In-vitro activity of lansoprazole against Helicobacter pylori

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Lansoprazole is a gastric parietal cell proton pump inhibitor that is also active against Helicobacter pylori in vitro. We aimed to investigate further the mechanism of its antimicrobial effect. The antimicrobial activity of lansoprazole and of its sulfenamide, a rearrangement product occurring spontaneously in acid environments, was studied by determining the MICs and MBCs for 11 cytotoxic and eight non-cytotoxic H. pylori strains and by measuring the rapidity of bacterial killing. The MIC\textsubscript{90} and MBC\textsubscript{90} were 2.5 mg/L and 10 mg/L, respectively, both for lansoprazole and for its sulfenamide. Cytotoxic strains were as susceptible as non-cytotoxic strains. The sulfenamide exhibited faster bactericidal activity. Lansoprazole did not inhibit the toxin-induced vacuolization of HeLa cells by a cytotoxic strain, hence its anti-H. pylori activity does not depend on inhibition of a v-ATPase-mediated, toxin-induced activity. Sulfenamide formation is likely to occur in vivo in the gastric environment, thus enhancing the bactericidal activity of the drug. Lansoprazole is likely to be useful, in association with antibiotics, in the treatment of H. pylori infection regardless of the cytotoxicity of the infecting strain.

Introduction

Benzimidazoles, such as omeprazole and lansoprazole, are gastric parietal cell proton pump inhibitors (PPIs) that can exert an antimicrobial activity against Helicobacter pylori in vitro.\textsuperscript{1,2} Recent studies have tried to explain the mechanism of action of PPIs against H. pylori. In-vitro studies have shown that omeprazole and lansoprazole also exert an inhibitory activity against the enzyme urease produced by H. pylori,\textsuperscript{3,4} suggesting that the enzyme could be the target of their antimicrobial activity. However, urease-negative strains are as susceptible as urease-positive strains in vitro and, at high concentrations, PPIs are also active against Helicobacter cinaedi and CLO type 3, which are naturally urease-negative\textsuperscript{5} (author’s unpublished observations), indicating that other bacterial substances or components are involved.

Certain H. pylori strains produce a toxin, VacA,\textsuperscript{5} that induces intracytoplasmic vacuolization in cells in culture and seems to be important in the development of peptic ulcers.\textsuperscript{6} The VacA-induced vacuolization depends on the stimulation of a v-type ATPase and is characterized by a progressive accumulation of protons in the endocytic compartment of cells in culture. VacA also alters in vitro the transmembrane ion trafficking mediated by a Na\textsuperscript{+},K\textsuperscript{+}-ATPase.\textsuperscript{7} Since PPIs inhibit the flux of protons at the level of cellular membranes, it is possible that VacA is a potential target for the antimicrobial activity they exert on H. pylori strains. However, in a recent study, we observed no differences in the levels of susceptibility to omeprazole shown by cytotoxic or non-cytotoxic H. pylori and no inhibition of vacuole formation could be observed microscopically.\textsuperscript{8} Lansoprazole has some differences from omeprazole in its interaction with the proton pump: it has greater liposolubility, i.e. better plasma membrane permeability, and therefore a possible increased intracellular bioavailability. It is also protonated more rapidly in the acidic environment and has three different binding sites on the gastric proton pump, whereas omeprazole has only two.\textsuperscript{9} It was of interest, therefore, to evaluate the potential effect of lansoprazole on VacA-positive and VacA-negative H. pylori strains, and on the vacuolating toxin.

Whatever the target, the antimicrobial activity of PPIs could depend on their acid conversion or ‘activation’, since exposure of lansoprazole to an acidic environment induces spontaneous protonation and enhances its inhibitory effect on acid secretion by gastric parietal cells.\textsuperscript{10} The permanent cation formed is a sulfenamide, in which the sulphur atom
binds to the nitrogen instead of to the carbon. Iwahi et al. reported that MICs of lansoprazole for H. pylori were higher than MICs of its sulfenamides. It is possible that the protonation in acidic environments leads to an increased affinity for the bacterial target, so modifying the potency and/or the kinetics of this interaction. The aim of this study was to determine the MICs, MBCs and kinetics of the killing activity of both acid-converted lansoprazole (the sulfenamide) and non-converted lansoprazole against 11 cytotoxic and eight non-cytotoxic H. pylori strains, and to investigate the possible effect of lansoprazole on the vacuolization induced in vitro by a cytotoxic bacterial filtrate.

