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Antimicrobial actions of benzimidazoles against oral streptococci

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Background/aim: Benzimidazoles, such as lansoprazole and omeprazole, are used extensively as proton-pump inhibitors to control stomach acid secretion and also have antimicrobial actions against *Helicobacter pylori*. Our objective was to determine whether they are potentially useful antimicrobials against oral bacteria.
Methods: *Streptococcus mutans* was our main test organism. It was grown in suspension cultures and biofilms. Standard physiologic assays were used to assess inhibitory actions of benzimidazoles.
Results: Benzimidazoles inhibited acid production by *S. mutans* in suspensions or biofilms. In pH-drop experiments, lansoprazole at a level of only 0.025 mM irreversibly inhibited acid production from glycolysis. Cell uptake of lansoprazole was found to be very pH sensitive and occurred mainly at pH values below about 5, indicating that the protonated form was taken up. Lansoprazole inhibition of glycolysis could be blocked by 2-mercaptoethanol, which suggests that disulfide bonds form between benzimidazoles and protein targets. Identified targets for benzimidazole inhibition included the phosphoenolpyruvate : sugar phosphotransferase system, the glycolytic enzymes aldolase, glyceraldehyde-3-phosphate dehydrogenase, and lactic dehydrogenase, and enzymes such as urease and arginine deiminase. Lansoprazole increased proton permeabilities of *S. mutans* cells but did not inhibit F-ATPases. Although cells in biofilms were somewhat less sensitive to the agents than those in suspensions, biofilm glycolysis was still markedly inhibited by 0.1 mM lansoprazole. Benzimidazoles are bactericidal, and the oral anaerobes *Fusobacterium nucleatum* and *Prevotella intermedia* were more sensitive to killing than was *S. mutans*.
Conclusion: Benzimidazoles appear to be useful inhibitors of oral bacteria in acid environments such as progressing caries lesions.

Key words: acid production; benzimidazoles; oral bacteria

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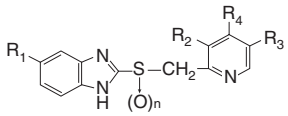
Benzimidazoles such as omeprazole and lansoprazole are used extensively to control stomach hyperacidity through inhibition of the acid-secreting (H^+/K^+) P-ATPase of parietal cells of the mucosa (21). The general structure of benzimidazoles is shown in Fig. 1, and the specific substituents for lansoprazole and omeprazole are indicated in the figure legend. In the acidified environment of the stomach, disulfide bond formation occurs involving the sulfur of the benzimidazole and a sulfhydryl group of (H^+/K^+) P-ATPase, resulting in inhibition of the enzyme, which can be slowly reversed. Stomach hyperacidity can lead to ulcers and, in the extreme, to cancer. *Helicobacter pylori* is a well recognized etiologic agent for stomach ulcers and is known to thrive in acidified environments. Treatments for stomach ulcers generally involve an antibiotic against *H. pylori* and a benzimidazole to reduce acid secretion. Benzimidazoles have been shown to have antimicrobial activity against *H. pylori* (14) and could possibly supplement the antibiotics used in treatment. It appears that the major target for benzimidazoles against *H. pylori* is NADH : ubiquinone oxidoreductase (20). Relatively little has been published on the modes of action of benzimidazoles against other bacteria, although antimicrobial potencies have



General formula of substituted benzimidazoles.

	R_1	R_2	R_3	R_4
Lansoprazole	H	CH_3	H	OCH_2CF_3
Omeprazole	OCH_3	CH_3	CH_3	OCH_3

Fig. 1. General formula for benzimidazoles.

been assessed, especially in regard to changes in potency related to various chemical substituents to the basic structure (4, 16).

Acid production by dental plaque bacteria is a major cause of oral disease, primarily dental caries involving acid dissolution of tooth mineral. The microbial etiology of caries involves opportunistic pathogens and ecological shifts in dental plaque in response to increased sugar challenge or decreased salivary flow in the mouth (18). The bacteria most closely associated with caries development are the mutans streptococci, noted for their high tolerance to acidification. Their virulence is related directly to their capacity to produce acids from glycolysis in acidified dental plaque at pH values of 4.0 or even lower. The major engine for acid tolerance in mutans streptococci is the membrane (H^+) F-ATPase. Differences in acid tolerance among oral bacteria can be related to levels of F-ATPase in cell membranes and also to pH-activity profiles for the enzymes from different bacteria (25). The more acid-tolerant organisms have greater F-ATPase activity and have enzymes with more acid pH optima or with broad pH optima extending into the acid range. In addition, the organisms generally can undergo phenotypic adaptation when grown at low pH values (2, 12). The adaptation involves increased levels of F-ATPase activity (23), changes in membrane fatty-acid composition (9), up-regulation of synthesis of DNA-repair enzymes (11) and chaperonin proteins (15), and a multitude of other changes.

Recently, Magalhães et al. (17) reported that lansoprazole inhibits an (H^+) P-ATPase isolated from the ATCC 25175 strain of *Streptococcus mutans*, which was also sensitive to orthovanadate, an inhibitor specific for P-ATPases. They proposed that P-ATPases are involved in the acid tolerance of the organism. Some years ago, we found that the GS-5 strain of *S. mutans* has very little ATPase activity sensitive to orthovanadate (26) and also found that omeprazole did not inhibit the F-ATPase of permeabilized cells of the organism (unpublished observations). In addition, Belli & Fryklund (1) found that the F-ATPase of *H. pylori* was not inhibited by omeprazole, nor was orthovanadate-sensitive ATPase activity. As described in this paper, we set out to determine whether benzimidazoles, specifically lansoprazole and omeprazole, are inhibitors of glycolysis and acid production by mutans streptococci, whether there are multiple targets for benzimidazoles, and whether they can be bactericidal for oral bacteria in suspensions or biofilms.

