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Cathelicidin LL-37 Killing of Methicillin-resistant *Staphylococcus epidermidis*: Foundations of Successful Therapy of Endocarditis

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Abstract

Methicillin-resistant *Staphylococcus epidermidis* (MRSE) endocarditis failing conventional therapy has been successfully treated with nafcillin plus daptomycin in the clinic. *In vitro* studies showed that nafcillin enhanced daptomycin killing of MRSE in both planktonic cells and biofilm. Nafcillin exposure also sensitized MRSE to killing by human neutrophils and cathelicidin antimicrobial peptide LL-37. Fluorescent microscopy showed increased daptomycin and LL-37 binding to the MRSE bacterial surface upon nafcillin treatment. Ceftriaxone also increased MRSE killing by daptomycin in planktonic cultures and biofilms, as well as daptomycin and LL-37 binding on the bacterial surface. Nafcillin, ceftriaxone, and possibly other β -lactams, may serve an important role in the therapy of MRSE endocarditis through augmentation of cationic peptide, the innate immune system, and daptomycin killing. Clinical studies will be needed to determine how early these regimens should be deployed to optimize clinical outcome.

Keywords

methicillin-resistant *Staphylococcus epidermidis*; endocarditis; antimicrobial peptide; combination therapy; daptomycin; nafcillin; cathelicidin

1. Introduction

Coagulase-negative staphylococci (CoNS) with biofilm-forming capability are emerging pathogens in native valve endocarditis, in the context of an increasing population of patients with long-term intravascular access devices [1,2]. Persistent bacteremia in CoNS endocarditis is associated with significant mortality risk, and risk of β -lactam resistance, congestive heart failure, and other medical comorbidities [2]. Therefore, shortening the

duration of bacteremia may improve patient outcomes. The pathologic lesions in infective endocarditis have micro-colonies embedded in an amorphous material, which is suggestive of biofilms [3]. This specific form of infection is particularly difficult to eradicate. Bacteria in biofilms are 100 to 1000 times more tolerant to antibiotics than bacteria in planktonic cultures because of the protective effect of the matrix and the reduction in the metabolic activity of bacteria [4].

In this work, we performed a series of in vitro experiments utilizing tricuspid valve endocarditis methicillin-resistant *Staphylococcus epidermidis* (MRSE) where conventional monotherapy therapy failed, but successful clinical and microbiological outcome was achieved with daptomycin plus nafcillin therapy. We found that β -lactams (nafcillin or ceftaroline) enhanced the microbicidal activities of daptomycin and human cathelicidin LL-37 against both planktonic and biofilm forms of infections.

2. Materials and Methods

2.1. Bacterial Isolates, susceptibility testing, and activity of antibiotics against planktonic cultures

Three clinical MRSE were selected for study from our laboratory repository. Strain 1 MRSE was from a clinical record of native tricuspid valve endocarditis (>1 cm vegetation via echocardiography) where bacteremia persisted on vancomycin (serum trough 15–20 mg/L) then daptomycin 8 mg/kg/day monotherapies. Utilizing a strategy previously described to treat patients with persistent methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia [5], intravenous nafcillin (2 g every 4 h) was added to daptomycin, and bacteremia cleared in 24 hrs. Strains 2 and 3 were isolates from uncomplicated MRSE bacteremia.

Susceptibility testing was performed according to CLSI methods [6] for nafcillin, ceftaroline, daptomycin, and vancomycin using both a standard (0.5×10^6 CFU/mL) and a high (5×10^6 CFU/mL) inoculum. The cathelicidin antimicrobial peptide LL-37 was also tested against MRSE strain 1. Kinetic killing assays were performed in cation-adjusted Mueller-Hinton broth (CA-MHB) supplemented up to 50 mg/L CaCl_2 when testing daptomycin, as previously described [5,7]. These assays utilized the β -lactam antibiotics, ceftaroline or nafcillin alone, or in combination with daptomycin, with all drugs tested at $\frac{1}{2}$ minimum inhibitory concentration (MIC) as measured with the high inoculum (5×10^6 CFU/mL). Bacteria were incubated with the drugs for up to 24 h, with aliquots collected over time, and plated on Todd-Hewitt agar (THA) plates after serial dilution in sterile saline solution.

