Cationic Antimicrobial Peptide LL-37 is Effective against both Extra- and Intra-Cellular *Staphylococcus aureus* *

Running Title: LL-37 effective against extra- and intra-cellular *S. aureus*

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ABSTRACT

The increasing resistance of bacteria to conventional antibiotics and the challenges posed by intra-cellular bacteria, which may be responsible for chronic and recurrent infections, have driven the need for advanced antimicrobial drugs for effective elimination of both extra- and intra-cellular pathogens. The purpose of this study was to determine the killing efficacy of cationic antimicrobial peptide (CAMP) LL-37 compared to conventional antibiotics against extra- and intra-cellular Staphylococcus aureus (S. aureus). Bacterial killing assays and a co-culture model of osteoblasts and S. aureus were studied to determine the bacterial killing efficacy of LL-37 and conventional antibiotics against extra- and intra-cellular S. aureus. We found that LL-37 was effective in killing extra-cellular S. aureus at nM concentrations, while lactoferricin-B was effective at μM concentrations and doxycycline and cefazolin at mM concentrations. LL-37 was surprisingly more effective in killing the clinical strain compared to the ATCC S. aureus. Moreover, LL-37 was superior to conventional antibiotics in eliminating intra-cellular S. aureus. The kinetics studies further revealed that LL-37 was fast in eliminating both extra- and intra-cellular S. aureus. Therefore, LL-37 was shown to be very potent and prompt in eliminating both extra- and intra-cellular S. aureus, and was more effective in killing extra- and intra-cellular S. aureus than commonly used conventional antibiotics. LL-37 could potentially be used to treat chronic and recurrent infections due to its effectiveness in eliminating not only extra- but also intra-cellular pathogens.
INTRODUCTION

Conventional antibiotics are becoming increasingly ineffective due to rapidly evolving multidrug resistant bacterial strains. The heavy use of antibiotics is causing bacteria to mutate and emerge as multidrug resistant “superbugs” such as methicillin resistant *S. aureus* (MRSA), vancomycin-resistant *S. aureus*, and vancomycin-intermediate *S. aureus* (5, 16, 48, 79). Recent studies reported that MRSA is posing a serious healthcare issue due to treatment failure, higher mortality, and increased healthcare costs (11, 38, 52). MRSA is now killing more people in the U.S. than acquired immune deficiency syndrome or AIDS (42). In 2009, the U.S. Centers for Disease Control and Prevention reported that bacterial infections, especially those caused by multidrug resistant *S. aureus*, are on the rise globally (15). Each year, approximately 19,000 people die due to recalcitrant and recurrent bacterial infections in the U.S. alone (42). Moreover, treating recurrent bacterial infections (17, 41) has become a daunting challenge due to the possible presence of intra-cellular bacteria (19, 28, 61); historically a high infection recurrence (~17%) was found in combat-related injuries (53). Therefore, the increasing resistance of bacteria to conventional antibiotics and the challenges posed by intra-cellular bacteria have driven the need for advanced or alternative antimicrobial drugs.

Cationic antimicrobial peptides (CAMPs) have recently emerged as an alternative to conventional antibiotic therapies (14, 49). They are produced by the innate immune system in both vertebrates and invertebrates as a first line of defense against microbial infections (30, 74, 83). They have broad-spectrum killing ability against pathogens (77, 82). In addition to their antibacterial and antifungal properties, CAMPs have also been described recently for their role in neutralization of endotoxins, chemokine-like activities, immunomodulating properties, induction
of angiogenesis, and wound repair (2, 37, 44, 68, 81). Currently, companies like HelixBiomedix are developing arrays of CAMPs in several pharmaceutical programs ranging from topical anti-infective to wound healing and cystic fibrosis (29), and several CAMPs and their derivatives are being investigated in pre-clinical and clinical trials (3, 4, 23, 29, 33, 78).

