STAPHYLOCOCCUS AUREUS BIOFILMS ARE HIGHLY TOLERANT TO TRADITIONAL ANTIBIOTICS BUT SUSCEPTIBLE TO ENGINEERED ANTIMICROBIAL PEPTIDE WLBU2

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ABSTRACT

Hospital associated infection remains a major problem which is responsible for ~100,000 deaths and over 10 billion dollars in health care costs annually in the US alone. Peri-prosthetic joint infection of total knee arthroplasties is an extremely challenging hospital associated infection to treat. These infections are challenging to treat due to the presence of established *S. aureus* bacterial biofilms on implant material and surrounding tissue. *S. aureus* biofilms secrete an extensive extracellular matrix structure, have altered stress response abilities, and have an increased proportion of metabolically inert "persister" cells. Antimicrobial peptides have been widely considered as a new class of antibiotic which could assist in the dilemma of drug tolerance bacterial biofilms. Engineered cationic amphipathic peptide WLBU2 is a rationally designed antimicrobial peptide which maximizes bacterial membrane attachment and disruption and decreases toxicity to mammalian cells. Here we hypothesize that *S. aureus* clinical isolate biofilms will display increased tolerance to clinically used antibiotics compared to planktonic cells *in vitro*. Additionally, we hypothesize that compared to cefazolin, WLBU2 has higher activity against *S. aureus* biofilms grown on implant material *in vitro* and can more effectively treat PJI in mice. We found that clinical isolates grown as biofilms were significantly more tolerant to all ten clinically administered antibiotics tested compared to the same isolates grown

as planktonic cells. Only rifampin, doxycycline, and daptomycin displayed activity against most bacterial biofilms tested. WLBU2 could kill over 99.9% of *S. aureus* biofilms grown on metal implant pieces in under two hours, while cefazolin failed to achieve this anti-biofilm activity over 24 hours. Finally, we demonstrated WLBU2 could more effectively treat periprosthetic joint infection in mice than cefazolin. In terms of public health significance, understanding how to better kill and clear established bacterial biofilms utilizing novel antimicrobial peptides will be crucial in decreasing the extensive morbidity and mortality from hospital and community associated infections.

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PREFACE

Thank you to my research advisor, Kenneth Urish, for permitting his technician to go back to school while continuing to work full time. To my other mentor Kurt Weiss, for allowing me to continue to work in cancer research during my education. My academic advisor Robbie Mailliard, who initially recruited me to IDM and has sagely counseled me along the way. Finally, to the entire IDM department, who has provided me with a quality and comprehensive educational experience.

Thank you to my mother and father, for all their love and support.

1.0 INTRODUCTION

Millions of patients will develop hospital associated infections annually, many which result from antibiotic tolerant pathogens. Infection remains an enormous problem in the field of orthopaedic surgery, where peri-prosthetic joint infections from *S. aureus* biofilms are difficult to completely treat with traditional antibiotics. Since the unique features of *S. aureus* biofilms prevent the complete action of antibiotics, new and specifically anti-biofilm compounds must be tested for therapy. Antimicrobial peptides are naturally made by the innate immune system, and more logically engineered derivatives could potentially serve as more effective anti-biofilm compounds for treating chronic infections.

1.1 *S. AUREUS* IN HOSPITIAL ASSOCIATED INFECTIONS

1.1.1 Hospital associated infections in the US

Roughly 2 million hospital associated infections (HAIs) occur annually in the US and will kill 75,000-90,000 patients during their hospitalization. HAIs mainly involve dangerous multidrug resistant ESKAPE pathogens, which are responsible for surgical site infections, ventilator-associated pulmonary infections, central line- associated bloodstream infections, and implant

prosthesis related infections (1–4). Currently in the US, the combined HAIs are estimated to cost 35-45 billion dollars in health care resources every year (5).

1.1.2 Total knee arthroplasty and peri-prosthetic joint infection

Total knee arthroplasties (TKAs) are among the largest major surgical procedures by volume in the US, with over 700,000 performed every year. These procedures are expected to increase over 500% by 2050 due to many factors including; an aging baby boomer generation staying in the workforce for longer, and an increase in diabetes as well as obesity in the population (6,7). An infected total knee arthroplasty, termed periprosthetic joint infection (PJI), occurs in 1.5-2% of patients undergoing joint replacement surgery. PJI treatment involves multiple subsequent surgical procedures and long-term antibiotic regimen. Treatment failure is over 60% and five-year mortality is ~25% (8–11).

1.1.3 *S. aureus* bacterial biofilms

Staphylococcus aureus is a common organism seen in HAIs and is the primary agent responsible for PJI in TKA. *S. aureus* in implant related infections regularly form and primarily exist as established biofilms. In contrast to freely floating planktonic cells, *S. aureus* biofilms are adherent aggregated populations of bacteria which are encased in secreted extracellular polymeric substances (EPS), which is known to contain polysaccharides, proteins, and DNA (12–16)

1.1.4 Biofilm antibiotic tolerance

Unique properties of established biofilms in relation to planktonic state results in an infection which is more virulent, can evade host immune clearance, and is highly tolerant to traditional antibiotic. A variety of biofilm drug tolerance mechanisms have been proposed, including impaired antibiotic penetration, altered stress responses, altered microenvironment, and reduced metabolic states in a sub-population of bacteria known as “persister cells” (13,17–19).

1.2 ANTIMICROBIAL PEPTIDES AS NOVEL ANTIBIOTICS

1.2.1 Natural antimicrobial peptides

Antimicrobial peptides can serve as an alternative strategy to traditional antibiotics. Antimicrobial peptides are found among may different plant and animal species. The human innate immune system consists of whole families of antimicrobial peptides, including cathelicidins and defensins (20,21). These cationic amphipathic peptides (CAPs) selectively bind to the strongly negatively charged bacteria and create pores in both gram-negative and -positive bacterial membranes. Cationic host defense peptides are CAPs that demonstrate the ability to kill bacteria regardless of resistance to traditional antibiotics. However, the use of natural cationic host defense peptides has been limited in the clinic due to suboptimal efficacy and systemic toxicity (22).

1.2.2 Engineered antimicrobial peptides

The limitation of naturally found antimicrobial peptides motivated the design of synthetic engineered cationic amphipathic peptides (eCAPs), resulting in the extensive characterization of WLBU2 as a candidate for potential clinical development. Natural antimicrobial peptides share unique structural characteristics with HIV-1 gp41 lentivirus lytic protein 1 (23). Peptides were derived from arginine and valine containing helical faces to yield a single functional lytic base unit (LBU). The combination of two lytic base units with specific tryptophan substitutions in the hydrophobic face resulted in the WLBU2. WLBU2 was rationally designed as an idealized helical peptide with optimized amphipathic structure to maximize bacterial membrane selectivity and minimize potential cytotoxicity toward the host (24).