Material and methods

Bacteria

The oral streptococci used for this study are from our laboratory culture collection – *Streptococcus gordonii* ATCC 10558, *S. mutans* GS-5, *S. mutans* UA159, *Streptococcus rattus* FA-1, *Streptococcus salivarius* ATCC 13419, and *Streptococcus sanguis* NCTC10904. The two strains of *S. mutans* showed very similar sensitivities to benzimidazoles, and so only data for the GS-5 strain are presented unless otherwise indicated. They are maintained with weekly subculture on tryptic-soy agar plates (Difco, Detroit, MI) to avoid selection of rapidly growing variants. Long-term storage was at −70°C in 50% glycerol solution. Cells for experiments with suspensions were routinely grown statically at 37°C in tryptone-yeast-extract (TY – 30 g Difco tryptone plus 5 g Difco yeast extract per liter) broth with 1% (w/v) glucose (55.6 mM), added so that growth was acid-limited rather than catabolite-limited. Cells were harvested by centrifugation from early stationary phase cultures, when the organisms would have undergone acid adaptation (2). For derepression-induction of the arginine deiminase system of *S. rattus*, TY broth was supplemented with only 0.1% glucose and with 1% arginine (47.5 mM) to allow derepression of the arginine deiminase system.

Fusobacterium nucleatum ATCC 25586 was grown in Brain-Heart-Infusion broth (Difco) supplemented with 5 mM glutathione in a Coy anaerobic incubator at 37°C in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. Cells were harvested during the exponential phase of growth by centrifugation and washed twice in buffer. Cell pellets were immediately transferred to the anaerobic chamber for experiments. *Prevotella intermedia* ATCC 25611 was grown in the BM medium of Shah et al. (24) and processed in the same manner as *F. nucleatum* except that the cells were washed with 2 mM potassium phosphate buffer, pH 7, containing 75 mM NaCl, 75 mM KCl, and 2 mM $MgCl_2$. *Candida albicans* 3153 A was obtained from Dr. Constantine Haidaris of the University of Rochester. It was grown aerobically at 37°C in yeast-extract-peptone-dextrose (YEPD) broth (Difco) and harvested by centrifugation during the exponential growth phase.

Biofilms

Mono-organism biofilms were grown on glass slides following procedures described previously (22). Basically, they were grown in TY-sucrose medium in fed-batch culture for 5–8 days at 37°C. The last feeding before use was with TY-glucose. The result was a dense biofilm with some 3×10^8 viable cells per mm^2 on slides with a total area of 18.75 cm^2.

Permeabilized cells

Cells were permeabilized following procedures described previously (2) involving treatment of cells with 10% (v/v) toluene and then subjecting them to two cycles of freezing and thawing before washing and use.

Assays of glycolytic activity

Glycolysis was assayed in two ways, either by pH drop associated with glycolysis by cells in unbuffered suspensions or biofilms, or by assessing glucose disappearance at constant pH. Both methods have been described in detail previously (22). Glucose disappearance was followed by enzymatic assay using glucose oxidase in assay kits purchased from Diagnostic Chemicals Ltd. (Charlottetown, P.E.I., Canada).

Assay of uptake of lansoprazole

Uptake of lansoprazole was determined by assessing decreases in absorbance of light of 400 nm wavelength in supernatant fluids from centrifuged samples of cell suspensions in salt solution (50 mM KCl plus 1 mM $MgCl_2$) at specified pH values. To determine whether lansoprazole spontaneously degraded under the experimental conditions, we also included control suspensions without cells. Changes in A_{400} of controls were negligible.

Proton permeability

Proton permeability was assessed using cell suspensions with 0.4 mg cell dry weight per ml as described previously (22). Basically, cells in suspensions (0.4 mg cell dry weight/ml) in the presence or absence of lansoprazole were initially incubated at a pH value of approximately 4.6; HCl was then added to drop pH rapidly to 4.4. The rise in pH associated with movements of protons into cells was monitored with a glass electrode, which can only detect protons outside of intact cells, not those within the electrically insulating cell membrane. Butanol 5% was added to the suspensions at 35 min to damage the cell membrane with resultant loss of pH across the cell membrane, as indicated by the rapid rise in suspension pH.

Enzyme assays

The phosphoenolpyruvate : glucose phosphotransferase system (glucose-PTS) was assayed in terms of the production of pyruvate from phosphoenolpyruvate in response to glucose addition, as described previously (22). Pyruvate was assayed with the use of lactic dehydrogenase. Two types of cell preparations were used. One was with suspensions of permeabilized cells. The other involved initially intact cells, which were treated with benzimidazoles, washed to remove unbound agent, and then permeabilized before PTS assay.

Assays of glycolytic enzymes were carried out as described previously (22). Aldolase was assayed spectrophotometrically by following the rate of change in absorbance of 340 nm wavelength light associated with NADH oxidation in the presence of glycerol-3-phosphate dehydrogenase and triose phosphate isomerase in a reaction with permeabilized cells initiated by addition of fructose 1,6-bisphosphate. Glyceraldehyde-3-phosphate dehydrogenase activity was assayed spectrophotometrically at 340 nm to follow the course of reduction of NAD to NADH by permeabilized cells in response to the addition of glyceraldehyde-3-phosphate. Lactic dehydrogenase was assayed in terms of changes in absorbance of 340 nm light associated with oxidation of NADH accompanying reduction of pyruvate to lactate.