Conventional antibiotics are relatively larger molecules compared to CAMPs and have different types of mechanisms in killing bacteria. Cefazolin, a beta-lactum and frequently used in orthopaedic infection treatment (73), has a very low permeability through cell membranes due to its hydrophilic nature and does not accumulate in the cytoplasm because of its rapid efflux (21). However, it binds to bacterial penicillin-binding proteins, thereby disrupting the synthesis of peptidoglycan, the integral part of the bacterial cell wall (67). Whereas doxycycline (tetracycline) and clindamycin (lincosamide) traverse bacterial membranes using the membrane transport system but they have to cross the threshold limit to interact with the ribosomes (67). Clindamycin was proven effective against intra-cellular bacteria (9, 67, 70).

Unlike conventional antibiotics, CAMPs have a different mode of action which is often more effective in destroying bacteria; they interact with bacteria through electrostatic forces (69, 80). CAMPs, including cathelicidin LL-37, are amphiphilic in nature and are comprised of hydrophobic and hydrophilic residues aligned on opposite sides of the peptides, facilitating their easy penetration through cell membranes (32, 55, 56, 66, 74). Their positively charged domain allows CAMPs to bind to bacterial membranes like magnets and the hydrophobic domain facilitates their penetration through phospholipid bilayers (65, 76). This mode of action results in bacterial death (59, 62).

Bacteria could develop resistance to conventional antibiotics by altering their antibiotic binding cell membrane receptors through mutations thereby making the antibiotics ineffective;
however, CAMPs target the lipid matrix of cell membranes whose lipid composition is highly unlikely to change due to bacterial mutation (54). Development of resistance against CAMPs by modifying membrane compositions of bacteria would compromise the bacteria’s viability (50) and thereby would not likely occur (14, 32, 55, 74). However, CAMPs may suffer proteolytic digestion (56), which could be minimized via a small alteration of the peptide structure to make them not be recognized or degraded by proteolytic enzymes (57).

Cathelicidin LL-37 is a CAMP that has recently attracted great interest (14, 26, 49). The objective of this study was to determine the antimicrobial properties of cathelicidin LL-37 compared to conventional antibiotics against extra- and intra-cellular S. aureus. We hypothesized that LL-37 can be effective not only in eliminating extra-cellular bacteria but also intra-cellular bacteria.

MATERIALS AND METHODS

A clinical strain of S. aureus obtained from a patient’s chronic wound at Ruby Memorial Hospital, Morgantown, WV and an American Type Culture Collection (ATCC, Manassas, VA) strain (25923) of S. aureus were investigated in this study; the susceptibility test outcomes were shown in the Supplementary Materials. S. aureus was chosen because it is one of the major pathogens responsible for most bacterial infections including orthopaedic infections (22, 24, 43, 58, 71) and is a potential cause of chronic and recurrent infections (10, 53). CAMPs (cathelicidin LL-37 and lactoferricin-B) and conventional antibiotics (cefazolin, doxycycline, and clindamycin) were purchased from Sigma-Aldrich (St. Louis, MO). The purity of LL-37
Extra-cellular antimicrobial activities of LL-37 and conventional antibiotics. The killing efficacies of LL-37, lactoferricin-B, doxycycline, and cefazolin were determined against extra-cellular *S. aureus* under the same experimental conditions. Sterile tryptic soy broth, prepared based on the manufacturer’s instruction, was used for bacterial cultures. Three colonies of *S. aureus* were inoculated into a sterile tube containing 5 mL of tryptic soy broth and incubated for 16 h at 37°C. The next day, 100 µL of 16 h *S. aureus* culture (stationary phase) was inoculated into a sterile tube containing 20 mL of fresh tryptic soy broth and was subjected to shaking (80 rpm) at 37°C for 2.5 h to acquire Log phase (exponential bacterial growth) using a reciprocal shaking bath, made by Precision (El Cajon, CA). The Log phase *S. aureus* inoculums were diluted to $1.0 \times 10^5$ colony forming units/mL (CFU/mL) with sterile phosphate buffered saline (PBS, pH 7.0). The assays were run with a total volume of 1 mL comprising *S. aureus* ($1.0 \times 10^5$ CFU/mL) and different molar concentrations (ranging from 10 nM to 100 mM) of cathelicidin LL-37, lactoferricin-B, doxycycline, and cefazolin, individually. The controls and the treated samples were incubated at 37°C for 30 min in a reciprocal shaking bath. The samples were then diluted and plated on 5% sheep blood agar plates. Dilutions of $10^1$, $10^2$, and $10^3$ were made for control and treated samples with sterile PBS. The drop plate method (8, 35) was used for viable bacterial enumeration and is described as follows: A sheep blood agar plate was divided into six sectors. A 20 µL bacterial suspension was pipetted and placed as a drop in each sector. After the drops dried, the plates were inverted and incubated at 37°C for 24 h. The procedure was repeated for each dilution. CFUs were determined using an Acolyte colony counter made by Synbiosis.
The killing efficacy of each drug was presented in terms of percentage killing at different molar concentrations. Percentage killing was calculated by dividing the difference between control and treated samples with a control value and then multiplying by 100. Data were averages of four samples.

