In Vitro Antibacterial Activity of Phx-3 against Helicobacter pylori

Tomoko HANAWA,* a Takako OSAKI,a Taki MANZOKU,bMinoru FUKUDA,cHayato KAWAKAMI,d Akio TOMODA,a and Shigeru KAMIYAb

a Department of Infectious Diseases, Division of Medical Microbiology, Kyorin University, School of Medicine; 6–20–2 Shinkawa, Mitaka, Tokyo 181–8611, Japan; bLaboratory for Electron Microscopy, Kyorin University, School of Medicine; cSecond Department of Anatomy, Department of Biochemistry, Kyorin University, School of Medicine; 6–20–2 Shinkawa, Mitaka, Tokyo 181–8611, Japan;
c Miyarisan Pharmaceutical Co., Ltd.; 1–10–3 Kamiyanamizu, Kita-ku, Tokyo 114–0016, Japan; d Tokyo Medical University; 6–1–1 Shinjuku, Shinjuku-ku, Tokyo 160–8402, Japan.

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Phx-3, one of the phenoxazine derivatives, is reported to have inhibitory effect on Mycobacterium species and Chlamydia pneumoniae but not Escherichia coli, Salmonella Typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, Listeria monocytogenes. The bactericidal activities of Phx-3 against Helicobacter pylori strains have not been assessed. Then, we measured minimum inhibitory concentration of Phx-3 for Helicobacter strains and assessed the morphological and biochemical effects of Phx-3 on H. pylori. In present study, it has shown that H. pylori strains including clarithromycin resistant strain and Helicobacter mustarvae were killed effectively by the treatment with Phx-3. Furthermore, severe morphological changes such as membrane blebbing and formation of hollows in H. pylori were detected. In addition, induction of heat shock protein 60 was observed. Taken together, Phx-3 has antibacterial activity against Helicobacter pylori.

Key words Helicobacter pylori; phenoxazine; bactericidal agent

MATERIALS AND METHODS

Bacteria and Cultivation We used three standard strains of H. pylori (American Type Culture Collection [ATCC] 43504, ATCC 43579, and The National Collection of Type Cultures NCTC [NCTC] 11638 and three clinical isolates (TK 1029, 1047, and 1402). Brucella broth (Difco Laboratories, Inc., Detroit, MI, U.S.A.) supplemented with 7% horse serum was used for the liquid culture of H. pylori strains. For the plate culture, 1.5% of bacto agar (Difco Laboratories, Inc., Detroit, MI, U.S.A.) was used. Bacteria were incubated at 37 °C under microaerobic atmosphere produced by the AnaeroPack®-MicroAero (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

Chemicals Phx-1, Phx-2 and Phx-3 were synthesized from 2-amino-5-methylphenol, 2-amino-4-methylphenol or o-aminophenol, respectively, with bovine hemoglobin.

Fig. 1. Chemical Structure of Phx-3 as Identified by Shimizu et al.7) The structure, 2-amino-phenoxazine-3-one is identical to that of questiomycin A.7)

* To whom correspondence should be addressed. e-mail: thanawa@ks.kyorin-u.ac.jp © 2010 Pharmaceutical Society of Japan
directly on the Phx-3-containing agar dilution plates. The MICs were determined after 3 d-cultivation. The sensitivities of these strains to CAM were measured by the E-test® according to the manufacturer’s instructions (AB Biodisk, Solna, Sweden).

**Electro Microscopy** The bacterial cells were collected and prefixed in an aqueous solution of glutaraldehyde in 50 mM phosphate buffer (pH 7.4) at 4°C. Following washing, the bacterial cells were fixed with OsO₄ in Veronal-acetate buffer (pH 6.0) for 16 h at room temperature. After treatment with 0.5% uranyl acetate, the samples were subsequently dehydrated in a graded series of alcohol. Micrographs with scanning electron microscope were observed with model JSM-5600LV (Japan Electron Optics Laboratory, Tokyo, Japan).

