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Phenoxazine Compounds Produced by the Reactions with Bovine Hemoglobin Show Antimicrobial Activity Against Non-tuberculosis Mycobacteria

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SHIMIZU, S., SUZUKI, M., TOMODA, A., ARAI, S., TAGUCHI, H., HANAWA, T. and KAMIYA, S. *Phenoxazine Compounds Produced by the Reactions with Bovine Hemoglobin Show Antimicrobial Activity Against Non-tuberculosis Mycobacteria.* Tohoku J. Exp. Med., 2004, **203** (1), 47-52 —— We studied the anti-microbial effects of phenoxazines produced by the reaction of *o*-aminophenol or its derivatives with bovine hemoglobin, on seven species of mycobacteria such as *Mycobacterium tuberculosis, Mycobacterium marinum, Mycobacterium intracellulare, Mycobacterium scrofulaceum, Mycobacterium fortuitum, Mycobacterium kansasii* and *Mycobacterium smegmatis* and some bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus, Listeria monocytogeneses*. These phenoxazines, including 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxzine-3-one (Phx-1),3-amino-1,4α-dihydro-4α,8-dimethyl-2H-phenoxazine-2-one (Phx-2), and 2-aminophenoxazine-3-one (Phx-3), prevented the proliferation of four non-tuberculosis mycobacteria including *M. scrofulaceum, M. kansasii, M. marinum*, and *M. intracellulare* dose-dependently, though the inhibitory effects of these phenoxazines differed according to the species of mycobacteria. However these phenoxazines failed to prevent the proliferation of *M. tuberculosis, M. fortuitum*, and *M. smegmatis*, and the concerned bacteria other than mycobacteria. The present results may contribute to development of novel antibiotics against non-

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Abbreviations: Phx-1, 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one; Phx-2, 3-amino-1,4α-dihydro-4α,8-dimethyl-2H-phenoxazine-2-one; Phx-3, 2-aminophenoxazine-3-one; MIC, minimum inhibitory concentration.

tuberculolsis mycobacteria. ——— phenoxazines; antimicrobial effects; *Mycobacterium*
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Though phenoxazines are found in the actinomycins (Brockmann and Muxfeldt 1956; Hollstein 1974), in various insect pigments called ommochromes (Butenandt et al. 1960), and in some microorganism metabolites (Anzai et al. 1960), and chemically synthesized by oxidative condensation of *o*-aminophenol and its derivatives (Gerber and Lechvalier 1964; Hishida et al. 1974), the biological effects of the phenoxazines remained obscure, except for actinomycin D, which shows strong anti-cancer effects by inhibiting DNA dependent RNA polymerase (Hollstein 1974). Chemically synthesized phenoxazines seemed to have little anti-microbial effects (Gerber and Lechevalier 1964) and anti-cancer effects (Motohashi et al. 1991), probably because of poor solubility in water. On the other hand, we found that novel phenoxazines such as 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx-1), 3-amino-1,4α-dihydro-4α,8-dimethyl-2H-phenoxazine-2-one (Phx-2) are biosynthesized by the reaction of 2-amino-5-methylphenol or 2-amino-4-methylphenol with human or bovine hemoglobin (Tomoda et al. 1991, 2001), are relatively soluble in water, and show anti-cancer effects both in vivo and in vitro (Shimamoto et al. 2001; Koshimu-Koizumi et al. 2002). We also reported that Phx-1 and Phx-2 have immunosuppressive activities (Akazawa et al. 2002; Gao et al. 2002).

We recently found that Phx-1 and Phx-2 show antiviral activity against the proliferation of poliovirus infected in Vero cells (Iwata et al. 2003). This result suggests that Phx-1 and Phx-2 may exert anti-microbial activities, and prompted us to investigate whether Phx-1 and Phx-2 may have anti-microbial effects on various species of bacteria, especially on mycobacteria. We furthermore studied the effects of 2-aminophenoxazine-3-one (Phx-3) having the most simple structure among phenoxazines, on the bacteria, as well, because questiomycin A, which shows anti-tuberculotic activity has been known as 2-aminophenoxazine-3-one (Anzai et al. 1960). The present manuscript is concerned with the anti-microbial effects of Phx-1, Phx-2 and Phx-3 on seven species of mycobacteria and several bacteria.

