Antimicrobial Activity of Saturated Fatty Acids and Fatty Amines against Methicillin-Resistant *Staphylococcus aureus*

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The objective of this study was to investigate the antimicrobial activities of saturated fatty acids and fatty amines against methicillin-resistant *Staphylococcus aureus* (MRSA). The antimicrobial activity of saturated fatty acids and fatty amines was determined by oxygen meters with multi-channels and disposable oxygen electrode sensors (DOX-96). Lauric acid, the most effective among the saturated fatty acids, showed antimicrobial activity at 400 μg/ml against methicillin-susceptible *Staphylococcus aureus* (MSSA) and MRSA. The minimal inhibitory concentration (MIC) of fatty amines depended on each hydrophobic chain length. The MIC of myristylamine was 1.56 μg/ml; most effective of the fatty amines. In time-kill curves, lauric acid and myristylamine produced a bactericidal effect and a bacteriostatic effect at 4-fold the MIC, respectively. The antimicrobial activities of lauric acid and myristylamine were decreased by human plasma. Cytotoxicity of 3 saturated fatty acids and 3 fatty amines was examined in cultured endothelial cells. Although cytotoxicity of fatty amines was severer than that of saturated fatty acids, myristylamine showed the highest value of apparent therapeutic index among them. DOX-96 was useful for screening antimicrobial substances, especially in the case of insoluble substances. We found that myristylamine showed anti-MRSA activity comparable to that of vancomycin and teicoplanin.

Key words methicillin-resistant *Staphylococcus aureus* (MRSA); oxygen meter; fatty acid; fatty amine; antimicrobial activity

Antimicrobial resistance among gram-positive bacteria has become increasingly prevalent and serious infections thus caused have become more widespread during the past 15 to 20 years.[1,2] Most nosocomial and community-acquired infections are caused by *Staphylococcus aureus* (*S. aureus*).[3,4] A recent investigation of isolates obtained in Nagasaki University Hospital of Medicine and Dentistry, an 869-bed hospital in Nagasaki, Japan reports that 60.2% of *S. aureus* isolates are methicillin-resistant. Methicillin-resistant *Staphylococcus aureus* (MRSA) rates were 54.5% according to data from the National Nosocomial Infections Surveillance System of the Centers for Disease Control and Prevention.[5] In addition, many strains of MRSA are resistant to all antimicrobial agents except glycopeptides (vancomycin and teicoplanin).[6] Alternatives to glycopeptides are sometimes necessary due to intolerance or treatment failure. *S. aureus* with glycopeptide-resistance has now been documented in the U.S.A.[7—9]

In order to discover which materials have anti-MRSA activity, various chemical compounds have been investigated.[10—14] Long-chain fatty acids are known as surface-active anionic detergents,[15] and in microbiology have a long history of more than eighty years.[16—18] In general, fatty acid sensitivity is considered to be a characteristic of gram-positive bacteria, with few gram-negative species being susceptible.[19] However, antimicrobial susceptibility testing against lipids is difficult because the lipid becomes turbid and insoluble. The antimicrobial activities of the lipids against MRSA have thus not been investigated systematically.

In our laboratory, Kitahara *et al.* recently confirmed that new oxygen meters with multi-channels and disposable oxygen electrode sensors (DOX-96; DAIKIN ENVIRONMENTAL LABORATORY, LTD., Tsukuba, Japan) may be applicable to antimicrobial susceptibility testing of clinical bacterial isolates. The minimum inhibitory concentration (MIC) determined by DOX-96 showed good agreement with MIC measured by the standard broth microdilution method. DOX-96 was also useful for turbid samples.[20] Therefore we measured the MIC of saturated fatty acids ($CH_3(CH_2)_nCOOH$) and fatty amines ($CH_3(CH_2)_{n+1}NH_2$) against MRSA by utilizing the characteristics of DOX-96 in measuring turbid samples.

In this study, we investigated *in vitro* antimicrobial activities of saturated fatty acids and fatty amines for useful compounds against MRSA.

