylori Helicobacter* species. Thanks also go to John May of Monash University for helpful advice and to Deborah Harrison for typing the manuscript.

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