Farnesol-Induced Disruption of the Staphylococcus aureus Cytoplasmic Membrane

Yoshihiro Inoue,* Naoko Togashi, and Hajime Hamashima

Showa Pharmaceutical University; Machida, Tokyo 194–8543, Japan.
Received May 15, 2015; accepted February 8, 2016

Farnesol, a sesquiterpene alcohol with an aliphatic carbon chain, inhibited the growth of Staphylococcus aureus and induced the leakage of potassium ions. We investigated the action of farnesol on the cytoplasmic membrane of S. aureus. No ion channels that would account for the loss of potassium ions were detected. Electron paramagnetic resonance measurements suggested that farnesol proceeds into the cytoplasmic membrane of S. aureus cells, where it induces the disordering and eventual disruption of the cytoplasmic membrane. This was supported by the result that the effects of farnesol decreased by the addition of carotenoid which was the stabilizing reagent for the lipid bilayer.

Key words  farnesol; cell membrane disordering; Staphylococcus aureus

Staphylococcus aureus is an opportunistic pathogen. Novel antibiotics against this organism are needed due to the depletion of therapeutic options resulting from the development of resistance to various antibiotics and, consequently, the increasing difficulty of curing nosocomial infections. We previously investigated plant-derived essential oils and their constituents and found that some terpene alcohols with aliphatic carbon chains, such as farnesol, exhibited antibacterial activity against S. aureus. The cell membrane of S. aureus was damaged after only a few minutes exposure to farnesol, but the underlying mechanism of this activity was not elucidated. The aim of the present study was to determine the mechanism of farnesol-induced damage to the cell membrane of S. aureus. The findings of this study could aid in safely and effectively treating infections of this bacterium and enhance efforts to develop more effective medicines for treating S. aureus infections.

MATERIALS AND METHODS

General  Farnesol, carotenoid, 5-doxyl stearic acid (5-NS), 16-doxyl stearic acid methyl ester (16-NMS) and melittin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Tetraethyl ammonium chloride and quinine hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). 16-doxyl stearic acid methyl ester (16-NMS) and melittin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). S. aureus FDA209P was used as the standard strain. Farnesol was added to the bacterial suspension at a concentration of 80 µg mL⁻¹, and 20 µL of the suspension was drawn into the capillary micropipette coated with spin-labeling reagent, immediately. One end of the capillary micropipette was sealed with EPR silent paste (TERUMO, Tokyo, Japan) and then the micropipette was incubated for 3 min at 37°C, after which EPR spectra were acquired using a JEX-RE 1X instrument (JEOL, Japan). Measurement conditions were as follows: temperature; 37°C, modulation amplitude; 1 G, scan range; 100 G, time constant; 0.3 s, scan speed; 100 G/4 min.

The order parameter (Sop) was calculated in the case of 5-NS as follows:

\[
Sop = \frac{(T_T - T_\perp)}{A}
\]

where \(T_T\) represents outer hyperfine splitting, \(T_\perp\) represents inner hyperfine splitting, and \(A\) represents a constant that is dependent on the system observed.

The apparent rotational correlation time, \(\tau\), was calculated in the case of 16-NMS as follows:

\[
\tau = 0.65 \times \Delta H_\rho \times (h_\rho / h_{1+1})^{1/2} \times 10^{-10}
\]

where \(\Delta H_\rho\) represents the peak-to-peak width of the center resonance line, \(h_\rho\) represents the peak-to-peak height of the center resonance line, and \(h_{1+1}\) represents the peak-to-peak height of the low-field resonance line.

Monitoring the Growth of S. aureus  The growth of S. aureus cells was monitored by observing changes in turbidity over time. The compound to be tested was added at the indicated concentration to 10-mL aliquots of brain heart infusion (BHI) broth (Becton Dickinson and Company) in an L-shaped test tube (internal diameter, 17 mm; length of arms, 180 mm and 70 mm) without any solubilizing agent or surfactant. An aliquot of an overnight culture of S. aureus was added to each sample to give approximately 10⁵ CFU mL⁻¹. The optical

* To whom correspondence should be addressed. e-mail: inoue@ac.shoyaku.ac.jp

© 2016 The Pharmaceutical Society of Japan
density at 660 nm (OD<sub>660</sub>) was determined with shaking using a biphotorecorder (TN-1520; Advantec, Tokyo, Japan).

**Detection of the Changes in the Concentrations of Potassium and Sodium Ions**

The concentration of potassium ions of the bacterial suspension was monitored by using the potassium-selective and reference electrodes. A silver/silver chloride electrode was used as the reference electrode. The potassium-selective electrode was prepared using valinomycin.7,8)

The concentration of sodium ions was measured by atomic spectrophotometry. The centrifugation and the filtration (ϕ: 0.2 µm (Millipore, MA, U.S.A.)) were made before the measurement.9)

Farnesol could contact with bacterial cells for one minute. The volume of bacterial suspension was 4 mL.