Urease activity of *S. salivarius* was assayed in terms of ammonia production with the use of commercial ammonia assay kits from Sigma Chemical Co. (St. Louis, MO) based on the reaction catalyzed by glutamate dehydrogenase. Ammonia production from arginine by *S. rattus* was assayed by the same method.

Killing assays

For assessing lethal actions of lansoprazole against biofilms of *S. mutans,* we titrated the biofilms on glass slides in salt solution (50 mM KCl + 1 mM $MgCl_2$) to pH values of 4.0 or 5.0 and treated them with 1.5 mM lansoprazole for 3 h at room temperature. At intervals, slides with attached biofilms were removed from the lansoprazole-containing solution and washed quickly by immersion in salt solution. Biofilms were scraped from the slides, and cells were dispersed by brief sonication with a Branson Sonifer Cell Disrupter 200 (Branson Sonic Power, Danbury, CT) at full power for 15 s without glass beads, as described previously (6). The dispersed cells were diluted in 1% Difco peptone broth and samples of the diluted suspensions were streak plated on tryptic-soy agar prior to incubation at 37°C for at least 48 h to allow for colony development.

Suspensions of cells of *F. nucleatum* or *P. intermedia* were treated with lansoprazole at a pH value of 5.0 in salt solution. Samples of the suspensions were then diluted in complete growth medium at an initial pH of 7 and incubated for at least 48 h to assess most probable numbers with five tubes for each dilution (19). The dilution was sufficient to reduce the lansoprazole level to well below the lethal dose.

Results

Inhibition of glycolysis by benzimidazoles

PH-drop experiments

As shown by the results from standard pH-drop experiments, lansoprazole inhibited glycolytic acid production from glucose by cells of *S. mutans* in suspension (Fig. 2A) or biofilms (Fig. 2B) and by cells of the yeast *C. albicans* in suspensions (Fig. 2C). In this type of assay, the organisms were given excess glucose so that glycolysis was limited by acidification and not by catabolite supply. When the acid produced was neutralized after 1 h (Fig. 2A), a second round of glycolysis and acid drop ensued. It was in this second round that the effects of lansoprazole were most evident. The delayed inhibitory effect may be related to the protonated forms of benzimidazoles being the active forms of the drug able to form disulfide bonds with sulfhydryl groups on target proteins (13). The pK_a value for omeprazole is 4.0 (7), and for lansoprazole approximately the same. Thus, in pH-drop assays, a period of acidification was required for the benzimidazoles to become activated for inhibition.

Glycolysis was quite sensitive to lansoprazole. As little as 0.025 mM (25 μM) lansoprazole had major inhibitory effects (Fig. 2A). Higher concentrations of lansoprazole were needed with biofilms (Fig. 2B). Here, about four times the level was required for biofilms, compared with cells in suspensions. With biofilms, at these higher concentrations, inhibitory effects were again hardly apparent in the first round of pH drop but were accentuated during the second round. Glycolysis by biofilms was slower than that by cell suspensions, presumably because of diffusion limitations.

It was not possible with *C. albicans* to decrease the suspension pH value to the low control values obtained with *S. mutans*, but lansoprazole and omeprazole were effective at reducing the rate and extent of acidification. They were less potent against the yeast compared with *S. mutans*, but they did show inhibitory activity against the acidogenic yeast. The results of experiments with other oral streptococci, including *S. gordonii*, *S. salivarius* and *S. sanguis*, indicated little difference in sensitivities among oral streptococci (data not shown). In these and other experiments, the antimicrobial potencies of lansoprazole and omeprazole were similar.

The inhibitory effects of benzimidazoles could be prevented, but not reversed, by 2-mercaptoethanol, but not by dithiothreitol or glutathione (data not shown). An example is presented in Fig. 2D for inhibition of pH drop by 0.25 mM lansoprazole. The inhibition could be almost completely stopped when 1 mM 2-mercaptoethanol was added at the same time as the lansoprazole. Once lansoprazole had reacted with the cells during the first round of pH drop, its inhibitory effects were not reversed by the addition of 2-mercaptoethanol.

Constant-pH experiments

In subsequent experiments, cell suspensions (0.4 mg cell dry weight per ml) treated with benzimidazoles were held at a constant pH of 4, 5, 6 or 7 under pH-stat conditions for set times. The exposed cells were then centrifuged, washed and suspended (1 mg cell dry weight per ml) at pH 7 with added glucose to assess their

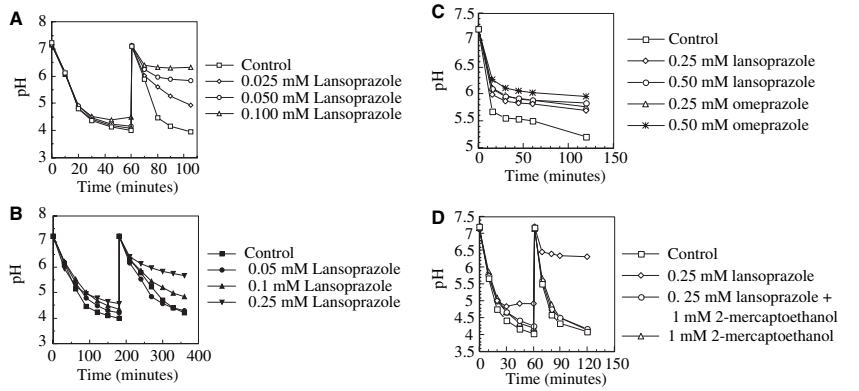


Fig. 2. Effects of lansoprazole or omeprazole on pH drop in suspensions of *S. mutans* (A), biofilms of *S. mutans* (B) and suspensions of *C. albicans* (C). D) Protection against benzimidazole damage by 2-mercaptoethanol added to cell suspensions of *S. mutans* at the same time as lansoprazole.

