Inhibitory Action of Lansoprazole and Its Analogs against *Helicobacter pylori*: Inhibition of Growth Is Not Related to Inhibition of Urease

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The proton pump inhibitors omeprazole and lansoprazole and its acid-activated derivative AG-2000, which are potent and specific inhibitors of urease of *Helicobacter pylori* (K. Nagata, H. Satoh, T. Iwahi, T. Shimoyama, and T. Tamura, Antimicrob. Agents Chemother. 37:769-774,1993), inhibited the growth of *H. pylori*. The growth was inhibited not only in urease-positive clinical isolates but also in their urease-negative derivatives which had no urease polypeptides. AG-1789, a derivative of lansoprazole with no inhibitory activity against *H. pylori* urease, also inhibited the growth of both strains even more strongly than the urease inhibitors lansoprazole and AG-2000. Furthermore, the antibacterial activity of omeprazole and lansoprazole was not affected by glutathione or dithiothreitol, which completely abolished the inhibitory activity of lansoprazole against *H. pylori* urease. These results indicated that the inhibitory action of these compounds against the growth of *H. pylori* was independent from the inhibitory action against urease.

Helicobacter pylori is a gram-negative, spiral-shaped organism whose niche is highly restricted to the gastric mucosa of humans and which is associated with both antral gastritis and duodenal ulcer disease (1, 4, 9, 15). *H. pylori* produces high levels of urease, which constitutes almost 6% of the soluble protein (11). Unlike that of other urease-positive bacteria, in which urease is located in the cytoplasm, *H. pylori* urease is located both in the cytoplasm and on the surface layer of *H. pylori* cells (3, 11), and it is reportedly one of the major surface layer proteins (7). Urease appears to be critical for *H. pylori* colonization of the gastric mucosa (8). The bacterium is quite sensitive to low pH in vitro (10), unless urea is present (14). It is postulated that the organism hydrolyzes urea, thereby releasing ammonia, which neutralizes acid, allowing survival of the bacterium and initial colonization.

We reported that antiulcer benzimidazole proton pump inhibitors (PPIs) such as lansoprazole and omeprazole, which inhibit H^+ and K^+ ATPase [(H^+/K^+)-ATPase] of stomach parietal cells, dose-dependently inhibit the urease activity in cell extracts of *H. pylori* (17). PPI inhibited *H. pylori* urease more potently than urease inhibitors such as acetohydroxamic acid, hydroxyurea, and thiourea with 50% inhibitory concentrations of 3.6 to 9.5 μM. The inhibition was higher under acidic conditions than under neutral conditions. PPIs also inhibited the urease activity of intact *H. pylori* and *Helicobacter mustelae* cells but not those of other bacteria such as *Proteus vulgaris*, *Proteus mirabilis*, and *Providencia rettgeri* (17). SH residues in the cell extract of urease-positive cells decreased after an incubation with lansoprazole, and SH-containing compounds such as glutathione (GSH) or dithiothreitol (DTT) completely abolished the inhibitory action, suggesting that the inhibitory mechanism of PPIs on *H. pylori* urease is due to a

blockage of SH residues in the cysteine of the *H. pylori* urease, which is similar to that which occurs in (H^+/K^+)-ATPase of parietal cells (18).

Iwahi et al. have reported that PPIs, especially lansoprazole and its analogs, inhibit the growth of *H. pylori* at concentrations of several micrograms per milliliter (12). This inhibition appeared to be specific to *H. pylori*, since the growth of more than 27 other bacterial species was not affected by lansoprazole even at 100 μg/ml (12). Other investigators also reported effective antimicrobial action of PPIs on *H. pylori* (2, 5). However, little has been reported concerning the antibacterial mechanism of these compounds against *H. pylori*.

Here, we examined the relationship between the inhibitory actions of PPIs upon urease activity and upon growth. We concluded that the inhibitory mode of PPIs on *H. pylori* growth was different from that on urease activity and that these effects were unrelated.

Plasmid pHPT54 carries part of the urease gene cluster of the *H. pylori* strain, UMAB41, and the cloned *ureB* gene is disrupted by the insertion of a Km^r gene (19). Among the five *H. pylori* strains used here, NCTC11637, CPY4110, and HPK5 were urease positive, whereas CPY4111 and HPT67 were urease negative. CPY4111 was isolated from CPY4110 by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (13), and HPT67 was constructed by allelic exchange mutagenesis through transformation of HPK5 to Km^r with pHPT54 as the donor DNA (19, 20). To confirm the exchange event, the chromosomal DNAs of HPK5 and HPT67 were digested with appropriate restriction enzymes and analyzed by Southern hybridization as described previously (19). This analysis clarified that HPT67 had no vector sequence of pHPT54 and that it carried at the chromosomal *ureB* gene a single copy of the Km^r gene that had been inserted by double-crossover-mediated homologous recombination (Fig. 1).

