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Electron-microscopic Study of the Bactericidal Effect of OPB-2045, a New Mono-biguanide Disinfectant Produced from Biguanide Group Compounds, against *Pseudomonas aeruginosa*

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Abstract

The bactericidal activity of OPB-2045 (1-(3,4-dichlorobenzyl)-5-octylbiguanide monohydrochloride hemihydrate) at several concentrations against *Pseudomonas aeruginosa* IFO 13275 was investigated morphologically by transmission and scanning electron microscopy.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of OPB-2045 against *P. aeruginosa* were the same, at 12·5 μg mL^{-1}, suggesting that it may be a suitable disinfectant for use in the medical field. Test bacteria were treated at concentrations of one half the MIC value (6·25 μg mL^{-1}), the MIC value (12·5 μg mL^{-1}), twice the MIC value (25 μg mL^{-1}) or ten times the MIC value (125 μg mL^{-1}) at 37°C for 30 min or 6 h and the cells were then examined by transmission and scanning electron microscopy. The cell damage evident after 6 h incubation was greater than observed after 30 min incubation. Especially, at one half the MIC, no cell damage was evident after 30 min incubation, but damaged cells were observed after 6 h incubation. The proportion of empty cells of *P. aeruginosa* increased as the concentration of added disinfectant was increased, and the release of intracellular components was also recognized. These results suggest that OPB-2045 acts on the cell membrane and cell wall of *P. aeruginosa*, and destroys their integrity at the level of the MIC (MBC). With the increase in OPB-2045 concentration and the increase in reaction time, the bactericidal effect increased markedly. Agglutination of the cells was observed at high concentrations of OPB-2045. This indicates that the bactericidal effect at high concentrations of OPB-2045 differs from that at low concentrations. A clear cell-damaging effect against the test strain was recognized which was dependent on the OPB-2045 concentration and the incubation time. From experiments concerning the relationship between the number of surviving bacteria and MIC values in soybean casein digest broth, the decrease in bacterial numbers was found to be dependent on the OPB-2045 concentration.

We conclude that it would be a useful contribution to the medical field to supply a new disinfectant to be employed in preventive countermeasures against infection caused by pathogenic bacteria.

Disinfectants have been used widely in preventive countermeasures against bacteria that cause infections such as *Pseudomonas aeruginosa*, methicillin-resistant*Staphylococcus aureus* (MRSA), and others. Recently, some modifications of disinfectant preparations including changes in the chemical structure of the active agent have been performed, and preparations with minor changes, based on the commercially available disinfectants, have appeared on the market, but it is still desirable to produce a new type of disinfectant so that preventive countermeasures against infectious bacteria might be performed more widely in the hospital. A new disinfectant, OPB-2045 (1-(3,4-dichlorobenzyl)-5-octylbiguanide monohydrochloride hemihydrate), has been developed containing a biguanide active base. In this report, we investigated the bactericidal activity of OPB-2045 against *P. aeruginosa*, a representative Gram-negative bacterium, by electron microscopy.

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Materials and Methods

Bacterial strain

P. aeruginosa IFO 13275, purchased from the Institute for Fermentation of Osaka, was used in this study.

Culture media

Soybean casein digest (SCD) broth (Nihon Pharmaceutical Co., Ltd.) was used for pre-incubation of the test bacteria. Muller-Hinton (MH) Agar (DIFCO) was used for determination of minimum inhibitory concentration (MIC), and Muller-Hinton (MH) broth (DIFCO) was used for determination of minimum bactericidal concentration (MBC).

Disinfectant

OPB-2045, a new disinfectant based on the biguanide group of compounds (Otsuka Pharmaceutical Co., Ltd), was used in this study.

MIC and MBC

The MIC of OPB-2045 against *P. aeruginosa* was determined by the agar dilution method described for Japan Society of Chemotherapy (Goto et al 1981) using micro-planters (Sakuma Seisakusyo Co., Ltd, Japan). The MBC of OPB-2045 against *P. aeruginosa* was determined by the method of Beck et al (1977).