1.2.3 WLBU2

WLBU2 has shown activity against planktonic MRSA in addition to a large panel of ESKAPE pathogens (25). Additional studies have demonstrated that systemic delivery of WLBU2 is effective against *P. aeruginosa* biofilms associated with cystic fibrosis with minimal toxicity (26,27), but activity against *S. aureus* antibiotic-resistant biofilms has not been shown. More importantly, this novel functional property has not been demonstrated in a translational model that can further advance the clinical development of WLBU2 as a superior therapeutic option to current antibiotic regimens.

2.0 STATEMENT OF THE PROJECT

Bacterial biofilms represent a major challenge in successfully treating a myriad of dangerous hospital associated infections. Establishment of biofilms in surgical sites can result in a chronic infection which cannot be completely treated and cleared by traditional antibiotics. Hospital associated biofilm infections, such as *S. aureus*, result in significant amount morbidity for patients and enormous costs to the health care system annually. These highly antibiotic tolerant bacterial biofilms, as well as the emergence of highly drug resistant mutant strains, have resulted in a large amount of work developing novel antibiotics which do not act against commonly targeted bacterial metabolic processes. Any new knowledge pertaining to the following aims will aid in further understanding of the failures of clinically used antibiotics against *S. aureus* bacterial biofilms and the potential utility of eCAP WLBU2 in successfully treating biofilm infections. These studies were supported in part by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS K08AR071494), the National Center for Advancing Translational Science (NCATS KL2TR0001856), the Orthopaedic Research and Education Foundation, and the Musculoskeletal Tissue Foundation.

3.0 SPECIFIC AIMS

Aim 1: Determine *S. aureus* PJI clinical isolate biofilm antibiotic tolerance compared to planktonic cultures

Sub-aim 1A: Determine *S. aureus* PJI clinical isolate planktonic cells antibiotic tolerance across clinically used antibiotic panel.

Sub-aim 1B: Determine *S. aureus* PJI clinical isolate biofilms antibiotic tolerance across clinically used antibiotic panel.

We hypothesize that PJI clinical isolate biofilms will have increased antibiotic tolerance compared to planktonic cells across all drugs tested.

Aim 2: To test and optimize WLBU2 activity against *S. aureus* biofilms grown on implant materials *in vitro*

Sub-aim 2A- Compare WLBU2 and cefazolin in timed killing of *S. aureus* planktonic and persister cells.

Sub-aim 2B- Optimize WLBU2 activity against *S. aureus* biofilms grown on metal implant material.

We hypothesize that WLBU2 shows improved activity against *S. aureus* biofilms compared to cefazolin, and activity will be enhanced in neutral buffered conditions *in vitro.*

Aim 3: To test WLBU2 efficacy in treating *S. aureus* biofilms in a murine model of periprosthetic joint infection.

Sub-aim 3A- Test WLBU2 efficacy in treating peri-prosthetic joint infection *in vivo* compared to traditional antibiotics.

Sub-aim 3B- Further study efficacy of WLBU2 in treating PJI using the D-enantiomer (D8) and utilizing a neutropenic mouse model.

We hypothesize WLBU2 systemic treatments will show efficacy in treating PJI *S. aureus* biofilms *in vivo*.

4.0 MATERIALS AND METHODS

4.1 CULTURE CONDITIONS AND BACTERIAL STRAINS

All strains were inoculated in Tryptic Soy Broth (TSB, Bectin Dickinson and Company) overnight at 37° C with shaking at 250 rpm. Strains were diluted in Mueller Hinton Broth (MHB; Bectin Dickinson and Company) to a final concentration of $0.5x10^6$ CFU/ml using the 0.5 MacFarland Standard (GFS Chemicals) and an Infinite M200 Spectrophotometer (Tecan). Assays were performed utilizing high throughput methods, all experiments were performed in 96 well plates. All experiments were performed at least in triplicate at three separate times with freshly inoculated cultures. Two lab strains, USA300 and SH1000, as well as 10 MRSA and 8 MSSA clinical isolates were tested. Institutional Review Board guidelines and regulations were followed in completing this study.

4.2 PLANKTONIC CULTURE MIC AND MBC

Both lab strains and clinical isolates of *S. aureus* were grown planktonically. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed using high throughput standardized CLSI assays. Fold dilutions of six antibiotics (vancomycin, rifampin, gentamicin, trimethoprim/sulfamethoxazole, doxycycline, and daptomycin) were

assessed in each isolate. MSSA samples were additionally tested with cefazolin and nafcillin, while MRSA samples were additionally tested with clindamycin and linezolid. Antibiotic concentrations of 125, 62, 31, 16, 8, 4, 2, 1, 0.5, 0.25, 0.13 µg/ml as well as untreated controls were tested. After 24 hours of treatment, MIC was assessed by staining treated cultures with PrestoBlue viability assay (Thermo-Fisher Scientific) according to manufacturer's instructions using a SynTek microplate reader. Prior to Presto Blue addition, well contents were plated on TSA II with 5% sheep blood CS100 plates and incubated overnight at 37º C. Colony forming unit (CFU) analysis on plates was performed; a 99.9% reduction in CFU's of the original plating density represented the MBC.

4.3 MATURE BIOFILMS MIC AND MBC

For bacterial biofilms treatment, *S. aureus* strains were planktonically grown for 24 hours, at which time fresh MHB was exchanged. At 48 hours, mature biofilms were treated with the same panel of antibiotics used in planktonic assays. Mature biofilms were washed with dPBS to remove background planktonic bacteria and treated with antibiotics diluted in MHB. Antibiotic concentrations were raised to 2000, 1000, 500, 250, 125, 62, 31, 16, 8, 4, 2 µg/ml as well as untreated controls. After 24 hours of treatment, the antibiotic supplemented broth was removed, biofilms were washed with dPBS, and 100 µl of dPBS was added to biofilms in wells. 96 well plates were manually scrapped for 1 minute to homogenize biofilms for minimum biofilm inhibitory concentration (MBIC) analysis. For scraping, sterile and autoclaved 0.1-10 µl micropipette tips were kept in holder within the hinged box container and taped securely in place. Pipette tips were inserted simultaneously into all 96 wells and the wells were scraped

vigorously. MBIC was assessed using PrestoBlue viability assay, increasing the incubation time to 1 hour. For minimum biofilm bactericidal concentration (MBBC), prior to PrestoBlue addition, scrapped biofilms well contents were plated onto blood agar plates and CFU analysis was performed.

4.4 BACTERIAL STRAINS AND CULTURE

S. aureus SH1000 was used for *in vitro* assays and the *in vivo* animal model. In addition, a series of *S. aureus* clinical strains were used for additional *in vitro* biofilm assays (5 methicillin-resistant strains, 4 methicillin-susceptible strains). All strains were inoculated in Tryptic Soy Broth (TSB, Bectin Dickinson and Company) overnight at 37 °C with shaking at 250 rpm. Strains were diluted in Mueller Hinton Broth (MHB; Bectin Dickinson and Company) to a final concentration of 0.5×10^6 CFU/ml using the 0.5 MacFarland Standard (GFS Chemicals) and an Infinite M200 Spectrophotometer (Tecan). All experiments were performed at least in triplicate at three separate times with freshly inoculated cultures. Institutional Review Board guidelines and regulations were followed for the use of clinically derived *S. aureus* strains.