Materials and Methods

Preparation of bovine hemolysates

Fresh bovine blood (1.6 l) obtained from a local abattoir, Tokyo, was treated with citrate as an anticoagulant, then centrifuged at 8000×*g* for 2 minutes. After removal of plasma and buffy coats, erythrocytes were suspended in 4 volumes of 0.9% NaCl solution, and centrifuged at 8000 ×*g* for 2 minutes. The pelleted erythrocytes were again suspended with the same solution and centrifuged. Then, the erythrocytes were lyzed with 5 volumes of distilled water. After standing for 10 minutes at room temperature, the lysates were centrifuged at 10 000×*g* for 20 minutes so as to remove erythrocyte membranes. About 3 l of hemolysates, including bovine hemoglobin, were obtained, and were used for the reaction with 2-amino-5-methylphenol or 2-amino-4-methylphenol, and *o*-aminophenol to obtain Phx-1 (Tomoda et al. 2001), Phx-2 (Tomoda et al. 1991) and Phx-3.

Preparation of Phx-3

Phx-3 was prepared principally based on the preparative method for Phx-1 (Tomoda et al. 2001), and was purified by using a column of Sephadex LH 20 (4 cm×50 cm), previously equilibrated with 50% ethanol, and eluted with 50% ethanol. The major eluates comparable to the absorption spectra of questiomycin A (Anzai et al. 1960) were collected and identified as Phx-3, by measuring UV and visible spectra, ^{1}H-nuclear

magnetic resonance (NMR) and ^{13}C-NMR spectra, and IR spectra. NMR and IR spectra were measured with JNM-ECP300 (JEOL, Tokyo), and IR700 (JASCO, Tokyo), respectively.

Evaluation of anti-microbial effects of Phx-1, Phx-2 and Phx-3 on various bacteria

Seven mycobacterial strains were precultured at 37°C for 1-3 weeks on 1% Ogawa medium (Nissui, Tokyo). *E. coli, P. aeruginosa, S.* Typhimurium, *S. aureus* and *L. monocytogenes* strains were precultured at 37°C in Mueller Hinton broth and brain heart infusion (BHI) broth, respectively, until its OD_{600} (optical density at 600 nm) reaches 1.0.

Minimum inhibitory concentration (MIC) of Phx-1, Phx-2 and Phx-3 against various bacteria

MIC of Phx-1, Phx-2 and Phx-3 against various bacteria was determined by microdilution method. Broth culture containing approximately 1.0×10^8 CFU (colony forming unit) ml of *E. coli, P. aeruginosa, S.* Typhimurium, *S. aureus* or *L. monocytogenes* was diluted to 200 fold, and each 100 μl suspension was inoculated onto 96-well microplate (approximately 5.0×10^4 CFU/well). Then, 100 μl of serially diluted Phx compounds was added and followed by 20-hour incubation at 37°C. One loopful of mycobacterial strains grown on 1% Ogawa medium was suspended in 4 ml of Myco broth (Kyokuto, Tokyo), resulting in approximately 0.8 of McFarland turbidity. Then, 10 μl of the suspension was inoculated into 90 μl of Myco broth in the presence of serially diluted Phx compounds followed by 7-day incubation at 37°C. Phx compounds were firstly solved in ethanol at 1-1.5 mg/ml and serially diluted in growth medium (Mueller Hinton broth, BHI broth or Myco broth) used for each bacterial strain. MIC was determined as the lowest concentration of Phx compounds showing a significant growth inhibition against each strain tested.