Transmission electron microscopy

Transmission electron microscopy (JEOL, JEM-1200EX) was performed according to the method of Hayat (1986). An SCD broth culture of *P. aeruginosa* was centrifuged at 2500 rev min^{-1} for 15 min, the supernatant was removed, and the residue was washed twice with sterilized physiological saline. Two millilitres of test sample solution at each concentration (control, 6·25, 12·5, 25 or 125 $\mu g\,mL^{-1}$) was added, and the samples were incubated at 37°C for 30 min or 6 h. After centrifugation (2500 rev min^{-1} for 15 min), 5 mL modified Karnovsky solution (a mixture of 1% paraformaldehyde and 1% glutaraldehyde; Karnovsky 1965) was added, and the samples were incubated for 2 h at 20°C. After removal of the modified Karnovsky solution, the samples were further processed for transmission electron microscopy by routine methods, and the morphology of the test bacteria was examined.

Scanning electron microscopy

Scanning electron microscopy (JEOL, JSM-T100) was performed according to the method of Hayat (1986). Glass filters overlaid with SCD agar (Nihon Pharmaceutical Co., Ltd) were inoculated with *P. aeruginosa*, and incubated overnight at 37°C. Each of the colonies on the glass filter was transferred to a screw-capped vial. Two millilitres of test sample solution at each concentration (6·25, 12·5, 25 or 125 $\mu g\,mL^{-1}$) or 2 mL control solution was added, and the samples were incubated for 30 min or 6 h at 20°C. The solution was discarded, 5 mL modified Karnovsky solution was added, and the samples were incubated for 2 h at 20°C. The samples were further processed for scanning electron microscopy by routine methods, and examined.

Relationship between the number of surviving bacteria and MBC values

OPB-2045 was diluted in distilled water or SCD broth (Nihon Pharmaceutical Co., Ltd), and 10 ml of test sample solution at each concentration (control, 6·25, 12·5, 25 or 125 $\mu g\,mL^{-1}$) was prepared. Ten millilitres of bacterial suspension (10^7 cells mL^{-1}) was added, and the cells were incubated at 37°C for 30 min or 6 h. Bacterial numbers in each test tube were determined by the agar dilution method using SCD agar.

Results

MIC and MBC of OPB-2045 against P. aeruginosa

The MIC and MBC of OPB-2045 against *P. aeruginosa* were the same, 12·5 $\mu g\,mL^{-1}$. The bactericidal effect of OPB-2045 as observed by transmission electron microscopy. Figures 1–6 show results demonstrating the bactericidal effect of OPB-2045 observed upon treatment of *P. aeruginosa*. No damage of control cells was found upon observing cells over a wide area at low magnification (Figure 1). Also, no change in the control group was found with respect to intracellular components, the cell membrane, or the cell wall, upon observation of ultra-thin sections of cells. At high magnification, three distinct layers, the cytoplasmic membrane, the peptidoglycan layer and the outer membrane, were clearly recognized (Figure 2). On the other hand, in the group treated with OPB-2045, the number of empty cells from which cell components had leaked, increased after treatment at 37°C for 30 min (Figure 3). At high magnification, irregular blebs were observed on the cell surface (Figure 4). Furthermore, the outer membrane had been destroyed and leakage of cell components was recognized (Figure 5). However, at an

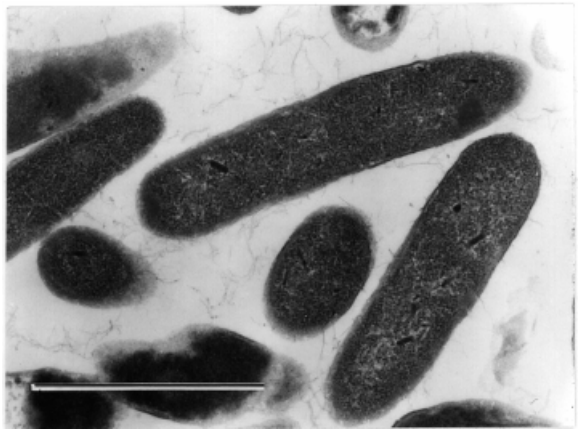

Figure 1. *Pseudomonas aeruginosa* (control) observed by ultra-thin section transmission electron microscopy (× 30 000 Scale 1 μm).