LL-37 was next tested from 50 nm to 100 µM (0.05, 0.25, 0.5, 1.0, 2.0, 3.0, 10.0, and 100 µM) concentrations for strain (ATCC strain vs. clinical strain; Log phase was studied) and phase (Log phase vs. stationary phase; ATCC strain was used) comparisons. The inoculums were diluted to 1.0 × 10^5 CFU/mL with sterile PBS. The experiments were carried out with a total volume of one mL comprising *S. aureus* (1.0 × 10^5 CFU/mL) and different molar concentrations (0.05, 0.25, 0.5, 1.0, 2.0, 3.0, 10.0, and 100 µM) of LL-37. The controls and the treated samples were incubated at 37°C for 30 min in a reciprocal shaking bath. The samples were then diluted and plated on 5% sheep blood agar plates using the drop plate method; the CFUs were determined and the percentage killing of LL-37 was calculated.

In addition, kinetic studies were conducted individually for LL-37 (250 nM), lactoferricin-B (25.0 µM), and cefazolin (1.0 mM) at given time intervals (5, 10, 15, and 30 min). LL-37 of 250 nM, lactoferricin-B of 25.0 µM, and cefazolin of 1.0 mM had approximately the same percentage killing from the aforementioned experiments. The kinetic experiments were run with a total volume of 1 mL comprising Log phase bacteria (1.0 × 10^5 CFU/mL) and LL-37 (250 nM), lactoferricin-B (25.0 µM), or cefazolin (1.0 mM) and incubated separately for 5, 10, 15 and 30 min at 37°C in a reciprocal shaking bath. At the pre-determined time, the control and treated samples were diluted and plated on 5% sheep blood agar plates using the drop plate method and the CFUs were determined. The percentage killing data were calculated and
normalized by assuming LL-37 (250 nM), lactoferricin-B (25.0 µM), and cefazolin (1.0 mM) had 100% killing at 30 min. Data were averages of four samples.

Intra-cellular antimicrobial activities of LL-37 and conventional antibiotics. A co-culture model of osteoblasts and *S. aureus* (1, 25, 31, 39, 64) was used to obtain intra-cellular *S. aureus*; *S. aureus* can internalize into osteoblasts and survive within them (1, 25, 31, 39, 64). The clinical strain of *S. aureus* in the Log phase was studied and a 500:1 ratio of *S. aureus* to osteoblasts was used. Experiments were conducted using a 12-well plate in a laminar flow hood under aseptic conditions. Dulbecco’s modified Eagle’s medium/F-12 (DMEM/F-12) and PBS were used for osteoblast cell culture. One mL of osteoblast cells (UMR-106, passage 2) with a cell density of $4 \times 10^5$ cells/mL were seeded in each well and incubated at 37°C with 5% CO₂ for 36 h to form a confluent monolayer. After 36 h, the wells were washed twice with 1 mL PBS to remove growth media. One mL of Log phase *S. aureus* ($2 \times 10^8$ CFU/mL) was then added to each well and the 12-well plate was incubated at 37°C. After co-culturing for 2 h, the wells were washed twice with 1 mL PBS; 50 μg of lysostaphin was added to each well and incubated for 2 h to eliminate extra-cellular *S. aureus*. Lysostaphin is an antimicrobial agent that does not penetrate eukaryotic cells and 50 μg/mL lysostaphin (Sigma-Aldrich) was found to be effective at eradicating any extra-cellular *S. aureus* (31, 75). The wells were washed twice with 1 mL PBS. Osteoblasts in three wells were immediately lysed with 0.1% Triton X-100 in PBS for 10 min at 37°C; the cell lysates were diluted in PBS, plated on blood agar plates overnight, and the intra-cellular *S. aureus* was $4 \times 10^4$ CFU. Different molar concentrations (10, 30, 50, and 100 μM) of LL-37 or plain DMEM were added to the remaining wells. After incubating at 37°C for 2 h, osteoblasts were rinsed twice with 1 mL PBS and then lysed with 0.1% Triton X-100 and the
intra-cellular *S. aureus* was plated on 5% sheep blood agar plates using the aforementioned drop plate method. Dilutions of $10^{-1}$, $10^{-2}$, and $10^{-3}$ were made for control and treated samples with sterile PBS. The colony numbers of viable intra-cellular *S. aureus* were determined. The same experiments were also carried out with conventional antibiotics including cefazolin and clindamycin at 100 µM for comparison; clindamycin was chosen due to its effectiveness against intra-cellular bacteria (9, 67, 70) and cefazolin due to its wide applications in orthopaedic infection treatment (73). Data were averages of four samples.