2.2. Activity of antibiotics against biofilms

Biofilms were grown in 96-well plates in Tryptic Soy Broth (TSB) supplemented with 0.5% NaCl and 0.25% glucose (TGN) and using a starting inoculum of approximately 10^7 CFU/mL, according to a previous method [8]. After incubation at 37°C for 24 h, biofilms were exposed for 24 h to nafcillin, ceftaroline, or daptomycin over a broad range of concentrations (10–3 to 103 mg/L) to obtain full concentration-response curves, or to

combinations of these drugs. Residual bacterial viability in the biofilm was evaluated by measuring the reduction of resazurin in fluorescent resorufin by viable bacteria within the biofilm [9]. Briefly, at the end of the incubation period, the medium was removed, and wells were washed twice with phosphate-buffered saline (PBS), then incubated with 10 mg/L resazurin in TSB for 30 min at room temperature in the dark. Resorufin fluorescence (excitation/emission wavelengths: 560/590 nm) was then measured. Concentration-response curve data were used to calculate pharmacodynamic parameters based on the equation of the sigmoidal regressions. Maximum efficacy (E_{max}) is the maximum reduction in metabolic activity extrapolated for an infinitely large antibiotic concentration, and relative potency (C_{50}) is the concentration that causes 50% reduction in resorufin fluorescence signal [8].

2.3. Killing assays with human cathelicidin LL-37 and neutrophils

Bacteria were grown overnight (14–16 h) in Todd-Hewitt broth (THB) with or without nafcillin at different subinhibitory concentrations, pelleted, washed using PBS, resuspended to an OD_{600nm} of 0.4 in PBS (approximately 2×10^8 CFU/mL) and diluted to 0.5×10^6 CFU/mL in RPMI medium–10% Luria-Bertani (LB) containing 32 μ M (1 \times MIC) human cathelicidin LL-37, then incubated at 37°C [5,7]. Aliquots were taken at time 0 and after 2 h of incubation and plated on THA after serial dilutions.

Neutrophil killing assays were performed as described previously [10]. Briefly, neutrophils were freshly isolated from the blood of healthy donors using PolyMorphPrep-kit after lysis of erythrocytes. Bacteria were grown to log phase either alone or in the presence of nafcillin subinhibitory concentrations, then incubated with an equivalent number of neutrophils in RPMI containing 2% of 70°C heat-inactivated fetal bovine serum. After 90 min of incubation at 37°C in a 5% CO₂ atmosphere, cells were lysed with 0.025% Triton X-100, and aliquots spread on THA to enable CFU enumeration. These studies were approved by the University of California San Diego Human Research Protections Program.

2.4. Daptomycin and LL-37 binding assay

A fluorescence microscopy approach adapted from a previously described method was used to quantify the binding of daptomycin and LL-37 to the bacteria [11]. An overnight culture of the MRSE clinical isolate was diluted to 1:100 in LB (daptomycin binding) or in phenol-free RPMI + 10% LB medium (LL-37 binding) and grown in a shaking incubator at 37°C and 200 rpm to an OD_{600nm} of 0.5–0.6. Bacteria were incubated for 45 min with nafcillin (5 mg/L) or ceftaroline (1 mg/L) and then labeled with TAMRA LL-37 at 5 mg/L or boron-dipyrromethene (BODIPY)-labeled daptomycin (BDP-daptomycin) at 8 mg/L for 20 min at 37°C with shaking at 200 rpm. Cells were washed three times with LB or phenol-free RPMI +10% LB respectively. The final wash contained 2 mg/L DAPI (4',6-diamidino-2-phenylindole) nucleic acid stain. Cells were visualized using a Delta Vision Deconvolution microscope [5].

