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In vitro activity of lauric acid or myristylamine in combination with six antimicrobial agents against methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

The objective of this study was to investigate the in vitro activities of lauric acid and myristylamine in combination with six antimicrobial agents against methicillin-resistant *Staphylococcus aureus* (MRSA). The combination effect of lipids and antimicrobial agents was evaluated by the checkerboard method to obtain a fractional inhibitory concentration (FIC) index. The effects of lauric acid + gentamicin (GM) and lauric acid + imipenem (IPM) combinations were synergistic against the clinical isolates in 12 combinations. An antagonistic FIC index was observed only with the myristylamine + GM combination. We investigated in detail the antimicrobial activity for two combinations that showed a synergistic effect. The cytotoxicity of lauric acid was not enhanced by the addition of GM and IPM. In time–kill studies, lauric acid + GM and lauric acid + IPM combinations at one-eighth of the minimum inhibitory concentration produced a bacteriostatic effect.

Keywords: Combination; Lipid; MRSA; Antimicrobial agent; In vitro

1. Introduction

Staphylococcus aureus is one of the major infectious disease-causing pathogens in humans. Methicillin-resistant *S. aureus* (MRSA) infection first emerged in early 1961 and has become increasingly prevalent, with serious infections becoming more widespread during the past 20–25 years [1,2]. According to data from the National Nosocomial Infections Surveillance System of the Centers for Disease Control and Prevention, MRSA rates are 54.5% [3]. In Japan, nosocomial infection due to MRSA became a problem in the 1980s when the use of third-generation cephalosporins became widespread [4]. The percentage of MRSA among nosocomial *S. aureus* in Japan is estimated to be 50–70% [5]. In addition, many strains of MRSA are resistant not only to β-lactam agents but also to fluoroquinolones, chloramphenicol, clindamycin, tetracyclines and aminoglycosides, but not glycopeptides (vancomycin and teicoplanin) [6]. Vancomycin and teicoplanin are glycopeptides with significant activity against Gram-positive bacterial pathogens. Vancomycin is widely used for the treatment of infections caused by MRSA [7]. Alternatives to glycopeptides are sometimes necessary owing to intolerance or treatment failure. *Staphylococcus aureus* with glycopeptide resistance has now been documented in the USA [7–9].

To identify agents with anti-MRSA activity, various chemical compounds have been investigated [10–14]. The broth microdilution method is the standard method for determining the minimum inhibitory concentration (MIC);

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however, it is not able to determine the antimicrobial susceptibility in turbid samples as it is based on the determination of optical absorbance. In our laboratory, we recently confirmed the antimicrobial activity of insoluble compounds such as saturated fatty acids and fatty amines against MRSA using new multi-channel oxygen meters and disposable oxygen electrode sensors (DOX-96; Daikin Environmental Laboratory, Ltd., Tsukuba, Japan) [15]. DOX-96 is useful for determining the antimicrobial activities of insoluble compounds [16] because it measures oxygen consumption by bacteria using oxygen electrodes. Among the saturated fatty acids and fatty amines, we found that lauric acid and myristylamine, respectively, were most effective against MRSA. We also found that myristylamine showed anti-MRSA activity comparable with that of vancomycin and teicoplanin; however, the antimicrobial activity of lauric acid and myristylamine was inhibited by plasma, and cytotoxicity was also demonstrated. It is necessary to reduce this cytotoxicity for the clinical application of lauric acid and myristylamine.

In this study, we investigated the in vitro antimicrobial activity of lauric acid and myristylamine in combination with existing antimicrobial agents against MRSA for clinical application.

2. Materials and methods

2.1. Materials

The following antimicrobial agents were used: oxacillin (Wako Pure Chemical Ind. Ltd., Osaka, Japan), ampicillin (ABPC; Meiji Seika Co., Tokyo, Japan), sulbactam/ampicillin (Pfizer Co., Tokyo, Japan), cefazolin (CEZ; Fujisawa Pharmaceutical Co., Osaka, Japan), cefotiam (Takeda Chemical Ind. Ltd., Osaka, Japan), cefmetazole (Sankyo Co., Tokyo, Japan), flumoxef (Shionogi Co., Osaka, Japan), cefpirome (Chugai Pharmaceutical Co., Tokyo, Japan), imipenem (IPM; Banyu Pharmaceutical Co., Tokyo, Japan), gentamicin (GM; Schering-Plough Co., Osaka, 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). Lauric acid and myristylamine were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Lauric acid and myristylamine were dispersed using a sonicator (NO5202; Ohtake Co., Tokyo, Japan). All other chemicals were of the highest purity available.

2.2. Bacterial strains

The three clinical isolates used in this study were MRSA strain numbers 5914, 2185 and 4536, collected in Nagasaki University Hospital of Medicine and Dentistry, Nagasaki, Japan.

2.3. DOX-96 system

The DOX-96 electrode is a 96-well plate with three electrodes embedded in each well. The oxygen in the sample is converted into a current by the following reaction: $4H^+ + O_2 + 4e^- \rightarrow 2H_2O$, and the current is drawn on a graph using a laptop computer [17]. As viable bacteria consume oxygen, the oxygen level in the sample decreases [16].

2.4. MIC determination

The MIC of antimicrobial agents was determined by a microdilution method with cation-adjusted Mueller–Hinton broth (BBL Microbiology Systems, Cockeysville, MD) according to the recommendations of the National Committee for Clinical Laboratory Standards [18].

Lauric acid and myristylamine were sufficiently suspended in Mueller–Hinton broth by a sonicator. The MICs of lauric acid and myristylamine were determined by DOX-96 owing to its usefulness in determining the antimicrobial activities of insoluble compounds [16]. The samples were diluted two-fold with Mueller–Hinton broth and dispensed into the wells (100 μL/well) of the electrode plate. All wells (except the negative controls) 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 lauric acid or myristylamine. The electrode plate was set on the DOX-96 and incubated for 999 min (16.65 h) at 35 °C. The current measurement in each well was taken as the oxygen consumption compared with their respective positive and negative controls. If bacteria existed in the sample, significant oxygen consumption was observed within 16.65 h owing to the proliferation of bacteria. This significant oxygen consumption was suppressed by the antimicrobial activity of lauric acid and myristylamine. The lowest concentration of lauric acid or myristylamine at which significant oxygen consumption was suppressed over 16.65 h was taken as the MIC [15].

2.5. Checkerboard study

The combined effects of two lipids (lauric acid and myristylamine) with six antimicrobial agents (ABPC, CEZ, IPM, MINO, GM and LVFX) were evaluated by the checkerboard method to obtain the fractional inhibitory concentration (FIC) index [19]. Six antimicrobial agents of different classes were selected for evaluation. Glycopeptides were not selected because of their strong activity against MRSA in the absence of lipids. The checkerboard consists of columns in which each well contains the same amount of antimicrobial agent diluted two-fold along the *x*-axis, and rows in which each well contains the same amount of lipid diluted two-fold along the *y*-axis on a 96-well plate for the DOX-96 [20]. The media, inocula and conditions were the same as in the DOX-96 method.