4.5 MINIMUM INHIBITORY AND BACTERICIDAL CONCENTRATION

The minimum inhibitory concentration (MIC) of cefazolin and WLBU2 for SH1000 in suspension was determined using CLSI assay protocols, incubating freshly plated cultures at 0.5×10^6 CFU/ml for 24 hours with serial dilutions of each antimicrobial and observing

inhibition of bacterial growth based on turbidity. Cefazolin concentrations ranged 0.044, 0.088, 0.18, 0.35, and 0.7 µM (0.02, 0.04, 0.08, 0.16, and 0.32 µg/ml). WLBU2 concentrations ranged 0.9, 1.8, 3.7, 7.5, and 15 µM (3.1, 6.2, 12.5, 25, and 50 µg/ml). Both antibiotics were diluted in MHB before addition to SH1000. The minimum bactericidal concentration (MBC) of cefazolin and WLBU2 for SH1000 in suspension was determined by incubating freshly plated cultures at 0.5×10^6 CFU/ml with antibiotics. Cefazolin concentrations ranged 0.15, 0.35, 0.7, 1.4, and 3.5 µM (0.08, 0.16, 0.32, 0.64, 1.6 µg/ml). WLBU2 concentrations ranged 4.5, 9, 18, 37, 74 µM (15, 31, 62, 125, 250 µg/ml). Well contents were tested at 0, 2, 8, 24, and 48 hours. After treatment, well contents were serial diluted into MHB, and CFU were determined using TSA II with 5% sheep blood CS100 plates that were incubated overnight at 37 °C. The limit of detection was 100 CFU/ml as 10 µl samples of the dilutions were plated. WLBU2 exhaustion assay was performed by subjecting WLBU2 at 10x MIC (250 µg/ml) to increasing inoculation densities to further assess bactericidal activity. SH1000 plated at 0.1, 1, 10, 100, and 1000×10^6CFU/ml in suspension for 30 minutes and quantified by serial dilution on blood agar plates

4.6 *IN VITRO* BIOFILM KILLING ASSAYS

Rods were prepared from 0.6 mm diameter stainless steel Kirschner wire (Synthes) and cut into 6 mm length, autoclaved, and plated in wells along with SH1000 and all clinical strains at 1×10^6 CFU/ml. After plating, fresh MHB media was exchanged at 24 hours. At 48 hours, wire with mature biofilms were either placed into fresh media, or treated with either cefazolin at 3.5 µM (1.6 µg/ml) or WLBU2 at 74 µM (250 µg/ml). At 0.5, 1, 6, and 24 hours Kirschner wire were taken from wells, placed into 1% Tween 20 in PBS and sonicated for 10 minutes. Resulting

sonication media was serial diluted into MHB and CFU were determined on blood agar plates. Sonicated rods were sterilely placed in fresh MHB for 72 hours and assessed for visual turbidity

4.7 PERSISTER CELL VIABILITY ASSAYS

SH1000 at 1×10^6 CFU/ml was pre-treated 90 minutes with carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) diluted to 12.5 µg/ml in MHB. Bacterial cultures were centrifuged, and pellet re-suspended in MHB before antibiotic treatment. Cefazolin treatment was at 3.5 µM (1.6 µg/ml), WLBU2 was at 74 µM (250 µg/ml). Percent survival was calculated from baseline bacterial cultures after pretreatment but before antibiotic addition. After 6 hours of treatment, serial diluted drop assays were performed on samples and plated on blood agar plates for CFU analysis.

4.8 VIABLE BACTERIAL BIOFILM MICROSCOPY

SH1000 was plated at 1×10^6 CFU/ml in 8 chambered slides (Lab-Tek), with wells replaced with fresh MHB 24 hours later. After 48 hours biofilms were treated with cefazolin and WLBU2 at 10xMIC (3.5 µM and 74 µM) and placed into 37 °C incubator. After 10 minutes, LIVE/DEAD BacLight Bacterial Viability Kit fluorescent stain (Invitrogen) was added to well contents and incubated at room temperature protected from light for 20 minutes. Fluorescence microscopy performed using a Nikon Eclipse TE2000 microscope with 20x objective, and a Q Imaging RETIGA EXi camera. Images captured and merged using Northern Eclipse software.

4.9 PERIPROSTHETIC JOINT INFECTION ANIMAL MODEL

All experiments were performed under approved IACUC animal protocol in University of Pittsburgh Division of Laboratory Animal Resources. Twelve-week-old B57BL/6 J female mice (Jackson) were used for all experiments. Mice where anesthetized by 2% isoflurane, hair was removed from leg and treated with betadine. With a scalpel, a medial parapatellar incision was made, and lateral displacement of the quadriceps-patellar complex allowed for visualization of the femoral intercondylar notch. With a 25-gauge needle, the femoral intramedullary canal was manually reamed. A 0.6 mm wide/6 mm long sterile Kirschner wire (Synthes) was inserted into the canal and was left protruding ~1 mm into the joint. The quadriceps-patellar complex was reduced back to midline and incision was closed using sutures. An inoculation volume of 10 µl with 1×10^6 CFU of SH1000 was injected into the joint space. Treatment group mice received either WLBU2 at 0.01 mg/kg-10 mg/kg, cefazolin at 50 mg/kg, or rifampin at 30 mg/kg) starting 24 hours after surgery and inoculation. Mice received antibiotic dose twice a day for three days. Mice were euthanized and Kirschner wire implant as well as a ~2 mm × 2 mm piece of distal femur were placed in 1% Tween 20 on ice. Implants were sonicated for 10 minutes; distal femur was mechanically homogenized for 30 seconds. Samples were serially diluted into MHB and 10 µl drop assays were performed on blood agar plates.

When comparing two groups, a two tailed Mann-Whitney test was performed, with $p < 0.05$ considered statistically significant. When comparing multiple groups, a two tailed Kruskal-Wallis test with Dunn's multiple comparisons post-test was performed, with $p < 0.05$ considered statistically significant.

4.10 STATISTICAL ANALYSIS

Antibiotic susceptibility and tolerance between planktonic and established biofilms of PJI clinical isolates were collected and compared. All graphical and statistical analysis was performed using GraphPad Prism. Since data is non-parametric, when comparing two groups, a Mann-Whitney test was performed. Matched sample analysis was performed using a Wilcoxon rank test, and multiple group variance testing was performed using a Kruskal-Wallis test with a Dunn's multiple comparisons posttest.

