In Vitro Studies on the Biocompatibility of Bis-Quaternary Ammonium Compounds

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INTRODUCTION

In recent years, various emerging and reemerging infectious diseases, such as new influenza A (H1N1), Ebola hemorrhagic fever, dengue fever, and Zika fever, have been reported in many parts of the world[1–3]. Many of these infectious diseases are zoonotic diseases, which can be transmitted from animals to humans and from humans to animals; consequently, they pose new health threats to humans worldwide. There are also growing global concerns about the spread of drug-resistant bacteria arising from the inappropriate use of antibiotic feed additives in food-producing animals or antibiotics for the treatment of human infectious disease[4,5]. Healthcare-associated infections (HAI) caused by antibiotic-resistant bacteria have become a major threat, particularly to humans[6–8]. The World Health Organization has developed a Global Action Plan on antimicrobial resistance (AMR) and has implemented effective sanitation, hygiene, and infection prevention measures as one of the objectives of its action plan[9].

At healthcare facilities, in order to reduce the incidence of HAI, several antiseptics and disinfectants are frequently used for the hand hygiene of healthcare workers (HCWs), as skin antisepsis for patients, and for the disinfection of touchable environmental surfaces. In particular, antimicrobial substances such as chlorhexidine gluconate (CHG)[10–12], polyhexamethylene biguanide (PHMB)[13–17], benzalkonium chloride (BAC)[10,18], and octenidine dihydrochloride (OCT)[19–22] are widely used as active ingredients in skin antiseptics for human use. Unlike antibiotics, these antiseptics do not lead to AMR problems; therefore, antiseptics are expected to be used for infection control[23].

However, from a safety perspective, antiseptics do not possess the selective toxicity observed with antibiotics, so they are toxic not only to microorganisms, but also to the human body. Therefore, these antiseptics are cytotoxic to human cells, and they are reported to cause adverse effects such as irritant or allergic contact dermatitis. For example, although CHG has been mainly used for the antisepsis of normal skin and mucous membranes, such as burn sites and the oral cavity, there are case reports of contact sensitization and allergic contact dermatitis[24,25]. In particular, allergic contact dermatitis resulting from CHG has been well known since the report of Calnan in 1962[23,26]. In addition, irritant contact dermatitis resulting from the use of CHG by HCWs has also been reported[27]. BAC, the most extensively used quaternary ammonium salt, is used for the disinfection of hard-surfaces and for topical antiseptic applications. Although BAC is also used clinically for hand hygiene and the antisepsis of mucous membranes, there are some reports of irritant or allergic contact dermatitis and conjunctivitis[28–30]. OCT and PHMB are relatively new antiseptics used in Europe. PHMB is used principally to treat pressure wounds and OCT is used for mucosal antisepsis, such as chronic wounds and burns. A study that compared the biocompatibility index (BI) of various antiseptics, as defined by antibacterial activity and cytotoxicity in murine fibroblasts showed that OCT and PHMB had greater biocompatibility than other tested antiseptics such as CHG and BAC[31]. However, reports on the adverse effects caused by OCT and PHMB describe several cases of allergic or contact dermatitis after application to human skin. According to these reports, aseptic tissue necrosis and chronic inflammation were observed after a wound antiseptic containing 0.1% OCT was used for the irrigation of penetrating hand wounds[32]. In

addition, a long-term adverse effect related to the skin was reported following the inappropriate use of a wound antiseptic containing 0.1% OCT[33]. In this case, Bauer et al. reported that following lavage of an abscess in the inguinal region with a wound antiseptic containing 0.1% OCT, a painful erythematous induration mimicking cellulitis persisted for several months. With respect to adverse effects on the skin, PHMB has been reported to be an uncommon contact allergen[34] and severe anaphylaxis has been reported[35,36].

These reports indicate that currently used antiseptics are toxic to human cells. Therefore, the confirmation of a history of allergic sensitivity and the proper use of antiseptic products are necessary to avoid their adverse effects. Moreover, in healthcare facilities, the risk of adverse effects, such as hand roughness and irritant contact dermatitis of HCWs, increases as the frequency of antiseptic use increases. Antiseptics are essentially toxic to both the microorganisms and the human body; moreover, there are no antiseptics that are both effective and safe. Therefore, the development of new antiseptic agents is desired to provide more options for skin antiseptics, not only from the perspective of efficacy improvements, such as improved antimicrobial activity and broadening the antibacterial spectrum, but also for the reduction of adverse effects such as contact dermatitis.

In this study, in order to obtain new antimicrobial substances with superior biocompatibility than that of currently used antiseptic agents, various bis-quaternary ammonium compounds (bis-QACs) were newly synthesized and reported in Chapter 1, and their antimicrobial activity and cytotoxic effect on normal human epidermal keratinocytes (NHEK) were investigated. Furthermore, the BI defined by antimicrobial

activity and cytotoxicity in NHEK cells was compared with that of conventional antiseptic agents such as OCT.

In Chapter 2, to examine the degree of skin irritation of newly synthesized bis-QACs in more detail, their cytotoxicity to several types of cultured human skin cells was compared. Subsequently, inflammatory cytokine expression in NHEK cells and three-dimensional reconstructed human epidermis models was compared, with a focus on the changes in the transcription or translation of the inflammation-related genes induced by the antiseptic agents.

CHAPTER 1.

Synthesis and Biocompatibility of Novel Bis-Quaternary Ammonium Compounds with Spacer Structure Derived from Pentaerythritol or Hydroquinone

Quaternary ammonium compounds (QACs) are one of the most widely antimicrobial agents, as they are commonly used in healthcare settings, the textile industry, water treatment, and the food industry[37]. A study that evaluated the bactericidal activity and cytotoxicity to human cells of several newly synthesized bis-QACs revealed them to be potential antiseptics for human use[38]. However, there are no reports of the suitability of these bis-QACs for human use, unlike the clinical use of OCT for the antisepsis of mucous membranes, such as chronic wounds.

In this chapter, we have described the synthesis of a series of bis-QACs for the purpose of developing new antimicrobial substances with better biocompatibility than those of existing antiseptic agents such as OCT. The antimicrobial activity of these bis-QACs and existing antiseptic agents was evaluated through the determination of the minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC), and a comparison of the cytotoxicity of these compounds. Moreover, the BI of these bis-QACs and existing antiseptic agents was compared. BI is the ratio of the concentration of these compounds that induced 50% cell death in NHEK cells to the MBC of the antiseptic agents against gram negative and gram-positive bacteria.

SECTION 1.

Synthesis of novel bis-quaternary ammonium compounds

The main bactericidal mechanism of QACs is a result of the ionic bonding of the positively charged hydrophilic sites of quaternary ammonium salt molecules to the negatively charged bacterial cell surface. The resulting neutralization of the negative charge on the bacterial cell wall causes the destruction of the cell wall and structural damage of the cell membrane, which destroys the vital functions of the cell. Bis-QACs, with two cationic hydrophilic moieties in the molecule, can act more quickly on the negatively charged bacterial cell surface than mono-QACs with only one cationic moiety. Therefore, bis-QACs exhibits bactericidal activity against a wide range of microorganisms at a lower concentration than mono-QACs. Furthermore, it is known that the antibacterial activity of bis-QACs is not readily affected by interfering substances such as proteins and lipids, or ion composition such as pH and calcium hardness. One of the objectives of this study was to obtain an antimicrobial substance with antimicrobial properties equal to or higher than that of OCT. We deduced that it is possible to change the hydrophilic-hydrophobic balance through the introduction of, for example, an ether group in the spacer moiety of the OCT molecule. Therefore, in this study, we first designed a bis-QAC with a spacer structure different from that of OCT and examined the method of synthesis.

1-1. MATERIALS AND METHODS

1-1-1. Chemicals

All chemicals for the synthesis of the novel bis-QAC derivatives were commercial products of reagent grade and were used without further purification.

1-1-2. General method

The chemical structure and purity of the synthesized compounds were evaluated by nuclear magnetic resonance (^{1}H-NMR) spectrometry and thin layer chromatography (TLC). TLC was performed using precast silica gel plates (Merck silica gel 60 F254) and the separated materials were visualized by using a UV-light apparatus at a wavelength of λ=254 nm. ^{1}H-NMR spectra were recorded at ambient temperature by using a Bruker Avance II 300 Spectrometer, with tetramethylsilane used as the internal standard.

1-2. RESULTS AND DISCUSSION

1-2-1. Synthetic procedure for novel bis-QAC derivatives

Three novel bis-QAC derivatives, N-alkyl{4,4'-(2,4,8,10-tetraoxaspiro [5.5]-undecan-3,9-diyl) dipyridinium dibromide (4TOSU-n), 3,3'-[1,4-phenylenebis(oxy)]bis(1-alkylpyridinium) dibromide (3PHBO-n), and 3-(3-hydroxy-2-(hydroxymethyl)-2-{[(1-alkylpyridinium-3-yl)oxy]methyl}propoxy)-1-alkylpyridinium dibromide (3HHDMP-n) were synthesized by N-alkylation of the pyridine derivatives of pentaerythritol or hydroquinone with alkyl halides.

The synthesis of 4TOSU-n followed the procedure shown in Fig. 1-1. The synthesis of 4TOSU-n involved a two-step process. In the first synthesis step, 4-pyridinecarboxaldehyde, pentaerythritol, and p-toluenesulfonic acid (TsOH) in 200 mL of toluene were refluxed in a 500 mL three-necked flask equipped with a mechanical stirrer, a Dean-Stark trap, and a thermometer. As a result of this condensation reaction, 4,4'-(2,4,8,10-tetraoxaspiro [5.5] undecane-3,9-diyl) dipyridine (diketal) was obtained at a yield of 90%. In the second synthesis step, diketal and alkyl bromides were added to a 250 mL three-necked flask equipped with a stirrer, a thermometer, and a dropping funnel. The quaternization reaction of these mixtures was conducted by the addition of a small amount of water under solvent-free conditions to obtain 4TOSU-n with the desired bis-type structure at a yield of 80%–90%.

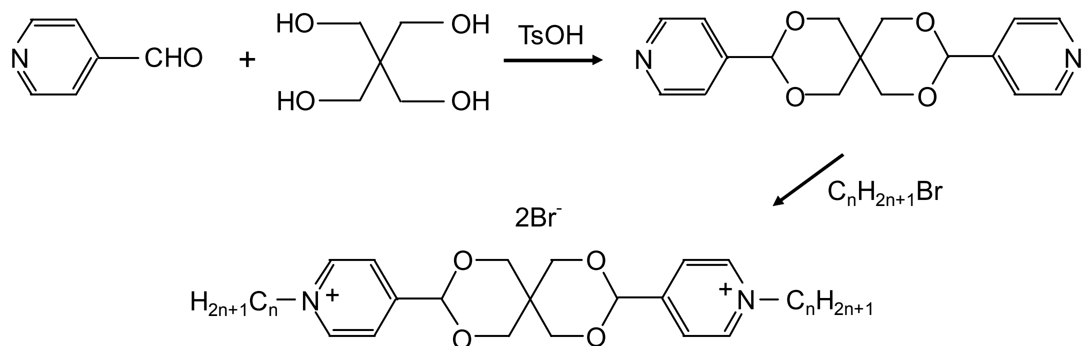


Fig. 1-1. Synthetic procedure of N-alkyl {4,4'-(2,4,8,10- tetraoxaspiro [5.5] -undecan-3,9-diyl) dipyridinium dibromide (4TOSU-n)

Subsequently, 3PHBO-n was synthesized according to the procedure shown in Fig. 1-2, which was also a two-step process. In the first step, 3-hydroxypyridine and 1,4-dibromobenzene were refluxed with stirring in dimethylformamide (DMF) in the presence of copper and potassium carbonate. This alkylation produced 3,3'-[1,4-phenylenebis (oxy)] dipyridine with a yield of 70%. In the second synthesis step, 3,3'-[1,4-phenylenebis (oxy)] dipyridine and alkyl bromides were introduced to a 250 mL three-necked flask equipped with a stirrer, a thermometer, and a dropping funnel. The quaternization reaction of these mixtures was conducted by the addition of a small amount of water in the absence of a solvent to obtain 3PHBO-n, with the desired bis type structure, at a yield of 60% to 70%.

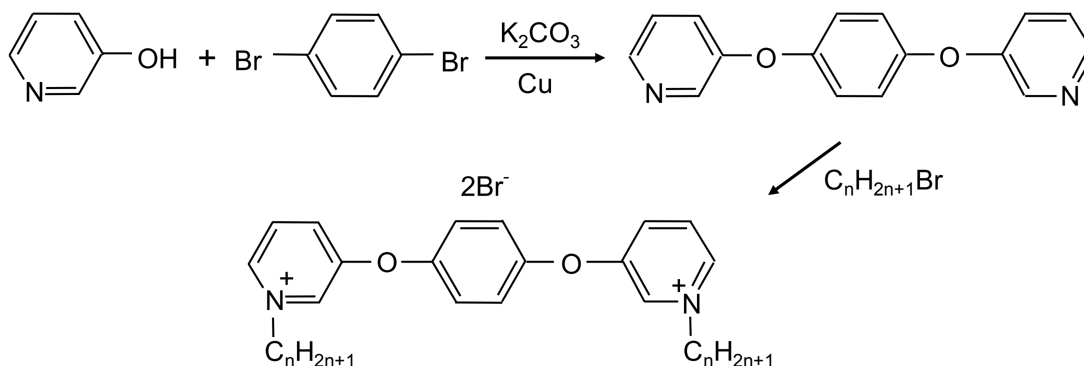


Fig. 1-2. Synthetic procedure of 3,3'-[1,4-phenylenebis (oxy)] bis (1-alkylpyridinium) dibromide (3PHBO-n)

The synthetic procedure of 3HHDMP-n is shown in Fig. 1-3. The synthesis of 3HHDMP-n was a five-step process. In the first step, pentaerythritol and benzaldehyde were reacted in toluene in the presence of TsOH to obtain a ketal diol at a yield of 75%. Subsequently, the ketal diol was reacted with p-toluenesulfonic chloride to obtain the ditosylate at a yield of 97%. The alkylation of 3-hydroxypyridine with the ditosylate in DMF in the presence of potassium carbonate produced 3,3'-[(2-phenyl-1,3-dioxane-5,5-diyl) bis (methyleneoxy)] at a yield of 88%. Furthermore, when the reaction compound was hydrolyzed in an aqueous solution of hydrochloric acid, 2,2-bis [(pyridine-3-yoxy) methyl] propane-1,3-diol was obtained at a yield of 78%. Finally, the quaternization of the reaction compound with alkylbromides was achieved by refluxing in 4-methylpentan-2-one to obtain 3HHDMP-n with the desired bis-QAC structures at a yield of 80% to 85%.