Cells of *H. pylori* were routinely cultured on Skirrow's me-
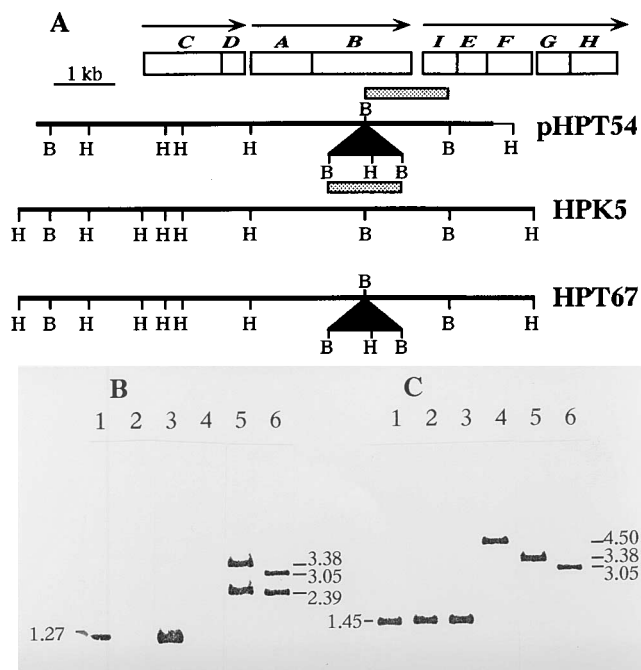
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FIG. 1. Physical structures of the *ure* regions. (A) Restriction maps of the *ure* regions of pHPT54, HPK5, and HPT67. Thick and thin lines represent the chromosomal and plasmid regions, respectively, and only a portion of the plasmid DNA relevant to this study is given for simplicity. Abbreviations and symbols; B, *Bam*HI site; H, *Hin*dIII site; solid triangle, Km^r determinant; boxes filled with dots, DNA fragments used as the probes in panels B and C. Plasmid pHPT54 carries part of the *ure* region of UMAB41 and has an insert of a Km^r determinant at the *Bam*HI site in the *ureB* gene (19). Above the pHPT54 map, the genetic organization of the *ure* region (6) is given, and the arrow indicates the transcriptional direction of the operon. Restriction maps of the HPK5 and HPT67 *ure* regions were determined on the basis of Southern analyses (panels B and C). The probes were a 1.27-kb *Bam*HI fragment containing the Km^r determinant (panel B) and a 1.45-kb *Bam*HI fragment containing a part of *ureI* (panel C) of pHPT54. *Bam*HI- and *Hin*dIII-digested samples were loaded in lanes 1 to 3 and 4 to 6, respectively. The samples in lanes 1 and 6 were derived from pHPT54 DNA, those in lanes 2 and 4 were derived from the chromosomal DNA of HPK5, and those in lanes 3 and 5 were derived from the chromosomal DNA of HPT67. The sizes of the DNA fragments in kilobases are indicated by the outermost lanes.

dium containing 7% (vol/vol) defibrinated horse blood for 3 days at 37°C in a microaerobic atmosphere (5% O_2, 15% CO_2, and 80% N_2). To assay urease levels and analyze the urease polypeptides by enzyme-linked immunosorbent assay (ELISA)

and Western blotting (immunoblotting), cells were cultured in 3% polypeptone broth (Becton Dickinson Microbiology Systems, Cockeysville, Md.) with shaking for 2 days at 37°C (16).

The cultured cells were harvested and disrupted by sonication, and then crude extracts were obtained by centrifugation at $10,000 \times g$ for 20 min. The assay of urease activity in cell extracts was as described previously (17). *H. pylori* urease is a multimeric complex of two subunits, UreA and UreB, which are the gene products of *ureA* and *ureB*, respectively. Urease subunit polypeptides were examined by ELISA and by Western blotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10 monoclonal antibodies (MAbs); 5 against UreA (S1 to S5) and 5 against UreB (L1 to L5) (16).