5.0 AIM 1 RESULTS

5.1 LARGE VARIATIONS IN CLINICAL ANTIBIOTIC ACTIVITY AGAINST *STAPHYLOCOCCUS AUREUS* BIOFILMS OF PERIPROSTHETIC JOINT INFECTION ISOLATES

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5.2 ABSTRACT

Staphylococcus aureus biofilms have a high tolerance to antibiotics, making the treatment of periprosthetic joint infection (PJI) challenging. From a clinical perspective, bacteria from surgical specimens are cultured in a planktonic state to determine antibiotic sensitivity. However, *S. aureus* exists primarily as established biofilms in PJI. To address this dichotomy, we developed a prospective registry of total knee and hip arthroplasty PJI *S. aureus* isolates to quantify the activity of clinically important antibiotics against isolates grown as biofilms. *S. aureus* planktonic minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were assessed using high throughput standardized CLSI assays of six antibiotics (vancomycin, rifampin, gentamicin, trimethoprim/sulfamethoxazole, doxycycline, and daptomycin). Mature biofilms of each strain were grown *in vitro*, after which biofilm MIC (MBIC) and biofilm MBC (MBBC) were determined. Overall, isolates grown as biofilms displayed larger variations in antibiotic MICs as compared to planktonic MIC values. Only rifampin, doxycycline, and daptomycin had measurable biofilm MIC values across all *S. aureus* isolates tested. Biofilm MBC observations complemented biofilm MIC observations; rifampin, doxycycline, and daptomycin were the only antibiotics with measurable biofilm MBC values. 90% of *S. aureus* biofilms could be killed by rifampin, 50% by doxycycline, and only 15% by daptomycin. Biofilm formation increased bacterial antibiotic tolerance nonspecifically across all antibiotics, in both MSSA and MRSA samples. Rifampin and doxycycline were the most effective antibiotics at killing established *S. aureus* biofilms.

5.3 INTRODUCTION

Staphylococcus aureus is a common organism responsible for orthopaedic related infections[1-2]. *S. aureus* in implant related infections regularly form and primarily exist as established biofilms[3-5] These infections are difficult to treat because bacterial cells in biofilms have a high tolerance to traditional antibiotics[2]. This antibiotic tolerance of biofilms is also seen in many other types of bacteria[6-8]. A variety of drug tolerance mechanisms have been proposed, including impaired antibiotic penetration, quorum-sensing regulation, and altered metabolic states[9].

Although biofilms are the primary state for bacterial cells in infections, standard antibiotic susceptibility testing uses bacteria grown as planktonic cultures[10-11]. This accurately quantifies antibiotic genetic resistance but fails to assess antibiotic activity against bacteria in a biofilm state. Antibiotic susceptibility and tolerance is much less understood when the bacteria are cultured as biofilms. There is a significant decrease in antibiotic sensitivity between planktonic bacteria and bacteria cells in biofilms because of the high tolerance of biofilms to antibiotics[12-13]. Therefore, testing of *in vitro* bacteria cultured as biofilms from patient isolates would provide a more accurate determination of antibiotic susceptibility by directly observing the bacteria phenotype that exists during infection.

There are limited studies that have evaluated the activity of antibiotics against *S. aureus* biofilms specifically using PJI clinical isolates. To address this, we developed a prospective clinical isolate registry of total knee arthroplasty (TKA) PJI samples to quantify the sensitivity of different antibiotics to clinical isolates of both methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) *in vitro* cultured biofilms. Both planktonic and biofilm MIC and MBC of a panel of commonly administered antibiotics were quantified across all

isolates. The objective was to determine the extent of variation in biofilm antibiotic sensitivity to clinically administered antibiotics.

5.4 MATERIALS AND METHODS

All strains were inoculated in Tryptic Soy Broth (TSB, Bectin Dickinson and Company) overnight at 37^{o} C with shaking at 250 rpm. Strains were diluted in Mueller Hinton Broth (MHB; Bectin Dickinson and Company) to a final concentration of $0.5x10^{6}$ CFU/ml using the 0.5 MacFarland Standard (GFS Chemicals) and an Infinite M200 Spectrophotometer (Tecan). Assays were performed utilizing high throughput methods, all experiments were performed in 96 well plates. All experiments were performed at least in triplicate at three separate times with freshly inoculated cultures. Two lab strains, USA300 and SH1000, as well as 10 MRSA and 8 MSSA clinical isolates were tested. Institutional Review Board guidelines and regulations were followed in completing this study.

5.4.1 Planktonic Culture MIC and MBC

Both lab strains and clinical isolates of *S. aureus* were grown planktonically. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed using high throughput standardized CLSI assays. Fold dilutions of six antibiotics (vancomycin, rifampin, gentamicin, trimethoprim/sulfamethoxazole, doxycycline, and daptomycin) were assessed in each isolate. MSSA samples were additionally tested with cefazolin and nafcillin, while MRSA samples were additionally tested with clindamycin and linezolid. Antibiotic

concentrations of 125, 62, 31, 16, 8, 4, 2, 1, 0.5, 0.25, 0.13 µg/ml as well as untreated controls were tested. After 24 hours of treatment, MIC was assessed by staining treated cultures with PrestoBlue viability assay (Thermo-Fisher Scientific) according to manufacturer's instructions using a SynTek microplate reader. Prior to Presto Blue addition, well contents were plated on TSA II with 5% sheep blood CS100 plates and incubated overnight at 37^{o} C. Colony forming unit (CFU) analysis on plates was performed; a 99.9% reduction in CFU's of the original plating density represented the MBC.

5.4.2 Mature Biofilms MIC and MBC

For bacterial biofilms treatment, *S. aureus* strains were planktonically grown for 24 hours, at which time fresh MHB was exchanged. At 48 hours, mature biofilms were treated with the same panel of antibiotics used in planktonic assays. Mature biofilms were washed with dPBS to remove background planktonic bacteria and treated with antibiotics diluted in MHB. Antibiotic concentrations were raised to 2000, 1000, 500, 250, 125, 62, 31, 16, 8, 4, 2 µg/ml as well as untreated controls. After 24 hours of treatment, the antibiotic supplemented broth was removed, biofilms were washed with dPBS, and 100 µl of dPBS was added to biofilms in wells. 96 well plates were manually scrapped for 1 minute to homogenize biofilms for minimum biofilm inhibitory concentration (MBIC) analysis. For scraping, sterile and autoclaved 0.1-10 µl micropipette tips were kept in holder within the hinged box container and taped securely in place. Pipette tips were inserted simultaneously into all 96 wells and the wells were scraped vigorously. MBIC was assessed using PrestoBlue viability assay, increasing the incubation time to 1 hour. For minimum biofilm bactericidal concentration (MBBC), prior to PrestoBlue

addition, scrapped biofilms well contents were plated onto blood agar plates and CFU analysis was performed.