Kinetic studies of LL-37 (100 µM) were also conducted against intra-cellular *S. aureus* at different time intervals (i.e. 0.5, 2, 12, and 24 h). Log phase *S. aureus* was internalized within the osteoblasts in a 12-well plate as described above in the osteoblast-*S. aureus* co-culture model. The extra-cellular *S. aureus* was eliminated using lysostaphin and the wells were washed twice with 1 mL PBS; 100 µM of LL-37 was added to each well and incubated at 37°C. Controls were run separately for each time point. After 0.5, 2, 12, and 24 h, osteoblasts were rinsed twice with 1 mL PBS and then lysed with 0.1% Triton X-100; the intra-cellular *S. aureus* was plated on 5% sheep blood agar plates. Percentage killing was calculated; data were averages of four samples.

**Statistical analysis.** Values of percentage killing were expressed as the mean ± standard deviation. Differences in percentage killing of extra-cellular *S. aureus* between the ATCC and clinical strains and between Log phase and stationary phase, and differences in percentage killing of intra-cellular *S. aureus* among cefazolin, clindamycin, and LL-37 were analyzed using JMP-V9 Statistical Visualization Software (SAS Institute Inc., Cary, NC). The data were transformed as the arcsin of the square-root of percentage killing and a t-test was run to compare the two groups; in the case where there were three groups, an ANOVA followed by Tukey’s honestly
significant difference (HSD) test was used to determine significance. A $p$ value $\leq 0.05$ was considered statistically significant.

**RESULTS**

**Extra-cellular bacterial killing efficacy vs. concentration of LL-37, lactoferricin-B, and conventional antibiotics.** *S. aureus* was treated with two CAMPs (i.e. cathelicidin LL-37 and lactoferricin-B) and their killing efficacy was compared with cefazolin and doxycycline, two commonly used antibiotics, under the same experimental conditions. Overall, LL-37 was effective in killing *S. aureus* at nM concentrations, while lactoferricin-B was effective at $\mu$M concentrations and doxycycline and cefazolin at mM concentrations (**Fig. 1**). LL-37 was found to exhibit over 90% killing efficacy at as low as 250 nM, over 99% at 500 nM, and 100% at 3.0 $\mu$M (**Fig. 1**). Whereas lactoferricin-B had approximately 2% killing potency at 250 nM, 15% at 500 nM, 67% at 3.0 $\mu$M, and over 90% at 25 $\mu$M. On the other hand, doxycycline and cefazolin were found to have significant killing abilities only at much higher concentrations; they had no killing efficacy at 3.0 $\mu$M, more than 90% killing efficacy at 1.0 mM, and 100% killing potency at 10 mM or higher (**Fig. 1**).