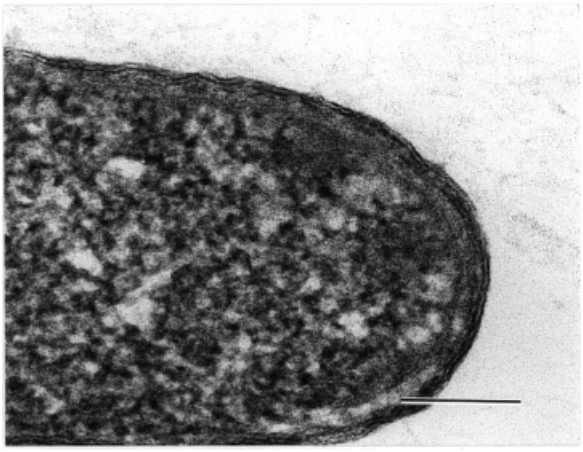

Figure 2. *Pseudomonas aeruginosa* (control) observed by ultra-thin section of transmission electron microscopy (× 160 000 Scale 0·1 μm).

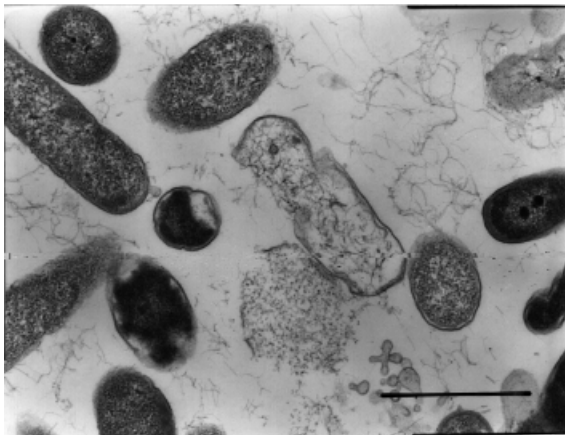

Figure 3. Bactericidal effect of OPB-2045 against *Pseudomonas aeruginosa* observed by ultra-thin section of transmission electron microscopy-OPB-2045 12·5 μg mL^{-1} treatment, 37°C, 30 min (20 000 Scale 1 μm).

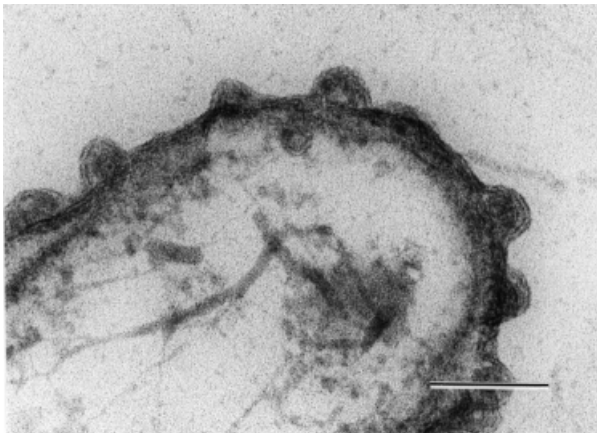

Figure 4. Bactericidal effect of OPB-2045 against *Pseudomonas aeruginosa* observed by ultra-thin section of transmission electron microscopy-OPB-2045 25 μg mL^{-1} treatment, 37°C, 30 min (× 160 000 Scale 0·1 μm).

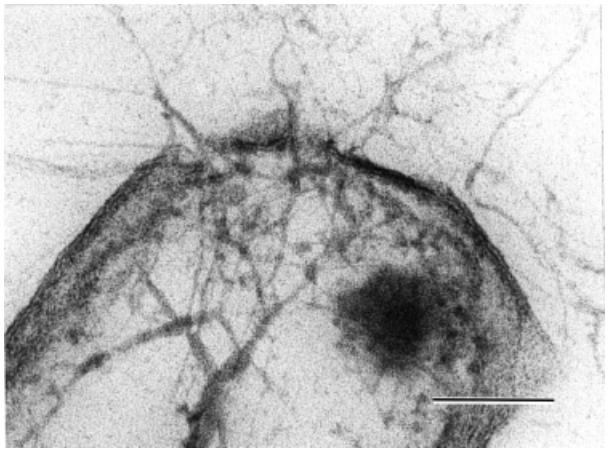

Figure 5. Bactericidal effect of OPB-2045 against *Pseudomonas aeruginosa* observed by ultra-thin section of transmission electron microscopy-OPB-2045 25 μg mL^{-1} treatment, 37°C, 30 min (× 160 000 Scale 0·1 μm).

