In Vitro Antibacterial Activity of Phx-3 against *Helicobacter pylori*

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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 musterae* 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

Phonoxyazine derivatives which are produced by microorganisms[1—3] and found in insect[4] possess various biological activities such as anti-tumor[2] and anti-microbial activities.[1,5—8] Although biological activity of actinomycin was elucidated, other phenoxyazine derivatives have not been examined well. We have been studied the anti-bacterial effects of Phx-1 (2-amino-4,4alpha-dihydro-4alpha-7-dimethyl-3*H*-phenoxazine-3-one), Phx-2 (3-amino-1,4alpha-dihydro-4alpha-8-dimethyl-2*H*-phenoxazine-2-one) and Phx-3 (2-amino-phenoxazine-3-one), which are produced by the reaction of *o*-aminophenol or its derivatives with bovine hemoglobin.[7] Additionally, the chemical structure of Phx-3 (Fig. 1) is identical to questiomycin A, which was isolated from the culture fluids of *Streptomyces* by Anzai *et al.*[1] as a compound with bactericidal activity against *Mycobacterium tuberculosis*. Subsequent studies showed that Phx-3 also inhibits the growth of non-tuberculosis *Mycobacterium* species.[7] In contrast, some other Gram-negative and Gram-positive bacteria were resistant to as much as 100 μg/ml of Phx-3[7] except for facultative intracellular bacteria *Chlamydia pneumoniae*.[8]

Helicobacter pylori, which infects approximately 50% of the population worldwide, is a causative agent for chronic gastroduodenal ulcer, and possibly gastric cancer. Triple therapy consisting of amoxicillin, clarithromycin (CAM) and a proton pump inhibitor has proven to be effective for the eradication of *H. pylori* so far.[9] As in the case of other bacteria, however, there has been an increase in the appearance of drug-resistant strains of *H. pylori*. It has been reported that failure of eradication of *H. pylori* infection with triple therapy was approximately 20% of infected patients in Japan.[10] With the increase in the frequency of CAM-resistant *H. pylori*, there is rising concern about the potential decline in the eradication rate of this infection.[11] Therefore, it is important to develop new drugs to combat this bacterium. In the present study for further investigation of possibility of phenoxazine derivatives as bactericidal compound, we examined the effects of Phx-3 on *H. pylori*.

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 supplemented to the medium. 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 according to the method described in.[12] Phx-3 was dissolved in methanol as a stock solution at the concentration of 25 mM and was stored at −20 °C until use. The chemical structure of Phx-3 is illustrated in Fig. 1.

Measurements of the Minimal Inhibitory Concentration (MIC) for *H. pylori* We evaluated the ability of Phx-3 to inhibit the growth of *H. pylori* by determining the MICs as described by the guidelines of the Clinical and Laboratory Standards Institute for Clinical Laboratory Standards.[13] Briefly, the 72-h old subcultures diluted and adjusted to the 1×10^7 to 1×10^8 colony forming units per ml were replicated
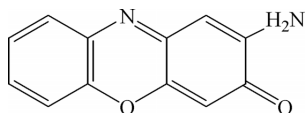

Fig. 1. Chemical Structure of Phx-3 as Identified by Shimizu *et al.*[7]

The structure, 2-amino-phenoxazine-3-one is identical to that of questiomycin A.[2]

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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 OsO_4 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 (Biodesign International, Saco, ME, U.S.A.) and H20[14] 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,[7] 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.[15—17] 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 micro-
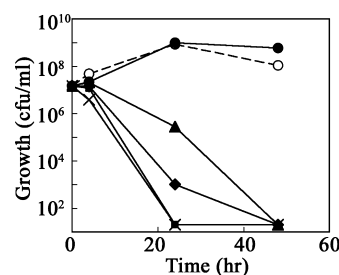

Fig. 2.   Bactericidal Effect of Phx-3 on *H. pylori* ATCC 43504

The cells were treated with Phx-3 at 0.5 μg/ml (solid circles), 1 μg/ml (solid triangles), 2 μg/ml (solid diamonds), 4 μg/ml (solid squares) or 8 μg/ml (crosses). The cellular viability was determined at 0, 3, 24, and 48 h by the viable cell counting on Brucella agar supplemented with 7% horse serum. The number of bacteria in the culture in the absence of Phx-3 was also measured (open circles). All experiments were performed in duplicate.
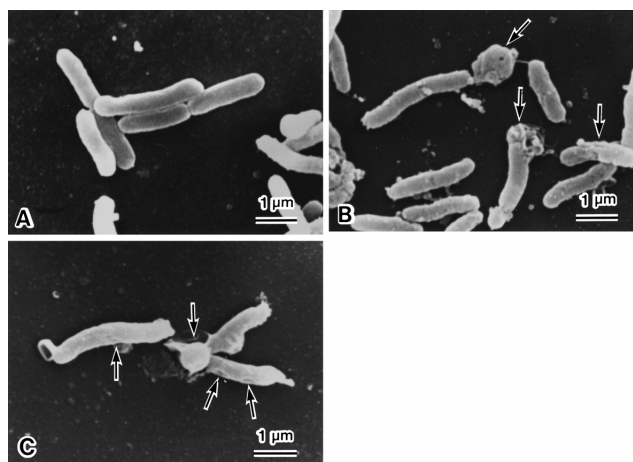

Fig. 3.   Scanning Electron Microscopy of *H. pylori* Treated with (B and C) or without (A) Treatment of 6 μg/ml Phx-3

Formation of spherical cells and membrane blebbing were observed (arrows) in Phx-3-treated cells. Bars represent 1 μm.

graphs 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 Opm19 proteins between Phx-3 treated and un-treated *H. pylori* (Fig. 4A). The result showed that the outer membrane was not exfoliated by the addition of Phx-3 contrary to our expectation. We also compared the amount of HSP60, general stress protein of *H. pylori* after treatment with Phx-3. It was shown that Phx-3 treated 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*.
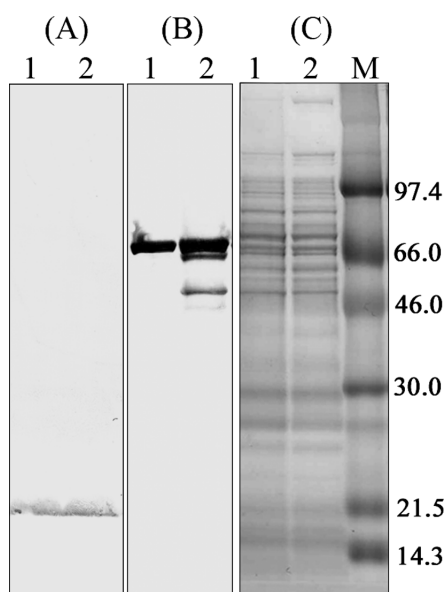
Fig. 4. Whole Cell Lysates of *H. pylori* ATCC 43504 with or without Treatment of Phx-3 Were Examined by Polyacrylamide Gel Electrophoresis, followed by Western Blotting with Anti-Omp19 (A) and Anti-HSP-60 Antibodies (B) or Staining with Coomassie Brilliant Blue (C)

Lane 1, *H. pylori* ATCC43504; lane 2 *H. pylori* ATCC43504 treated with Phx-3; lane M: protein molecular weight markers. Numbers at the left of lane M indicate the molecular weights of the markers.

Yamaguchi and coworkers[14] 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 s arming 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 swarming 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 exten-

sion 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*. Specie 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.[8] 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.

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