**Western Blotting** *H. pylori* ATCC 43504 strain was cultured for 8 h, and 12 μg/ml of Phx-3 was added. Following the 6-h, bacteria cells were harvested by centrifugation at 8500×g for 10 min and washed with phosphate buffered saline (PBS). The bacterial cells were disrupted by ultrasonic waves (Sonifier, Bronson Ultrasonics Co., Danbury CT, U.S.A.). Cell debris was removed by centrifugation at 8500×g for 20 min. The cell lysates were developed with 12.5% acrylamide gel and transfer to the polyvinylidene difluoride (PVDF) membrane. Following to the reaction with primary and secondary antibodies, reacted proteins with primary antibodies were detected with DAB (3,3′-diaminobenzidine). The anti-Omp19 (Biodies International, Saco, ME, U.S.A.) and H2014) monoclonal antibodies were used for the detection of Omp19 and HSP60, respectively.

**RESULTS AND DISCUSSION**

We assessed the anti-microbial effect of Phx-3 on *H. pylori* ATCC 43504, ATCC 43579, NCTC 11638, TK 1029, 1047, and 1402. None of *H. pylori* strains were able to grow in the presence of 1 μg/ml or more of Phx-3 (data not shown). Among examined strains, *H. pylori* TK 1047 was considered CAM-resistant according to the CLSI guidelines as the MIC of CAM against this strain was 1.5 μg/ml for CAM. Therefore, Phx-3 was able to prevent the growth of CAM resistant strain as well as sensitive strains.

In addition, we found that the MIC of Phx-3 against *Helicobacter musterae* ATCC 43772 was 0.5 μg/ml, which was similar to the MICs of Phx-3 for the *H. pylori* strains. Therefore, it appears that Phx-3 inhibits the growth of *Helicobacter* species including *H. pylori*.

To further examine the antibacterial effect of Phx-3 on *H. pylori*, we counted the number of colonies of bacteria in the culture at selected time points after the addition of various concentrations of Phx-3. As shown in Fig. 2, Phx-3 killed *H. pylori* at the concentrations equal to or higher than the MIC. After 48 h, Phx-3 reduced the numbers of bacteria to below the limit of detection (Fig. 2). Unlike other bacteria to which Phx-3 has weak antibacterial activity, our results suggest that *Helicobacter* species are highly susceptible to Phx-3.

**Anti-*H. pylori*** agents have been shown to cause a variety of morphological changes. We therefore examined the morphological changes of *H. pylori* ATCC 43504 cells after a 6-h culture in the presence or absence of 6 μg/ml of Phx-3 using scanning electron microscopy. The electron micrographs shown in Fig. 3 revealed that Phx-3 caused severe morphological changes, including membrane blebbing, the formation of hollows and the formation of spherical cells. Interestingly, no coccoid cells were observed until 8 h after treatment with Phx-3. Because the membrane-like structures were attached to the surface of the bacterial cells treated with Phx-3, the exfoliation of outer membrane was suspected.

We next examined the localization of outer membrane protein in the *H. pylori* cells. The lysates were separated on polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (Fig. 4C). The Omp19 protein was detected using monoclonal antibody against Omp19. We detected no differences in the amounts of Omp19 proteins between Phx-3 treated and untreated cells produced more heat shock protein 60 than untreated cells (Fig. 4B). The damages of the bacterial cells caused by Phx-3 may be generated in not only the surface of the cells but also whole cells. As shown in Fig. 4B (lane 2), H20 monoclonal antibody used in this study to detect HSP60 of *H. pylori* reacted with unidentified proteins of *H. pylori*.
Yamaguchi and coworkers\(^4\) purified *H. pylori* HSP60 using affinity chromatography with H20 monoclonal antibody. After loading of the purified HSP60 fraction in Western blot analysis, several proteins except for the HSP60 were still detected with H20 monoclonal antibody as minor bands. These results suggest that they may be degraded HSP60 protein or cross-reacting antigen induced by various stresses.