MATERIALS AND METHODS

Materials The following antimicrobial agents were used: Oxacillin (MPIPC; Wako Pure Chemical Ind. Ltd., Osaka, Japan), ampicillin (ABPC; Meiji Seika Co., Tokyo, Japan), sulbactam/ampicillin (S/A; Pfizer Co., Tokyo, Japan), cefazolin (CEZ; Fujisawa Pharmaceutical Co., Osaka, Japan), cefotiam (CTM; Takeda Chemical Ind. Ltd., Osaka, Japan), cefmetazole (CMZ; Sankyo Co., Tokyo, Japan), flomoxef (FMOX; Shionogi Co., Osaka, Japan), cefpirome (CPR; Chugai Pharmaceutical Co., Tokyo, Japan), imipenem (IPM; Banyu Pharmaceutical Co., Tokyo, Japan), gentamicin (GM; Schering-Plough Co., Osaka, Japan), arbekacin (ABK; Meiji Seika Co., Tokyo, Japan), minocycline (MINO; Wyeth-Lederle Co., Tokyo, Japan), levofloxacin (LVFX; Daiichi Pharmaceutical Co., Tokyo, Japan), vancomycin (VCM; Shionogi Pharmaceutical Co., Osaka, Japan), and teicoplanin (TEIC; Fujisawa Pharmaceutical Co., Osaka, Japan). Octanoic acid, decanoic acid, lauric acid, myristic acid, palmitic acid, stearic acid, octylamine, decylamine, laurylamine,

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myristylamine, and stearylamine were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Hexadecylamine was purchased from Nacalai Tesque (Kyoto, Japan). All saturated fatty acids and fatty amines were dispersed with a sonicator (NO5202, Ohtake Co., Tokyo, Japan). All other chemicals were of the highest purity available.

Bacterial Strains *S. aureus* ATCC29213 and 5 clinical isolates were used in the present study. ATCC29213 was methicillin-susceptible *S. aureus* (MSSA) and 5 clinical isolates were MRSA. The clinical isolates (strain numbers 4952, 6849, 3818, 352, and 5914) were collected in Nagasaki University Hospital of Medicine and Dentistry, Nagasaki, Japan.

DOX-96 System The electrode for DOX-96 was a 96-well plate with three electrodes embedded in each well. The oxygen amount in the sample was converted into current by the following reaction, $4H^+ + O_2 + 4e^- \rightarrow 2H_2O$, and the current was drawn on a graph with a laptop computer.[21] As viable bacteria consumed the oxygen, the oxygen amount in the sample decreased.

MIC Determination MIC of antimicrobial agents was determined by a microdilution method with cation-adjusted Mueller–Hinton broth (BBL Microbiology Systems, Cockeysville, MD, U.S.A.) according to the recommendations of the National Committee for Clinical Laboratory Standards.[22] MIC of saturated fatty acids and fatty amines was determined by DOX-96. The saturated fatty acids and fatty amines were adequately suspended in Mueller–Hinton broth by sonicator because they are insoluble. The samples were diluted two fold in Mueller–Hinton broth, and dispensed into the wells (100 μl/well) of an electrode plate. Fresh Mueller–Hinton broth (90 μl) was added to the wells. All the wells except the negative control wells were inoculated with 10 μl of each bacterium in Mueller–Hinton broth to yield a final inoculum size of 1×10^5 colony-forming units (CFU)/ml. The negative control wells received 10 μl of Mueller–Hinton broth only. The positive control wells received Mueller–Hinton broth instead of fatty acids or fatty amines. The electrode plate was set on DOX-96 and was incubated for 999 min (16.65 h) at 35 °C. The measurement of the current in each well when compared with their respective positive and negative controls was taken as oxygen consumption. If bacteria existed in the sample, remarkable oxygen consumption was observed within 999 min (16.65 h) by proliferation of bacteria. The remarkable oxygen consumption was suppressed by antimicrobial activity of some fatty acid or fatty amine. The lowest concentration of fatty acid or fatty amine at which the remarkable oxygen consumption was suppressed during 999 min (16.65 h) was taken to be the MIC. MIC of saturated fatty acids and fatty amines in the presence of human plasma was also determined by DOX-96. Human blood was collected from healthy volunteers after informed consent. Human plasma was obtained by centrifugation of human blood at $1000\times\boldsymbol{g}$ for 5 min. MIC of saturated fatty acids and fatty amines after addition of human plasma (not inactivation) was determined by the method described above.