5.4.3 Statistical Analysis

Antibiotic susceptibility and tolerance between planktonic and established biofilms of PJI clinical isolates were collected and compared. All graphical and statistical analysis was performed using GraphPad Prism. Since data is non-parametric, when comparing two groups, a Mann-Whitney test was performed. Matched sample analysis was performed using a Wilcoxon rank test, and multiple group variance testing was performed using a Kruskal-Wallis test with a Dunn's multiple comparisons posttest.

5.5 RESULTS

5.5.1 Variations in Antibiotic Activity Against *S. Aureus* Planktonic Cultures and Mature Biofilms

CLSI protocol and a mature biofilm *in vitro* model was used to quantify variations in planktonic MIC, planktonic MBC, biofilm MIC, and biofilm MBC of different antibiotics across *S. aureus* isolates. Gentamicin, trimethoprim/sulfamethoxazole, and vancomycin displayed larger variations (~1.5 log spread) in planktonic MICs across all isolates, while rifampin, doxycycline and daptomycin displayed smaller variations (~0.5 log spread) (Fig 1A-blue). Alternatively, planktonic MBCs of all antibiotics displayed a large amount of variation (2 log spread) across all

isolates (Fig 1B-blue). Rifampin showed superior antimicrobial action against planktonic cultures compared to other antibiotics, with MBC values ranging from 0.13 to 8 µg/ml. Next, we determined variations in MBIC and MBBC of antibiotics tested against all isolates. Most antibiotics showed a large variability (2-3 log spread) in MBIC across all isolates, except for daptomycin which displayed a variation of only one order of magnitude (Fig 1A-red). Many isolate biofilms showed no sensitivity to trimethoprim/sulfamethoxazole, gentamicin, or vancomycin despite normal sensitivity observed in planktonic cultures. Only rifampin, doxycycline, and daptomycin could kill mature biofilms, with biofilm MBCs ranging from 80 to 2000 µg/ml (Fig1B-red).

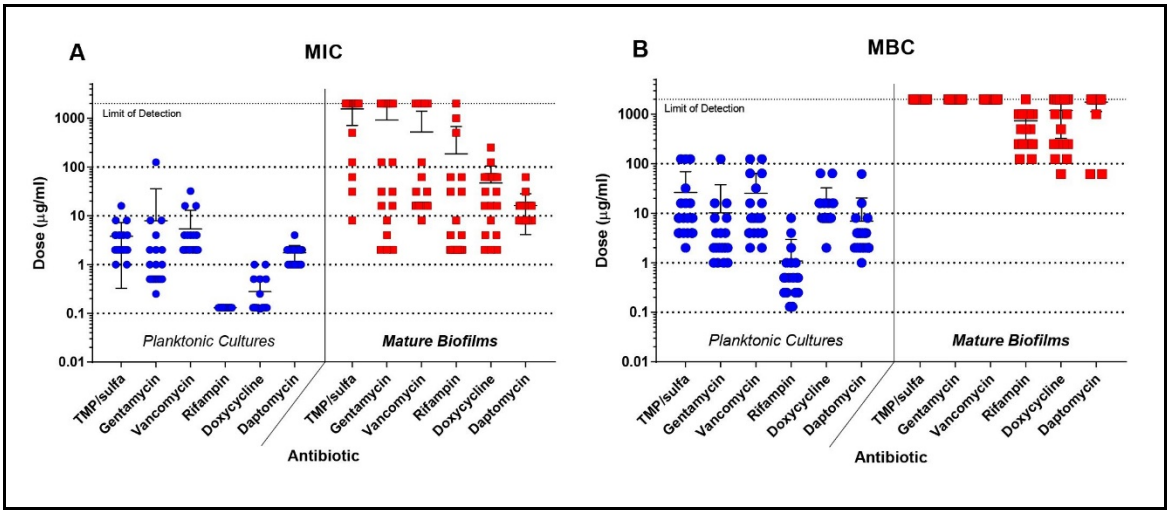

Figure 1. PJI *S. aureus* biofilms show decreased antibiotic sensitivity and increased tolerance to killing.

Across all clinical isolates, antibiotic planktonic MICs (blue) and antibiotic mature biofilm MICs (red) were determined using a PrestoBlue viability assay (A). Antibiotic MICs of mature biofilms showed a much larger variation across all isolates compared to planktonic cultures. Across isolates, antibiotic planktonic MBCs (blue) and antibiotic mature biofilm MBCs (red) were determined by CFU analysis using blood agar plates (B). Clinical isolate biofilms were highly tolerant to antibiotics.

5.5.2 Both MSSA and MRSA Biofilms Display Decreased Sensitivity Across all Antibiotics

We assessed planktonic MIC and biofilm MIC (MBIC) of additional antibiotics cefazolin and nafcillin for MSSA isolates, as well as clindamycin and linezolid for MRSA isolates. 78% of MSSA isolate biofilms displayed sensitivity to cefazolin under our limit of detection (Fig 2A-red), while ~83% MRSA isolate biofilms displayed no sensitivity to clindamycin (Fig 2B-red). MIC values for established biofilms were more variable across isolates compared to planktonic cultures. In both MSSA and MRSA isolates, rifampin MIC for all 20 *S. aureus* strains was 0.125 μg/ml, while the MBICs ranged dramatically from 2-2000 μg/ml (Fig 2A and B). Daptomycin was the only antibiotic for which biofilm MIC did not display dramatic variation in sensitivity across all isolates. In every antibiotic tested, there was a significant increase in MBIC compared to planktonic MIC in both MSSA (Fig 2A) and MRSA (Fig 2B) isolates. Rifampin, doxycycline, and daptomycin could inhibit growth in all isolate biofilms at the doses tested. Matched sample analysis confirmed that the MIC for each individual strain did significantly increase in the biofilm phenotype compared to planktonic for both MSSA and MRSA (Fig 2C and D).