The MICs of omeprazole, lansoprazole, and lansoprazole analogs upon growth of urease-negative mutants and their parent strains were determined. Lansoprazole (AG-1749), its acid-activated analog (AG-2000), and AG-1789 were synthesized by the Pharmaceutical Research Division of Takeda Chemical Industries, Ltd., Osaka, Japan. The chemical structures of these compounds are described elsewhere (12). Omeprazole was provided by Fujisawa-Astra Co. Ltd., Osaka, Japan. These compounds were dissolved in 100% ethanol or distilled water and diluted 10- and 100-fold with 3% polypeptone broth (Becton Dickinson Microbiology Systems), and then aliquots with or without GSH (Wako Pure Chemicals Co., Osaka, Japan) or DTT (Sigma Chemicals, St. Louis, Mo.) were put into wells of 24-well-tissue culture plates (Corning Glass Works, Corning, N.Y.). One milliliter of brucella agar medium (Becton Dickinson Microbiology Systems) supplemented with 7% (vol/vol) defibrinated horse blood was added to each well. *H. pylori* cells were cultured in a liquid medium with shaking at 37°C as described previously (17), and then 10^5 cells in the logarithmic phase were inoculated into each well. After a 3- or 4-day incubation in a microaerobic atmosphere (5% O_2, 15% CO_2, and 80% N_2), the MICs were determined and are presented as a range of results from several experiments.

Table 1 summarizes the biochemical characteristics of urease in the *H. pylori* strains used in this study. In the crude extracts of CPY4110 and HPK5, we detected urease activity and both UreA and UreB subunit polypeptides by ELISA using 10 MAbs. On the other hand, the crude extracts of CPY4111 and HPT67 showed no urease activity, UreB, or UreA subunits. SDS-PAGE (Fig. 2A) and Western blotting (Fig. 2B) further confirmed that both UreB and UreA subunit polypeptides with molecular sizes of around 64 and 30 kDa,

TABLE 1. Biochemical characteristics of urease in crude extracts of *H. pylori* strains used in this study

Strain	Derivation and phenotype	Activity[a] (U/mg of protein)	Urease			
			Subunit polypeptides			
			ELISA[b]		Western blot[c]	
			UreA	UreB	UreA	UreB
NCTC11637	Wild type, Ure$^+$	46.3	+	+	+	+
CPY4110	Wild type, Ure$^+$	25.3	+	+	+	+
CPY4111	CPY4110 derivative,[d] *Ure$^-$*	<0.01	−	−	−	−
HPK5	Wild type, Ure$^+$	33.3	+	+	+	+
HPT67	HPK5 derivative,[e] *Ure$^-$*	<0.01	−	−	−	−

[a] The activity of urease in crude extracts was assayed as described previously (17). One unit of urease activity indicates 1 μmol of ammonia liberated per min per mg of protein at 25°C.
[b] Urease subunit polypeptides were examined by ELISA using 10 MAbs.
[c] Urease subunit polypeptides were examined by Western blotting after SDS-PAGE (cf. Fig. 2).
[d] CPY4111 generated by NTG mutagenesis (13).
[e] HPT67 constructed by allelic exchange mutagenesis to disrupt the *ureB* gene (cf. Fig. 1).
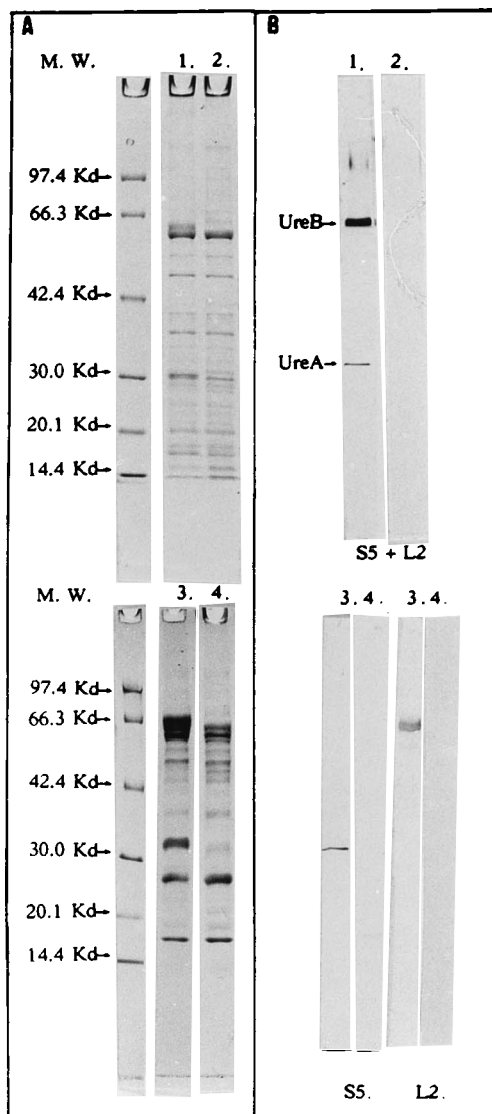
FIG. 2. SDS-PAGE profiles of crude extracts from wild-type and urease-negative strains (A) and Western blots with MAbs of S5 and L2 raised against UreA and UreB subunit peptides of *H. pylori* urease, respectively (B). Crude extracts derived from CPY4110, CPY4111, HPK5, and HPT67 were resolved by electrophoresis on a 10 to 20% gradient SDS-polyacrylamide gel. Resolved bands were electrotransferred onto a nitrocellulose-polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) and reacted with MAbs S5+L2 or S5 and L2 as described previously (16). Lane 1, CPY4110; lane 2, CPY4111; lane 3, HPK5; lane 4, HPT67.