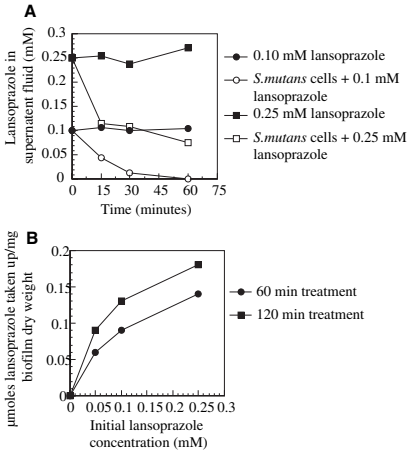


Fig. 4. Kinetics of uptake of lansoprazole by *S. mutans* cells. A) Lansoprazole remaining in supernatant fluids originally with 0.1 mM benzimidazole after incubation times shown without (●) or with (○) addition of cells (0.4 mg cell dry weight per ml), or in supernatant fluids originally containing 0.25 mM lansoprazole without (■) or with (□) addition of cells. B) Uptake of lansoprazole by *S. mutans* biofilms after exposures at pH 4 for 60 min (●) or 120 min (■) to lansoprazole at the indicated concentrations.

glycolytic capacities. An example of the type of data obtained is shown in Fig. 3A for the rate of inactivation by 0.25 mM lansoprazole of the glycolytic capacity of cells of *S. mutans* GS-5 at a constant pH value of 4. The glycolytic capacity was reduced by nearly 50% following 10 min of exposure at pH 4; by 30 min, the capacity was almost totally abolished. Reductions in glycolytic capacity were not reversed when cells were washed repeatedly, even in the presence of reducing agents such as dithiothreitol or β-mercaptoethanol, prior to assays at pH 7 of glycolysis. Lansoprazole 0.25 mM produced little or no inactivation of the glycolytic system of intact cells at pH values of 6 or 7, at least during a 1-h exposure, but that inactivation was nearly complete in 1 h at treatment pH values of 4 or 5 (Fig. 3B). Similar data were obtained for omeprazole.

The glycolytic capacities of mono-organism biofilms could be reduced by lansoprazole (or omeprazole) at acid pH values. For example, in a set of experiments, mature biofilms of *S. mutans* were exposed to lansoprazole at pH values of 6 or 4 for 1 h and washed rapidly by immersion in salt solution (50 mM KCl plus 1 mM $MgCl_2$) to remove unbound agent. The intact biofilms were then placed in salt solution with glucose, and glucose utilization was monitored over 4 h. Biofilms exposed to lansoprazole at pH 6 showed little inhibition compared with control, unexposed films. However, those exposed to the agent at pH 4 were inhibited by an average of 55%, 62%, and 77% by 0.05, 0.10, and 0.25 mM lansoprazole, respectively, compared with the control value of 1.1 µmol glucose catabolized per mg biofilm dry weight. No lansoprazole inactivation of glycolysis occurred for biofilms incubated at a pH value of 6, even for periods of hours.

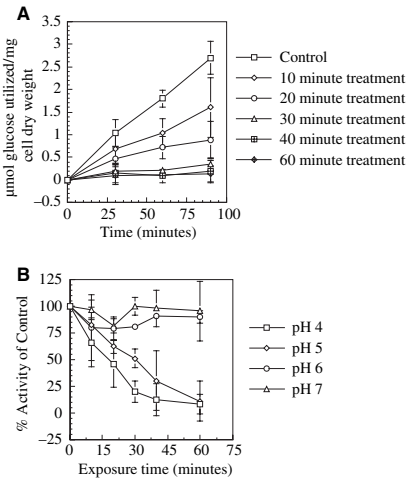


Fig. 3. Inactivation of glycolytic capacities of intact cells of *S. mutans* treated at pH 4 with 0.25 mM lansoprazole prior to being washed and suspended in glucose-containing medium at a pH value of 7 (A). B) Kinetics of inactivation at pH values of 4, 5, 6, and 7. Error bars indicate standard deviations with $n = 3$.

Uptake of lansoprazole by *S. mutans*

When bacterial cells were treated with lansoprazole or omeprazole at pH values below 5, they turned a purple color, which was not readily washed out. The benzimidazoles alone also undergo a color change at acid pH values from colorless to yellow. This color change was used as the basis for a quantitative assay for uptake of the benzimidazoles by *S. mutans* cells. Basically, the decrease in absorption of light of 400 nm wavelength in supernatant fluids from cell suspensions at pH adjusted to 4 was used as a measure of decreased concentrations of lansoprazole or omeprazole. All of the lansoprazole in a 0.10 mM solution was taken up by cells of *S. mutans* in a suspension with 0.4 mg cell dry weight per ml during a 60-min exposure at pH 4 (Fig. 4A). This highly concentrative uptake was presumably due to formation of covalent disulfide bonds between benzimidazoles and cell targets. The total uptake at 60 min was 0.25 µmol per mg cell dry weight, equivalent to some 50 µmol per ml cell volume. The uptake under the same conditions from 0.25 mM lansoprazole was higher, 0.43 µmol per mg cell dry weight, but nearly a third of the benzimidazole remained outside the cells. At the higher concentration, uptake was rapid and nearly complete in 20 min at pH 4. Uptake appeared to be limited at the higher concentration by the numbers of cell reactive sites. The benzimidazoles are weak bases (7) and movement into cells in relation to pH across the cell membrane, with the interior less acid than the surrounding medium, would not occur spontaneously. The normal pH across cell membranes of mutans streptococci would act to favor the compounds staying in the suspending medium, where they would be more highly protonated at the lower pH value there. However, disulfide bond formation would effectively lower internal