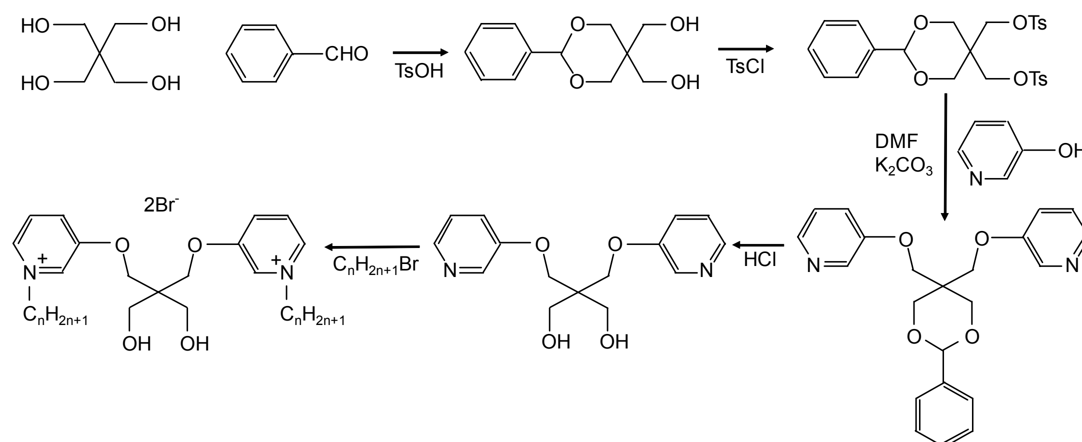


Fig. 1-3. Synthetic procedure for 3-(3-hydroxy-2-(hydroxymethyl)-2-{[(1-alkylpyridinium-3-yl) oxy] methyl} propoxy)-1-alkylpyridinium dibromide (3HHDMP-n)

The ^{1}H-NMR spectra for 4TOSU-n, 3PHBO-n, and 3HHDMP-n are shown in Table 1-1. The chemical shifts and integration values obtained were consistent with the expected structures of 4TOSU-n, 3PHBO-n, and 3HHDMP-n.

Table 1-1. ^{1}H-NMR Data for new compounds 4TOSU-n, 3PHBO-n, and 3HHDMP-n

Compounds	^{1}H-NMR (CD_3OD) δ(ppm)
4TOSU-8	0.84 (t, J = 7.0 Hz, 6H, CH_3), 1.18-1.32 (m, 20H, CH_2), 1.84-1.96 (m, 4H, CH_2), 3.81-4.13 (m, 6H, CH_2), 4.52-4.60 (m, 2H, CH_2), 4.66 (t, J = 7.4 Hz, 4H, N^+-CH_2), 5.88 (s, 2H, CH), 8.18 (d, J = 6.7 Hz, 4H, CH, Py), 9.21 (d, J = 6.7 Hz, 4H, N-CH, Py).
4TOSU-10	0.91 (t, J = 7.0 Hz, 6H, CH_3), 1.22-1.48 (m, 28H, CH_2), 1.96-2.12 (m, 4H, CH_2), 3.84-4.16 (m, 6H, CH_2), 4.62-4.78 (m, 6H, 2 N^+-CH_2, CH_2), 5.88 (s, 2H, CH), 8.21 (d, J = 6.4 Hz, 4H, CH, Py), 9.08 (d, J = 6.4 Hz, 4H, N-CH, Py)
4TOSU-12	.91 (t, J = 7.0 Hz, 6H, CH_3), 1.24-1.48 (m, 36H, CH_2), 1.96-2.12 (m, 4H, CH_2), 3.86-4.16 (m, 6H, CH_2), 4.62-4.80 (m, 6H, 2 N^+-CH_2, CH_2), 5.88 (s, 2H, CH), 8.20 (d, J = 6.4 Hz, 4H, CH, Py), 9.08 (d, J = 6.4 Hz, 4H, N-CH, Py)
3PHBO-8	0.88 (t, J = 7.0 Hz, 6H, CH_3), 1.26-1.56 (m, 20H, CH_2), 2.02-2.16 (m, 4H, CH_2), 4.75 (t, J = 7.4 Hz, 4H, N^+-CH_2), 7.48 (s, 4H, Ar), 8.13 (dd, J_1 = 8.8 Hz, J_2 = 6.0 Hz, 2H, CH, Py), 8.34 (dd, J_1 = 8.8 Hz, J_2 = 2.2 Hz, 2H, CH, Py), 8.83 (d, J = 6.0 Hz, 2H, N-CH, Py), 9.13 (d, J = 2.2 Hz, 2H, N-CH, Py)
3PHBO -10	0.88 (t, J = 7.0 Hz, 6H, CH_3), 1.24-1.54 (m, 28H, CH_2), 2.00-2.14 (m, 4H, CH_2), 4.74 (t, J = 7.4 Hz, 4H, N^+-CH_2), 7.47 (s, 4H, Ar), 8.07-8.17 (m, 2H, CH, Py), 8.29-8.35 (m, 2H, CH, Py), 8.79-8.86 (m, 2H, N-CH, Py), 9.10-9.16 (m, 2H, N-CH, Py)
3PHBO-12	0.87 (t, J = 7.0 Hz, 6H, CH_3), 1.24-1.56 (m, 36H, CH_2), 1.98-2.10 (m, 4H, CH_2), 4.73 (t, J = 7.4 Hz, 4H, N^+-CH_2), 7.46 (s, 4H, Ar), 8.11 (dd, J_1 = 8.6 Hz, J_1 = 6.0 Hz, 2H, CH, Py), 8.32 (dd, J_1 = 8.6 Hz, J_1 = 1.7 Hz, 2H, CH, Py), 8.80 (d, J = 6.0 Hz, 2H, N-CH, Py), 9.10 (d, J = 1.7 Hz, 2H, N-CH, Py)
3HHDMP-8	0.91 (t, J = 6.7 Hz, 6H, CH_3), 1.22-1.50 (m, 20H, CH_2), 2.00-2.16 (m, 4H, CH_2), 3.84 (s, 4H, O-CH_2), 4.43 (s, 4H, Py-O-CH_2), 4.66 (t, J = 7.5 Hz, 4H, N^+-CH_2), 8.03 (dd, J_1 = 8.6 Hz, J_2 = 5.8 Hz, 2H, CH, Py), 8.28 (dd, J_1 = 8.6 Hz, J_2 = 1.8 Hz, 2H, CH, Py), 8.64 (d, J = 5.8 Hz, 2H, N-CH, Py), 8.95-9.05 (m, 2H, N-CH, Py).
3HHDMP-10	0.91 (t, J = 6.7 Hz, 6H, CH_3), 1.18-1.50 (m, 28H, CH_2), 2.00-2.16 (m, 4H, CH_2), 3.84 (s, 4H, O-CH_2), 4.43 (s, 4H, Py-O-CH_2), 4.65 (t, J = 7.7 Hz, 4H, N^+-CH_2), 8.03 (dd, J_1 = 8.8 Hz, J_2 = 5.9 Hz, 2H, CH, Py), 8.27 (dd, J_1 = 8.8 Hz, J_2 = 1.9 Hz, 2H, CH, Py), 8.64 (d, J = 5.9 Hz, 2H, N-CH, Py), 8.95-9.05 (m, 2H, N-CH, Py).
3HHDMP-12	0.91 (t, J = 6.7 Hz, 6H, CH_3), 1.18-1.50 (m, 36H, CH_2), 2.00-2.16 (m, 4H, CH_2), 3.82 (s, 4H, O-CH_2), 4.41 (s, 4H, Py-O-CH_2), 4.66 (t, J = 7.4 Hz, 4H, N^+-CH_2), 8.02 (dd, J_1 = 8.5 Hz, J_2 = 5.8 Hz, 2H, CH, Py), 8.27 (dd, J_1 = 8.5 Hz, J_2 = 2.0 Hz, 2H, CH, Py), 8.64 (d, J = 5.8 Hz, 2H, N-CH, Py), 8.96-9.06 (m, 2H, N-CH, Py).

The yields of 4TOSU-n, 3PHBO-n, and 3HHDMP-n and their solubility in water at 20°C are shown in Table 2. 4TOSU-n and 3HHDMP-n showed high yields of more than 80%, respectively. With regard to the solubility in water at 20°C, the solubility of 3HHDMP-n was higher than that of 4TOSU-n and 3PHBO-n.

Table 1-2. Yields and solubility of compounds 4TOSU-n, 3PHBO-n, and 3HHDMP-n

Compounds	Yield (%)	Formula	Solubility (mg/ml)
4TOSU-8	80	$C_{33}H_{52}Br_2N_2O_4$	24
4TOSU-10	82	$C_{37}H_{60}Br_2N_2O_4$	15
4TOSU-12	90	$C_{41}H_{68}Br_2N_2O_4$	10
3PHBO-8	63	$C_{32}H_{46}Br_2N_2O_2$	24
3PHBO-10	65	$C_{36}H_{54}Br_2N_2O_2$	18
3PHBO-12	69	$C_{40}H_{62}Br_2N_2O_2$	14
3HHDMP-8	80	$C_{31}H_{52}Br_2N_2O_4$	370
3HHDMP -10	83	$C_{35}H_{60}Br_2N_2O_4$	135
3HHDMP -12	85	$C_{39}H_{68}Br_2N_2O_4$	110

SECTION 2.

Biocompatibility of Novel bis-QACs

In the previous section, we have detailed the synthesis of three series of bis-QACs to allow the development of a new antimicrobial substance with better biocompatibility than that of commonly used antiseptics. There are several studies on biocompatibility calculated from antimicrobial activity and the cytotoxicity of antimicrobial substances used as antiseptics[31,39,40]. In these studies, the antibacterial activity of the antiseptic agents was evaluated at MIC, MBC, and the concentration required for a 3 log reduction in microbial count. In addition, various cell cultures of human or animal origin were used in the cytotoxicity assay. In this section, we first compared the basic antimicrobial activity of these bis-QACs with existing antiseptic agents through the measurement of MIC and MBC. Furthermore, the cytotoxicity of newly synthesized bis-QACs on NHEK cells was compared with that of existing antiseptic agents. We conducted a further comparison of the bis-QACs with existing antiseptic agents through the calculation of their BI.

2-1. MATERIALS AND METHODS

2-1-1. Chemicals

4TOSU-8, 4TOSU-10, 4TOSU-12, 3PHBO-8, 3PHBO-10, 3PHBO-12, 3HHDMP-8, 3PHBO-10, and 3PHBO-12, which were synthesized as described in the previous section, were used. For the existing antiseptic agents, CHG was purchased from Sagami Chemical Industry Co., Ltd (Tokyo, Japan) and BAC was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). In addition, OCT was obtained from Dishman Pharmaceuticals & Chemicals Ltd (Tokyo, Japan), and PHMB was obtained from Lonza Japan Ltd (Tokyo, Japan).

2-1-2. Bacterial strains

The antimicrobial activity of each compound was determined in four species of gram-negative bacteria, such as *Escherichia coli* ATCC 25922 (*E. coli* ATCC 25922), five species of gram-positive bacteria such as *Staphylococcus aureu*s ATCC 6538 (*S. aureus* ATCC 6538), and one species of fungus (Table 1-3).

Table 1-3. Microorganisms employed for antimicrobial assay

Gram negative bacteria	*Escherichia coli* ATCC 25922
	Pseudomonas aeruginosa ATCC 27853
	Acinetobacter baumannii JCM 6841
	Burkholderia cepacia JCM 5964
Gram positive bacteria	*Staphylococcus aureus* ATCC 6538
	Staphylococcus aureus ATCC 700698 (MRSA)
	Staphylococcus epidermidis ATCC 12228
	Enterococcus hirae ATCC 10541
	Enterococcus faecalis ATCC 29212
Fungus	*Candida albicans* ATCC 10231

2-1-3. Preparation of cell suspension used for minimum inhibitory concentration measurement

Bacteria were inoculated into nutrient broth (NB) medium by using a platinum loop and preincubated overnight at 37°C. The cell suspension was diluted with sterilized physiological saline to produce a solution with an optical density (OD600) of 0.1 to 1.0. The fungus was inoculated on potato dextrose agar plate and incubated at 30°C for 2 days, suspended in 10 mL of sterilized physiological saline, and collected in a test tube. Then, each cell suspension or a spore suspension was adjusted to 1×10^6 CFU/mL with NB medium or dextrose peptone broth.

2-1-4. Preparation of cell suspension used for minimum bactericidal concentration measurement

The test bacteria were inoculated into 10 mL of NB medium and preincubated at 37°C for 24 h. The cell suspension was adjusted to 1×10^7 CFU/mL with sterilized

physiological saline and used as a test microorganism suspension.

2-1-5. Measurement of minimum inhibitory concentration

MIC was measured in accordance with the microbroth dilution method. A 20 g/L stock solution of each antimicrobial substance was prepared by dilution with ethanol and then final concentrations of 256 mg/L to 0.25 mg/L were obtained by 2-fold serial dilutions using NB or dextrose peptone broth. After mixing a 150 μL portion of each serial dilution with an equal volume of the microorganism suspension described above in each well of a 96-well plate, the mixtures were incubated for 24 h at 37°C. After incubation, the MIC of each test antimicrobial substance was visually determined based on turbidity.

2-1-6. Measurement of minimum bactericidal concentration

A 20 g/L stock solution of each antimicrobial substance was prepared by dilution with ethanol and then serial dilutions were made with sterilized water. After mixing a 4.5 mL portion of each serial dilution with 0.5 mL of the microbial suspension described above, the mixtures were incubated at 20°C for 10 min. After 10 min, one loopful of each mixture was inoculated into 4.5 mL of NB containing neutralizer (10% Tween 80 + 3% lecithin). The inoculated broth was incubated at 37°C for 24 h and then plated onto agar medium. After incubation of the agar plates at 37°C for 24 h, the MBC of each antimicrobial substance was determined by visual inspection.

2-1-7. Measurement of the hydrophobicity of bis-QACs

The molecular hydrophobicity (R_M) of the test compounds was calculated from the equation $R_M = \log (1/R_f - 1)$[41]. The R_f value represents the flow rate in reversed-phase partition chromatography. The R_M of bis-QACs was determined by partition chromatography on reversed-phase thin layer silica gel by using an acetonitrile-ethyl alcohol-water (10:9:1) as the developing solvent. After the development of the solvent in a chromatographic chamber saturated with solvent vapor, the R_f of the test compounds was measured under irradiation with UV light.