Time-Kill Curve Time-kill curves were performed with lauric acid and myristylamine at concentrations of 1, 2, and 4 fold the MIC. The mid-logarithmic-phase preparation was appropriately diluted in lipid-containing Mueller–Hinton broth test tubes (2 ml) to achieve a final inoculum of 1×10^5 CFU/ml. The same inoculum was added to lipid-free Mueller–Hinton broth as a growth control. The tubes were incubated at 37 °C for 24 h. Samples were taken at 0, 1, 2, 4, 6, and 24 h; suitable dilutions were made in Mueller–Hinton broth; and 10 μl was plated on tryptic soy agar. The plates were incubated at 37 °C for 24 h, and colony counts were performed. Time-kill curves were constructed by plotting $\log_{10}$ CFU/ml against time over 24 h.[23]

Cytotoxicity Assay Human endothelial cell line EA.hy 926 was kindly donated by the First Department of Internal Medicine, Nagasaki University Hospital of Medicine and Dentistry. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37 °C under an atmosphere of 5% CO_2 in air. FBS was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). DMEM, antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml), and other culture reagents were obtained from GIBCO BRL (Grand Island, NY, U.S.A.). Cytotoxicity tests on EA.hy 926 cells were carried out using a WST-1 (Dojindo Laboratories Inc., Kumamoto, Japan) commercially available cell proliferation reagent. The assay is based on cleavage of the tetrazolium salt WST-1 by active mitochondria to produce a soluble colored formazan salt. Since the conversion occurred only with viable cells, it directly correlated with the cell number. The EA.hy 926 cells were plated at 4×10^3 cells/well in 96-well microtiter plates (Becton-Dickinson, Franklin, NJ, U.S.A.). Twenty-four hours after plating, the growth medium was removed and replaced with the test solutions (100 μl). After 16.5 h exposure the reaction medium was removed, and 100 μl fresh growth medium and 10 μl cell proliferation reagent WST-1 were added in each well. The cells were incubated for 2 h at 37 °C in a humidified atmosphere with 5% CO_2, and the absorbance was measured at a wavelength of 450 nm with a reference wavelength of 630 nm, using a microplate reader (Spectra 1, TECAN Austria Ges. m.b.H., Grodig, Austria). The results were expressed as percent optical density of treated *vs*. control untreated, serum-containing cultures.[24]

RESULTS

Susceptibility of *S. aureus* to Antimicrobial Agents The MIC of 15 antimicrobial agents, commonly used and of different classes, against 6 strains of *S. aureus* were determined by the microdilution method and are shown in Table 1. MSSA was susceptible to all of the antimicrobial agents tested except for ABPC. On the other hand, 5 strains of MRSA were resistant to penicillins. In addition, MRSA 6849 was resistant to GM and LVFX, MRSA 3818 was resistant to GM, MRSA 352 was resistant to CEZ, CTM, CPR, and LVFX, and MRSA 5914 was resistant to all cephems, IPM, GM, MINO, and LVFX. Although 5 strains of MRSA had different resistances to various antimicrobial agents, VCM and TEIC were effective against all strains used in the present study.

Antimicrobial Activities of Saturated Fatty Acids and Fatty Amines against MRSA Table 2 shows MIC of saturated fatty acids against 6 strains of *S. aureus* measured by DOX-96. We confirmed that the presence of lipid itself didn't affect the process of oxygen consumption. Lauric acid, most

Table 1. MIC of 15 Antimicrobial Agents against 6 *Staphylococcus aureus*

Antimicrobial agents	MIC (μg/ml)					
	MSSA	MRSA				
	ATCC 29213	4952	6849	3818	352	5914
MPIPC	≦0.5	16	>16	16	>16	>16
ABPC	2	16	>16	>16	>16	>16
S/A	1	16	16	16	>16	>16
CEZ	≦0.5	4	4	8	>16	>16
CTM	1	4	4	4	>16	>16
CMZ	1	8	16	8	8	>16
FMOX	≦0.5	4	8	4	8	>16
CPR	≦0.5	4	4	4	>16	>16
IPM	≦0.5	≦0.5	≦0.5	≦0.5	≦0.5	>16
GM	≦0.5	1	>16	>16	≦0.5	>16
ABK	≦0.5	2	2	4	≦0.5	2
MINO	≦0.5	≦0.5	≦0.5	≦0.5	≦0.5	16
LVFX	≦0.5	≦0.5	16	1	>16	8
VCM	1	2	1	1	1	2
TEIC	≦0.5	≦0.5	≦0.5	1	≦0.5	4

Each compound was measured three times.

Table 2. Antimicrobial Activity of Saturated Fatty Acids against 6 *Staphylococcus aureus*

Fatty acids (Carbon number)	MIC (μg/ml)					
	MSSA	MRSA				
	ATCC 29213	4952	6849	3818	352	5914
Octanoic acid (C8)	>1600	>1600	>1600	>1600	>1600	>1600
Decanoic acid (C10)	800	800	800	800	800	800
Lauric acid (C12)	400	400	400	400	400	400
Myristic acid (C14)	1600	>1600	800	1600	>1600	>1600
Palmitic acid (C16)	>1600	>1600	>1600	>1600	>1600	>1600
Stearic acid (C18)	>1600	>1600	>1600	>1600	>1600	>1600

Each compound was measured three times.