2.5. Curve fitting and statistical analyses

Curve fitting (non-linear regression and calculation of E_{max} and C_{50} in biofilms) and statistical analysis were performed with GraphPad Prism (version 9.0.0; GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Activity of antibiotics against planktonic cultures

The MICs of antibiotics (in mg/L) at low/high inocula were 0.5/2, 0.25/1, 0.5/2 (daptomycin), 0.25/0.5, 0.5/0.5, 0.25/0.5 (ceftaroline), 1/1, 32/32, 1/1 (nafcillin), and 1/2, 0.5/1, 1/1 (vancomycin) for strains 1, 2, and 3, respectively. In time kill-curve experiments (Figure 1), all antibiotics were essentially bacteriostatic when used alone at $\frac{1}{2}$ MIC measured at high inoculum. In contrast, a rapid killing was observed when daptomycin was combined with each of the β -lactams, with a 2 to 3–4 log₁₀ decrease in CFU counts that was observed for all strains after 4–8 h and was maintained when the incubation time was prolonged to 24 h.

3.2. Activity of antibiotics against biofilms

Biofilms play a critical role in endocarditis [3,12]; therefore, the activity of daptomycin combined with nafcillin or ceftaroline was tested against biofilms of the MRSE strain 1. Biofilms (24-h old) were first exposed for 24 h to nafcillin, ceftaroline or daptomycin over a broad range of concentrations (10–3 to 103 mg/L) to obtain full concentration-response curves (Figure 2A, left panels) to enable calculation of pharmacodynamic parameters (E_{max} and C₅₀; Figure 2B). All drugs showed a concentration-dependent activity, with daptomycin causing an almost complete reduction in the resorufin fluorescence signal at the highest concentration tested, and β -lactams only an approximately 50% reduction in this signal. Beta-lactams were then combined with two fixed concentrations of daptomycin (1 and 10 mg/L, approximating free minimum and maximum concentrations [C_{min} and C_{max}, respectively] in human serum; Figure 2A, right panels). The combination of daptomycin with nafcillin was markedly more efficient than either drug alone (higher E_{max}) and was also more potent (lower C₅₀), particularly when daptomycin was used at 10 mg/L (C₅₀ below the lowest concentration of nafcillin tested). When daptomycin was combined with ceftaroline, there was an increased E_{max} (significant only for daptomycin at 1 mg/L) and a modest reduction in C₅₀ when daptomycin was used at 10 mg/L.

3.3. Influence of nafcillin on bacterial killing by LL-37 and neutrophils

Beta-lactams enhance killing of MRSA by innate immune mechanisms, such as cationic antimicrobial peptides or neutrophils [7,10]. To assess whether this was also the case with MRSE, MRSE strain 1 was incubated overnight with or without nafcillin at subinhibitory concentrations, after which bacteria were subjected to killing by the human cathelicidin antimicrobial peptide LL-37 at its MIC (Figure 3A) or by neutrophils (Figure 3B) for 2 h. The effect of LL-37 alone was modest; however, there was a marked decrease in CFU count in the presence of sub-MIC concentrations of nafcillin. Similarly, a significantly increased susceptibility to neutrophil killing was observed when bacteria were preincubated with nafcillin. In both assays, the effect of nafcillin was concentration-dependent.

3.4. Influence of β -lactams on daptomycin and LL-37 binding to bacteria

Binding of daptomycin or human cathelicidin LL-37 to MRSE strain 1 was examined after pulsing bacteria during 45 min with 5 mg/L nafcillin or 1 mg/L ceftaroline (Figure 3C

and D). The level of daptomycin and LL-37 binding to MRSE was low but significantly increased when the bacteria were pre-exposed to nafcillin or ceftaroline.