2-1-8. Cytotoxicity assays

The cytotoxicity assay was performed by using normal human neonatal epidermal keratinocytes (NHEK (NB)) cells purchased from Kurabo Industries Ltd (Osaka, Japan). EpiLife® Medium supplemented with HKGS (Prod. No. M-EPI-500-CA and S-001-5, Life Technologies, Tokyo, Japan) was used as an antibiotic-free NHEK (NB) cell culture medium. Suspensions of NHEK (NB) were prepared at 1×10^5 cells/mL were seeded in 96-well plates at 100 μL/well and cultured in a 5% CO_2 incubator at 37ºC for 3 days. On the third day of culture, the old medium was replaced with serial dilutions of each antimicrobial substance prepared with fresh medium (100 μL/well) and the cultures were maintained in a CO_2 incubator at 37ºC for 48 h. The assay was performed in triplicate for each concentration of antimicrobial substance. After incubation, the medium containing the antimicrobial substance in each well was replaced with 100 μL of 0.5 mg/mL 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and the cells were incubated at 37ºC for 2 h. After a reaction time of 2 h, the

medium was completely removed from each well and replaced with 100 μL of isopropyl alcohol to solubilize the reaction product. Subsequently, the absorbance of each well at 570 nm was measured in an automatic plate reader (Tecan Spectra Thermo, Austria). The relative cell viability (%) was determined from the relative absorbance of the cells treated with the test compounds to the value of the untreated control cells. The IC_{50} value was defined as the applied concentration of an antimicrobial agent at which 50% of the cells survived.

2-2. RESULTS AND DISCUSSION

2-2-1. Bacteriostatic activity and antimicrobial spectrum of novel bis-QACs

To determine the antimicrobial activity of the newly-synthesized bis-QACs, fundamental bacteriostatic activity was first evaluated through the measurement of MIC values for gram-negative and gram-positive bacteria. The MICs of these compounds against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 6538 are shown in Table 1-4. For comparison, the existing antiseptic agents of CHG, PHMB, BAC, and OCT were used as reference compounds. The results showed that 4TOSU-10, 3PHBO-10, 3PHBO-12, 3HHDMP-10, and 3HHDMP-12 with a C10 or C12 alkyl chain length had greater bacteriostatic activity against *E. coli* ATCC 25922 than the reference compounds. Furthermore, 3PHBO-10 and 3PHBO-12 exhibited excellent bacteriostatic activity against *P. aeruginosa* ATCC 27853 compared with the reference compounds. The MICs of 4TOSU-10, 3PHBO-8, 3PHBO-10, 3PHBO-12, 3HHDMP-10, and 3HHDMP-12 against *S. aureus* ATCC 6538 indicated their superior bacteriostatic activity to commonly used antiseptics.

The bis-QACs that showed superior bacteriostatic activity compared with the commonly used antiseptics against all tested bacteria were 3PHBO-10 and 3PHBO-12. In particular, these bis-QACs showed more potent bacteriostatic activity than that of mono-QACs, such as BAC. In general, mono-QACs are known to be less effective against gram-negative bacteria. In contrast, several studies have reported that bis-QAC possesses broad-spectrum antimicrobial activity and exhibits potent bacteriostatic activity against both gram-negative and gram-positive bacteria[42–46]. The antimicrobial

properties of the newly synthesized bis-QACs were also consistent with the trend in bis-QACs properties described in these studies. Furthermore, the bacteriostatic activity of the bis-QACs with an alkyl chain length of C10 or C12 tended to be superior to that of bis-QACs with an alkyl chain length of C8. In particular, the bacteriostatic activity of the bis-QACs with alkyl chain lengths of C10 or C12 was equal to or higher than that of OCT; these results suggested that these compounds had wide-ranging bacteriostatic activity.

Table 1-4. MICs of 4TOSU-n, 3PHBO-n, 3HHDMP-n, and the reference compounds

Compounds	MIC (mg/L) [a]		
	E. coli ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 6538
4TOSU-8	4.0±3.5	37±24	2.3±1.5
4TOSU-10	1.0±0.0	9.3±6.1	0.83±0.29
4TOSU-12	8.0±6.9	32±0.0	2.3±1.5
3PHBO-8	4.0±0.0	128±0.0	1.0±0.0
3PHBO-10	1.0±0.0	2.0±0.0	0.5±0.0
3PHBO-12	2.0±0.0	4.0±0.0	1.0±0.0
3HHDMP-8	256±0.0	-	43±18
3HHDMP-10	2.7±1.2	43±18	1.7±0.58
3HHDMP-12	1.7±0.58	21±9.2	1.7±0.58
CHG	4.0±0.0	9.3±6.1	4.0±0.0
PHMB	3.3±1.2	21±9.2	5.3±2.3
BAC	11±5.0	128±0.0	2.0±0.0
OCT	4.0± 0.0	8.0±0.0	2.0±0.0

- No activity was observed up to 256 mg/L. a) All experiments were performed in triplicate; each value represents the mean ± standard deviation (n=3).

Therefore, the antimicrobial activity of bis-QACs with an alkyl chain of C10 or C12, which showed potent bacteriostatic activity against three species of bacteria, was subsequently tested against more species of gram-negative bacteria, gram-positive bacteria, and fungus. As shown in Table 1-5, 4TOSU-10, 12, 3PHBO-10, 12, and 3HHDMP-10, 12 exhibited potent bacteriostatic activity against all gram-negative bacteria, gram-positive bacteria, or fungi, demonstrating their wide-spectrum antibacterial activity.

Table 1-5. MICs of 4TOSU-10, 12, 3PHBO-10, 12, 3HHDMP-10, 12, and the reference compounds

Strain	MIC (mg/L) [a]									
	4TOSU -10	4TOSU -12	3PHBO -10	3PHBO -12	3HHDMP -10	3HHDMP -12	CHG	PHMB	BAC	OCT
A. baumannii JCM 6841	32±0.0	11±4.6	16±0.0	11±4.6	64±0.0	16±0.0	53±18	43±18	21±9.2	13±4.6
B. cepacia JCM 5964	8.0±0.0	19±12	4.0±0.0	27±9.2	16±0.0	64±0.0	149±98	256±0.0	213±74	16±0.0
E. hirae ATCC 10541	1.0±0.0	5.3±2.3	2.0±0.0	2.7±1.2	6.7±2.3	16±14	16±0.0	21±9.2	8.0±0.0	11±4.6
E. faecalis ATCC 29212	1.7±0.6	6.7±2.3	2.0±0.0	5.3±2.3	4.0±0.0	19±12	27±9.2	8.0±0.0	8.0±0.0	16±0.0
S. aureus ATCC 700698 (MRSA)	1.7±0.6	11±4.6	2.0±0.0	6.7±2.3	2.0±0.0	8.0±0.0	12±6.9	8.0±0.0	16±0.0	9.3±6.1
S. epidermidis ATCC 12228	1.0±0.0	5.3±2.3	1.0±0.0	9.3±6.1	0.83±0.29	9.3±6.1	11±4.6	4.0±0.0	4.0±0.0	8.0±0.0
C. albicans ATCC 10231	16±0.0	13±4.6	8.0±0.0	19±12	64±0.0	27±9.2	107±37	8.0±6.9	27±9.2	6.7±2.3

a) All experiments were performed in triplicate; each value represents the mean ± standard deviation (n=3).

2-2-2. Bactericidal activity of novel bis-QACs

To compare the bactericidal activity of the three series of bis-QACs with that of existing antiseptic agents, we measured the MBCs against *E. coli*, *P. aeruginosa*, and *S. aureus* (Table 1-6). The MBC values of 4TOSU-8 and 3PHBO-12 against *E. coli* were comparable with that of OCT, which had the lowest MBC value of all common antiseptic agents. In the bactericidal activity against *P. aeruginosa*, the MBC values of 4TOSU-12, 3PHBO-12, and 3HHDMP-12, which are bis-QACs with an alkyl chain length of 12, were lower than those of existing antiseptic agents. The MBC values of 4TOSU-12 and 3PHBO-12 against *S. aureus* were comparable with that of OCT, which had the lowest MBC value of all existing antiseptic agents. The bis-QAC that showed MBC values equal to or superior to that of the existing antiseptic agents against all tested bacteria was 3PHBO-12.

Table 1-6. MBCs of 4TOSU-n, 3PHBO-n, 3HHDMP-n, and the reference compounds

Compounds	MBC (mg/L)		
	E. coli ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 6538
4TOSU-8	5.0±0.0	25±0.0	-
4TOSU-10	33±14	75±43	83±29
4TOSU-12	18±12	8.3±2.9	8.3±2.9
3PHBO-8	-	-	-
3PHBO-10	42±14	42±14	100±0.0
3PHBO-12	6.7±2.9	13±10	13±10
3HHDMP-8	-	-	-
3HHDMP-10	25±0.0	100±0.0	-
3HHDMP-12	15±8.7	8.3±2.9	33±14
CHG	25±0.0	-	-
PHMB	100±0.0	42±14	-
BAC	25±0.0	25±0.0	13±10
OCT	5.0±0.0	27±23	12±12

-: No activity was observed up to 100 mg/L. All experiments were performed in triplicate; each value represents the mean ± standard deviation (n=3).

The MBC measurement results showed a trend similar to the MIC measurement results, which showed that antimicrobial activity increased as the alkyl chain length increased from 8 to 12. It should be noted that as the MBCs of 3PHBO-8 and 3HHDMP-8 against all tested bacteria were greater than 100 mg/L, the exact MBC could not be measured. The spacer structure crosslinking the two cationic pyridinium rings in bis-QACs is known to affect the antimicrobial activity[47–49]. For example, the antimicrobial activity of bis-QACs with a longer methylene spacer is less susceptible to changes in the alkyl chain length. In contrast, the antimicrobial activity of bis-QACs with a short methylene spacer was significantly affected by the change in alkyl chain length.

As a possible explanation for this phenomenon, Shirai et al.[49] reported that the extension in the methylene chain length of bis-QACs contributed to an increase in their conformers owing to an increase in steric structure diversity. They speculated that as the number of conformers of bis-QAC increased, the influence of the methylene chain spacer on the bactericidal activity was enhanced and that of the alkyl chain was suppressed. As the spacers of the three series of bis-QACs synthesized by us are pentaerythritol or hydroquinone structures, there is no variation in their steric structures. Similarly, in the case of bis-QACs with a short methylene chain length, their bactericidal activity is thought to be restricted by the alkyl chain length. However, the bactericidal activity of 4TOSU-12, 3PHBO-12, and 3HHDMP-12 with a C12 alkyl chain against all tested bacteria was greater than that of CHG and PHMB. Moreover, these bis-QACs exhibited a potent bactericidal activity, comparable to that of OCT. These results indicated that bis-QACs with a C12 alkyl chain had potent bactericidal activity against both gram-negative and gram-positive bacteria.

2-2-3. Influence of molecular hydrophobicity on bactericidal activity

We then compared the relationship between the bactericidal activity (1/log MBC) and the molecular hydrophobicity (R_M) of the three series of bis-QACs and common antiseptic agents. The bactericidal activity against *E. coli* ATCC 25922 (Fig. 1-4a), *P. aeruginosa* ATCC 27853 (Fig. 1-4b), and *S. aureus* ATCC 6538 (Fig. 1-4c), corresponding to the molecular hydrophobicity (R_M) of bis-QACs, is shown in Figure 1-4. The bactericidal activity of 3PHBO-n and 3HHDMP-n against all tested bacteria tended to be greater in the compounds with higher molecular hydrophobicity. These

results revealed that the bactericidal activity of 3PHBO-n and 3HHDMP-n depended on their molecular hydrophobicity. Although the bactericidal activity of 4TOSU-n against *S. aureus* ATCC 6538 increased with an increase in molecular hydrophobicity, the activities against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were not correlated with the molecular hydrophobicity. These results suggested that the molecular hydrophobicity of 4TOSU-n exerted little influence on the bactericidal activity against gram-negative bacteria, but had an effect on the bactericidal activity against gram-positive bacteria.

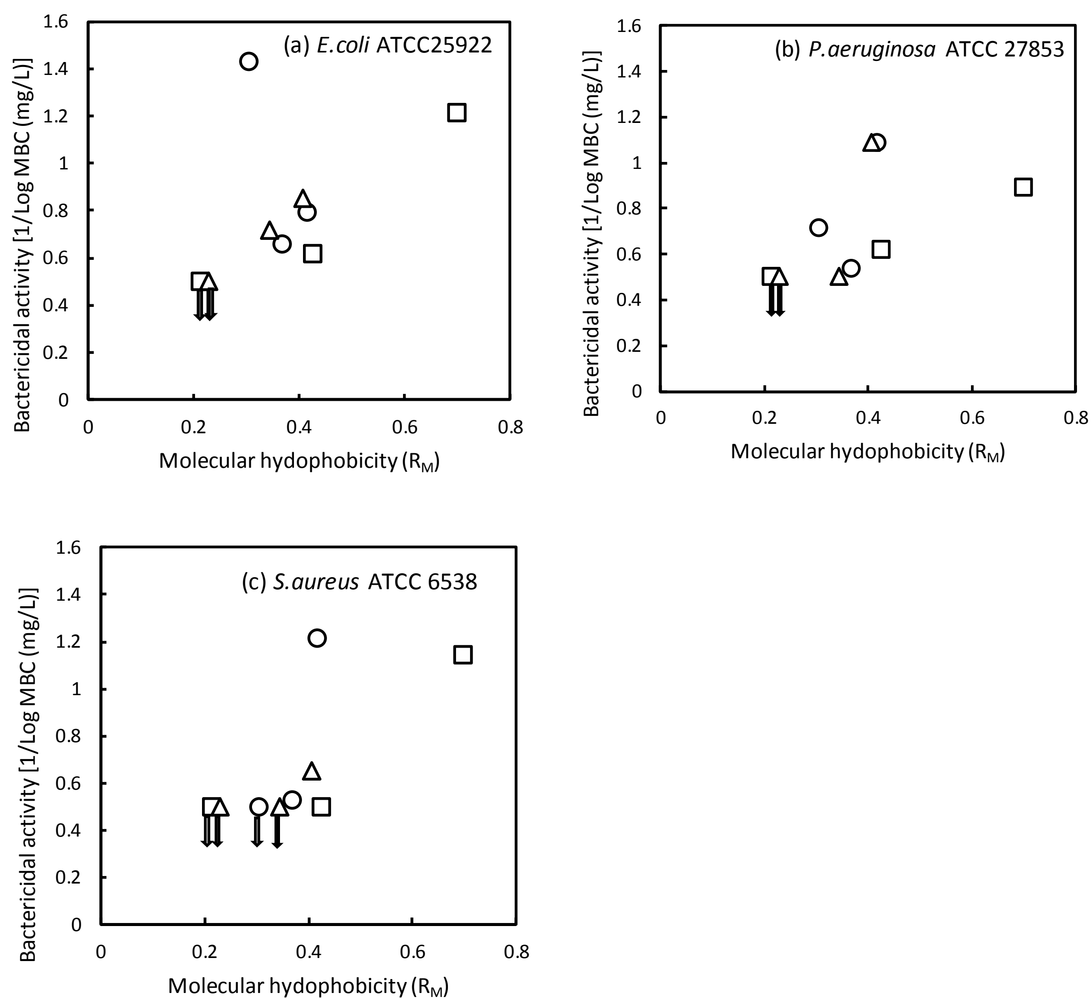


Fig. 1-4. The correlation between molecular hydrophobicity and bactericidal activity of 4TOSU-n, 3PHBO-n, and 3HHDMP-n

Symbols: ○, 4TOSU-n; □, 3PHBO-n; △, 3HHDMP-n; ↓, Bactericidal activity (1/Log MBC) = < 0.5.