Table 3. Antimicrobial Activity of Fatty Amines against 6 *Staphylococcus aureus*

Fatty amines (Carbon number)	MIC (μg/ml)					
	MSSA	MRSA				
	ATCC 29213	4952	6849	3818	352	5914
Octylamine (C8)	400	400	400	400	400	400
Decylamine (C10)	100	100	100	100	100	100
Laurylamine (C12)	6.25	6.25	6.25	6.25	6.25	6.25
Myristylamine (C14)	1.56	1.56	1.56	1.56	1.56	1.56
Hexadecylamine (C16)	3.13	3.13	3.13	6.25	1.56	3.13
Stearylamine (C18)	12.5	12.5	12.5	25	12.5	25

Each compound was measured three times.

effective among the saturated fatty acids, showed antimicrobial activity at 400 μg/ml against all 6 strains of *S. aureus* that are gram-positive bacteria. None of the saturated fatty acids were so effective against MRSA compared with antimicrobial agents.

Table 3 shows MIC of fatty amines against 6 strains of *S. aureus* measured by DOX-96. The MIC of fatty amines depended on the hydrophobic chain length. The MIC of myristylamine was 1.56 μg/ml; this was the most effective of the fatty amines. The efficacy of myristylamine was approximately 250 fold that of lauric acid. Furthermore it showed extremely high antimicrobial activity against all strains of MRSA, almost equal to those of VCM and TEIC.

Time-Kill Curve Figures 1A and B present the killing curves of lauric acid and myristylamine, respectively, at concentrations equal to 1, 2 and 4 fold the MIC. The results are expressed as log 10 changes in the number of surviving bacteria in the antimicrobial tests at various time intervals com-

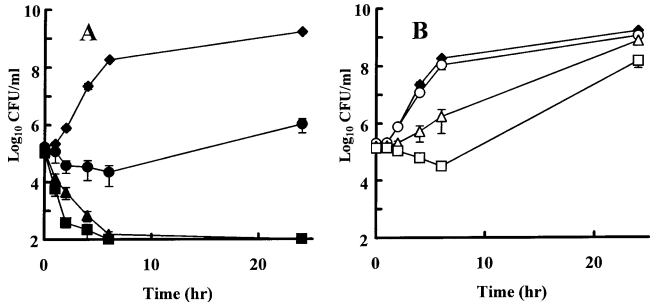

Fig. 1. Time-Kill Curves of Lauric Acid (A) and Myristylamine (B) against MRSA 5914

Bacteria were incubated without lipids (growth control, ◆), with lauric acid (MIC, ●), (2×MIC, ▲), (4×MIC, ■) and with myristylamine (MIC, ○), (2×MIC, △), (4×MIC, □). Data points are mean values±S.E.M. of three experiments.

Table 4. Effect of Plasma on the Antimicrobial Activity of Lauric Acid and Myristylamine against MRSA 5914

	MIC (μg/ml)		Inhibitory index ($MIC_{Lipid}/MIC_{Lipid+Plasma}$)
	Lipid	Lipid+plasma	
Lauric acid	400	800	0.50
Myristylamine	1.56	25	0.063

Each compound was measured three times.

pared with lipid-free control. The initial control count was 5 log CFU/ml, which increased to 9 log CFU/ml after 24 h. Lauric acid showed a bacteriostatic effect against MRSA at the concentration of MIC. The suppressive effect of lauric acid on bacterial regrowth depended on its concentration. At 2 and 4 fold the MIC, lauric acid produced a bactericidal effect after 6 h of incubation. The suppressive effect of myristylamine on bacterial regrowth also depended on its concentration. Myristylamine had bacteriostatic effect against MRSA below 4 fold the MIC.

Effect of Plasma on Antimicrobial Activity Table 4 shows the effect of plasma on antimicrobial activities of lauric acid and myristylamine. The MIC of lauric acid and myristylamine against MRSA were determined in Mueller–Hinton broth with 10% human plasma by oxygen consumption using DOX-96. The MIC of lauric acid and myristlamine increased to 800 μg/ml and 25 μg/ml, respectively, in the presence of 10% plasma. The MIC was divided by that with 10% plasma and the ratios are listed in Table 4 as inhibitory indices. The antimicrobial activity of myristylamine was decreased to 6.3% by human plasma from the standpoint of inhibitory index, although that of lauric acid was decreased to 50%.