concentrations and enhance uptake into the cell.

The data presented in Fig. 4B are for biofilms over a range of initial lansoprazole concentrations with two sampling times, 1 and 2 h, at pH 4. They indicate that uptake only approached saturation with an initial concentration of 0.25 mM lansoprazole. Longer incubation times were chosen with biofilms to allow for diffusion of the agent into the films. The shapes of the uptake curves suggest two processes involved in uptake, one saturated at a fairly low concentration of about 0.1 mM and another process not readily saturated. The limited solubility of lansoprazole in water limited experiments with higher concentrations of the compound. However, the concentrations tested were within the range for the inhibitory effects described.

Effects on proton permeability

The pH-drop curves of Fig. 1 suggest that lansoprazole may have an effect on the acid tolerance as well as the acidogenesis of *S. mutans*; in other words, net membrane permeability to protons could be affected. As shown by the proton permeability curves of Fig. 5, lansoprazole did act to increase the permeability of cells of *S. mutans* GS-5 to protons, especially at a concentration of 0.1 mM. The permeability being assessed is a dynamic permeability (3). Protons from the environment diffuse inward across the cell membrane after acidification of the suspension but then can be extruded by the F-ATPase of the cell membrane. The rise in pH in the control suspension was slow, indicating that the F-ATPase was effective in reversing the flow of protons into the cytoplasm. Permeabilities were increased for the cells exposed to lansoprazole, suggesting that F-ATPase could be inhibited. However, attempts to show that isolated F-ATPase was inhibited by lansoprazole were largely unsuccessful, except when very high concentrations of the benzimidazole, about 1 mM, were applied to permeabilized cells at a pH value of 4. It would appear that the increases in proton permeability caused by lansoprazole are due to effects on barrier functions of the membrane rather than inhibition of F-ATPase. There could have been inhibition of P-ATPases in the membrane, but the effect is not likely to be major for cells of the GS-5 strain. As indicated previously, the benzimidazoles are weak bases and would therefore act to move protons out of, rather than into, cells. However, if they reacted with sulfhydryl groups of membrane proteins, they could disrupt barrier functions. In the standard proton permeability experiments, the range of pH for exposure of the benzimidazoles was limited by the need to maintain sufficient pH across the cell membrane to facilitate proton movements to the cytoplasm.

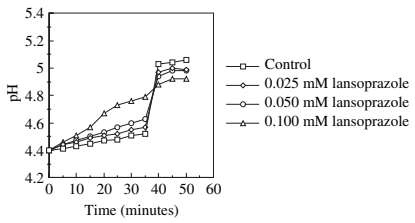


Fig. 5. Proton permeability of cells of *S. mutans* in suspensions (0.4 mg cell dry weight per ml) increased by action of lansoprazole. 5% butanol was added at 35 min to disrupt cell membranes.

Inhibition of glycolytic enzymes

Sugar uptake by oral streptococci is primarily by means of the PTS, which involves a phospho-relay system with terminal components, Enzymes IIC, embedded in the cell membrane. The system catalyzes transfer of phosphate to an incoming sugar and translocation across the cell membrane to yield a sugar phosphate in the cytoplasm. The glucose-PTS of permeabilized cells of *S. mutans* was inhibited by exposure for 30 min to lansoprazole at pH 5 with a 50% inhibitory concentration (IC_{50}) of ca. 0.09 mM (Fig. 6A). Permeabilized cells were exposed and then washed to remove unbound lansoprazole before being assayed at pH 7 for PTS phosphorylation activity. When intact cells at pH 4 were treated similarly with lansoprazole, washed and then permeabilized, there was some reduction in sensitivity, but 50% inhibition could still be achieved with about 0.20 mM lansoprazole (Fig. 6B). It appears that inhibition of PTS transport of glucose into intact cells may play a significant role in benzimidazole inhibition of glycolysis.

Inhibition of glycolysis by lansoprazole and omeprazole also appeared to involve glycolytic enzymes in the cytoplasm. The glycolytic enzymes found to be the most sensitive to benzimidazoles were aldolase, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase. Data are presented in Fig. 7 for intact cells treated at pH 5 with 0.5 mM omeprazole or lansoprazole for 1 h before washing, pH adjustment to 7.0, and standard assays of enzyme activity. Aldolase was the most sensitive, glyceraldehyde-3-phosphate dehydrogenase also was very sensitive, and lactate dehydrogenase was the least sensitive. Enzymes in intact cells or even in biofilms could be inhibited by the benzimidazoles. For example, glyceraldehyde-3-phosphate dehydrogenase in intact biofilms was inhibited by about 75% after treatment with 1 mM lansoprazole for 1 h at a pH value of 5.0. The benzimidazoles can apparently cross the cell membrane to affect intracellular targets, although passage probably follows initial damage to the cell membrane, which appears to protect partially cytoplasmic enzymes from damage.