**Extra-cellular bacterial killing efficacy of LL-37 vs. *S. aureus* strains.** LL-37 was tested on both clinical and ATCC *S. aureus* strains with different molar concentrations, ranging from 0.05 $\mu$M to 100 $\mu$M for strain comparison. LL-37 exhibited 100% killing on both strains at higher concentrations (10 and 100 $\mu$M). However, at concentrations lower than 3 $\mu$M, LL-37 was surprisingly more effective in killing the clinical strain compared to the ATCC strain (**Fig. 1**).
There was a 24% increase in the killing ability toward the clinical strain compared to the ATCC strain at 1.0 µM; the difference was more prominent (over 40%) at lower concentrations, e.g. 0.5, 0.25, and 0.05 µM (Fig. 2).

**Extra-cellular bacterial killing efficacy of LL-37 vs. *S. aureus* phases.** LL-37 seemed to kill significantly more *S. aureus* in the stationary phase compared to *S. aureus* in the Log phase at concentrations at or lower than 1.0 µM; no differences in percentage killing were observed at concentrations higher than 2.0 µM (Fig. 3).

**Extra-cellular bacterial killing kinetics of LL-37.** The extra-cellular bacterial killing kinetics of LL-37 were compared with lactoferricin-B and cefazolin. Incredibly, LL-37 was able to eliminate more than 70% of *S. aureus* within just 5 min and more than 90% within 15 min (Fig. 4). On the contrary, lactoferricin-B and cefazolin had much slower kinetics and showed almost no bacterial killing within the first 5 min and less than 40% killing within 15 min (Fig. 4).

**Intra-cellular antimicrobial activities of LL-37.** The killing potency of LL-37 was determined against intra-cellular *S. aureus* at different molar concentrations (10, 30, 50, and 100 µM). LL-37 was found to be very effective in eliminating intra-cellular *S. aureus*. The intra-cellular bacterial percentage killing increased with increasing LL-37 concentration and 100 µM of LL-37 completely killed the intra-cellular *S. aureus* (Fig. 5). In contrast, at the same concentration (i.e. 100 µM), cefazolin and clindamycin eliminated only 2% and 23% of the intra-cellular *S. aureus*, respectively (Fig. 6). Kinetic studies further showed that LL-37 killed
approximately 50% of the intra-cellular S. aureus within 30 min and all bacteria within 2 h (Fig. 7).

DISCUSSION

It is well known that a wide variety of pathogens including bacteria and viruses are capable of internalizing into human cells thereby causing intra-cellular diseases like human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), hepatitis, and tuberculosis (TB) (reviewed in reference 6). One of the critical challenges in treating these types of infections is the intra-cellular nature of the pathogens, which may protect the pathogens from a variety of antibiotic therapies and host immune responses. Antibiotics such as aminoglycosides and ß-lactams have limited cellular penetration whereas antibiotics like fluoroquinolones or macrolides have poor retention within cells and thereby are inefficient at killing intra-cellular pathogens (13). Moreover, some bacteria such as S. aureus, which has long been considered an extra-cellular pathogen, have now been found to be able to internalize and survive within host cells, e.g. osteoblasts (28, 36, 39, 51, 60, 63), and may contribute to chronic and recurrent infections (24). Therefore, advanced drugs for effectively destroying both extra- and intra-cellular pathogens are needed in order to reduce or prevent chronic and recurrent infections. In this study, the potential bacterial killing activities of LL-37 against intra-cellular S. aureus were examined and compared with conventional antibiotics. The bacterial killing activities of LL-37 against extra-cellular bacteria were also investigated and compared with conventional antibiotics.

Our studies indicated that LL-37 is very potent and fast (Figs. 1 and 4) at eliminating extra-cellular S. aureus, the common culprit of many bacterial infections. Among LL-37,
lactoferricin-B, doxycycline, and cefazolin, LL-37 was apparently foremost in eliminating extra-cellular *S. aureus*. LL-37 was remarkably potent in killing more than 90% of *S. aureus* even at 250 nM (Fig. 1). Our experiments showed that a substantially smaller quantity of LL-37 (100 times vs. lactoferricin-B; 4,000 times vs. doxycycline and cefazolin) was needed to eliminate extra-cellular *S. aureus* (Fig. 1). Moreover, LL-37 was not only potent but also expeditious in eliminating extra-cellular *S. aureus*. LL-37 was found to be much faster in killing extra-cellular *S. aureus* compared to lactoferricin-B and cefazolin (Fig. 4).