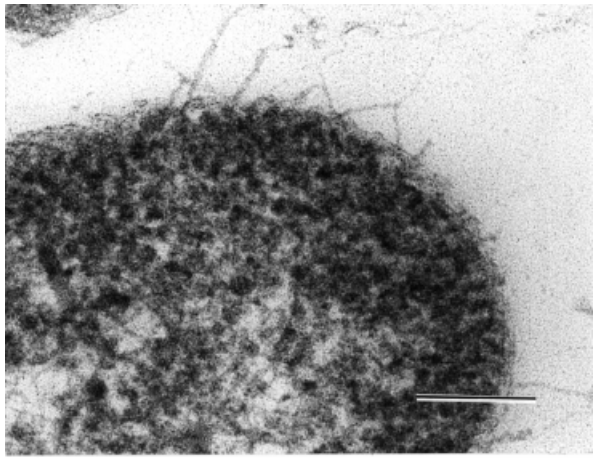

Figure 6. Bactericidal effect of OPB-2045 against *Pseudomonas aeruginosa* observed by ultra-thin section of transmission electron microscopy-OPB-2045 125 μg mL^{-1} treatment, 37°C, 30 min (× 160 000 Scale 0·1 μm).

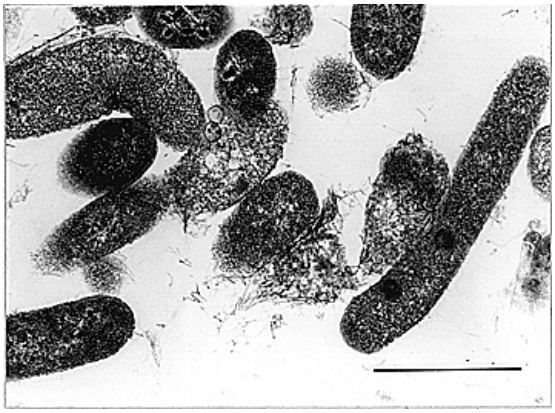

Figure 7. Bactericidal effect of OPB-2045 against *Pseudomonas aeruginosa* observed by ultra-thin section of transmission electron microscopy-OPB-2045 125 μg mL^{-1} treatment, 37°C, 30 min (× 20 000 Scale 1 μm).

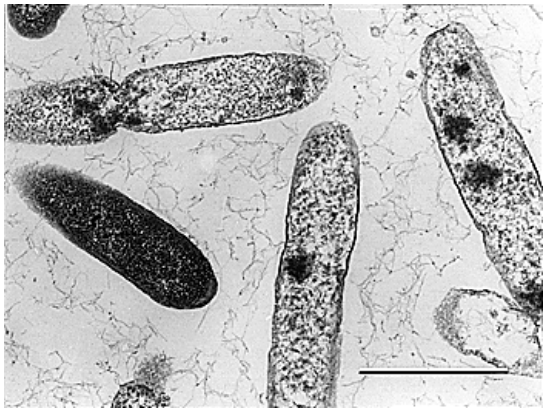

Figure 8. Bactericidal effect of OPB-2045 against *Pseudomonas aeruginosa* observed by ultra-thin section of transmission electron microscopy-OPB-2045 6·25 μg mL^{-1} treatment, 37°C, 6 h (× 20 000 Scale 1 μm).

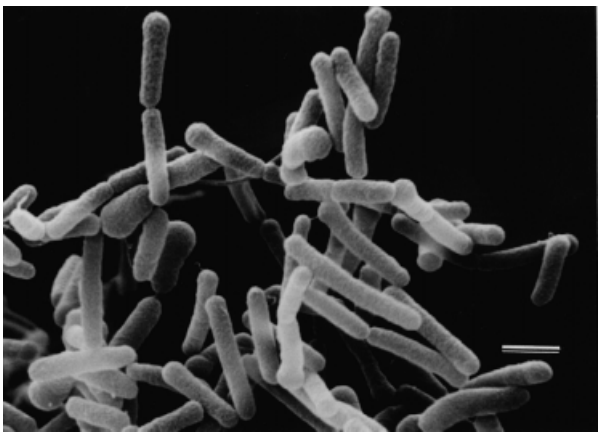

Figure 9. *Pseudomonas aeruginosa* (control) observed by scanning electron microscopy (× 5000 Scale 1 μm).