Motility of *H. pylori* during colonization in the gastric mucosa is an important factor for the pathogenesis following *H. pylori* infection.\(^18\) We therefore assessed the possibility that Phx-3 affects the motility of *H. pylori* by measuring swimming motility on the soft agar medium containing 0.4% agar. We found that pretreatment or simultaneous treatment with Phx-3 at concentrations below the MIC did not alter the swimming of *H. pylori* (data not shown).

The three-dimensional structure of chemical substances sometimes provides clues to their mechanism of action. Phx-3 is a planar molecule with 2-amino and 3-ketone residues. These features provide structural rigidity, which could allow Phx-3 to intercalate into the double-stranded DNA. Phx-3 could form hydrogen bonds with deoxyguanosine between the GpC pairs. We therefore extracted the genomic DNA of *H. pylori* and examined the DNA patterns by agarose gel electrophoresis. By staining with ethidium bromide, we did not detect any changes until 8 h after the treatment with Phx-3 (data not shown).

Furthermore, we examined the bactericidal effect of Phx-1 and Phx-2 on *H. pylori* strains, and the MICs of these compounds against tested nine *H. pylori* strains and *H. mustelae* strain were between 50 µg/ml and 100 µg/ml, and 20 µg/ml and 100 µg/ml, respectively. These results indicate no bactericidal activities of Phx-1 and Phx-2 against *Helicobacter*. Difference of structures between Phx1 or Phx-2, and Phx-3 was the presence of methyl residues which give to the extension to three-dimensional change. It is possible that the planar structure of Phx-3 is important to its bactericidal activity. Bendic and Volanschi evaluated the intermolecular interactions between the drug-nucleic acid complexes by modeling based on molecular mechanics optimization.\(^{19}\) Their molecular modeling of questiomycin A (Phx-3) suggested that it can bind in the minor groove after intercalation in double-stranded DNA. The bactericidal activity of Phx-3 might be therefore due to interaction with the genomic DNA of *H. pylori*.

It is not clear why Phx-3 shows the specificity of the bactericidal activity for *Helicobacter*. Species specific intrinsic drug resistance is thought to be determined by several factors such as size of porin(s), efflux pump, modification of drug, affinity of active point and surface charge. Exner et al.\(^ {20} \) identified four pore forming outer membrane proteins related to porin, and the channel size of porin in *H. pylori* was relatively small. This could contribute to the resistance of *H. pylori* to hydrophilic antibiotics. Among them, the efflux of the toxic drugs is an important factor controlling the intrinsic sensitivity. *H. pylori* is highly sensitive to many hydrophilic and hydrophobic agents despite relatively low susceptibilities to the polycation polymyxin B and cationic antimicrobial peptides.\(^ {21} \) Bina et al.\(^ {22} \) proposed that reasonable uptake of hydrophobic substances coupled with nonfunctioning efflux systems could explain the relatively high susceptibility of *H. pylori* to hydrophobic antibiotics. Phx-3 might therefore behave like other hydrophobic antibiotics with respect to uptake into and efflux from bacterial cells. After accumulation of Phx-3, the molecules may intercalate into the double-stranded DNA and inhibit transcription.

In summary, we demonstrated the specific bactericidal activity of Phx-3 against *H. pylori in vitro*. This specific sensitivity of *H. pylori* seems to be due to the genus specific structure of cell-surface. And, possible mechanism of bactericidal activity of Phx-3 is interaction with bacterial DNA and inhibition of its transcription. On the other hand, effects of Phx-3 on the eukaryote cells were previously assessed by Yamaguchi and colleagues.\(^3 \) They described that concentrations of less than 10 µM of Phx-3 did not show cytotoxicity to HEP-2 and THP-1 cells.

These results obtained provide important information for development of novel anti-*H. pylori* drugs.

### REFERENCES