LL-37 furthermore exhibited a strain specific higher killing ability toward the clinical strain compared to the ATCC strain at concentrations lower than 3.0 µM (Fig. 2). These findings indicated that the *S. aureus* clinical strain was surprisingly more susceptible to LL-37 than its ATCC strain; the reason is unknown. In our previous in vivo studies, we found that the *S. aureus* clinical strain was much more virulent in inducing infections compared to the ATCC strain (47).

LL-37 also presented a phase specific response (Fig. 3) at concentrations lower than 1.0 µM, with a higher killing ability toward the stationary phase than the Log phase. This may suggest that it is relatively easier to eliminate stationary bacteria than Log phase bacteria.

More interestingly, we found that LL-37 was very effective in eliminating intra-cellular pathogens. LL-37 had remarkable intra-cellular killing ability against *S. aureus* compared to conventional antibiotics like cefazolin and clindamycin; the latter was reported to have potent antimicrobial properties against intra-cellular *S. aureus* due to its good penetration, retention, and distribution properties in eukaryotic cells (9, 67). Our results indicated that 100 µM concentration of LL-37 completely eliminated intra-cellular *S. aureus* within just 2 h, whereas cefazolin and clindamycin eliminated only 2% and 23%, respectively (Fig. 6). However, due to the intra-cellular nature of the pathogen, a much higher (100 µM vs. 3 µM) concentration of LL-
37 was needed (Figs. 1 and 5) and relatively slower kinetics were observed (Figs. 4 and 7) in killing intra-cellular *S. aureus* compared to extra-cellular *S. aureus*. Note that 10 mM of cefazolin and doxycycline were needed to completely eliminate extra-cellular *S. aureus* alone (Fig. 1).

The current study therefore demonstrated that LL-37 is very potent and fast at eliminating both extra- and intra-cellular *S. aureus* compared to conventional antibiotics. Moreover, LL-37 may exhibit synergistic antibacterial activities with β-defensin and lysozyme in both neutral and acidic environments (18). However, the antibacterial properties of LL-37 may be reduced by serum proteins. It was reported that certain biological fluids containing glycosaminoglycans and serum may hamper the antibacterial properties of LL-37 (7). Serum proteins such as apolipoproteins could bind to LL-37 and reduce its antimicrobial efficacy (7, 34, 40). Interestingly, the removal of N-terminal hydrophobic amino acids from LL-37 may reduce the effect of serum without compromising its antimicrobial properties (20).

One limitation of this study is that the potential toxicity of LL-37 was not examined. It was reported that LL-37 could prevent sepsis in neonatal rats (27), and a low dose (100 µg/kg) of LL-37 did not induce observable toxicity but a high dose (3000 µg/kg) resulted in adverse effects and appeared to be toxic to organs affected by sepsis (27). It is noteworthy that studies on human cathelicidin analogs reveal that removal of hydrophobic amino acids from the N-terminal end of native LL-37 could decrease its cytotoxicity without compromising the peptide’s antimicrobial efficacy toward both gram positive and gram negative bacteria (20). Wang et al (72) recently mapped and unmasked the potential roles of cationic residues of human cathelicidin LL-37 against different bacterial strains. The cationic side chains of the major antimicrobial region of human cathelicidin LL-37 were fragmented and their functional roles were studied in detail. The
GF-17 fragment comprising the residues 17 to 32 was found to be more potent against methicillin resistant *S. aureus* in vitro compared to intact LL-37. It also indicated that the conversion of amino acids from lysines (K) to arginines (R) increased the ability of the peptide to kill *S. aureus*. Therefore, the use of the GF-17 fragment of LL-37 may lead to lower dosages and thereby reduced toxicity (72).