Results

The chemical structure of phenoxazine compounds, Phx-1, Phx-2 and Phx-3 used in the present study, is shown in Fig.1. Since a phenoxazine compound obtained by the reaction of o-aminophenol with bovine hemoglobin solution has not been identified in detail, we measured IR spectra, ^{1}HNMR and ^{13}CNMR spectra and UV and visible spectra of the compound prepared by the reaction of o-aminophenol with bovine hemoglobin solution. Consequently, the results showed that the phenoxazine compound is 2-aminophenoxazine-3-one, being identical to questiomycin A (Anzai et al. 1960). Measured data are: IR(KBr): 3401, 3308, 1656, 1588, 1495, 1463, 1291, 1272, 1203, 1173 cm^{-1}; ^{1}HNMR (300MHz, DMSO-d_6): δ6.37 (s, IH), 6.38 (s, 1H), 6.82 (bs, 2H), 7.37-7.53 (m, 3H), 7.71 (dd, 1H); ^{13}CNMR (76MHz, DMSO-d_6): δ 180.3, 149.0, 148.3, 147.4, 142.0, 133.8, 128.9, 128.0, 125.4, 116.0, 103.5, 98.4; APCI-MS: 213 (MH^+).

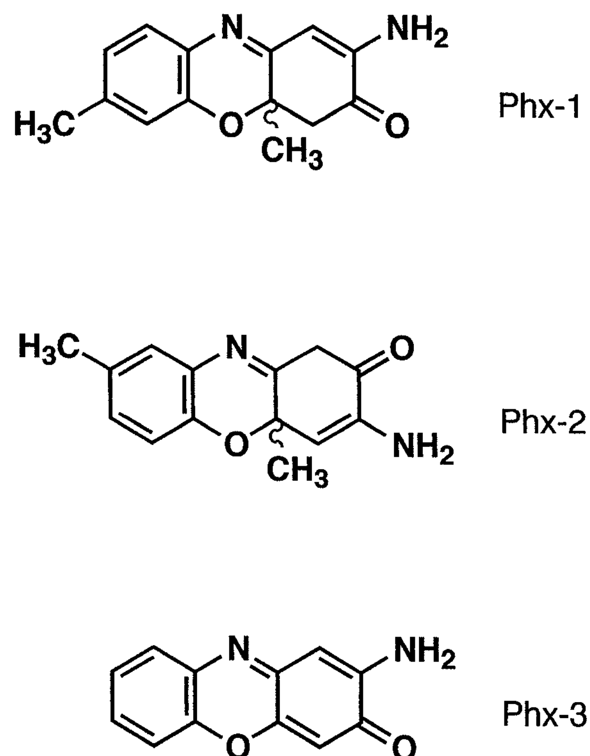

Fig. 1. Chemical structure of Phx-1, Phx-2 and Phx-3.

TABLE 1. *Antimicrobial activity of Phx-1, Phx-2 and Phx-3 against mycobacterial strains*

Test organisms	Strain No.	Minimum inhibitory concentration (μg/ml)		
		Phx-1	Phx-2	Phx-3
Mycobacterium scrofulaceum	ATCC19981	2.8	1.4	2.8
Mycobacterium kansasii	ATCC12478	22.5	11.3	> 45
Mycobacterium marinum	JM-1	> 45	> 45	11.3
Mycobacterium intracellulare	ATCC15984	> 45	> 45	5.6
Mycobacterium Tuberculosis	H37Ra	> 45	> 45	> 45
Mycobacterium fortuitum	ATCC19542	> 45	> 45	> 45
Mycobacterium smegmatis	No. 1	> 45	> 45	> 45

Table l summarizes minimum inhibitory concentration (MIC) of Phx-1, Phx-2 and Phx-3 against on seven mycobacteria. These phenoxazines exerted antimicrobial effects on four species of mycobacteria. Namely, proliferation of *M. scrofulaceum* was most remarkably inhibited by Phx-1, Phx-2 and Phx-3 (MIC was 2.8 μg/ml for Phx-1, 1.4 μg/ml for Phx-2 and 2.8 μg/ml for Phx-3). Proliferation of *M. kansasii* was significantly inhibited by Phx-1 and Phx-2 (MIC was 22.5 μg/ml for Phx-1 and 11.3 μg/ml for Phx-2), but was not effectively affected by Phx-3. Proliferation of *M. marinum* and *M. intracellulare* was significantly inhibited by Phx-3 (MIC was 11.3 μg/ml and 5.6 μg/ml, respectively), but was not affected by Phx-1 and Phx-2. In spite of these facts, *M. tuberculosis*, *M. fortuitum*, and *M. smegmatis* were not affected by these phenoxazines. Phx-1, Phx-2 and Phx-3 exerted no antimicrobial effect on *E.coli*, *P. aeruginosa*, *S.* Typhimurium, *S. aureus*, and *L. monocytogenes*, though not shown in the table.