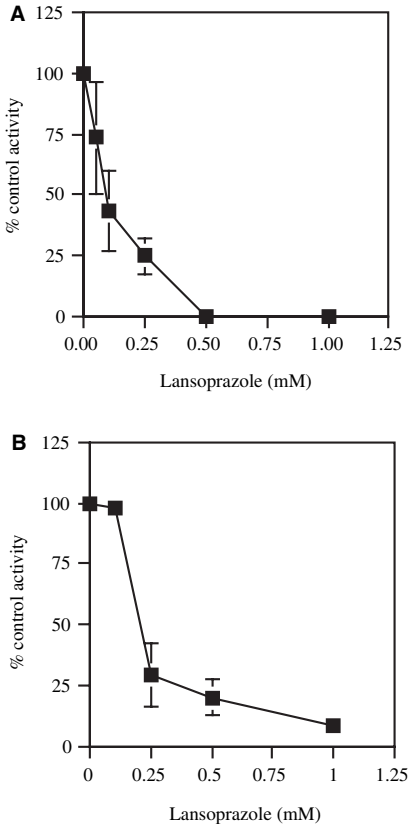


Fig. 6. Lansoprazole inhibition of glucose-PTS activity of permeabilized (A) or intact (B) cells of *S. mutans*. The cells in suspensions were treated with lansoprazole at a pH value of 4.0 for 1 h. Permeabilized cells were then washed and suspended at pH 7.0 for assay of PTS activity. Control (100%) activity = 1.1 μmol pyruvate produced from phosphoenolpyruvate in response to glucose per mg cell dry weight per hour. Intact cells were similarly exposed to lansoprazole, washed, and then permeabilized before assay at pH 7.0 for glucose-PTS activity. 100% activity = 2.3 μmol pyruvate per mg cell dry weight per hour. Error bars indicate standard deviations with $n = 3$.

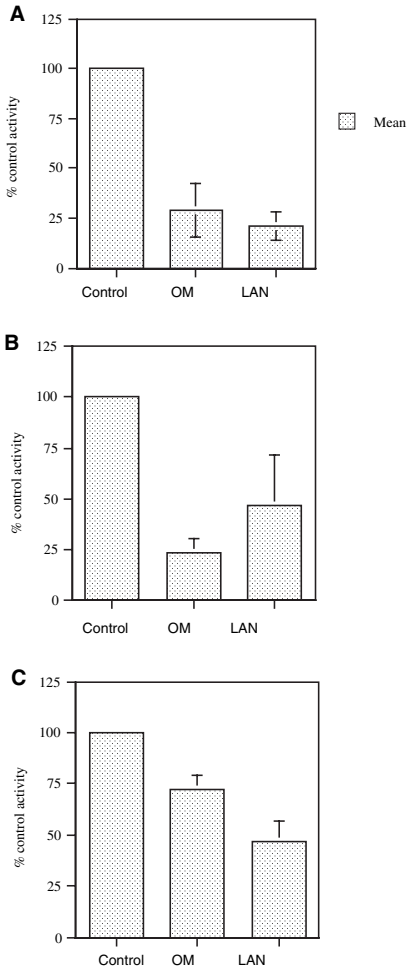


Fig. 7. Inhibition of cytoplasmic enzymes of glycolysis in intact cells of *S. mutans* by omeprazole (OM) or lansoprazole (LAN). Intact cells in suspensions were treated for 1 h at a pH value of 5.0 with 0.5 mM OM or LAN before being washed and permeabilized for assay of the enzyme activities at pH 7.0. Data are shown for aldolase (A), glyceraldehyde-3-phosphate dehydrogenase (B), and lactic dehydrogenase (C). Control (100%) activities were, respectively, 0.05, 0.009, and 0.042 μmol NADH per mg cell dry weight per min. Error bars indicate standard deviations with $n = 3$.

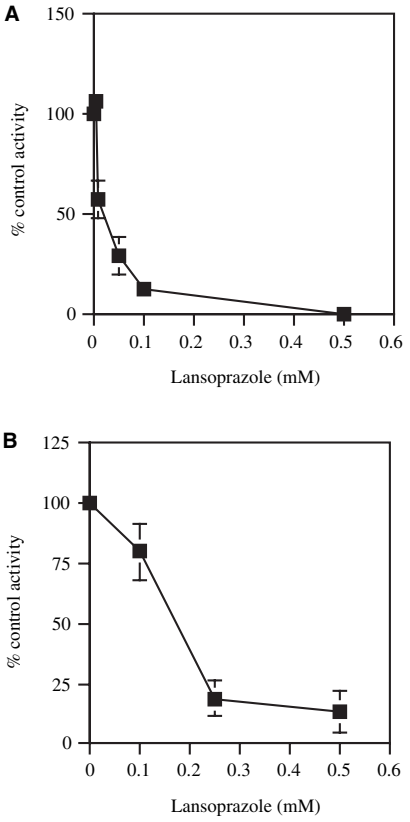


Fig. 8. Lansoprazole inhibition of urease in permeabilized (A) or intact (B) cells of *S. salivarius*. Control activity = 0.12 and 0.07 μmol NH_3 produced per minute per mg cell dry weight, respectively, for permeabilized and intact cells. Error bars indicate standard deviations with $n = 3$.