Materials and methods

Strains and growth conditions

Strains of H. pylori used for lansoprazole susceptibility tests included the type strain (NCTC 11637) and 18 strains isolated from patients who had undergone endoscopy for dyspepsia. The pathology of patients from whom strains were isolated is reported in Table I. Gastric mucosa biopsies were cultured on Columbia agar containing 7% horse blood, 0.1% dimethyl-β-cyclodextrin (Teijin Ltd, Japan), 10 mg/L trimethoprim, 10 mg/L vancomycin, 5 mg/L cefsulodin, 20 U/mL polymyxin B and 5 mg/L amphotericin B. Plates were incubated in a microaerobic environment obtained by using an anaerobic jar with a gas-generating kit for microaerophilic atmosphere (Oxoid, Unipath, Garbagnate Milanese, Italy). Plates were incubated at 37°C for 5 days. Colonies resembling H. pylori were identified by Gram's stain and rapid urease, oxidase and catalase tests. Strains were stored in Wilkins-Chalgren broth with 20% glycerol at -70°C.

Determination of cytotoxin production

Strains stored at -70°C were thawed and cultured on Columbia agar with 7% horse blood and 0.1% dimethyl-β-cyclodextrin. Brucella broth with 10% fetal bovine serum was inoculated and incubated in a microaerobic environment at 37°C, at 150 rpm for 48 h. A after centrifugation at 4°C at 10,000 g for 20 min, the cell-free supernatant was filtered through 0.22 μm pore filters and tested on HEp-2 cells in culture at 1:2, 1:3, 1:5, 1:10 and 1:20 dilutions. Cells were inspected microscopically after 12, 18 and 24 h for the presence of intracytoplasmic vacuoles. A after 24 h, vacuolization was verified by the Neutral Red concentration method. Non-cytotoxic broth culture filtrates were concentrated 20-fold with 50% saturated ammonium sulphate at 4°C overnight. A after centrifugation, the pellet was dissolved in phosphate-buffered saline (PBS) pH 7.4, dialysed against PBS, and tested on cells. The strains were considered cytotoxic if more than 50% of cells were vacuolated at any dilution. MIC determinations

Lansoprazole was dissolved in dimethylsulphoxide (DMSO) at 10 mg/mL and diluted in citrate pH 3 or phosphate buffer pH 7 at 4°C for 120 min. Exposure to low pH transforms the drug into its protonated ‘active’ metabolite, the sulfenamide. During this reaction, the colourless drug suspension becomes a blackish colour. The drugs were incorporated in Columbia agar with 7% horse blood at concentrations ranging from 0.3 to 40 mg/L. Bacterial suspensions were prepared in Brucella broth with 10% fetal bovine serum from 2-day cultures on Columbia blood agar up to an optical density of 0.5 (at 590 nm). Dilutions were made on log basis to 10⁻⁷, and 10 μL of each dilution was seeded in duplicate on to Columbia blood agar and spread over the entire surface. After incubation for 5 days, the number of colonies was counted and expressed in cfu/mL. Such suspensions contained approximately 2 x 10⁸ cfu/mL. Five microlitre suspensions of each H. pylori strain containing approximately 10⁶ organisms were inoculated on to prepared plates containing antibiotics and incubated in a microaerobic environment at 37°C for 5 days. The MIC₉₀ was taken as the lowest concentration of lansoprazole at which the growth of at least 90% of strains tested was completely inhibited. Each test was performed in triplicate and the result was expressed as the median of the MIC₉₀ obtained.

MBC determinations

Once the MICs had been recorded, the surface of agar, where H. pylori suspensions had been inoculated and no growth had been observed, was scraped with a loop and subcultured on plain Columbia blood agar (without any drug). A after 5 days of incubation in a microaerobic atmosphere at 37°C, plates were inspected for bacterial growth. The MBC was considered to be the lowest concentration of drug in the first series of plates at which no bacterial growth was visible in the subcultured plates. Each test was performed in triplicate and the result was expressed as the median of the MBCs obtained.