DISCUSSION

Tomoda et al. (1987, 1991, 2001) reported that three phenoxazine compounds were produced by the reaction of o-aminophenol and its derivatives with human or bovine hemoglobin. The biological activity of these phenoxazines still remains clarified, though Phx-1 has been recently characterized by its anti-cancer activity (Shimamoto et al. 2001; Koshibu-Koizumi et al. 2002), immunosuppressive activity (Akazawa et al. 2002; Gao et al. 2002) and antiviral activity (Iwata et al. 2003). The chemical structure of these phenoxazines are listed in Fig.1. Anzai et al. (1960) found that questiomycin A, a phenoxazine compound, produced by the *Streptomyces* isolated from the soil in Tokyo, was identified as 2-aminophenoxazine-3-one and was demonstrated to exert antibiotic activity against *M. tuberculosis*. However, further investigation for the biological effects of questiomycin A has not been undertaken. According to the present results, the chemical structure of Phx-3 was identical to that of questiomycin A (Fig.1). Therefore, we expected that Phx-3 would inhibit the proliferation of *M. tuberculosis*, however, it showed no inhibitory effects, though it had antimicrobial activity against *M. scrofulaceum, M. intracellulare*, and *M. marinum* (Table 1). One plausible explanation is that *M. tuberculosis* H37Ra strain used in our present experiments is different in terms of drug sensitivity to phenoxazine compounds from *M. tuberculosis* strains (BCG and H-2) used in the experiment by Anzai et al. (1960). In addition, Anzai et al. (1960) applied Kirchner's medium for *M. tuberculosis*, and determined the antimicrobial effect of questiomycin A at 21 days after inoculation. It is possible that the differences in the medium and cul-

ture period for *M. tuberculosis* between the experiments by Anzai et al. (1960) and us might be associated with the disaccording results.

Though Phx-1, Phx-2 and Phx-3 showed considerable antimicrobial activity against non-tuberculosis mycobacterial strains such as *M. scrofulaceum, M. kansasii, M. marinum* and *M. intracellurale*, these phenoxazines did not show any significant inhibitory effects against *M. tuberculosis, M. fortuitum*, and *M. smegmatis*, and other bacteria tested in this study, suggesting that phenoxazine compounds can be used for treatment of the patients with non-tuberculosis mycobacterial infection. Cell wall structure of mycobacteria is unique and different from other Gram-positive and Gram-negative bacteria. It consists of innermost electron-dense layer (peptidoglycan and arabinogalactan layers), intermediate electron-transparent zone (mycolate layer) and outermost electron-dense layer composed of assorted lipoglycans, free polysaccharides, glycolipids and phospholipids (Vissa and Brennan 2002; Brennan 2003). This characteristic structure of the cell wall of mycobacterial strains might be related with the higher sensitivity to phenoxazine compounds than other bacteria such as *E. coli, P. aeruignosa, S.* Typhimurium*, S. aureus* and *L. monocytogenes*. In the present study, *M. scrofulaceum* was shown to be the most sensitive mycobacterial strain to phenoxazine compounds. Interestingly, *M. marinum and M. intracellulare* were sensitive to Phx-3 only. In contrast, *M. kansasii* was relatively sensitive to Phx-1 and phx-2, but not to Phx-3. The reason why several non-tuberculosis mycobacterial species were sensitive to some type of phenoxazine compound is unclear at present, because there seems little relationship of action and the chemical structure among three phenoxazines. However, it is possible that biosynthesis of mycobacterial cell wall including pathways, regulation and assembly of cell wall components might be different among various mycobacterial species, resulting in different sensitivity to phenoxazine compounds. The details of this assumption should be clarified in future.

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