TABLE 2. MICs of omeprazole, lansoprazole, and lansoprazole analogs against clinical isolates and their urease-negative mutants of *H. pylori*

Strain	MIC (µg/ml) (range)			
	Lansoprazole	Omeprazole	AG-2000	AG-1789
CPY4110	3.15–6.25	50–100	6.25–12.5	1.56
CPY4111	3.13–6.25	50–100	6.25	1.56
HPK5	3.13–6.25	50–100	6.25	1.56
HPT67	3.13–6.25	50–100	6.25	0.78–1.56

mutants CPY4111 and HPT67 was inhibited dose dependently by PPIs. The MICs of lansoprazole and omeprazole against CPY4111 and HPT67 were similar to those against their parent strains, CPY4110 and HPK5 (Table 2). In accordance with our previous results (12), the MICs of omeprazole against these strains were about 10 times higher than that of lansoprazole. Analogs of lansoprazole, AG-2000 and AG-1789, also inhibited the growth of both the wild-type and the urease-negative mutant strains for which MICs were similar to those of lansoprazole (Table 2). In contrast to growth inhibition, urease inhibition by lansoprazole and its analogs was variable. Lansoprazole and AG-2000 inhibited the urease activity of CPY4110, HPK5, and NCTC11637 with 50% inhibitory concentrations of 8 to 12 µM, whereas AG-1789 at concentrations above 1 mM did not inhibit urease activity. On the basis of findings that the growth of urease-negative mutants is inhibited by these compounds and that this inhibition of urease activity of *H. pylori* is not correlated with growth inhibition, we concluded that urease is not involved in the ability of lansoprazole or its analogs to inhibit growth. This notion was also supported by the following facts. We demonstrated that the inhibitory action of lansoprazole and omeprazole on *H. pylori*-urease activity was abolished by SH-containing compounds such as GSH or DTT; the concentrations for 50% restoration of the inhibition by 50 µM lansoprazole were 15 and 17 µM for GSH and DTT, respectively (17). Moreover, their inhibitory action was more effective under acidic conditions (17). These properties were very similar to those of stomach (H^+/K^+)-ATPase (18), and it was suggested that the target of PPIs is a cysteine residue(s) near the active site of *H. pylori* urease (17). In this study, the addition of 50 or 100 µM GSH or the same dose of DTT to the tested medium and changes of pH (6 or 7) in the medium did not affect the MICs of lansoprazole (data not shown). These results suggest that cysteine groups are not involved in the growth inhibition of *H. pylori* by lansoprazole and its analogs.

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respectively, were absent from CPY4111 and HPT67. HPT67 had an insertion of a Km^r gene in the *ureB* gene, and its null urease activity is consistent with the fact that the UreB subunit contains the catalytic site for the enzyme activity (6). It has been reported that *ureB* forms an operon with *ureA* so that transcription starting from the promoter located upstream of *ureA* proceeds to *ureB* (6), therefore leading to our notion that HPT67 produces the UreA subunit. Although we do not know the reason why we did not detect the UreA subunit in the cell extract of HPT67, that synthesized in *H. pylori* might have been degraded rapidly in the absence of the UreB subunit.

Growth of the wild-type and mutant strains CPY4110 and CPY4111 or HPK5 and HPT67 did not differ under the growth conditions described above. The growth of the urease-negative

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