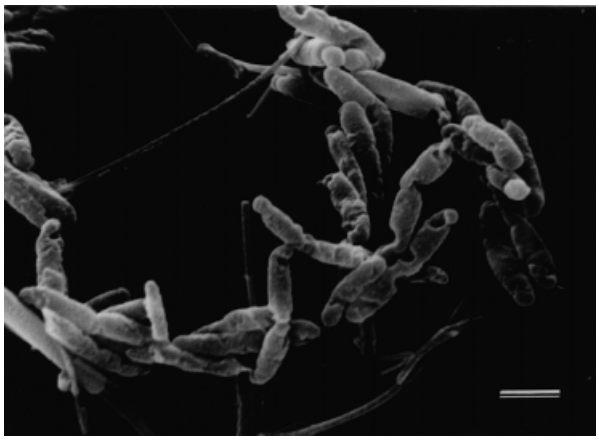

Figure 10. Bactericidal effect of OPB-2045 against *Pseudomonas aeruginosa* observed by scanning electron microscopy-OPB-2045 6·25 μg mL^{-1} treatment, 37°C, 30 min (× 5000 Scale 1 μm).

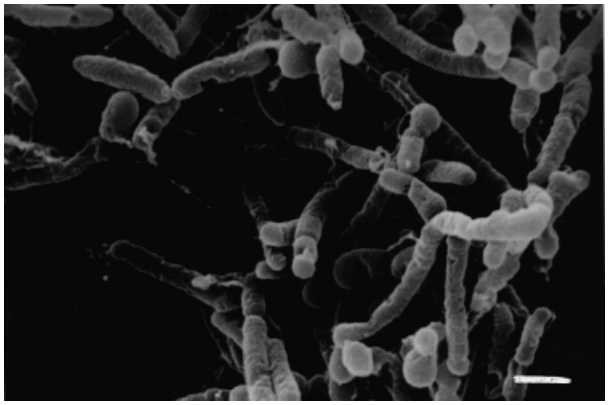

Figure 11. Bactericidal effect of OPB-2045 against *Pseudomonas aeruginosa* observed by scanning electron microscopy-OPB-2045 25 μg mL^{-1} treatment, 37°C, 30 min (× 5000 Scale 1 μm).

Figure 12. Bactericidal effect of OPB-2045 against *Pseudomonas aeruginosa* observed by scanning electron microscopy-OPB-2045 6·25 μg mL^{-1} treatment, 37°C, 6 h (× 5000 Scale 1 μm).

OPB-2045 concentration of ten times the MIC, the cell membrane and cell wall had been destroyed (Figure 6) and many agglutinated cells were recognized (Figure 7). After treatment at 37°C for 6 h, no damage was found in the control cells, but in the test group treated with OPB-2045 at one half the MIC, empty cells were clearly evident (Figure 8).

Bactericidal effect of OPB-2045 as observed by scanning electron microscopy

Figures 9–12 show results demonstrating the bactericidal effect of OPB-2045, including the morphological changes at the cell surface, observed upon treatment of *P. aeruginosa*. In the control group, no evidence of damaged cells, such as the appearance of holes or rough areas on the cell surface, was found after incubation for 30 min at 37°C (Figure 9). In the test groups, cells with such holes were found (Figure 10). With the increase in OPB-2045 concentration, the number of leaking cells increased markedly. At OPB-2045 concentrations of twice the MIC and ten times the MIC, many empty cells were observed (Figure 11). After incubation for 6 h at 37°C, in cells of the control group no change was observed. In the test groups, marked changes in the morphology of the cells were evident as compared with those incubated for 30 min at 37°C (Figure 12). With the increase in OPB-2045 concentration, the number of damaged cells increased markedly.

Relationship between the number of surviving bacteria and MIC values

Figure 13 shows results concerning the relationship between the number of surviving bacteria and MIC values. In experiments in which the cells were suspended in distilled water, although bacterial numbers in the control group did not change in the course of the 6-h incubation period, no viable bacteria were detected in the test groups treated with OPB-2045 at one half the MIC, the MIC, twice the MIC or ten times the MIC after 30 min or 6 h incubation. In experiments in which the cells were suspended in SCD broth, the bacterial numbers in the control group had increased after 30 min or 6 h incubation, but in the test groups treated with OPB-2045 a decrease in bacterial numbers was recognized and the extent was dependent on the OPB-2045 concentration.