The value is the mean of three independent experiments.

2-2-4. Cytotoxicity of novel bis-QACs

To compare the cytotoxicity of the three series of bis-QACs and existing antiseptic agents, we performed an MTT assay using cultured cells. On the presumption that the antiseptic agents are topically applied to the skin, we used NHEK (NB) cells for the cytotoxicity assay. The cytotoxic effects of 4TOSU-n, 3PHBO-n, 3HHDMP-n, and the existing antiseptic agents on NHEK (NB) cells were presented as IC_{50} values determined by MTT assays based on both the mass/volume and molar concentration and are shown in Table 1-7. The toxicity of the tested compounds, in terms of mass/volume concentration, was ranked in decreasing order as follows: OCT > 4TOSU-8 > 3PHBO-10 > 3PHBO-8 > BAC > 4TOSU-10 > 4TOSU-12 > 3HHDMP-10 > 3PHBO-12 > 3HHDMP-12 > CHG > PHMB > 3HHDMP-8. When the IC_{50} values were calculated on the basis of their molar concentration, the order of cytotoxicity changes was as follows: OCT > 4TOSU-8 > 3PHBO-10 > 3PHBO-8 > PHMB > 4TOSU-12 > BAC > 4TOSU-10 > CHG > 3PHBO-12 > 3HHDMP-10 > 3HHDMP-12 > 3HHDMP-8.

These results revealed that in the conditions of the cytotoxicity assay in the present study, all tested bis-QACs were less cytotoxic than OCT. In particular, the cytotoxicity of 4TOSU-10, 3PHBO-12, 3HHDMP-8, 3HHDMP-10, or 3HHDMP-12 was lower than that of mono-QACs, such as BAC, and comparable to that of biguanide-based compounds, such as CHG and PHMB. In addition, based on the IC_{50} values calculated from the mass/volume concentration, the cytotoxicity of the existing antiseptics tested was ranked in order from the highest to the lowest as follows: OCT > BAC > CHG > PHMB. This result revealed that QACs tended to have slightly higher cytotoxicity than

biguanide-based compounds. In addition, we calculated IC_{50} values by using the molar concentration, since PHMB has a relatively high molecular weight among the antiseptic agents investigated. When this method was used, the cytotoxicity of PHMB was higher than that of BAC. In contrast, OCT was the most cytotoxic of the tested compounds when analyzed by either mass or molecular weight.

Table 1-7. IC_{50} values of 4TOSU-n, 3PHBO-n, 3HHDMP-n, and the reference compounds on NHEK (NB) cells determined by MTT assay

Compounds	MTT IC_{50} [a)]		
	Molecular weight	mg/L	mmol/L
4TOSU-8	700.6	1.82±0.89	0.0026
4TOSU-10	756.7	5.97±0.12	0.0079
4TOSU-12	812.8	6.21±0.26	0.0076
3PHBO-8	650.5	2.33±0.02	0.0036
3PHBO-10	706.6	2.24±0.01	0.0031
3PHBO-12	762.7	6.40±0.07	0.0084
3HHDMP-8	676.6	60.50±1.08	0.0894
3HHDMP -10	732.7	6.36±0.11	0.0087
3HHDMP -12	788.8	7.05±0.21	0.0089
CHG	897.8	7.46±1.08	0.0083
PHMB	2700	18.3±4.14	0.0068
BAC	354.0	2.71±0.30	0.0077
OCT	623.8	1.33±0.87	0.0021

a) mean ± standard deviation (n=3)

2-2-5. Biocompatibility of novel bis-QACs

The BI against each microorganism tested of the three bis-QAC series and the existing antiseptic agents was defined as the ratio of the IC_{50} in NHEK (NB) cells to

each MBC after 10 min exposure at 20ºC; these values are presented in Fig. 1-5. An accurate BI could not be calculated for any of the tested compounds, as the MBCs against three bacterial species of 3HHDMP-8, with the lowest cytotoxicity among the tested compounds, were over 100 mg/L. The BI values for the tested compounds calculated from the IC_{50} values in NHEK (NB) cells and the MBC values against *E. coli* ATCC 25922 were ranked in the following order 3PHBO-12 > 3HHDMP-12 > 4TOSU-8 > 4TOSU-12 > CHG > 3HHDMP-10 > PHMB > 4TOSU-10 > BAC > OCT > 3PHBO-10 > 3PHBO-8. In particular, 4TOSU-8, 4TOSU-12, 3PHBO-12, and 3HHDMP-12 showed greater BIs against *E. coli* ATCC 25922 than the existing antiseptic agents.

In addition, the BIs based on the IC_{50} in NHEK (NB) cells and the MBC against *P. aeruginosa* ATCC 27853 were ranked in the following order: 3HHDMP-12 > 4TOSU-12 > 3PHBO-12 > PHMB > BAC > 4TOSU-10 > 4TOSU-8 > 3HHDMP-10 > 3PHBO-10 > OCT > 3PHBO-8 > CHG. The BIs of bis-QACs with an alkyl chain length of C12, such as 4TOSU-12, 3PHBO-12, and 3HHDMP-12, against *P. aeruginosa* ATCC 27853 were greater than those of the existing antiseptic agents.

The order of the BI values of the tested compounds calculated from the IC_{50} in NHEK (NB) cells and the MBC value against *S. aureus* ATCC 6538 was 4TOSU-12 > 3PHBO-12 > 3HHDMP-12 > BAC > OCT > 4TOSU-10 > 3PHBO-10. The accurate BIs of 4TOSU-8, 3PHBO-8, 3HHDMP-10, CHG, and PHMB against *S. aureus* ATCC 6538 could not be calculated for the same reason as for 3HHDMP-8 mentioned above. However, it was clear that the BIs of these antiseptic agents against *S. aureus* ATCC 6538, except PHMB, were much lower than 0.1.

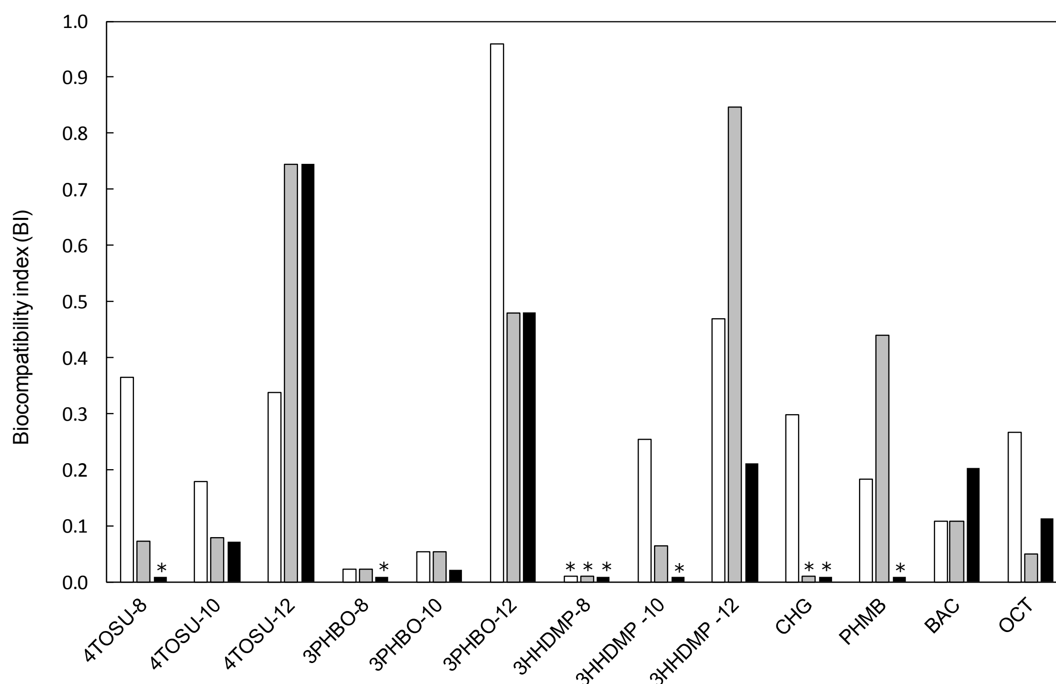


Fig. 1-5. Biocompatibility Index (BI) of the three bis-QAC series and the reference compounds

The BI represents the ratio of the IC_{50} in NHEK (NB) cells to the MBC value after 10 min exposure at 20ºC against bacteria such as *E. coli*, *P. aeruginosa*, and *S. aureus.* White; $BI_{E.\ coli}$: Light gray; $BI_{P.\ aeruginosa}$: Black; $BI_{S.\ aureus}$. *, not calculable.

The comparison of BI values indicated that the newly synthesized bis-QACs, such as 4TOSU-12, 3PHBO-12, and 3HHDMP-12, had equal or greater biocompatibility than the investigated antiseptic agents. In contrast, some of the existing antiseptic agents had lower BI values against some of the tested microorganisms than the novel bis-QACs, and were suggested to present a greater risk of adverse effects when applied to the skin than the novel bis-QACs. Thus, the results of these studies on the bacteriostatic activity, bactericidal activity, cytotoxicity, and biocompatibility all indicated the acceptability of

the newly synthesized bis-QACs for application to human skin.

Several studies have evaluated the antimicrobial activity and cytotoxicity of antimicrobial substances. Damour et al.[39] determined the order of cytotoxicity of the antimicrobial substances by using the ratio of the CD_{50} in human fibroblasts and keratinocytes to the MIC for antibiotics and the MBC for antiseptic agents. In their experiments, cultured human fibroblasts and keratinocytes were exposed to an antimicrobial substance for 15 min, which was a brief exposure period compared with that used in our cytotoxicity assay. Nevertheless, for the CD_{50}/MBC ratio measurement, they reported that the rank order of cytotoxicity for BAC was higher than that of CHG, which was consistent with our results. In another study, Müller and Kramer[31] reported the BI of various topical antiseptic agents, which was defined as the ratio of the antibacterial activity against *E. coli* and *S. aureu*s and cytotoxicity on cultured murine fibroblasts. Their results reported the rank order of BI was OCT > PHMB > CHG > BAC. Although these results differ from our studies, we consider that the discrepancy arises from the differences in the procedures of the antimicrobial activity tests and the MTT assay. In their study, the experiments to evaluate the antimicrobial activity by the concentrations required to achieve a microbial reduction of 99.9% were performed in the cell culture medium with 10% fetal bovine serum (FBS), which is more reflective of the composition of wound fluids. In the MTT assay, they also diluted the test antiseptic agents with 10% FBS-containing cell culture medium, and evaluated cytotoxicity by allowing the diluted antiseptic agents to act on murine fibroblasts for 30 min. In our study, the antimicrobial substances and microorganisms were mixed in sterilized water to evaluate the lowest concentration at which 1×10^6 CFU/mL of the test

microorganisms could be completely killed. Moreover, in order to reduce the influence of the medium on antimicrobial substances in the MTT assay, we diluted the antimicrobial substances with FBS-free cell culture medium and the NHEK (NB) cells were exposed to the dilutions for 48 h.

In an investigation of the toxicity indices for commercially available skin and wound cleansers, such as hydrogen peroxide, sodium hypochlorite, and povidone iodine, Wilson et al. reported that some of the antimicrobial substances were more toxic in phosphate-buffered saline (PBS) than in medium containing FBS[40]. Nagamune et al.[38] evaluated the antimicrobial activity of bis-QACs and the acute cytotoxic effects on several types of human cells, including fibroblasts, keratinocytes, erythrocytes, and lymphomas. They reported that the IC_{50} values of bis-QACs varied depending on the cell types. Their results showed that the NHEK (NB) cells were more susceptible to the tested QACs than other types of human cells.

It should be noted that the results of cytotoxicity evaluation by the MTT assay using cells, as reported in these studies, can be affected by the evaluation conditions. It is therefore necessary to assess the biocompatibility of the antimicrobial substances under conditions that better simulate "real life" situations. For example, it is conceivable that additional experiments should be conducted using a three-dimensional reconstructed human epidermis model known to exhibit good correlation with in vivo skin irritation. This assessment system might provide more accurate information on the BI rank order for antiseptic agents. In the present study, BI was evaluated in NHEK (NB) cells, which are more susceptible to bis-QACs than human-derived cells such as fibroblasts, erythrocytes, and lymphoma. NHEK (NB) cells were also exposed to the antimicrobial

substances for 48 h in the FBS-free cell culture medium, to eliminate the influence of the components of the culture medium as far as possible. From these results, it was suspected that the examination conditions of this study provided sensitive detection of the cytotoxicity of the test compounds.

SECTION 3.

Conclusion

The purpose of this study was to develop a topical antiseptic agent with better biocompatibility than existing antiseptic agents; thus, we synthesized three novel bis-QAC derivatives of 4TOSU-n, 3PHBO-n, and 3HHDMP-n. Through the evaluation of the antibacterial activity of these bis-QACs by the examination of the MIC and the MBC, it was found that some of the bis-QACs exhibited a broad and potent antimicrobial spectrum comparable to that of OCT.