4. Discussion

MRSE are emerging pathogens in native valve endocarditis and are associated with poor outcomes, in line with the 25–30% mortality seen in MRSA endocarditis [2]. Adopting the strategy reported for MRSA bacteremia [5,7], the addition of the anti-staphylococcal β -lactam, nafcillin successfully cleared within 24 h bacteremia that had persisted for 6 days on standard monotherapy.

The results from in vitro studies of the MRSE endocarditis strain 1 indicate three potential mechanisms for this observation. First, nafcillin markedly enhances the killing activity of daptomycin against *S. epidermidis*, with enhanced daptomycin binding to the cell membrane, as demonstrated against MRSA or vancomycin-resistant enterococci [7,13]. The exact mechanism of this synergy is still unknown but may include a reduced cell wall cross-linking and net surface charge following release of teichoic acid by β -lactams [5,14], which may enable greater daptomycin access to the cell membrane. Second, nafcillin enhances daptomycin bacterial killing within biofilms, which is highly relevant in this case of endocarditis with a large vegetation present. Again, this enhanced antibiotic activity was reported in a more complex dynamic model of biofilm caused by MRSA or by *Enterococcus faecium* [15,16]. Third, nafcillin, even at subinhibitory concentrations, enhances innate immune-killing activities of the cationic host defense peptide cathelicidin LL-37 and neutrophils, as previously observed against MRSA [7,10]. The daptomycin plus ceftaroline combination showed synergy and may be preferred by clinicians over nafcillin alone, which had no in vitro activity against MRSE [17].

This study has some important limitations. Although three MRSE strains showed synergy with daptomycin plus β -lactam in classic in vitro assays, only one isolate was extensively evaluated in vitro, and no in vivo assays were conducted. A larger number of isolates is needed to determine if the findings described herein are strain-specific, or generalizable to most or all MRSE. Whether bacteremia clearance was the direct result of nafcillin cannot be determined with certainty, but this is inferred based on the laboratory findings and published data with MRSA [5].

5. Conclusion

The current study extends to MRSE observations previously made for MRSA that daptomycin plus beta-lactam (including ceftaroline) therapy is a potent combination for treating endocarditis. Our findings, coupled with published data on MRSA, justify further study of daptomycin plus β -lactam in the treatment of endovascular CoNS infections, including animal models of MRSE infective endocarditis and, subsequently, in clinical trials.

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Competing Interests:

GS received speaking fees from Abbvie, and Paratek, and consulting fees from Paratek. VN has received research grant support from Roche Pharma and is on the Scientific Advisory Board of Cidara Therapeutics.

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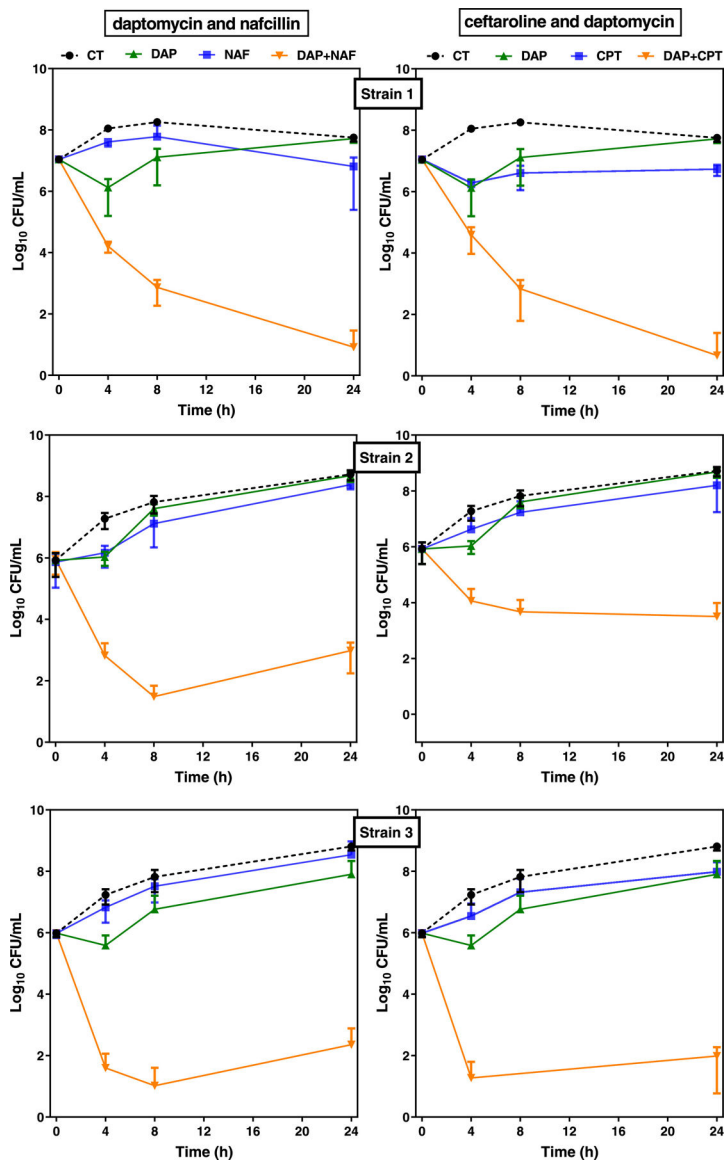