Discussion

There have been many reports concerning the bactericidal activity of disinfectants as demonstrated by electron microscopy (Arimura & Kitagawa1973; Richards & Cavill 1976, 1979a, b; Hayat 1978; Gelinas & Goulet 1983; Bobichon & Bouchet 1987). Such studies have good visual impact allowing us to understand the bactericidal activity of the disinfectant. Transmission electron microscopy is usually employed to observe cell damage such as empty cells or leaking cells, and the overall cell damage is assessed by scanning electron microscopy. In this report, we used both types of electron microscopy to examine the effects of a new disinfectant OPB-2045. The morphological changes in *P. aeruginosa* cells resulting from treatment with OPB-2045 for 30 min or 6 h at concentrations of one half the MIC, the MIC, twice the MIC and ten times the MIC were studied in an effort to clarify the mechanism of action against *P. aeruginosa*. Results of transmission electron microscopy showed that in the control group there was no cell damage, such as changes in intracellular components, the cell membrane or the cell wall, after incubation at 37°C for 30 min or 6 h. In contrast, in the test groups treated with OPB-2045 the number of empty cells, from which the cell contents had leaked, increased in a concentration-dependent manner upon treatment at 37°C for 30 min. At the OPB-2045 concentration of ten times the MIC, agglutination of the cells in which the cell membrane and cell wall had been destroyed, was recognized. This phenomenon was similar to findings with chlorhexidine digluconate reported by Hugo & Longworth (1965). In the test groups treated with OPB-2045 at one half the MIC at 37°C for 6 h, empty cells were clearly evident, as compared with 30-min incubation. The results of scanning electron microscopy showed that in the control group no damaged cells, such as those with a hole or rough area on the cell surface, were present after 30 min or 6 h at 37°C, but in the test group such cells with holes in the cell envelope were found. The number of leaking cells increased markedly with the increase in OPB-2045 concentration and at concentrations of twice and ten times the MIC many empty cells were observed. The morphological changes in the test cells evident after 6-h incubation were more extensive than those observed after 30-min incubation. Especially at one half the MIC, no damaged cells were found after 30-min incubation, but damaged cells were observed after 6-h incubation. These results suggest that the number of damaged cells increases in accordance with the increase in incubation time in the presence of OPB-2045. Thus, the effectiveness of disinfection would increase with prolonged incubation. The proportion of empty cells of *P. aeruginosa* increased with the increase in con-

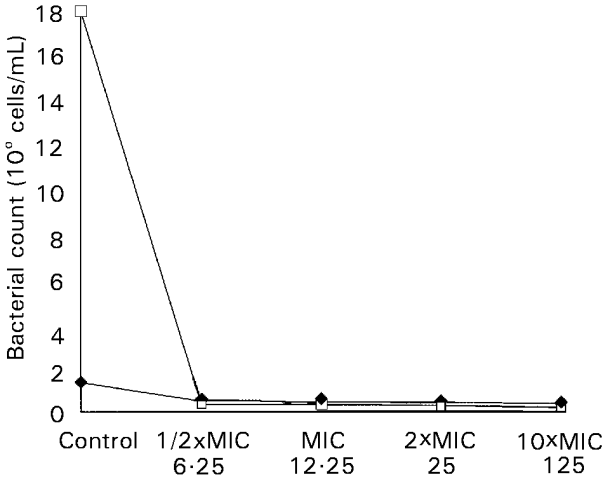

Figure 13. Relationship between the number of surviving bacteria in SCD broth and MIC values.

centration of disinfectant added, and the release of cell membrane components was also recognized. These results suggest that OPB-2045 acts on the cell membrane and cell wall of *P. aeruginosa* and destroys their integrity at the level of the MIC (MBC). The agglutination of cells was found at a high concentration of OPB-2045. This indicates that the bactericidal effect of OPB-2045 at high concentrations differs from that at low concentrations. A clear cell-damaging effect against the test strain was recognized and the extent of damage was dependent on the OPB-2045 concentration and the incubation time. Furthermore, in experiments concerning the relationship between the number of surviving bacteria and MBC values in SCD broth, a decrease in bacterial numbers was recognized and the extent was dependent on the concentration of OPB-2045 added. This time, we did not examine the bactericidal action of OPB-2045 with a short time period, such as 5 min. However, from our results, it seems likely that OPB-2045 would act to kill the *Pseudomonas aeruginosa* strain tested upon short-time incubation. From the above results, it is suggested that OPB-2045 displays suitable bactericidal action against *P. aeruginosa* and it may be an effective disinfectant for clinical use. However, further studies of its usefulness will be necessary to confirm whether it displays bactericidal effectiveness under field conditions such as in the presence of organic material. We finally emphasize that it would be a useful contribution to the medical field to supply a new disinfectant to be employed in preventive countermeasures against infection caused by pathogenic bacteria.

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