In preliminary tests we compared the loop scraping technique with the replica plating method for the determination of MBC of colloidal bismuth subcitrate for ten H. pylori strains; we obtained the same results (data not shown). Here we also determined MBCs of non-acid-converted lansoprazole for three H. pylori strains (NCTC 11637, G21 and G50) and compared the results obtained using our technique with those obtained with the replica plating method. In brief, two series of plates containing two-fold dilutions of lansoprazole were prepared. MICs of lansoprazole were determined as reported above in duplicate. A after MICs had been recorded, MBCs were determined by the scraping technique using one series of plates. Each plate of the other series was pressed lightly on sterile velvet covering the flat of a cylindrical
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block. Columbia agar plates without lansoprazole were then pressed on the velveteen block. Subcultured and replicated plates were incubated in a microaerobic atmosphere at 37°C for 5 days. The MBC in each case was determined as above.

Susceptibility of the strains by disc diffusion

Four strains were tested, NCTC 11637, G32, G21 and G50. Assays were carried out with plates allowed to dry in a sterile cabinet with lids removed for 2 h, and also with wet plates (i.e. soon after preparation) in order to see whether the humidity of the surface of the agar could influence the zones of inhibition. Strains were suspended in Brucella broth at a density corresponding approximately to McFarland 4 opacity standard, and seeded on to Columbia agar plates with 10% horse blood. Discs (6 mm) were charged with 50 μg lansoprazole previously exposed to pH 3 or pH 7 and were placed on the agar surface. Plates were incubated in a microaerobic environment at 37°C. After 5 days, the diameters of the zones of inhibition were measured.

Killing curves

Dilutions of lansoprazole and its sulfenamide ranging from 40 mg/L to 0.6 mg/L were prepared in brain-heart infusion broth containing 10% fetal bovine serum and 0.1% dimethyl-β-cycloextrin. Each dilution was inoculated with 10^7 cfu (final) of H. pylori NCTC 11637 grown previously for 2 days on Columbia blood agar. Vials were incubated in a tissue culture incubator with 10% CO_2 for 42 h. At 0, 3, 12, 18 and 42 h, samples were removed and diluted on a log basis, and 10 μL of each dilution was plated on to Columbia agar with 7% horse blood and 0.1% dimethyl-β-cycloextrin. After incubation under microaerophilic conditions at 37°C for 5 days, colonies were counted. The lower limit of colony detection was 100 cfu/mL.

Inhibition of H. pylori vacuolating activity

A concentrated broth culture filtrate of the cytotoxic strain H. pylori NCTC 11637 was prepared by precipitating a culture supernatant with 50% saturated ammonium sulphate as described. This filtrate was mixed with lansoprazole or sulfenamide at concentrations ranging from 1 mg/L to 128 mg/L and added, in triplicate, to HeLa cells cultured in Dulbecco’s modified Eagle’s medium in 96-well plates at a density of approximately 3 × 10^3 cells per well. The concentrated filtrate was used at twice the vacuolating titre (the highest dilution that induced vacuolization of >50% of cells after 24 h of incubation). The inhibition of vacuolization was verified after 24 h incubation by inspecting cells microscopically and by the Neutral Red method. Cells were treated with 100 μL per well of 0.05% Neutral Red in PBS pH 7.4 at 25°C for 4 min. After washing three times with 200 μL of PBS containing 0.2% bovine serum albumin, the dye was extracted with acid alcohol (70 mL ethanol, 29 mL distilled water, 1 mL fuming HCl), and the intensity of the colour was measured spectrophotometrically at 530 nm.

Results

Cytotoxic and non-cytotoxic strains

Eleven of the 18 H. pylori strains studied (including the type strain NCTC 11637) were cytotoxic, while eight strains were non-cytotoxic (Table I). The broth culture filtrates of non-cytotoxic strains had no vacuolating activity against HeLa cells, even after concentration.