In addition, the cytotoxicity of these bis-QACs on NHEK (NB) cells was lower than that of QACs (BAC and OCT) and comparable with that of biguanide-based compounds (CHG and PHMB). Furthermore, we compared the bis-QACs with existing antiseptic agents, in terms of their BI, which was defined as the ratio of the IC_{50} values in NHEK (NB) cells and the MBC values against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 6538. As a result, novel bis-QACs such as 4TOSU-12, 3PHBO-12 and 3HHDMP-12 showed superior BI values equal to or greater than existing antiseptic agents. Thus, these results strongly suggested that the biocompatibility of 4TOSU-12, 3PHBO-12, and 3HHDMP-12 was equal to or higher than that of the existing antiseptic agents; therefore, bis-QACs are expected to be useful antiseptic agents for topical application to the skin.

CHAPTER 2.

In vitro evaluation of skin irritation caused by novel bis-QACs and commonly used skin antiseptics by cell culture methods

In the previous chapter, Section 1, we examined the synthesis of novel bis-QACs with a spacer structure derived from pentaerythritol or hydroquinone. We also reported the BI value, which was defined as the ratio of the bactericidal activity of these novel bis-QACs and the cytotoxicity in NHEK (NB) cells in Section 2. Our results showed that some of these new bis-QACs had BI values equal to or greater than that of existing antiseptic agents which are applied to human skin, such as OCT, BAC, PHMB, and CHG. Thus, it was suggested that these bis-QACs were promising agents for application to the human skin surface.

However, it was also necessary to consider that the cytotoxicity assay using cultured cells may not correlate exactly with in vivo skin irritation. In the cytotoxicity assay, the test compound is dissolved in the culture medium and exposed to the cultured cells; however, depending on the type of the antimicrobial substances, the test compound may react with the components contained in the culture medium and prevent accurate assay evaluation[40]. Furthermore, it has been reported that the susceptibility of human cells to antiseptic agents varies depending on the type of human cells used for the cytotoxicity assay[38]. Therefore, to predict the skin irritation caused by antiseptic agents, although an assay of cell death may be used as an index, a more accurate evaluation must be performed by the combination of multiple appropriate evaluation methods. There are many studies on the prediction of skin irritation of chemical substances such as

surfactants; some have focused on the mRNA expression and protein production of inflammatory cytokines in cultured cells[50,51]. However, in order to predict the irritation caused by skin antiseptic agents, few reports have compared the effects of several antiseptic agents on the expression of inflammatory cytokines in cultured cells. In the first section of this chapter, we aimed to compare the skin irritation properties of newly synthesized bis-QACs and antimicrobial substances conventionally used as antiseptics, through the combination of multiple evaluation methods. To achieve this objective, we first performed the cytotoxicity assays in several types of cultured cells that constitute human skin. Subsequently, we focused on the transcription of inflammation-related genes induced by the antiseptic agents and compared the expression levels of inflammatory cytokine-related genes in NHEK cells.

However, it has also been reported that the response of two-dimensional monolayer cultures such as NHEK cells has a low correlation with the in vivo skin response[52–54]. This is caused by the absence of a stratum corneum functioning as a barrier of the skin in NHEK cells, which are susceptible to much lower concentrations of antiseptic agents than used in practice. Therefore, as described in the first section of this chapter, bis-QACs and existing antiseptic agents were applied to NHEK cells at a concentration that did not reduce the viable cell ratio or notably suppress gene expression, and the expression levels of cytokine-related genes were compared. In contrast, as the three-dimensional reconstructed human epidermis model has a stratum corneum with barrier function, antiseptic agents can be applied at the same concentration used in practice. Accordingly, as described in Section 2 of this chapter, bis-QACs and existing antiseptic agents at approximately 0.1% to 0.2%, which are the clinically used

concentrations of existing antiseptics, were applied to a three-dimensional reconstructed human epidermis model, and their cytotoxicity was compared. In addition, we evaluated the mRNA and protein expression of inflammatory cytokines in the three-dimensional reconstructed human epidermis model.

SECTION 1.

Evaluation of skin irritation of novel bis-QACs using cultured cells

In the previous chapter, we described the synthesis of 3 series of bis-QACs (4TOSU-n, 3PHBO-n, and 3HHDMP-n), which differ in the spacer structure crosslinking the two cationic pyridinium rings, and compared their BIs, expressed as a ratio of cytotoxicity to antibacterial activity, with existing antiseptic agents. Our results showed that, among these bis-QACs, 3PHBO-12 and 3HHDMP-12 had BI values equal to or higher than those of existing antiseptic agents; they are expected to be clinically useful.

However, for the cytotoxicity assay, it has been reported that the test results are influenced by the difference in the susceptibility of the cells used to the antiseptic agents[38,40]. In this section, to evaluate skin irritation of these bis-QACs in more detail, we performed a cytotoxicity assay on four types of human-derived cells and compared the IC_{50} values of 3PHBO-12, 3HHDMP-12, and the existing antiseptic agents. To mimic the topical application of antiseptic agents to normal skin, mucosa, and damaged skin, we performed the cytotoxicity assay in skin cells such as epidermal keratinocytes and fibroblasts, and human monocytic cell lines such as THP-1 and HL-60.

We then evaluated the effect of antimicrobial substances on the mRNA expression of inflammatory cytokines in NHEK cells. Several studies have reported the involvement of cytokines produced from NHEK cells with the inflammatory reactions caused by skin irritation from chemical substances[55,56]. Therefore, each antimicrobial substance was exposed to NHEK cells at a concentration lower than the IC_{50} and the expression of

inflammatory cytokine genes was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

1-1. MATERIALS AND METODS

1-1-1. Chemicals

The chemical structures of 3PHBO-12 and 3HHDMP-12, which are the novel bis-quaternary ammonium salts used in this chapter, are shown in Fig. 2-1.

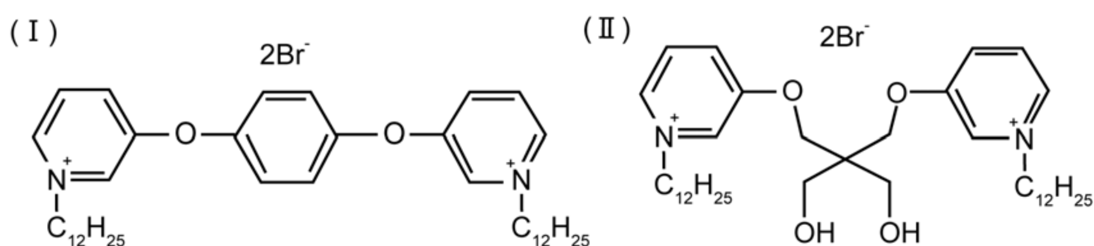


Fig. 2-1. Chemical structures of new bis-QACs. (I): 3PHBO-12; (II): 3HHDMP-12

1-1-2. Reference compounds

BAC was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). OCT, CHG, and PHMB were purchased from Dishman Pharmaceuticals and Chemicals Ltd (Tokyo, Japan), Sagami Chemical Industry Co., Ltd (Tokyo, Japan), and Lonza Japan Ltd (Tokyo, Japan), respectively.

1-1-3. Preparation of test solution

The novel bis-QACs and existing antiseptic agents were dissolved in 95% ethanol to produce a 100 g/L stock solution. Subsequently, the working solutions were prepared by diluting the stock solutions (at least to 1:2000 dilution) in cell culture medium to the

required concentrations. Therefore, the final concentration of ethanol contained in the working solution was 0.05% (v/v) or less.

1-1-4. Cell culture

Adult normal human epidermal keratinocytes (NHEK (AD)) and adult normal human dermal fibroblasts (NHDF (AD)) were obtained from Kurabo Industries Ltd (Osaka, Japan). DermaLife basal medium, supplemented with a DermaLife K LifeFactors Kit (Prod. No. LL-0007, Lifeline Cell Technology, Frederick, MD, USA), was used as a culture medium for NHEK (AD) cells. NHDF (AD) cells were cultured in FibroLife basal medium supplemented with a FibroLife K LifeFactors Kit (Prod. No. LL-0011, Lifeline Cell Technology, Frederick, MD, USA). THP-1 cells and HL-60 cells were obtained from Riken BRC (Tsukuba, Japan) and cultured in RPMI 1640 (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS, 100 U/mL penicillin G, and 0.1 mg/mL streptomycin.

1-1-5. Cytotoxicity assays

The cytotoxicity assays in NHEK (AD), NHDF (AD), THP-1, and HL-60 cells were performed by using a Cell Counting Kit-8 (CCK-8) obtained from Dojindo Laboratories (Kumamoto, Japan). The CCK-8 is a colorimetric assay kit based on the water-soluble compound, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST), which produces a water-soluble formazan dye in the presence of electron carriers through the reduction in live cells. NHEK (AD) and NHDF (AD)

cells prepared at 10^5 cells/mL were seeded in 96-well culture plates at 100 µL/well and cultured in a 5% CO_2 incubator at 37ºC for 3 days. Subsequently, the culture medium was replaced with serial dilutions of each antimicrobial substance diluted in culture medium (100 µL/well) and the plate was returned to the incubator for 3 days. For the cytotoxicity assay in THP-1 and HL-60 cells, each cell suspension prepared at 5.55×10^5 cells/mL in RPMI-1640 was plated into the wells of 96-well plates at 90 µL/well. Ten microliters of the serially diluted solutions of each antimicrobial substance was added to each well and cultured in a 5% CO_2 incubator at 37ºC for 3 days. Then, 10 µL of CCK-8 solution was added to each well and incubated for 3 h at 37°C. Finally, the absorbance of the mixture in each well was measured at 450 nm by using an automated plate reader (Tecan Infinite F50, Austria). The assay was performed in triplicate for each concentration. The absorbance of the cells treated with each antimicrobial substance relative to the untreated control cells was expressed as a percentage and reported as the cell viability (%). The IC_{50} value was defined as the concentration of the antimicrobial substance at which 50% of the cells survived.

1-1-6. RNA extraction and real-time PCR analysis

The effects of bis-QACs and clinically used antiseptics on cytokine gene expression in NHEK (AD) cells were evaluated by using qRT-PCR. NHEK (AD) cells prepared at 1.6×10^4 cells/mL were seeded in 6-well plates (1.5 mL/well) and cultured in an incubator with 5% CO_2 at 37ºC. The medium was exchanged as required and NHEK (AD) cells were cultured until sub-confluent and exposed to each antimicrobial substance at concentrations below the IC_{50} for 72 h. Untreated cells and cells treated

with medium containing ethanol at concentrations less than 0.05% were used as the control.

Total RNA was extracted from the treated and untreated cells by using a NucleoSpin® RNA kit (Macherey-Nagel, Dueren, Germany) in accordance with the manufacturer's protocol. The absorbance at 260 nm was measured to quantify the total RNA concentration. Reverse transcription reaction was performed on 100 ng total RNA in accordance with the protocol of PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio Inc., Shiga, Japan). Quantitative RT-PCR was performed using the StepOne realtime PCR system (Applied Biosystems, Carlsbad, CA) with Express SYBR GreenER qPCR SuperMix Universal (Invitrogen) and specific target gene primers. The primer sequences of each gene are listed in Table 2-1. All results were normalized to the expression of GAPDH, a housekeeping gene, by using the ΔΔCt method and reported as the relative mRNA expression.

Table 2-1. Primer sets for qRT-PCR

Primer name	Primer sequence (5'-3')	Size (bp)
GAPDH	Fw ; GTC TTC ACC ACC ATG GAG AAG GCT Bw ; CAT GCC AGT GAG CTT CCC GTT CA	398
IL-1α	Fw ; GGA GAG CAT GGT GGT AGT YAG CAA C Bw ; CAA CAC TGC ACA AGT GAG ACA AGT G	649
IL-1β	Fw ; ATG GCA GAA GTA CCT GAG CTC GC Bw ; ACA CAA ATT GCA TGG TGA AGT CAG TT	802
IL-6	Fw ; GGC ATC TCA GCC CTG AGA AAG GAG A Bw ; CAC CAG GCA AGT CTC CTC ATT GAA TCC	628
IL-8	Fw ; ATG ACT TCC AAG CTG GCC GTG CT Bw ; TCT CAG CCC TCT TCA AAA ACT TCT C	289
TNF-α	Fw ; GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A Bw ; GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC T	444

1-1-7. Statistical analysis

Statistical analyses were computed by using Ekuseru-Toukei 2010 software (Social Survey Research Information Co., Ltd, Tokyo, Japan). The data were analyzed by one-way ANOVA followed by the Dunnett's multiple comparison test.

1-2. RESULTS AND DISCUSSION

1-2-1. Topical antiseptic agent-induced cytotoxicity

The cytotoxicity of the two bis-QACs on NHEK (AD), NHDF (AD), THP-1 and HL-60 cells was reported at the IC_{50} values determined from the WST assay (Table 2-2). CHG, PHMB, BAC, and OCT, which are used as skin antiseptics, were used as reference compounds. The cytotoxicity of the tested compounds in NHEK (AD) cells was ranked in descending order as follows: OCT > BAC > 3PHBO-12 > CHG > 3HHDMP-12 > PHMB. The cytotoxicity in NHDF (AD) cells was ranked in the following order: OCT > BAC > 3PHBO-12 > 3HHDMP-12 > CHG ≥ PHMB. The cytotoxicity in THP-1 and HL-60 cells was ranked in the following order: OCT > BAC > 3 PHBO-12 > 3HHDMP-12 > CHG > PHMB.

Table 2-2. Cytotoxicity of 3PHBO-12, 3HHDMP-12, and existing antiseptic agents on various human cells, presented as IC_{50} values determined from the WST assay

Compounds	IC_{50} (mg/L)[a)]			
	NHEK (AD)	NHDF (AD)	THP-1	HL-60
3PHBO-12	3.3 ± 0.0	3.1 ± 0.0	1.6 ± 0.2	1.7 ± 0.1
3HHDMP-12	3.5 ± 0.2	4.0 ± 0.1	2.0 ± 0.1	3.7 ± 0.1
OCT	0.8 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
BAC	1.2 ± 0.0	3.0 ± 0.1	1.2 ± 0.1	0.5 ± 0.0
CHG	3.4 ± 0.1	15.8 ± 0.9	3.3 ± 0.1	3.9 ± 0.1
PHMB	7.6 ± 0.8	15.9 ± 0.1	5.0 ± 0.5	4.0 ± 0.2

a) mean ± standard deviation (n=3)

Our results showed that 3PHBO-12 and 3HHDMP-12 were less cytotoxic than the existing QACs, such as OCT and BAC, in all cells used. In particular, the cytotoxicity of 3HHDMP-12 was the lowest among the QACs tested and the cytotoxicity to NHEK (AD) cells was comparable with that of biguanide-based compounds such as CHG. This result was consistent with the results presented in the previous chapter, which investigated the cytotoxicity of new bis-QACs on NHEK (NB) cells. Moreover, the cytotoxicity of the existing antiseptic agents followed the order OCT > BAC > CHG ≥ PHMB, regardless of the cell type, and QAC tended to be more cytotoxic than biguanide-based compounds.