Figure 1. Time kill-curves of the clinical *S. epidermidis* by daptomycin, nafcillin or their combination (left) or daptomycin, ceftaroline or their combination (right). The initial inoculum was approx. 5×10^6 CFU/mL and antibiotics were used at a concentration corresponding to $\frac{1}{2}$ MIC obtained with this high inoculum. Data are mean \pm SD of 24 replicates.

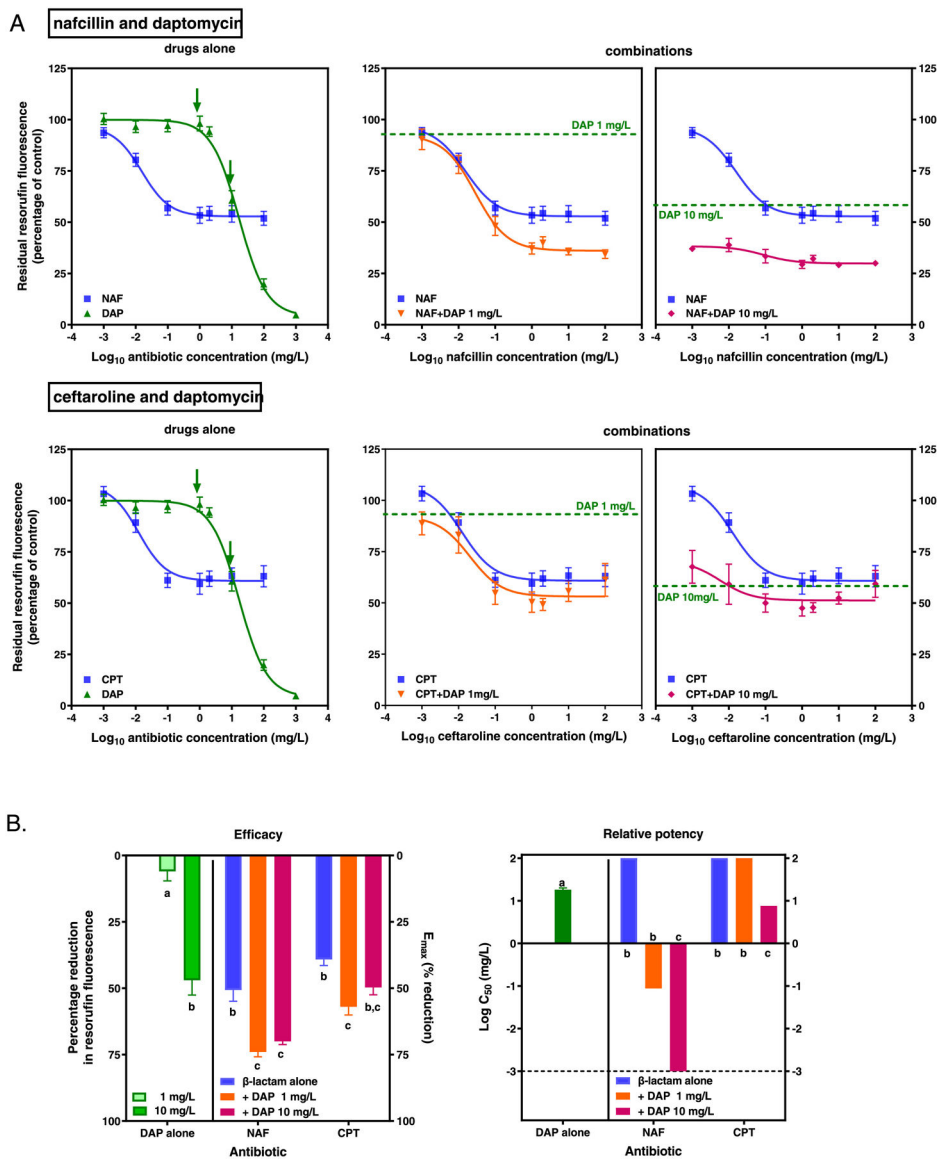


Figure 2.
A. Bacterial viability of MRSE Strain 1 in biofilms after 24 h of incubation with antibiotics. Drugs alone: concentration-response for daptomycin (DAP), nafcillin (NAF; top), or ceftaroline (CPT; bottom) alone. Combinations: nafcillin and ceftaroline were used over the same range of concentrations as in the left panels and combined with daptomycin at 1 mg/L (middle) or 10 mg/L (right). The curves for β-lactams alone are reproduced in these panels for comparison. The arrows in the left panels point to the concentrations of daptomycin used in combination. The dotted line in the combination panels shows the effect obtained for daptomycin alone at the concentration used. Data are mean ± SEM of 2–3 experiments performed in triplicate. **B.** Pharmacodynamic parameters of the activity of antibiotics alone or in combination calculated from the hill equation of the concentration-response curves shown in panel A. Left: antibiotic efficacy. The graph shows the percentage of reduction in resorufin fluorescence (compared with control biofilms) obtained after 24 h incubation with

daptomycin alone at 1 or 10 mg/L (left axis; green bars) and the maximal efficacy (E_{\max} ; right axis) calculated from the equation of the concentration response curve for β -lactams alone (blue bars) or combined with daptomycin at 1 mg/L (orange bars) or 10 mg/L (pink bars). Right: antibiotic relative potency. The graph shows the concentration of antibiotic (in log scale) needed to reduce the fluorescence signal of resorufin by 50% (compared with control biofilms) after 24 h of incubation with daptomycin alone (green bar), β -lactams alone (blue bars) or combined with daptomycin 1 mg/L (orange bars) or 10 mg/L (pinks bars). Statistical analysis (one-way ANOVA with Tukey post-hoc test); conditions with different letters are significantly different from one another ($P < 0.05$).