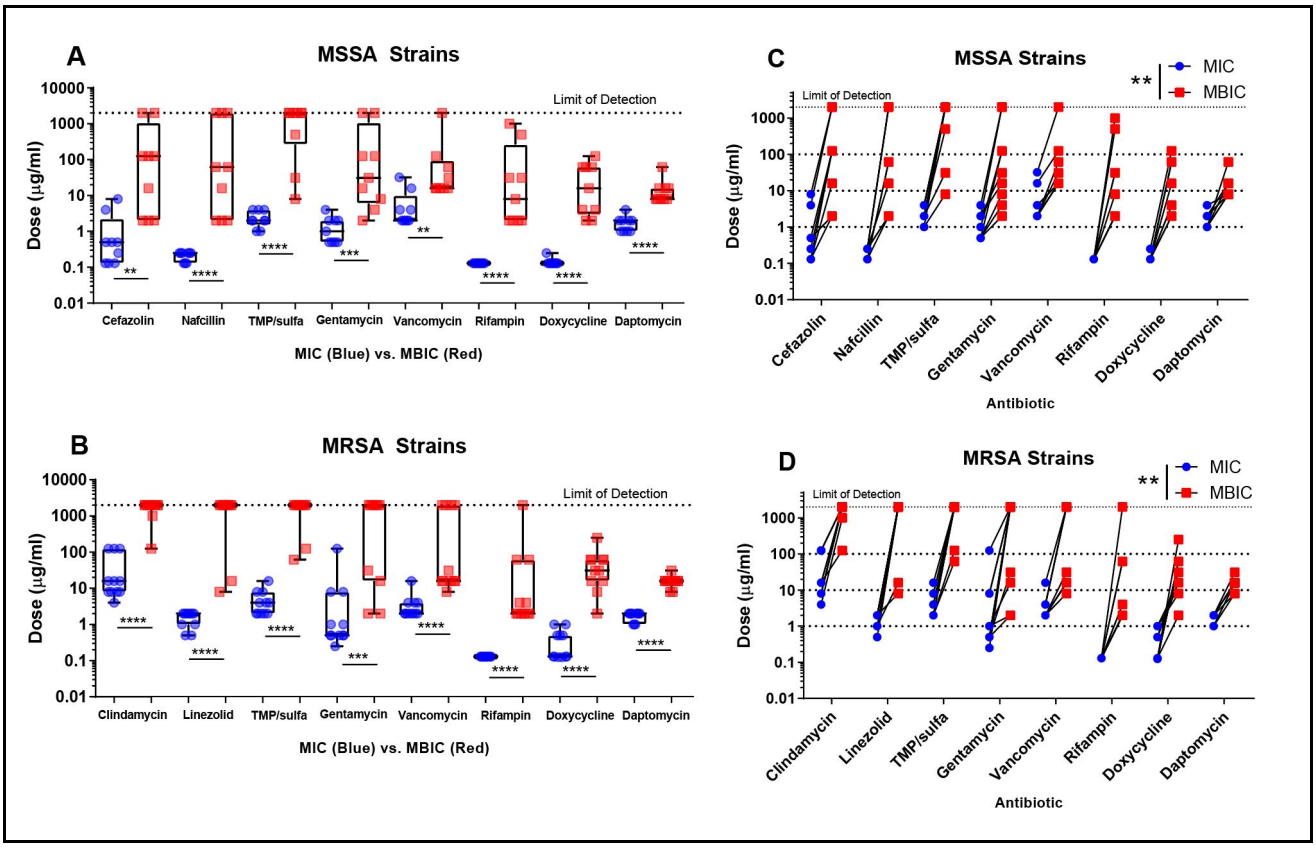

Figure 2. Both MSSA and MRSA biofilms demonstrate decreased sensitivity across all clinically used antibiotics.

MSSA and MRSA biofilms both show less antibiotic sensitivity with mature biofilm MICs (MBICs) significantly higher ($p<0.001$ ****, $p<0.005$*** $p<0.01$ **) than planktonic MIC in between every antibiotic tested using a Mann-Whitney Test (A-B). Matched analysis of *S. aureus* planktonic and biofilm MICs confirms a statistically significant increase ($p<0.01$ **) in MBIC compared to MIC in every strain tested using a Wilcoxon Test (C-D).

5.5.3 Cefazolin Fails to Eliminate MSSA Biofilms and Vancomycin Fails to Eliminate MRSA Biofilms

Planktonic MBC and biofilm MBC (MBBC) of isolates were determined. Cefazolin, nafcillin, clindamycin, linezolid, trimethoprim/sulfamethoxazole, gentamicin, and vancomycin could not eliminate any isolate biofilms up to our limit of detection (Fig 3A and B-red). In all antibiotics tested, there was a significant increase in MBBC compared to planktonic MBC in MSSA (Fig

3A) and MRSA (Fig 3B) isolates. Measurements of biofilm MBC complements MIC observations; rifampin, doxycycline, and daptomycin were best at killing biofilms *in vitro*. 90% of *S. aureus* biofilms could be eliminated by rifampin, 50% by doxycycline, and only 15% by daptomycin. MBBC values of each antibiotic against both MSSA (Fig 3A) and MRSA (Fig 3B) were compared to determine which antibiotic eliminated biofilms most effectively. In MSSA biofilms, rifampin and doxycycline MBBC values were significantly lower than MBBCs of cefazolin, nafcillin, trimethoprim/sulfamethoxazole, gentamicin, and vancomycin (Fig 3C). In MRSA biofilms, only rifampin MBBC values were significantly lower across all antibiotics tested (Fig 3D).