Several studies have evaluated the cytotoxicity in NHEK or NHDF cells as a measure of the skin irritation of antiseptic agents[31,39,40]. Müller et al.[31] conducted a cytotoxicity assay using neutral red and MTT as colorimetric indicators and reported the order of the IC_{50} values of antiseptic agents in cultured murine fibroblasts. Although their examination conditions, such as cell type and application time of antiseptic agents, was different to our study, the order of cytotoxicity they obtained was consistent with our results. This provided a strong indication that our assay system accurately assessed the cytotoxicity of antimicrobial substances. Although the susceptibility of THP-1 and HL-60 cells to antiseptic agents tended to be slightly higher than NHEK (AD) and NHDF (AD) cells, the difference between the IC_{50} values was small. The different susceptibility of cell types was thought to result from the application of the antimicrobial substances to human-derived cells for the relatively long time of 72 h in the present cytotoxicity test.

1-2-2. Effects of antiseptic agents on cytokine gene expression in NHEK (AD) cells

We evaluated the effect of 3HHDMP-12, 3PHBO-12, and the existing antiseptic agents on the mRNA expression of inflammatory cytokines in NHEK (AD) cells. To avoid a drastic decrease in cell viability due to the cytotoxicity of test compounds, NHEK (AD) cells were exposed to antimicrobial agents at concentrations below the IC_{50} for 24 to 72 h and the mRNA expression of IL-1α is shown in Figure 2-2. The mRNA expression of IL-1α significantly increased in cells treated with 0.5 mg/L OCT and 0.8 mg/L BAC for 48 h or more and in cells treated with 2 mg/L 3PHBO-12 for 72 h compared with that in the untreated control cells. In particular, the mRNA expression of IL-1α was strongly induced by existing QACs, such as OCT and BAC, with a significant increase observed 48 h after stimulation with these antiseptic agents. In contrast, even when other antimicrobial substances were applied to NHEK (AD) cells for 48 h, the mRNA expression of IL-1α was not significantly altered. There was no significant difference in IL-1α mRNA expression between untreated control and ethanol vehicle control.

In general, it is thought that the release of primary cytokines from NHEK cells after exposure to irritants is involved in local inflammatory reactions such as erythema and edema[55,56]. The expression of inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, and TNF-α has also been reported to be induced by the application of various chemicals in keratinocytes or reconstructed human epidermis models[50,56-58]. In particular, IL-1α is known to be an inflammatory mediator produced by NHEK cells in the initial stages of inflammation. Our results showed that an increase in IL-1α mRNA expression was

strongly induced over time by existing QACs such as OCT and BAC, but not by the novel bis-QACs. As OCT and BAC were also highly cytotoxic to NHEK (AD) cells, IL-1α mRNA expression may be correlated with cytotoxicity. In addition, 3HHDMP-12, a new bis-QAC, did not induce a significant increase in IL-1α mRNA, which suggested that it may cause milder skin irritation than other antiseptic agents.

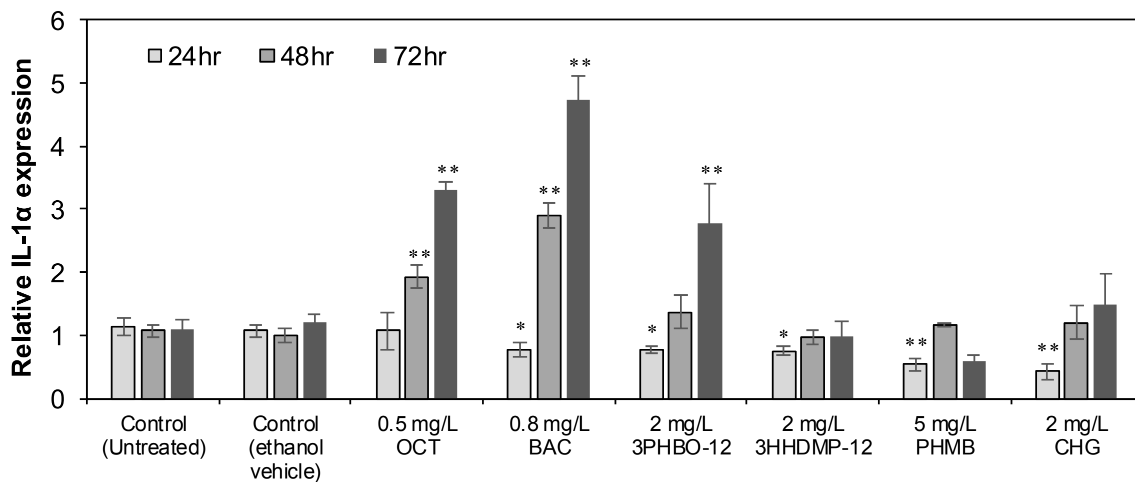


Fig. 2-2. The mRNA expression of inflammation-related genes (IL-1α) in NHEK (AD) cells treated with 3PHBO-12, 3HHDMP-12, and existing antiseptic agents for up to 72 h

The mRNA expression levels determined by qRT-PCR were normalized to GAPDH and plotted relative to those of untreated cells (control). All assays were performed in triplicate; the data are shown the mean ± standard deviation.

***P* < 0.01, **P* < 0.05 compared with control (untreated cells).

SECTION 2.

Evaluation of skin irritation of novel bis-QACs in a three-dimensional reconstructed human epidermis model

The purpose of this experiment was to achieve more accurate prediction of the skin irritation of antimicrobial substances through the appropriate combination of multiple evaluation methods, rather than using only a cytotoxicity assay based on cell death as an index. The three-dimensional reconstructed human skin model has a multi-layered epidermal structure and is known to exhibit a good correlation with in vivo skin irritation[52,59]. In the present study, the skin irritation of the new bis-QACs were investigated by using a three-dimensional reconstructed human epidermis model LabCyte EPI-MODEL 24 (LabCyte), which is listed as a recognized “In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method” by the Organization for Economic Co-operation and Development. The structure of the LabCyte cultures is morphologically similar to that of human skin and includes a stratum corneum with barrier functionality. Therefore, the skin irritation test using LabCyte cultures can be evaluated in similar conditions to actual use, and antiseptic agents of the clinically used concentrations can be applied. Thus, in the present study, 3PHBO-12, and 3HHDMP-12, and existing antiseptic agents were applied to LabCyte cultures at practical concentrations and the cytotoxicity of each antimicrobial substance on LabCyte cultures was compared. In addition, we detected the expression of inflammatory cytokines and their secretion into assay medium by qRT-PCR and enzyme-linked immunosorbent assay (ELISA).

2-1. MTERIALS AND METHODS

2-1-1. Chemicals

As in the previous section, 3PHBO-12 and 3HHDMP-12, which are novel bis-quaternary ammonium salts, were used.

2-1-2. Reference compounds

BAC was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). OCT, CHG, and PHMB were purchased from Dishman Pharmaceuticals & Chemicals Ltd (Tokyo, Japan), Sagami Chemical Industry Co., Ltd (Tokyo, Japan), and Lonza Japan Ltd (Tokyo, Japan), respectively.

2-1-3. Preparation of test solution

3PHBO-12, 3HHDMP-12, BAC, and OCT were dissolved in 95% ethanol to produce a 100 g/L stock solution. These test antiseptic stock solutions were diluted with sterilized distilled water (DW) to prepare a final concentration of 1000 mg/L test compound/0.95% ethanol aqueous solution. In contrast, stock solutions of CHG and PHMB were prepared in DW. The working solution for these existing antiseptic agents was prepared by the same method to yield concentrations of 2000 mg/L CHG and 2000 mg/L PHMB. A solution of 95% ethanol diluted 100-fold with DW was used as the control.

2-1-4. Cell culture

LabCyte EPI-MODEL 24, composed of NHEK cells forming a multilayered structure, was purchased from Japan Tissue Engineering Co., Ltd (Aichi, Japan) and cultured in accordance with the manufacturer's instruction manuals. Each well of the 24-well assay plate was filled with 500 μL of assay medium and the culture inserts containing the human epidermal tissue were transferred into the wells of the assay plate. The assay plate was placed in a 5% CO_2 incubator and incubated overnight at 37°C.

2-1-5. MTT assay in LabCyte cultures

We applied 100 μL of the working solutions containing each test compound or control (DW) onto the epidermal tissue surface of the LabCyte culture for 15 min. LabCyte cultures were also treated with 100 μL of 0.95% ethanol aqueous solution. The test compounds were aspirated from the culture inserts, the epidermal tissues were washed three times with 500 μL PBS, and the LabCyte cultures were placed in a 5% CO_2 incubator and cultured at 37°C for 24 h.

MTT medium was prepared by dissolving MTT at 0.5 mg/mL in the assay medium. The assay medium of each well was aspirated, replaced with 0.5 mL MTT medium, and cultured in a 5% CO_2 incubator at 37°C for 3 h. The epidermal tissue was removed from the culture insert using forceps and transferred to a microcentrifuge tube containing 300 μL isopropyl alcohol. The epidermal tissue was extracted in a dark place at 20-25 °C (room temperature) for 3 h. After the dye was sufficiently extracted from the epidermal tissue, a 150 μL aliquot of the extracted solution was transferred into each well of a 96-well plate. The absorbance of each well at 570 nm was measured by using an automated plate reader (Tecan Infinite F 50, Austria). The cell viability was calculated

from the relative value of the A570 of the extract from the epidermal tissue treated with the test compound against that of the control and expressed as a percentage.

2-1-6. RNA extraction and real-time PCR analysis

As in the previous section, 100 μL of each test compound solution diluted to the actual-use concentration was applied onto the epidermal tissue surface. LabCyte cultures were also untreated or treated with DW or 0.95% ethanol aqueous solution. After incubation for 15 min, the test compounds were removed from the culture inserts by aspiration. Subsequently, the epidermal tissues were washed three times with PBS and cultured in a CO_2 incubator at 37°C for 24 h. After the incubation period, the assay medium was collected into 1.5 mL microcentrifuge tubes and stored at -80°C for ELISA analysis. The epidermal tissues were washed three times with 200 μL PBS, removed from the culture insert by using forceps, and transferred into a 1.5 mL microcentrifuge tube containing 250 μL lysis buffer. The epidermal tissue was homogenized by using a closed system tissue homogenizer (NIPPI Inc., BioMasher II, Tokyo, Japan) and a further 100 μL of lysis buffer was added. The cell lysates were clarified by centrifugal filtration through NucleoSpin filters (Macherey-Nagel, NucleoSpin® RNA, Dueren, Germany) for 2 min at 12000×g. The supernatant was transferred into a new 1.5 mL microcentrifuge tube and 350 μL of 70% ethanol was added and mixed by vortexing. The subsequent procedures of total RNA extraction from the epidermal tissues, reverse transcription of the total RNA, and qRT-PCR were performed as described in Chapter 2, Section 1.

2-1-7. Cytokine measurement

The levels of cytokines released into the assay medium of LabCyte cultures were quantified by using human ELISA kits for IL-1α (Thermo Scientific, Waltham, MA, USA) and TNF-α (Novex, Thermo Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. As an example, the test method for the IL-1α human ELISA kit is briefly described below.

First, 50 μL of the standard, control, and test samples was added to the appropriate human IL-1α antibody precoated wells and incubated for 2 h at room temperature. Subsequently, 50 μL biotinylated antibody reagent was added to each well and the plate was incubated for 1 h at room temperature. The reaction mixtures were removed and the wells were washed three times with wash buffer. Then, 100 μL streptavidin-HRP solution was added into each well and incubated at room temperature for 30 min. The solution was removed, the wells were washed three times with wash buffer, 100 μL of premixed TMB substrate solution was added to each well, and the plate was placed in the dark and incubated at room temperature for 30 min. The reaction was stopped by the addition of 100 μL of stop solution to each well. The absorbance of each well at 450 nm was measured by using an automated plate reader (Tecan Infinite F50, Austria).

2-1-8. Statistical analysis

Statistical analyses were computed by using Ekuseru-Toukei 2010 software (Social Survey Research Information Co., Ltd, Tokyo, Japan). The data were analyzed by one-way ANOVA followed by the Dunnett's multiple comparison test.

2-2. RESULTS AND DISCUSSION

2-2-1. Cytotoxic effects of antiseptic agents in LabCyte cultures

For the prediction of the skin irritation induced by topical antimicrobial substances at practical use concentrations, we compared their cytotoxicity in a three-dimensional reconstructed human epidermis model. The cell viability of LabCyte cultures treated with each antimicrobial substance or DW (control), as determined by the MTT assay, is shown in Figure 2-3. Our results showed a sharp decrease in cell viability only when the LabCyte cultures were treated with 1000 mg/L BAC or 1000 mg/L OCT. However, 3PHBO-12, 3HHDMP-12, CHG, and PHMB were not cytotoxic to LabCyte cultures under the conditions used in this experiment.

The cytotoxicity of BAC to LabCyte cultures was the highest among the tested antimicrobial substances, although BAC was less cytotoxic than OCT to monolayer cultured cells such as NHEK (AD). The discrepancy may be attributable to the difference in skin permeability between BAC and OCT; that is, the permeability of BAC through the stratum corneum of LabCyte cultures was greater than that of OCT, which increased the cytotoxicity of BAC. In addition, unlike the results in monolayer cultured cells such as NHEK (AD), the new bis-QACs, PHMB, and CHG were not cytotoxic to the LabCyte cultures. This result is also assumed to result from the low permeability of these tested antimicrobial substances through the stratum corneum of the LabCyte cultures.