Inhibition of alkali production by oral streptococci

Some oral streptococci may act to reduce the cariogenicity of dental plaque by producing alkali (5); for example, production of ammonia from urea via the urease system or from arginine via the arginine deiminase system or by the agmantine pathway (10). *S. salivarius* is urease-positive and is able to degrade urea secreted in parotid saliva, which may contain as much as 10 mM urea. As shown by the dose–response curve in Fig. 8A, urease of permeabilized cells of *S. salivarius* ATCC 13419 was highly sensitive to inhibition by lansoprazole at a treatment pH of 5 for 30 min with an ID_{50} concentration close to 0.02 mM. The ID_{50} value for omeprazole was similar. However, as shown in Fig. 8B, urease within intact cells was more resistant. Even when intact cells were treated with lansoprazole at pH 4 for 1 h before being washed and permeabilized for urease assay at pH 7, the ID_{50} concentration was about 0.19 mM. Urea is known to be able to enter intact cells without being permeabilized. The findings presented here indicate again that the intact cell membrane protects against benzimidazole inhibition of cytoplasmic urease but is not a complete barrier.

Ammonia production from arginine by *S. rattus* FA-1 via the arginine deiminase system was slightly less sensitive to lansoprazole in experiments of the type carried out for ureases. The ID_{50} value for inhibition of arginine deiminase in permeabilized cells was approximately 0.15 mM at a pH value of 5 with exposure to the agent for 30 min. After treatment with lansoprazole the cells were washed and arginine deiminase activity assayed at pH 7. If intact cells were treated with lansoprazole or omeprazole and then washed and permeabilized before assays of residual arginine deiminase activity, the ID_{50} value was only slightly higher than that obtained for cells permeabilized before exposure to the benzimidazoles. Bacterial cells are not freely permeable to arginine, and benzimidazole inhibition of arginolysis likely involves inhibition of membrane enzymes catalyzing arginine uptake.

Killing

Benzimidazoles are bactericidal for *S. mutans* cells even those in biofilms. An example is presented in Fig. 9A of killing of *S. mutans* cells in biofilms by 1.5 mM lansoprazole at pH values of 5 and 4. Cells of oral streptococci in suspensions were much more sensitive to killing by the benzimidazoles, and the levels of the agents that were highly inhibitory for the PTS of intact cells (0.25 mM) were also lethal for the bacteria. The gram-negative, oral anaerobes *F. nucleatum* and *P. intermedia* were even more sensitive to killing by benzimidazoles. At a concentration of only 0.1 mM lansoprazole and a pH value of 5, average *D*-values (time for killing of 90% of the population) of 20 and 9 min, respectively, were obtained (Fig. 9B, C). There was a lag before killing began with lower concentrations of lansoprazole, but subsequent killing was rapid.

Discussion

The potency of lansoprazole and omeprazole for inhibiting acid production by intact cells of *S. mutans* was found to be somewhat lower than that for inhibition of hog gastric mucosa (H^+/K^+) P-ATPase of isolated membranes. Acid production in pH-drop experiments by intact *S. mutans* cells was inhibited by some 50% at lansoprazole concentrations of about 25 μM, whereas inhibition of the P-ATPase occurred at concentrations close to 10 μM (8). Magalhães et al. (17) found that 50 μM lansoprazole inhibited the P-ATPase of *S. mutans* by 44% for the membrane-associated enzyme or by 91% for the isolated enzyme. Thus, the levels of lansoprazole needed for the inhibitory effects described here for cells in suspensions or for enzymes in permeabilized cells are similar to those required for P-ATPase inhibition. However, there are always

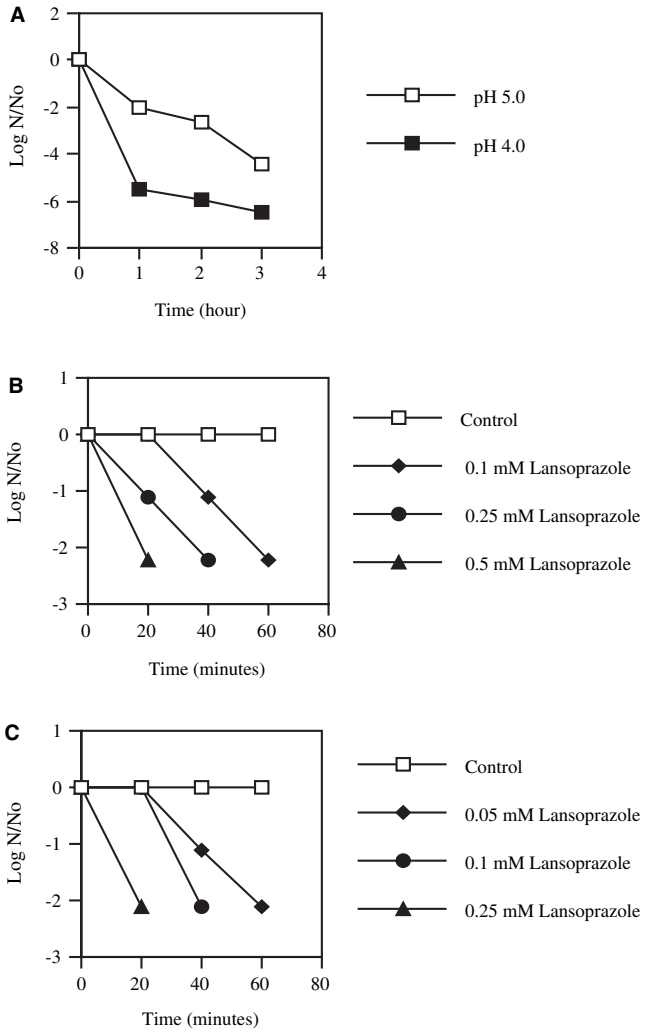


Fig. 9. Bactericidal actions of lansoprazole for biofilms of *S. mutans* treated at pH values of 4.0 and 5.0 with 1.5 mM lansoprazole (A), or for suspensions of *F. nucleatum* (B) or *P. intermedia* (C) treated at pH 5.0 with the indicated concentrations of lansoprazole.