MICs and MBCs of lansoprazole

Cytotoxic and non-cytotoxic H. pylori strains were tested for their susceptibility to lansoprazole and its protonated metabolite. The MICs of lansoprazole and its sulfenamide ranged from 0.6 mg/L to 2.5 mg/L, and MBCs ranged from 2.5 mg/L to 10 mg/L (Table II). In most cases, the MBC was two to four times higher than the MIC. The susceptibilities of cytotoxic strains to lansoprazole did not differ significantly from those of non-cytotoxic strains (Table II). The number of colonies grown on subcultured

Table I. H. pylori strains tested, cytotoxin production and disease of patients from whom strains were isolated

<table>
<thead>
<tr>
<th>Strain</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxic</td>
<td></td>
</tr>
<tr>
<td>NCTC 11637</td>
<td>- (type strain)</td>
</tr>
<tr>
<td>G32</td>
<td>gastric ulcer</td>
</tr>
<tr>
<td>G33</td>
<td>antral gastritis</td>
</tr>
<tr>
<td>G104</td>
<td>active chronic gastritis</td>
</tr>
<tr>
<td>NFD24</td>
<td>gastric cancer</td>
</tr>
<tr>
<td>NFD29</td>
<td>gastric cancer</td>
</tr>
<tr>
<td>NFO42</td>
<td>duodenal ulcer</td>
</tr>
<tr>
<td>NFO59</td>
<td>moderate superficial chronic gastritis</td>
</tr>
<tr>
<td>NFO62</td>
<td>erosive gastritis</td>
</tr>
<tr>
<td>NFO63</td>
<td>active atrophic chronic gastritis</td>
</tr>
<tr>
<td>NFO64</td>
<td>duodenal ulcer</td>
</tr>
<tr>
<td>Non-cytotoxic</td>
<td></td>
</tr>
<tr>
<td>G21</td>
<td>moderate superficial chronic gastritis</td>
</tr>
<tr>
<td>G12</td>
<td>gastric ulcer</td>
</tr>
<tr>
<td>G25</td>
<td>erosive gastritis</td>
</tr>
<tr>
<td>G50</td>
<td>antral gastritis</td>
</tr>
<tr>
<td>G204</td>
<td>duodenal ulcer</td>
</tr>
<tr>
<td>NFD01</td>
<td>moderate superficial chronic gastritis</td>
</tr>
<tr>
<td>NFD02</td>
<td>moderate superficial chronic gastritis</td>
</tr>
<tr>
<td>NFO57</td>
<td>moderate superficial chronic gastritis</td>
</tr>
</tbody>
</table>
plates using our procedure was 30–100% of the number of colonies grown using the replica plating technique (Table III), indicating that the loop scraping technique we used in our work to determine MBCs of lansoprazole was valid.

### Susceptibility of the strains by disc diffusion

These tests were performed to determine whether the susceptibility to lansoprazole and its sulfenamide could be investigated by the Kirby–Bauer method, which is more manageable than MIC determination. The zones of inhibition ranged from 30 mm to 56 mm for both drugs. The growth inhibition was highly dependent on the degree of humidity of the plates; differences of 6–15 mm were found with the four strains tested, depending on whether the plates were allowed to dry or they were used wet, soon after preparation.

### Killing curves

The aim of this test was to investigate whether the protonation of lansoprazole could influence its bactericidal activity in broth. The killing curves relative to drug concentrations of 5 mg/L and 2.5 mg/L are shown in Figure 1. At the concentration of 2.5 mg/L, the sulfenamide produced, over a period of 18 h, a decrease in cfu which was ≥3.5 log_{10} units higher than the decrease induced by the same concentration of non-converted lansoprazole. At concentrations of 10 mg/L of both the agents, no viable organisms were detected at 12 h (>99.9% of the inoculum killed; data not shown).

### Vacuolization inhibition test

Potential inhibition of vacuolization by lansoprazole was examined microscopically and by the Neutral Red method (Figure 2). The results obtained with the sulfenamide were similar (data not shown). Microscopically, the drug did not hinder the activity of the toxin, and HeLa cells appeared vacuolated at any concentration of lansoprazole up to 20 mg/L. At 40 mg/L, the drug had a cytopathic effect. Fewer cells were seen in the wells with 20 and 10 mg/L lansoprazole than in the control wells without drug. Aessment of potential vacuolization inhibition by Neutral Red uptake showed that, at concentrations of lansoprazole of 5 mg/L, there was no inhibition of vacuolization, while at higher concentrations, the reduction in optical density (OD) was most probably because of the inhibitory effect of the drug on cell growth (Figure 2).