In summary, *S. aureus* and *S. aureus* internalized within osteoblasts were treated with LL-37 and conventional antibiotics. LL-37 was found to have rapid and robust killing efficacy against both extra- and intra-cellular *S. aureus*, one of the most common causes of bacterial infections. In eliminating extra-cellular *S. aureus*, LL-37 is 100 times more potent than lactoferricin-B and 4,000 times more potent than conventional antibiotics such as doxycycline and cefazolin. LL-37 also eliminates the majority (more than 70%) of *S. aureus* within just 5 min compared to almost no killing by lactoferricin-B and cefazolin at the same timepoint. The efficacy of LL-37 was found to be bacterial strain and phase specific. Surprisingly, LL-37 was more effective at killing the clinical strain versus the ATCC strain of *S. aureus*. In eliminating intra-cellular *S. aureus*, 100 µM of LL-37 killed approximately 50% of intra-cellular *S. aureus* within the first 30 min and completely eradicated the bacteria within 2 h. However, at the same concentration, cefazolin and clindamycin only eliminated 2% and 23% of the intra-cellular *S. aureus*, respectively, within 2 h. Therefore, we conclude that LL-37 has rapid and remarkable killing abilities toward both extra- and intra-cellular *S. aureus* compared to conventional antibiotics. In future studies, we will examine the *in vivo* antimicrobial activities of LL-37 in our animal model (12, 45, 46) and may evaluate *in vitro* whether LL-37 will induce resistance.
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REFERENCES


**Figure Legend**

**FIG 1** Killing potencies of LL-37, lactoferricin-B, and conventional antibiotics (i.e. cefazolin and doxycycline) against extra-cellular *S. aureus* (clinical strain) in the Log phase.

**FIG 2** Strain specific killing efficacy of LL-37 against *S. aureus* (ATCC vs. clinical strains) in the Log phase. Incubation time was 30 min. *p<0.05 compared to ATCC strain at the same concentration.

**FIG 3** Killing efficacy of LL-37 against *S. aureus* in Log and stationary phases. Incubation time was 30 min. *p<0.05 compared to Log phase at the same concentration.

**FIG 4** Kinetics of LL-37 killing against extra-cellular *S. aureus* (clinical strain) in the Log phase.

**FIG 5** Intra-cellular killing efficacy of LL-37 against *S. aureus* (clinical strain) within osteoblasts. Incubation time was 2 h.

**FIG 6** Intra-cellular killing efficacy of cefazolin, clindamycin, and LL-37 against *S. aureus* (clinical strain) within osteoblasts. The concentration of cefazolin, clindamycin, and LL-37 was 100 μM; incubation time was 2 h. (A) Percentage killing; (B) Images at 10^5 dilution: (a) control, (b) cefazolin, (c) clindamycin, and (d) LL-37. *p<0.05 compared to cefazolin and clindamycin; **p<0.05 compared to cefazolin.

**FIG 7** Kinetics of LL-37 killing against intra-cellular *S. aureus* (clinical strain) within osteoblasts. Concentration of LL-37 was 100 μM.
FIG 1 Killing potencies of LL-37, lactoferricin-B, and conventional antibiotics (i.e. cefazolin and doxycycline) against extra-cellular *S. aureus* (clinical strain) in the Log phase.
FIG 2  Strain specific killing efficacy of LL-37 against *S. aureus* (ATCC vs. clinical strains) in the Log phase. Incubation time was 30 min. *p<0.05 compared to ATCC strain at the same concentration.

FIG 3  Killing efficacy of LL-37 against *S. aureus* in Log and stationary phases. Incubation time was 30 min. *p<0.05 compared to Log phase at the same concentration.
**FIG 4** Kinetics of LL-37 killing against extra-cellular *S. aureus* (clinical strain) in the Log phase.

**FIG 5** Intra-cellular killing efficacy of LL-37 against *S. aureus* (clinical strain) within osteoblasts. Incubation time was 2 h.
FIG 6 Intra-cellular killing efficacy of cefazolin, clindamycin, and LL-37 against *S. aureus* (clinical strain) within osteoblasts. The concentration of cefazolin, clindamycin, and LL-37 was 100 μM; incubation time was 2 h. (A) Percentage killing; (B) Images at 10^{-1} dilution: (a) control, (b) cefazolin, (c) clindamycin, and (d) LL-37. *p<0.05 compared to cefazolin and clindamycin; **p<0.05 compared to cefazolin.
FIG 7 Kinetics of LL-37 killing against intra-cellular *S. aureus* (clinical strain) within osteoblasts. Concentration of LL-37 was 100 μM.