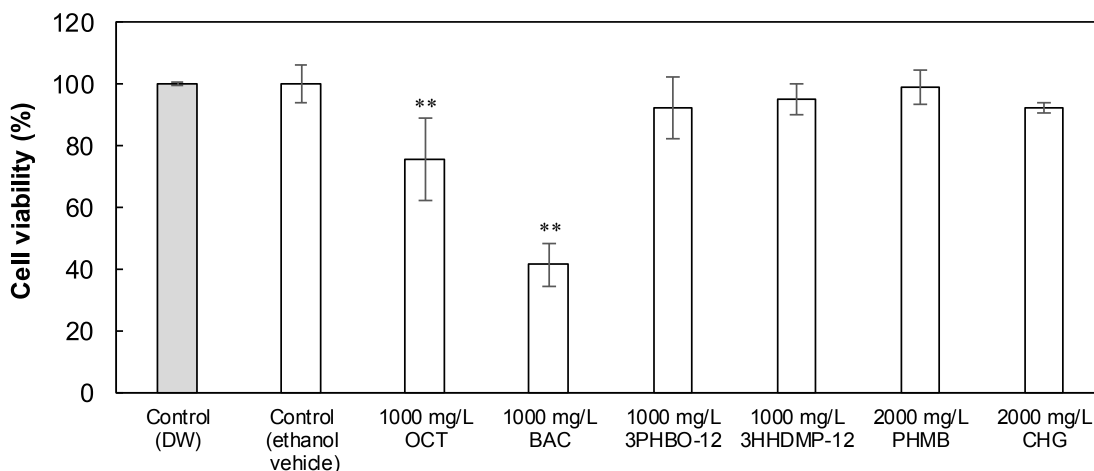


Fig. 2-3. Cytotoxicity of 3PHBO-12, 3HHDMP-12, and the reference antiseptic agents in LabCyte cultures

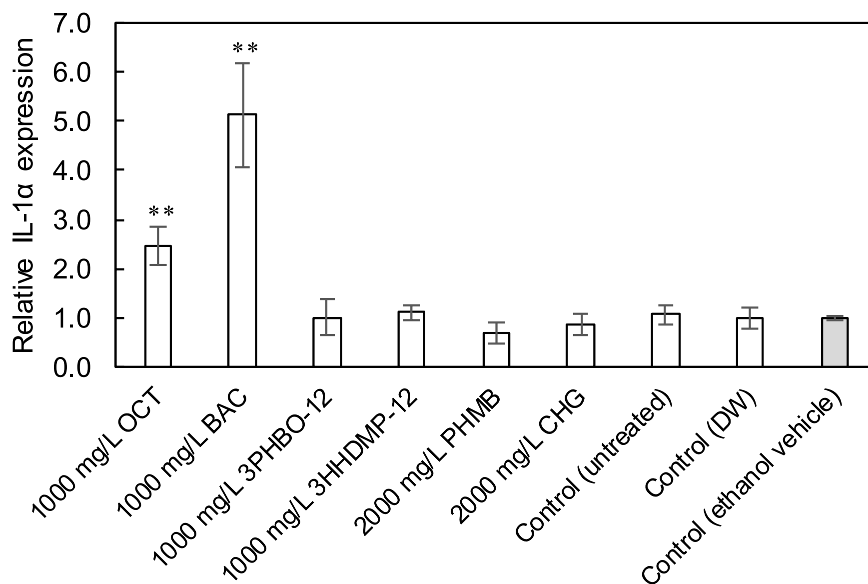
LabCyte cultures were exposed to DW (control), 0.95% ethanol aqueous solution, the indicated concentration of novel bis-QACs and various antiseptic agents, and cell viability was determined by MTT assay. All assays were performed in triplicate; the data are presented as the mean ± standard deviation.

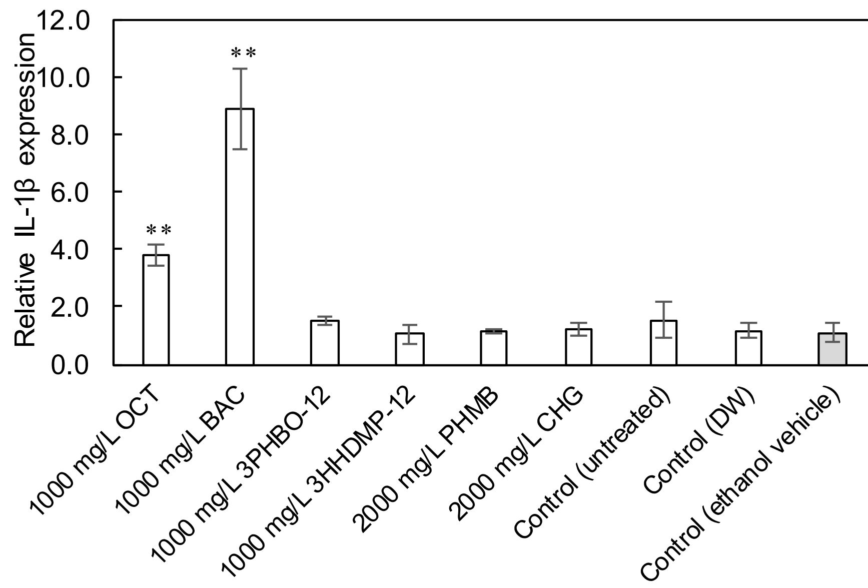
**P < 0.01 compared to control (DW).

2-2-2. Effects of antiseptic agents on cytokine gene expression in LabCyte cultures

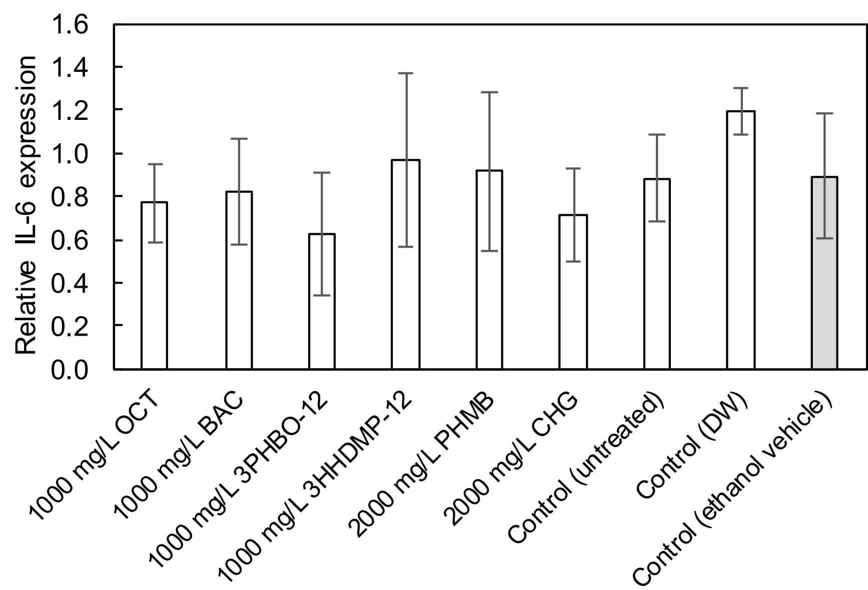
To investigate the changes in cytokine gene expression in LabCyte cultures treated with a practical concentration of antimicrobial substances for 15 min, qRT-PCR targeted to selected inflammatory cytokine gene was performed. These inflammatory cytokines are commonly used in in vitro experiments as biomarkers for skin irritation responses. As shown in Figure 2-4, the mRNA expression of inflammatory cytokines was strongly induced by existing QACs such as OCT and BAC. Our results showed that 1000 mg/L OCT significantly induced IL-1α, IL-1β, and TNF-α expression compared with ethanol vehicle-treated control cultures. In addition, 1000 mg/L BAC significantly induced the expression of four inflammatory cytokine genes (IL-1α, IL-1β, IL-8, and TNF-α)

compared with ethanol vehicle-treated control cultures. In particular, 1000 mg/L BAC markedly induced the mRNA expression of IL-1α, IL-1β, and IL-8 compared with OCT-treated LabCyte cultures. However, 1000 mg/L 3PHBO-12, 1000 mg/L 3HHDMP-12, 2000 mg/L PHMB, and 2000 mg/L CHG did not significantly alter the expression level of any of the tested inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8 and TNF-α) in the LabCyte cultures. There was no significant difference in mRNA expression of tested inflammatory cytokines among untreated cultures, DW-treated cultures, and ethanol vehicle-treated cultures.





Relative IL-1β expression
12.0
10.0
8.0
6.0
4.0
2.0
0.0
**
**
1000 mg/L OCT
1000 mg/L BAC
1000 mg/L 3PHBO-12
1000 mg/L 3HHDMP-12
2000 mg/L PHMB
2000 mg/L CHG
Control (untreated)
Control (DW)
Control (ethanol vehicle)



Relative IL-6 expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1000 mg/L OCT
1000 mg/L BAC
1000 mg/L 3PHBO-12
1000 mg/L 3HHDMP-12
2000 mg/L PHMB
2000 mg/L CHG
Control (untreated)
Control (DW)
Control (ethanol vehicle)

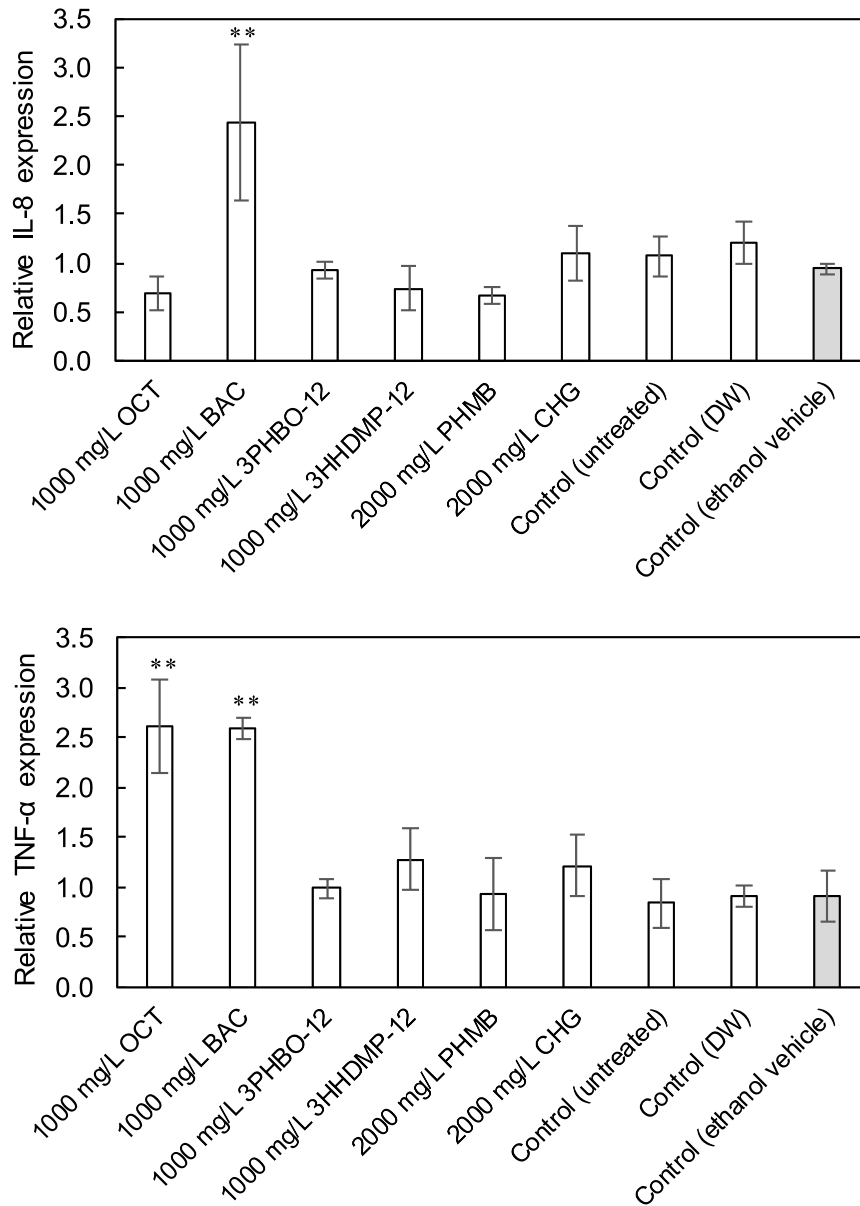


Fig. 2-4. The mRNA expression of inflammation rerated genes (IL-1α, IL-1β, IL-6, IL-8, and TNF-α) in LabCyte cultures treated with 3PHBO-12, 3HHDMP-12, and existing antiseptic agents

The mRNA expression levels determined by qRT-PCR were normalized to those of GAPDH and plotted relative to those in cells treated with 0.95% ethanol aqueous solution (control). All assays were performed in triplicate; the data are shown as the mean ± standard deviation.

***P* < 0.01 compared to ethanol vehicle (0.95% ethanol aqueous solution) control.

2-2-3. Effects of antiseptic agents on secretion of cytokines in LabCyte cultures

As the antimicrobial substance-induced inflammatory cytokine production was suggested to occur in the LabCyte cultures, we also measured the quantity of inflammatory cytokines such as IL-1α and TNF-α released into the medium from LabCyte cultures 24 h post incubation. It is well known that IL-1α and TNF-α are very important inflammatory mediators produced at the initiation of inflammation. It has also been reported that genetic polymorphisms of IL-1α and TNF-α affect the susceptibility of the skin to inflammation and the development of irritant contact dermatitis[60,61]. For these reasons, to assess skin irritation caused by new bis-QACs and existing antiseptics, we measured the protein levels of IL-1α and TNF-α, which are highly correlated with the risk of irritant contact dermatitis. Figure 2-5 show the cytokine content of the medium, determined by ELISA. IL-1α secretion into the culture medium significantly increased after treatment with 1000 mg/L OCT, 1000 mg/L BAC, or 2000 mg/L CHG compared to that induced by ethanol vehicle treatment. As for the results of qRT-PCR analysis, 1000 mg/L BAC induced the highest IL-1α secretion among the test antimicrobial substances.