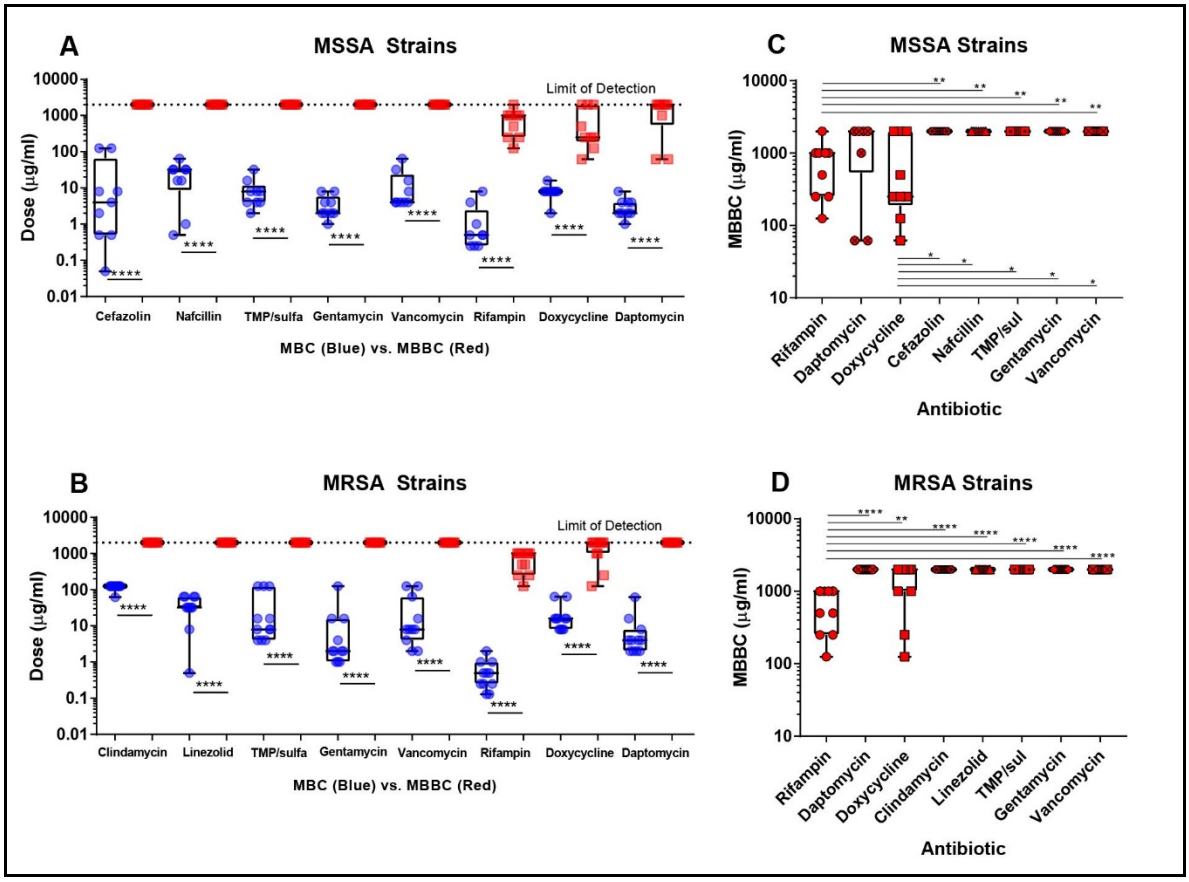

Figure 3. Clinically important antibiotics are unable to effectively kill MSSA and MRSA *S. aureus* biofilms. *S. aureus* planktonic minimum bactericidal concentration (MBC) was compared to minimum biofilm bactericidal concentration (MBBC) for MSSA (A) and MRSA (B). All strains show increased tolerance to all antibiotics tested

in our panel with MBBC values all statistically significantly higher ($p<0.0001$****) than the MBC values (A-B). In MSSA isolate biofilms, the rifampin and doxycycline treatment groups had a significantly lower MBC ($p<0.05$ *, $p<0.0001$ ****) compared to the remaining antibiotics (C). In MRSA isolate biofilms, only the rifampin treatment group had significantly lower MBC ($p<0.0001$ ****) compared to remaining antibiotics (D).

5.6 DISCUSSION

S. aureus is a virulent and extremely challenging pathogen to treat in orthopaedic implant associated infections. The major criterion for antibiotic selection in the treatment of infection is based on susceptibility testing of clinical isolates cultured in a planktonic state[11]. However, in a clinical infection, *S. aureus* primarily exists as established biofilms[14]. Based on this dichotomy, we were interested in determining variations in antibiotic sensitivity between clinical *S. aureus* isolates when cultured as established biofilms compared to the typical planktonic culture. We observed a remarkably large variation in biofilm MIC as compared to planktonic MIC in the clinically important antibiotics cefazolin, vancomycin, and rifampin.

The large decrease in antibiotic sensitivity of *S. aureus* biofilms as compared to planktonic cells was nonspecific across all drugs tested. Rifampin, doxycycline, and daptomycin demonstrated a superior ability to inhibit biofilm growth compared to other antibiotics tested based on relative comparison of MIC. This is in close agreement with other groups showing a clear tolerance to traditional antibiotics by bacterial biofilms[12,15]. Rifampin is the only traditionally administered antibiotic that has shown high and reliable anti-biofilm activity in *S. aureus*[5]. Suppressive doxycycline therapy for a small and high risk PJI cohort showed reasonable effectiveness of the antibiotic for successful treatment[16]. Daptomycin is a more recently developed lipopeptide which disrupts the cellular membrane of bacteria, rapidly lysing cells

independent of metabolic activity. This antibiotic has been shown to be highly effective against a panel of MRSA clinical isolate biofilms [15,17]. To our knowledge, this is the first-time doxycycline has been demonstrated to be as effective as rifampin or daptomycin at killing *S. aureus* mature biofilms from an *in vitro* perspective.

Comparison of antibiotic activity in mature biofilms and planktonic cultures is complicated by intra-strain growth and biofilm characteristic differences. Our observations were limited due to differences in biophysical properties of the secreted extracellular matrix, metabolic output, and the population density within biofilms grown from each individual isolate. Despite these limitations, we clearly show bacterial biofilms remained after treatment with extremely high doses of clinically important antibiotics. This study displays a clear loss of antibiotic activity against bacterial biofilms compared to planktonic cells. Rifampin, the optimal anti-biofilm antibiotic screened, could only effectively kill biofilms *in vitro* at doses not achievable in human patients secondary to overt toxicity.

Established bacterial biofilms have a remarkable tolerance to antibiotics. Our data suggests that antibiotic treatment in *S. aureus* knee and hip PJI and other orthopaedic infections are a critical but incomplete part of treatment. Surgical debridement and the host immune system play vital roles in successful treatment of orthopaedic related biofilm infections. Our results should not be interpreted in the context that standard planktonic antibiotic sensitivity testing should be replaced. Standard CLSI testing for MIC provide invaluable clinical data on genetic antibiotic resistance. This work does suggest that there is a phenotypic and non-specific change in tolerance to antibiotics that occurs between planktonic and biofilm phenotypes of bacteria. Further, it suggests that from an *in vitro* perspective, rifampin and doxycycline may have a

stronger ability to control implant associated infections. Further clinical studies are warranted to confirm these results.

5.7 ACKNOWLEDGEMENTS

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6.0 AIM 2 AND AIM 3 RESULTS

6.1 ELIMINATION OF ANTIBIOTIC RESISTANT SURGICAL IMPLANT BIOFILMS USING AN ENGINEERED CATIONIC AMPHIPATHIC PEPTIDE WLBU2

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6.2 ABSTRACT

Antibiotics are unable to remove biofilms from surgical implants. This high antibiotic tolerance is related to bacterial persisters, a sub-population of bacteria phenotypically tolerant to antibiotics secondary to a reduced metabolic state. WLBU2 is an engineered cationic amphipathic peptide designed to maximize antimicrobial activity with minimal mammalian cell toxicity. The objective of this study was to test the ability of WLBU2 to remove Staphylococcus aureus surgical implant biofilms. WLBU2 effectively treated S. aureus biofilms formed by a variety of clinical MSSA and MRSA strains and created culture-negative implants in the in vitro

biofilm model. Blocking bacterial metabolism by inhibiting oxidative phosphorylation did not affect WLBU2 killing compared to decreased killing by cefazolin. In the surgical implant infection animal model, WLBU2 decreased biofilm mass as compared to control, untreated samples. WLBU2 could rapidly eliminate implants in vitro and had sufficient efficacy in vivo with minimal systemic toxicity.

6.3 INTRODUCTION

Infection remains an enormous clinical challenge in the field of surgery, and greatly increases the risk of morbidity and mortality for the patient. Total knee arthroplasty or knee replacement surgery provides an example of this dilemma. Given its success and cost feasibility, total knee arthroplasty has become one of the largest major surgical procedures by volume in the United States[1-2]. However, infection remains the most serious and costly reason for total knee arthroplasty failure[3-4]. An infected total knee arthroplasty, termed periprosthetic joint infection, is a devastating diagnosis. Treatment options are few and require repeat surgical intervention with long-term antibiotic regimen[5]. Five-year mortality for periprosthetic joint infection is approximately 25%, higher than three of the most common cancers of melanoma, breast, and prostate[6-7]. The most common organism in surgical site infection and periprosthetic joint infection is *Staphylococcus aureus*[8-9]. First-line treatment for these infections include first generation cephalosporins such as cefazolin for methicillin-susceptible strains and vancomycin for methicillin-resistant strains[5].