**Time-Kill Assay**

Cells from an overnight culture of S. aureus were washed twice by centrifugation with phosphate-buffered saline (PBS; pH 7.0). The cell pellet was resuspended in 1 mL of PBS at approximately 10<sup>7</sup> CFU mL<sup>-1</sup>. An aliquot (100 µL) of this suspension of cells was added to buffer (10 mL) that contained farnesol (80 µg mL<sup>-1</sup>) with or without an inhibitor and incubated at 37°C. The buffer did not contain any solubilizer. Samples were removed for determination of viable cell counts after 0, 2, 4 and 6 h. Serial ten-fold dilutions of the bacterial suspension (10<sup>-1</sup> to 10<sup>-4</sup>) were then prepared in PBS buffer. Aliquots (50 µL) of each diluted sample were plated on agar-solidified BHI with a spiral plater (Autoplate 3000; Spiral Biotech, Bethesda, MD, U.S.A.). The plates were incubated at 37°C for 24 h and then colonies were counted. Killing curves were constructed by plotting numbers of viable cells against time.

**RESULTS AND DISCUSSION**

Exposure of S. aureus cells to some terpene alcohols with aliphatic carbon chains, such as farnesol, results in a drastic increase in the potassium concentration of the suspension.2) The increase in the potassium ion concentration could be the result of damage to the bacterial cell membrane. The degree of potassium ion leakage from the cells depends on the length of the terpene alcohol carbon chain. As S. aureus does not possess an outer membrane, the passage of compounds is controlled by the cytoplasmic membrane.

The migration of potassium ions across the lipid bilayer membrane is mediated by several mechanisms, such as ionophores, potassium channels, Na<sup>+</sup>/K<sup>+</sup> ATPase, and disruption of the membrane by reactive oxygen species. In the first three above mentioned mechanisms, the integrity of the lipid bilayer membrane is retained, but this is not the case with reactive oxygen species. To determine whether the integrity of the lipid bilayer membrane of S. aureus is retained upon exposure to farnesol, changes in the fluidity of the membrane were evaluated using EPR measurements (Fig. 1). The degree of disorder in the membrane was determined as the relative ratio of fluidity in the presence of farnesol to that in the absence of farnesol. The relative ratio of fluidity was defined as 100% when the bacterial cell was affected by no reagent.10) Melittin was used as the positive control to damage the bacterial cell membrane and increased the relative fluidity to 250% (superficial layer) and 180% (deeper layer). The probe molecules 5-NS and 16-NMS reflect the state of the superficial layer and deeper layer, respectively. The fluidity of the superficial and
deeper layers increased by 182 and 118%, respectively, following exposure to farnesol, indicating that farnesol increases the degree of disorder in the membrane of *S. aureus*.

Concentration of potassium ions in the bacterial cell is higher than the one of outside of the cell when bacteria are alive and grow. In the case of sodium ions, the concentration of outside of the cell is higher than the one in the bacterial cells. The changes in the concentrations of potassium and sodium ions of the bacterial suspension were observed at the addition of farnesol into the bacterial suspension (Fig. 2). The concentration of potassium ions was increased and the concentration of sodium ions was decreased. Their differences were statistically significant (*p* < 0.05). These results indicated that the potassium ions existing in the bacterial cell flowed out to the outside of the cell and the sodium ions existing at outside of the bacterial cell flowed into the inside of the cell. It means that the bacterial membrane was damaged and lost the membrane homeostasis.

Figures 3 and 4 show growth curves of *S. aureus* in medium containing farnesol and the potassium channel inhibitors quinine and tetraethyl ammonium chloride.11,12) Neither quinine nor tetraethyl ammonium chloride addition caused a shift in the growth curve of cells incubated in the presence of only farnesol compared with cells incubated in the absence of farnesol, suggesting that the antibacterial activity of farnesol does not involve disruption of potassium channels. Based on these data, ionophore activity, potassium channel disruption, and Na\(^+\)/K\(^+\) ATPase disruption were excluded as mechanisms underlying the drastic leakage of potassium ions from cells exposed to farnesol.

To determine whether reactive oxygen species were involved, an NBT assay was carried out (Fig. 5). Addition of farnesol did not increase the absorbance at 575 nm, indicating that farnesol does not induce the formation of reactive oxygen species.

Figure 6 shows growth curves for *S. aureus* cultured in medium containing farnesol and carotenoid. The addition of carotenoid led to a concentration-dependent shift in the growth curve toward that of cells incubated in the absence of farnesol, indicating that carotenoid suppressed the antibacterial activity of farnesol. Taylor pointed out that carotenoid decreases the fluidity of the lipid bilayer by insertion between the phospholipids, thus stabilizing the cell membrane.13) As
the carbon chain of carotenoid is similar in length to that of bacterial membrane phospholipids, its insertion into the membrane can stabilize the membrane and prevent disorder. Kranenburg et al. reported that the degree of cell membrane fluidity increases as the length of the carbon chains of the phospholipids composing the cell membrane decrease.14 It was also reported that phase transition is induced by the insertion of carotenoid into the bacterial cell membrane, where it forms a gap in the membrane. This result also supported the results mentioned above. These results supported the results of Figs. 3, 4 and 6. There was little change in the bacterial cell membrane fluidity when carotenoid added into the bacterial suspension containing farnesol (Fig. 1). This result also supported the results mentioned above.

Based on the results of the present study and other reports, we conclude that farnesol is absorbed into the lipid bilayer of the bacterial cell membrane, where it forms a gap in the membrane that alters the fluidity of the lipid bilayer. As a result, the bacterial membrane loses its resistance to pressure from the inside of the cell and eventually bursts as the number of gaps formed by farnesol reaches a critical threshold.

**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**