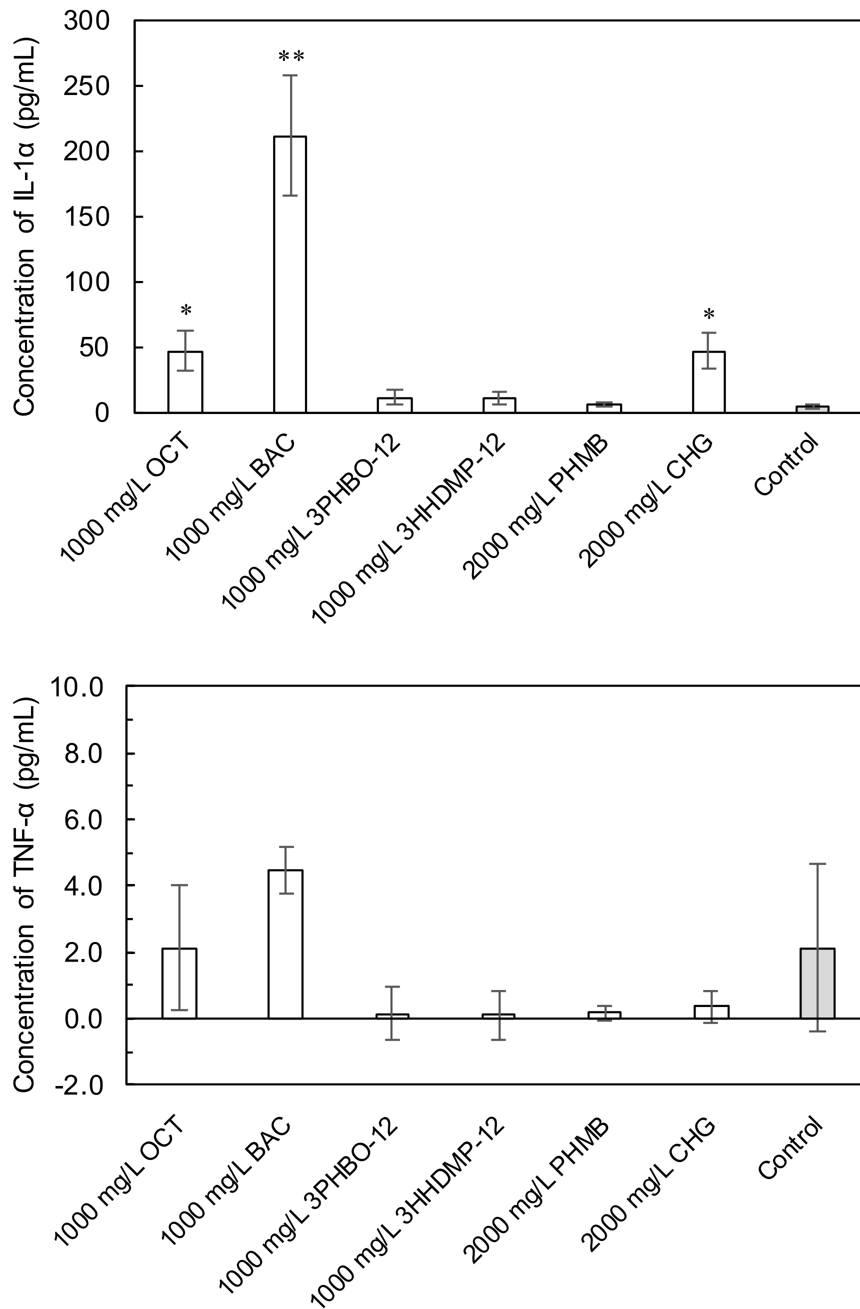


Fig. 2-5. Cytokine secretion from LabCyte cultures treated with 3PHBO-12, 3HHDMP-12, and the existing antiseptic agents

The levels of IL-1α or TNF-α in the assay medium were determined by ELISA. All assays were performed in triplicate and the data are shown as the mean ± standard deviation.

**$P < 0.01$, *$P < 0.05$ compared to ethanol vehicle (0.95% ethanol aqueous solution) control.

It has been reported that IL-1α secretion increased with decreased dose-dependent cell viability in a BAC-treated reconstructed human epidermis model[62]. As seen in this study, we showed that the inflammatory cytokine mRNA expression and IL-1α secretion were correlated to some extent with the cytotoxicity of the antimicrobial substance. Furthermore, it was revealed that IL-1α mRNA expression correlated with IL-1α secretion in OCT- or BAC-treated LabCyte cultures. In contrast, CHG did not alter IL-1α mRNA expression in LabCyte cultures, and only increased IL-1α secretion from epidermal tissues. As previously reported, mRNA expression varies over time in cultured cells responding to specific stimulus[63]. In the present study, various antimicrobial substances were applied to the LabCyte cultures for 15 min, after which they were washed with PBS and the cultures then incubated for a 24 h. We analyzed cytokine mRNA expression and IL-1α production by using samples of epidermal tissue and culture medium collected at the end of this incubation period. It was inferred that CHG-induced IL-1α mRNA expression has a short half-life and that mRNA expression decreased during late-stage culture.

In contrast, no significant TNF-α secretion was observed for LabCyte cultures treated with any of the antimicrobial substances tested. Conversely, OCT and BAC did not increase TNF-α secretion, although they did increase mRNA levels of TNF-α in LabCyte cultures. Similarly, Bernhofer et al. also reported that TNF-α mRNA levels increased in their examination of the irritation of consumer products using three-dimensional cultured skin, whereas TNF-α secretion from the tissues was not detected[54]. These results suggested that some regulatory mechanism restricts the translation stage of the TNF-α gene, in three-dimensional cultured skin treated with

OCT and BAC.

In any case, novel bis-QACs, such as 3PHBO-12 or 3HHDMP-12, and biguanide-based compounds, such as PHMB, did not enhance the expression of inflammatory cytokines at both mRNA and protein levels. The three-dimensional reconstructed human skin model has a morphologically similar structure to human skin and has been reported to exhibit a good correlation with in vivo skin irritation[52,59]. Therefore, it is predicted that the skin irritancy of 3PHBO-12 and 3HHDMP-12 is equal to or less than that of other antiseptic agents, even if these novel bis-QACs are applied to human skin at practical-use concentrations.

In addition, the three-dimensional reconstructed human epidermis model, LabCyte, which we used as an alternative to evaluate in vivo skin irritation does not reflect all the functions of human skin tissue. In recent years, a sensitive human skin model with immature stratum corneum has been marketed as a three-dimensional reconstructed human epidermis model for the evaluation of irritation to sensitive skin or in infants. Moreover, in healthcare facilities, as antiseptic agents may be used frequently and over a long period of time, the skin irritation caused by antiseptic agents should be evaluated under test conditions simulating more practical cases. Therefore, further investigation is necessary for the accurate prediction of the risk of adverse effects, such as skin irritation, of novel bis-QACs.

SECTION 3.

Conclusion

In this chapter, the novel bis-QACs 3PHBO-12 and 3HHDMP-12 were compared with commonly-used antiseptics, BAC, OCT, CHG, and PHMB, to predict their potential to cause skin irritation. The cytotoxicity of these compounds in various cultured cells, as well as their effect on the expression of inflammatory cytokine genes such as IL-1α were evaluated.

The cytotoxicity of these novel bis-QACs on NHEK (AD), NHDF(AD), HL-60, and THP-1 cells derived from humans was lower than that of existing QACs (BAC and OCT), although the novel bis-QACs showed higher cytotoxicity than the biguanide-based compounds (CHG and PHMB). In addition, mRNA expression of inflammatory cytokines was more strongly induced by BAC and OCT than by the novel bis-QACs after treatment with concentrations below the IC_{50} obtained with NHEK (AD) cells. Furthermore, we compared the gene expression and secretion for the inflammatory cytokines in antimicrobial substances-treated LabCyte EPI-MODEL at the actual use concentration. The inflammatory cytokine gene expression and IL-1α secretion significantly increased in OCT- or BAC-treated LabCyte cultures. However, even at the actual therapeutic concentration, the novel bis-QACs did not alter inflammatory cytokine mRNA expression or IL-1α secretion. These data suggested that the skin irritation potential of novel bis-QACs such as 3PHBO-12 and 3HHDMP-12 was comparable to or less than that of existing antiseptic agents, and that these agents can safely be used as a skin antiseptics with few adverse effects.

EXECUTIVE SUMMARY

Antiseptic agents are extensively used for hand hygiene by HCWs and for skin antisepsis of patients to control and prevent the spread of HAI. Antiseptic agents, such as CHG, PHMB, BAC, and OCT, are used widely as active ingredients in antiseptics. However, there are several reports on their cytotoxicity in human cells and adverse effects, such as irritant contact dermatitis or allergic contact dermatitis, caused by these antiseptic agents. In addition, these antiseptic agents cannot kill all types of microorganisms and each has its own inherent antimicrobial spectrum. Therefore, the development of new antimicrobial substances that can be used as a topical skin antiseptic is expected to create more options for skin antisepsis, not only in terms of increased antimicrobial activity, but also in terms of reducing adverse effects such as contact dermatitis.

In addition, most studies on the conventional toxicity evaluation of antiseptic agents evaluated only cytotoxicity; there are few studies focusing on changes in the transcription and translation of inflammation-related genes induced by the antiseptic agents. Therefore, in order to predict the skin irritation potential of antiseptic agents, not only a cytotoxicity assay of cell death was used, but a more accurate evaluation was performed through the combination of multiple evaluation methods.

To meet these objectives, in the first chapter of this study, we examined the synthesis method of various bis-QACs, in order to obtain the molecular structure of a novel antimicrobial substance with greater biocompatibility than OCT, which is known to have excellent bactericidal activity. Three series of novel bis-QACs were obtained by

linking two molecules of pyridinium salt with a spacer structure such as pentaerythritol or hydroquinone, followed by N-alkylation with alkyl halides. The antimicrobial activity of the newly synthesized bis-QACs, 4TOSU-n, 3PHBO-n and 3HHDMP-n, and the existing antiseptic agents was compared through the examination of the MIC and MBC. Our results indicated that 4TOSU-10, 12, 3PHBO-10, 12, and 3HHDMP-10, 12 exerted potent bacteriostatic activity against gram-negative bacteria, gram-positive bacteria, and fungi, which demonstrated their broad antibacterial spectra. Furthermore, the measurement of MBC to evaluate the bactericidal activity of the novel bis-QACs revealed excellent MBC values as the alkyl chain length increased from 8 to 12. In particular, as the bactericidal activity of bis-QACs with an alkyl chain length of C12 was comparable with that of OCT, which showed the best bactericidal activity among the existing antiseptic agents, it was determined that these compounds had potent bactericidal activity.

To compare the cytotoxicity of the novel bis-QACs with existing antiseptic agents, an MTT assay was performed in NHEK (NB) cells. The cytotoxic effect of 4TOSU-10, 3PHBO-12, 3HHDMP-8, 3HHDMP-10, and 3HHDMP-12 was lower than that of existing QACs and was comparable with that of biguanide-based compounds. The comparison of BIs revealed that novel bis-QACs, such as 4TOSU-12, 3PHBO-12 and 3HHDMP-12, had equal or greater biocompatibility than the existing antiseptic agents tested. Thus, the biocompatibility of 4TOSU-12, 3PHBO-12, and 3HHDMP-12 was equal to or greater than that of existing antiseptic agents; therefore, these bis-QACs were expected to be useful as topical skin antiseptics.

In Chapter 2, to predict the potential skin irritation of novel bis-QACs more

accurately, we focused on the inflammatory cytokines induced by antiseptics and compared their expression at both the mRNA and protein level. In this study, novel bis-QACs such as 3PHBO-12, 3HHDMP-12, which showed particularly excellent BI values as reported in the first chapter, were used for the test. The cytotoxicity of 3PHBO-12 and 3HHDMP-12 in NHEK (AD), NHDF (AD), THP-1 and HL-60 cells was evaluated by using a WST assay. The cytotoxicity of these bis-QACs was lower than that of the existing QACs, but higher than that of the biguanide-based compounds. Subsequently, each antimicrobial substance at a concentration lower than the IC_{50} obtained from the cytotoxicity assay was applied to NHEK (AD) cells for 24 to 72 h to compare the mRNA expression levels of inflammatory cytokines. Our results showed that BAC or OCT induced an increase in inflammatory cytokine expression over time compared with the novel bis-QACs. In particular, 3HHDMP-12 did not induce the mRNA expression of inflammatory cytokines, which suggested that skin irritation may be milder than existing antiseptic agents.

Therefore, we evaluated the potential irritancy of the novel bis-QACs in more detail by using a reconstructed human skin model, LabCyte EPI-MODEL which is known to exhibit a good correlation with in vivo skin irritation. 3PHBO-12 and 3HHDMP-12 were applied to the LabCyte cultures at the actual use concentration and their cytotoxicity was compared. Cytotoxicity was observed only when cultures were treated with OCT and BAC, and not when treated with novel bis-QACs. In addition, the expression of inflammatory cytokines in the LabCyte cultures treated with each antimicrobial substance was compared at both the mRNA and protein level. As seen in the results of the cytotoxicity assay, inflammatory cytokine gene expression and IL-1α

secretion was significantly increased in OCT or BAC-treated LabCyte cultures. In contrast, even at actual use concentrations, the novel bis-QACs did not alter inflammatory cytokine mRNA expression or IL-1α secretion. From the results of experiments using LabCyte cultures, it was suggested that the skin irritancy of 3PHBO-12 and 3HHDMP-12 was equal to or less than that of existing antiseptic agents.

In summary, this paper is a comparative study of the efficacy and safety of newly synthesized bis-QACs and existing antiseptic agents and has clarified the antimicrobial properties, cytotoxicity, biocompatibility, and potential skin irritancy of these bis-QACs. Unlike antibiotics, which are intended to have selective toxicity to microorganisms, skin antiseptics are toxic not only to microorganism cells, but also to human skin cells. This is an essential property and there are therefore no antiseptic agents that completely overcome the problems of both efficacy and safety. However, the development of new antimicrobial substances is expected to create more options for skin antisepsis, not only through increased antimicrobial activity, but also through a reduction in adverse effects such as contact dermatitis. In particular, the biocompatibility and potential skin irritation of 3PHBO-12 and 3HHDMP-12 were equal to or less than those of existing antiseptic agents. Therefore, were considered that these novel bis-QACs were applicable antiseptics to human skin, which prevent many HAI, including drug-resistant bacteria.

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LIST OF PUBLICATIONS

Ⅰ **Yamamoto, M., Matsumura, R., Hirata, Y., Nagamune, H.**, 2019. A Comparative Study of Skin Irritation Caused by Novel Bis-quaternary Ammonium Compounds and Commonly used Antiseptics by using Cell Culture Methods. *Toxicology in Vitro.* 54, February, 75-81.
https://doi.org/10.1016/j.tiv.2018.09.009

Ⅱ **Yamamoto, M., Takami, T., Matsumura, R., Dorofeev, A., Hirata, Y., Nagamune, H.**, 2016. In vitro evaluation of the biocompatibility of newly synthesized bis-quaternary ammonium compounds with spacer structures derived from pentaerythritol or hydroquinone. *Biocontrol Sci*. 21 (4), 231–241. https://doi.org/10.4265/bio.21.231

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