Cytotoxicity of Saturated Fatty Acids and Fatty Amines Figure 2 shows cytotoxicity of saturated fatty acids and fatty amines on endothelial cells. Saturated fatty acids and fatty amines showed a dose-dependent cytotoxicity. Cytotoxicity of fatty amines was severer than that of saturated fatty acids. Cytotoxicity of saturated fatty acids decreased with increasing carbon number. The 50% lethal concentrations (LC_{50}) of lipids on endothelial cells were estimated from data in Fig. 2, and are listed in Table 5 with apparent therapeutic indices (LC_{50}/MIC). Myristylamine showed the highest value of apparent therapeutic index among 3 saturated fatty acids and 3 fatty amines.

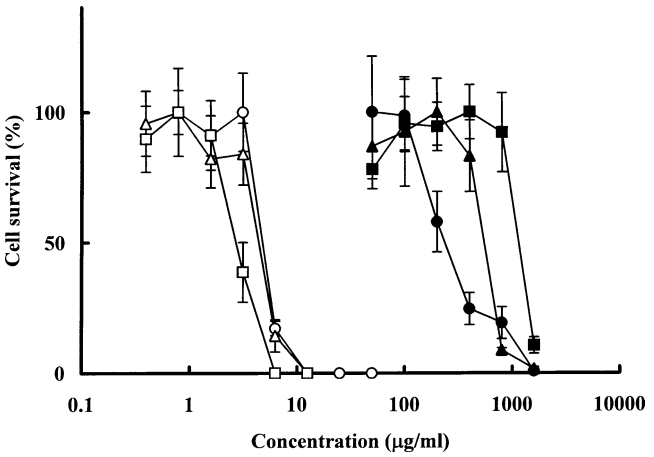

Fig. 2. Cell Survival of EA.hy 926 Endothelial Cells Treated with Saturated Fatty Acids and Fatty Amines

Cells were incubated with decanoic acid (●), lauric acid (▲), myristic acid (■), laurylamine (○), myristylamine (△), and hexadecylamine (□). Data points are mean values±S.E.M. of six experiments.

Table 5. Cytotoxicity of 3 Saturated Fatty Acids and 3 Fatty Amines

Lipids	LC_{50} (μg/ml)[a]	Apparent therapeutic index (LC_{50}/MIC)
Decanoic acid	296.0±24.5	0.37
Lauric acid	592.6±29.7	1.48
Myristic acid	1252.8±64.0	<0.78
Laurylamine	4.9±0.5	0.78
Myristylamine	4.8±0.4	3.08
Hexadecylamine	3.0±0.5	0.48

a) Values are the mean±S.E.M. of six experiments.

DISCUSSION

MRSA poses an increasingly serious health care problem in many parts of the world. Several surveillance studies have shown an increase in the prevalence of methicillin-resistance among *S. aureus* isolates, although there is considerable variation between countries.[25,26] MRSA expresses methicillin-resistance by producing a specific penicillin-binding protein, PBP2′, that has a decreased binding affinity to β-lactam antibiotics.[27,28] Actually, all clinical isolates of MRSA used in the present study showed resistance to penicillins such as MPIPC, ABPC, and S/A (Table 1). In addition, 5 strains of MRSA showed various resistances to antimicrobial agents except for penicillins. They were, however, susceptible to VCM and TEIC. Consequently, we investigated the antimicrobial activities of saturated fatty acids and fatty amines against 1 strain of MSSA and 5 strains of MRSA by DOX-96. DOX-96 was reported to be useful to determine the antimicrobial activities for insoluble compounds[20] because it measures oxygen consumption by bacteria using oxygen electrodes.

Inhibitory properties of fatty acids against bacteria were reported to become more pronounced for longer and more unsaturated compounds.[29] We examined the antimicrobial activities of saturated fatty acids with various chain lengths. Generally, long-chain fatty acids were reported to have