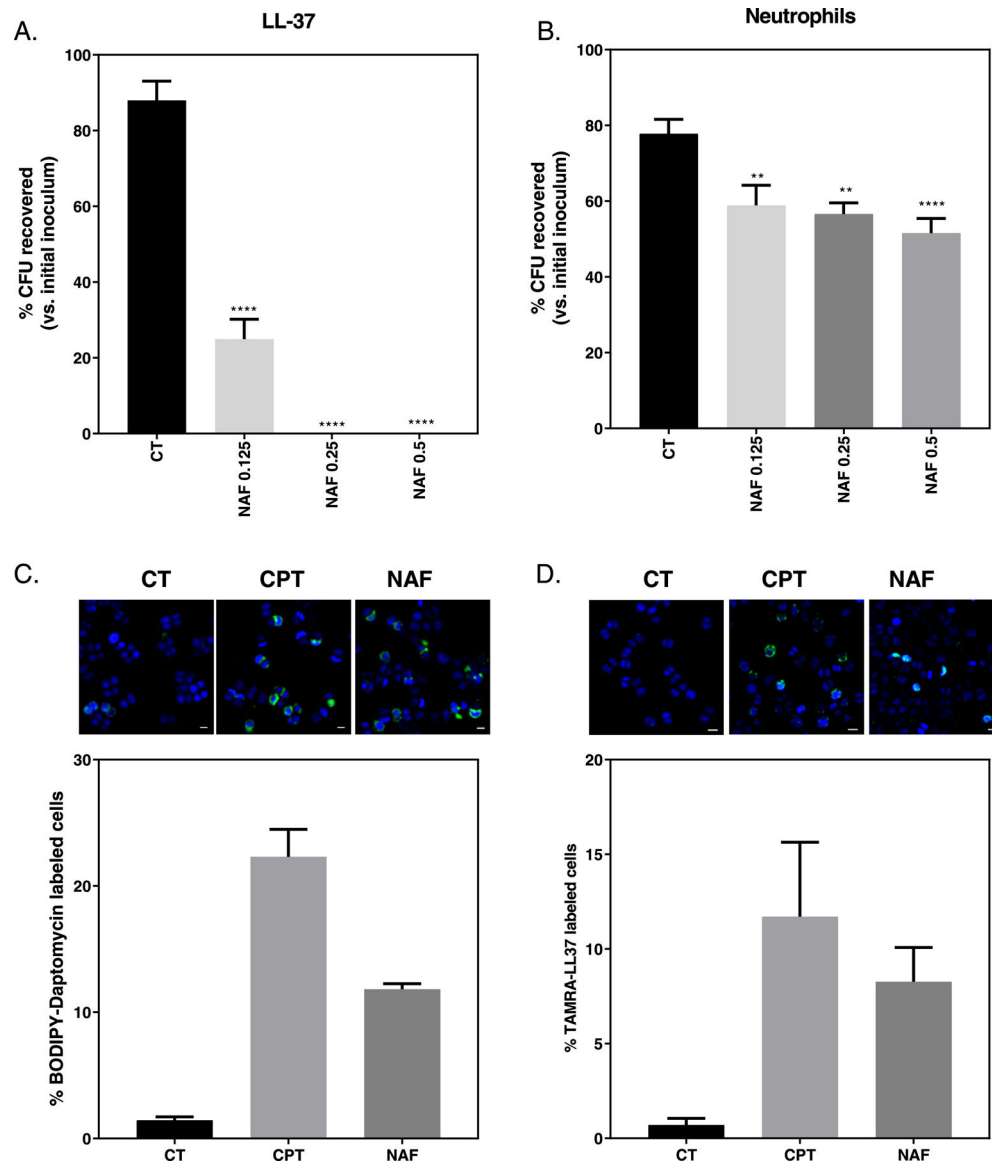


Figure 3. A-B. Killing of Strain 1 by human cathelicidin LL-37 (32 μ M) (A) or neutrophils (B) after 2 h of incubation. Bacteria were preincubated overnight in antibiotic-free medium (control; CT) or media containing nafcillin (NAF) at concentrations of 0.125, 0.25 or 0.5 mg/L. C-D. Binding of BODIPY-labeled daptomycin (BDP-DAP) or TAMRA-labeled LL37 to Strain 1. Cells were preincubated for 45 min with nafcillin (NAF 1 mg/L) or ceftaroline (CPT 5 mg/L) or in control conditions (CT), then reincubated for 20 min with BODIPY-DAP at 8 mg/L (C) or TAMRA-LL-37 at 5 mg/L. Results are from experiments performed in duplicate and reported as mean \pm SEM from three different fields.