difficulties in relating results obtained with fractions from lysed cells to intact cell effects. For example, inhibition of urease in permeabilized cells of *S. salivarius* showed an ID_{50} of about 20 μM, whereas for intact cells the ID_{50} was about 10 times higher. The reactivity of benzimidazoles with protein sulfhydryl groups in our studies did not appear to be highly specific for P-ATPases but occurred with many cell enzymes, including the PTS for sugar uptake. In fact, we conclude that benzimidazole inhibition of glycolysis by oral streptococci involves multiple targets. Membrane targets are likely to be more important than cytoplasmic targets simply because they are directly exposed to the agents added from the outside. Benzimidazole antibacterial potency is also affected by experimental conditions, especially pH, and to a lesser extent, also by biomass concentration. However, biofilms with high biomass density could be inhibited with the agents. The dependence of potency on cell biomass concentration is common with antimicrobial agents and can be related to the multiple sites for interaction between cells and agents.

The basic mechanism by which lansoprazole and omeprazole inhibited glycolysis appears to be the same or similar to that for inhibition of proton-pump P-ATPases, with formation of disulfide bonds between the protonated form of the agent and target proteins. The pH-activity profiles for inhibition of glycolysis clearly indicate that the protonated forms of the agents are the active forms. Both lansoprazole and omeprazole are weak bases with pK_a values of approximately 4.0 and were found to be active glycolytic inhibitors only at pH values below about 5.0. Amelioration of inhibition by β-mercaptoethanol is likely to be due to β-mercaptoethanol reacting with the benzimidazoles to neutralize them, especially as the agents had to be added together for β-mercaptoethanol to prevent glycolytic inhibition. The inhibition appeared to be irreversible, at least under the conditions of our experiments. A highly desirable characteristic of the benzimidazoles as anticaries agents is that they become active only at the time of major cariogenic challenge when the environmental pH drops. Also, at low pH values, the agents are concentrated from the environment by bacterial cells, apparently because of disulfide bond formation involving target proteins. Uptake is then not readily reversible, although it is possible that the agents could be released by enzymes that catalyze cleavage of disulfide bonds. The levels of benzimidazoles required for inhibition of biofilms were somewhat higher than those needed for the same effects on cells in suspensions. However, biofilm functions could still be inhibited, and the irreversibility of inhibition would lead to progressively greater damage during repeated exposures.

Glycolysis is the major function of acid-tolerant, cariogenic bacteria such as the mutans streptococci causing direct damage to the host. Growth of the organisms occurs during the phases of the pH cycle of dental plaque when sugar challenge is low and plaque pH high, above about 5. Even mutans streptococci can not reproduce at pH values below this. However, they can carry out glycolysis uncoupled to growth at environmental pH values as low as 4, or even somewhat lower for some strains. Glycolysis at low pH values is key for cariogenicity. Therefore antimicrobials such as the benzimidazoles able to inhibit glycolysis at low pH values have potential as anticaries agents.

F-ATPases appear not to be inhibited by benzimidazoles, even those of *H. pylori* (1). In our current experiments we were able to show that some inhibition of F-ATPase activity could occur, but only at very high concentrations of lansoprazole, close to molar levels, which are supersaturating. It seems that inhibitory effects on F-ATPases are not important for sensitizing glycolysis to acidification. Lansoprazole did enhance proton permeabilities of cells. However, this effect is likely due to interaction of the agent with proteins, other than the F_0 portion of the F-ATPase, in the cell membrane and to reduction in the barrier functions of the membrane. A major target for benzimidazoles in *H. pylori* is NADH : ubiquinone

oxidoreductase (20), but this enzyme is not likely to be present in membranes of oral streptococci because the organisms are not known to have *trans*-membrane electron-transport systems.

Overall, it seems that benzimidazoles are potentially useful agents against cariogenic oral bacteria and possibly other pathogenic microbes They have multiple cell targets for damage, including membrane enzymes such as those of the PTS or various P-ATPases, but also multiple cytoplasmic enzymes in cells with damaged membranes. Therefore, the type of resistance involving changes in antimicrobial targets is not likely to develop quickly during benzimidazole use. Also, since the agents become covalently bonded to their targets, multidrug transporters are not likely to be effective in moving benzimidazoles out of the cell. For the agents to be effective, the pH must drop to values of 5 or below. Thus, cariogenic dental plaque is a good example of a locus in the body where the agents would be effective. In sites of infection with inflammation, pH values commonly drop, and the agents may have wider applicability, although probably not in periodontal disease sites, where the pH tends to be elevated because of extensive proteolysis. However, pH values in phagolysosomes generally fall sufficiently to allow protonation and activation of benzimidazoles to kill ingested bacteria. Biofilms have only a slightly greater resistance than cells in suspensions. Therefore, benzimidazoles may also be effective against pathogenic biofilms, which regularly become acidified because of diffusion limitations and are difficult to control with the standard antimicrobial agents used currently.

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