higher antimicrobial activities against gram-positive bacteria than gram-negative bacteria, although most organisms are resistant to saturated fatty acids in small concentrations.[19,30] Actually lauric acid, most effective among the saturated fatty acids, showed antimicrobial activity at 400 μg/ml against 6 strains of *S. aureus* that are gram-positive bacteria (Table 2). This species difference of the fatty acid sensitivity appears to be closely related with the structural differences of the bacterial surface and the mechanism of the antimicrobial effect. The cytoplasmic membrane is certainly the target point attacked by long-chain fatty acids for killing. The biophysical and biochemical effects of fatty acids on the molecular structure of the cytoplasmic membrane might be speculated that the hydrocarbon chain of added long-chain fatty acids is inserted into the phospholipid bilayer of the membrane, thus increasing destabilization of the menbrane. The outer membrene of gram-negative bacteria behaves as a entry barrier against fatty acids, but the cell wall of gram-positive bacteria may adsorb and transport fatty acids into the inner membrane.[31,32] At 4 fold the MIC, lauric acid produced a bactericidal effect after 6 h of incubation (Fig. 1A). Although lauric acid was not so effective compared with antimicrobial agents, it may be a potential antimicrobial material and ointment base for infection control.

Cationic surface-active detergents such as benzalkonium chloride and benzethonium chloride are clinically used as strong disinfectants. It is well known that cationic surface-active detergents are effective on both gram-positive bacteria and gram-negative bacteria.[33] In the preliminary experiment, the MIC of benzalkonium chloride was approximately 6.25 μg/ml on MRSA. Benzalkonium chloride was reported to exhibit a biocidal action that appears to result from the disruption of the cell membrane, inactivation of enzymes, and denaturation of cell proteins.[34] Myristylamine also showed antimicrobial activity at low concentrations (Table 3). The MIC of myristylamine was 1.56 μg/ml, most effective among the fatty amines. The efficacy of myristylamine was approximately 250 fold that of lauric acid. It is also worth noting that myristylamine showed extremely high antimicrobial activity against all strains of MRSA used in the present study. VCM and TEIC were effective at low concentrations against the majority of gram-positive bacteria including MRSA by its selective action on bacterial cell wall peptidoglycan. Myristylamine might show antimicrobial activity by the disruption of the cell membrane, inactivation of enzymes, and denaturation of cell proteins because myristylamine is similar to cationic surface-active detergents. The activity of myristylamine was almost equal to those of VCM and TEIC, although there must be a big difference between them in antimicrobial mechanism.

Antimicrobial activity of benzalkonium chloride derivatives was reported to have a bactericidal effect dependent on the hydrophobic chain length.[35] The hydrophobic chain length may partly affect the detergent activity on the cytoplasmic membrane. The MIC of fatty amines also depended on hydrophobic chain length. Myristylamine, however, showed a bacteriostatic effect against MRSA below 4 fold the MIC (Fig. 1B). This difference of antimicrobial mechanism between benzalkonium chloride and myristylamine might be explained by their structural differences. Further study of antimicrobial mechanisms is necessary for myristylamine.

Benzalkonium chloride was reported to be inactivated by human plasma and biological components.[36] The influences of human plasma on the antimicrobial activities of lauric acid and myristylamine were examined and are presented in Table 4. The antimicrobial activity of myristylamine was decreased to 6.3% by human plasma from the standpoint of MIC value although that of lauric acid was decreased to 50%. The antimicrobial activities of lauric acid and myristylamine might be neutralized by adsorption on plasma proteins. But the details of reduced antimicrovial activities were not investigated, so we need additional investigations.

Finally we investigated the cytotoxicity of saturated fatty acids and fatty amines (Fig. 2). Saturated fatty acids and fatty amines showed a dose-dependent cytotoxicity. Although cytotoxicity of fatty amines was severer than that of saturated fatty acids, myristylamine showed the highest value of apparent therapeutic index among 3 saturated fatty acids and 3 fatty amines (Table 5). The external application is supported by the fact that saturated fatty acids and fatty amines show a significant cytotoxicity against endothelial cells. From the therapeutic point of view it may have an adverse effect. On the other hand, endothelial cells specific effect could be used in the field of cancer angiogenesis.

In the present study, we investigated the antimicrobial activities of saturated fatty acids and fatty amines against MRSA using DOX-96. It was indicated that DOX-96 was useful for screening antimicrobial substances, especially in the case of insoluble substances. We found that myristylamine showed anti-MRSA activity comparable to that of VCM and TEIC. The detergent-like properties may allow attacking the biofilms consisting of bacteria. Additionally, inexpensive antimicrobial compounds are very useful and available for infection control and medical attendance in hospitals. We expect that these lipids would be useful for an external application, or otherwise may be delivered systematically in a form of liposomes. But inhibition of antimicrobial activity of myristylamine by plasma and severe cytotoxicity was also demonstrated. Further study is necessary for the clinical application of lipids including unsaturated fatty acids and fatty amines.

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