### Discussion

Proton pump inhibitors (PPIs), such as omeprazole and lansoprazole, are active against Helicobacter spp., espe-
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The bacterial target of these compounds is not known. One possible target for benzimidazoles could be the vacuolating toxin, since this protein is produced by approximately 50% of *H. pylori* isolates, and has partial homology with certain ATPases that regulate the flux of ions through cell membranes. A further study demonstrated that the toxin stimulated a v-type ATPase. Whatever the mechanism of toxicity, it was important to verify whether cytotoxic strains and in-vitro vacuole formation could be influenced by benzimidazoles. Previous studies showed that omeprazole had no direct effect on in-vitro cytotoxicity and cytoxin production, and no strain variation in omeprazole susceptibility was observed. The facts that cytotoxic and non-cytotoxic strains were susceptible to lansoprazole to a similar extent and that vacuolation was not prevented by the drug, indicate that bacterial structures or substances other than the cytoxin ought to be the target, and that lansoprazole does not have any activity on v-type ATPase.

Mauch et al. found an ATPase activity at the surface of *H. pylori* that was inhibited by benzimidazoles, including lansoprazole, but only after their acid-conversion. This ATPase is a p-type proton pump, uncommon in bacteria, but widespread in eukaryotic cells, including parietal cells. This observation could indicate that the mechanism of the antimicrobial effect of lansoprazole is the same one that accounts for its antisecretory activity: the inhibition of a p-type ATPase. However, this interpretation is questioned by our findings that MICs and MBCs of lansoprazole and of its sulfenamide are similar (Table II). It is possible that the factor that improved lansoprazole activity in the experiment performed by Mauch et al. was the acidic environment per se.

MICs of lansoprazole obtained in this study were lower than those reported by Mégraud et al. who reported a MIC of 16 mg/L, and by Iwahi et al. who reported MICs ranging from 3.13 to 12.5 mg/L (MIC = 6.25 mg/L). Methodological differences could account for these variations, but these researchers all agree that lansoprazole is approximately four times more active than omeprazole against *H. pylori* in vitro. Iwahi et al. reported an increased activity of acid-converted forms of lansoprazole, the sulfenamides AG-2000 and AG-1812 (MIC = 3.13 mg/L). However, in this study we found no difference in the levels of activity of lansoprazole or its sulfenamide (MIC = 2.5 mg/L for both drugs), although we did observe a more rapid antimicrobial effect of the sulfenamide in broth. Since both MICs and MBCs of the two drugs were similar (Table II), we hypothesized that the acid-converted lansoprazole could have a higher affinity for the bacterial target, which could account for the faster bactericidal activity in broth, and that a prolonged incubation would lead to a similar degree of saturation of *H. pylori* receptor sites for lansoprazole.

Benzimidazoles localize specifically in the gastric mucosa, but it is not known whether they can also reach antimicrobial concentrations in the mucus layer where at least 90% of *H. pylori* resides. According to a radioautographic study in rats, lansoprazole reaches high concentrations at the luminal membrane of the parietal cells that could also produce high levels of drug within the lumen. Even if benzimidazoles do not diffuse into the mucus, they could possibly constitute a defence barrier against *H. pylori* along the gastric mucosa and especially in the glandular lumen. Gastric cells have a short life (only 3–4 days); when they desquamate, the bacteria that adhere to them are expelled into the gut. The organisms living in the mucus...
have to colonize the new cells in order to maintain the epithelial inflammation. Inflammation increases the transudation, from the bloodstream to the lumen, of serum and other substances which may be metabolically important for the bacteria. Omeprazole and lansoprazole are able to inhibit in vitro the activity of urease produced by H. pylori. Urease is essential for H. pylori to initiate colonization because it protects the organisms from the gastric acidity during initial colonization. A dialtonally, urease may have an important metabolic function and can favour bacterial adhesion. It is possible, therefore, that both the antimicrobial and the antiurease activities of lansoprazole, although exerted only at the surface of the gastric epithelium, could modify the host-bacterial interactions.

In conclusion, the anti-H. pylori activity of lansoprazole does not depend on v-ATPase-mediated, toxin-induced activity inhibition. The selective activity of lansoprazole against H. pylori, its antiurease effect, and its stability in acidic and neutral environments are likely to be important factors in the treatment of H. pylori infection. The acidic activation, likely to occur in vivo in the gastric environment, enhances the bactericidal activity of the drug. Lansoprazole is likely to be useful, associated with antibiotics, in the treatment of H. pylori infection regardless of the cytotoxicity of the infecting strain.

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References


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