The poor outcomes with infected surgical implants are a result of the high antibiotic tolerance of biofilms established on the implant[8,10-11]. It has been well established that traditional

antibiotics are unable to eliminate approximately 5–10% of bacterial biofilms[12]. This tolerance is believed to be achieved, in part, through bacterial persisters, a small sub-population of bacteria cells in biofilms, which have a reduced metabolic state[13-16]. This renders the bacteria tolerant to antibiotics, as there is no active metabolic or cell division pathway for the antibiotic to disrupt.

Antimicrobial peptides serve as a potential alternative strategy to traditional antibiotics. Cationic amphipathic peptides (CAPs) selectively bind to bacteria and create pores in both gram-negative and -positive bacterial membranes. Cationic host defense peptides are CAPs that demonstrate the ability to kill bacteria regardless of resistance to antibiotics. However, the use of natural cationic host defense peptides has been limited in the clinic due to suboptimal efficacy and systemic toxicity[17]. Such limitations are indicative of the contextual activity of CAPs, reflective of their evolution as effector molecules of the innate immunity with the ability to prevent infections by specific pathogens interacting with the host in specific environments. As a result, pathophysiological conditions resulting in acidic pH and abnormal salt concentrations may reduce the effectiveness of these CAPs. More importantly, they tend not to work in systemic circulation likely because of the presence of divalent cations and binding of plasma proteins, which restrict their potential use to topical applications. Hence, efforts to develop these CAPs for clinical applications are hampered by the lack of systemic *in vivo* efficacy in animal models.

These limitations motivated the design of synthetic engineered cationic amphipathic peptides (eCAPs), resulting in the extensive characterization of WLBU2 as a lead candidate for potential clinical development. WLBU2 was rationally designed as an idealized helical peptide with optimized amphipathic structure to maximize bacterial membrane selectivity and minimize potential cytotoxicity toward the host[18-19]. To specifically address the limitations of CAPs, we initially demonstrated the broad-spectrum activity of WLBU2 against both gram-positive and -

negative bacteria in the presence of saline and divalent cations. With respect to the failure of CAPs to retain activity in systemic circulation, we first developed an *ex-vivo* bacteremia model indicating the potential systemic efficacy of WLBU2. We showed that WLBU2 subsequently displayed efficacy in a murine model of *P. aeruginosa* sepsis. Thus, unlike naturally occurring CAPs, WLBU2 maintains activity under complex biological conditions[20-21] against common multidrug-resistant (MDR) pathogens, and with minimal toxicity in animal models[22]. WLBU2 has shown activity against planktonic MRSA in addition to a large panel of ESKAPE pathogens[23]. However, despite all these advances compared to overall CAP limitations, the clinical development of WLBU2 would be best justified in the context of the failure of clinically used antibiotics. With the enormous burden of biofilm-associated infections on health care such as medical implants, trauma, and other surgical site infections, more recent studies have been focused on the potential of WLBU2 to either prevent or disrupt bacterial biofilms. Hence, we and others have demonstrated that systemic delivery of WLBU2 is effective against *P. aeruginosa* biofilms associated with cystic fibrosis with minimal toxicity[22,24], but activity against *S. aureus* antibiotic-resistant biofilms has not been shown. More importantly, this novel functional property has not been demonstrated in a translational model that can further advance the clinical development of WLBU2 as a superior therapeutic option to current antibiotic regimens.

We reasoned that if the activity of eCAP WLBU2 was independent of metabolism, it should be able to eliminate antibiotic tolerant biofilms on surgical implants more effectively than traditional antibiotics. The goal of this study was to determine differences in WLBU2 activity against *S. aureus* in planktonic growth state and in biofilms on surgical implant material as

compared to the common clinically used antibiotic, cefazolin, using both *in vitro* and *in vivo* models.

6.4 MATERIALS AND METHODS

6.4.1 Bacterial strains and culture

S. aureus SH1000[25] was used for *in vitro* assays and the *in vivo* animal model. In addition, a series of *S. aureus* clinical strains were used for additional *in vitro* biofilm assays (5 methicillin-resistant strains, 4 methicillin-susceptible strains). All strains were inoculated in Tryptic Soy Broth (TSB, Bectin Dickinson and Company) overnight at 37 °C with shaking at 250 rpm. Strains were diluted in Mueller Hinton Broth (MHB; Bectin Dickinson and Company) to a final concentration of 0.5×10^6 CFU/ml using the 0.5 MacFarland Standard (GFS Chemicals) and an Infinite M200 Spectrophotometer (Tecan). All experiments were performed at least in triplicate at three separate times with freshly inoculated cultures. Institutional Review Board guidelines and regulations were followed for the use of clinically derived *S. aureus* strains.

6.4.2 Minimum inhibitory and bactericidal concentration

The minimum inhibitory concentration (MIC) of cefazolin and WLBU2 for SH1000 in suspension was determined using CLSI assay protocols, incubating freshly plated cultures at 0.5×10^6 CFU/ml for 24 hours with serial dilutions of each antimicrobial and observing inhibition of bacterial growth based on turbidity. Cefazolin concentrations ranged 0.044, 0.088,

0.18, 0.35, and 0.7 μM (0.02, 0.04, 0.08, 0.16, and 0.32 μg/ml). WLBU2 concentrations ranged 0.9, 1.8, 3.7, 7.5, and 15 μM (3.1, 6.2, 12.5, 25, and 50 μg/ml). Both antibiotics were diluted in MHB before addition to SH1000.

The minimum bactericidal concentration (MBC) of cefazolin and WLBU2 for SH1000 in suspension was determined by incubating freshly plated cultures at 0.5×10^6 CFU/ml with antibiotics. Cefazolin concentrations ranged 0.15, 0.35, 0.7, 1.4, and 3.5 μM (0.08, 0.16, 0.32, 0.64, 1.6 μg/ml). WLBU2 concentrations ranged 4.5, 9, 18, 37, 74 μM (15, 31, 62, 125, 250 μg/ml). Well contents were tested at 0, 2, 8, 24, and 48 hours. After treatment, well contents were serial diluted into MHB, and CFU were determined using TSA II with 5% sheep blood CS100 plates that were incubated overnight at 37 °C. The limit of detection was 100 CFU/ml as 10 μl samples of the dilutions were plated. WLBU2 exhaustion assay was performed by subjecting WLBU2 at 10x MIC (250 μg/ml) to increasing inoculation densities to further assess bactericidal activity. SH1000 plated at 0.1, 1, 10, 100, and 1000×10^6CFU/ml in suspension for 30 minutes and quantified by serial dilution on blood agar plates.

6.4.3 *In vitro* biofilm killing assays

Rods were prepared from 0.6 mm diameter stainless steel Kirschner wire (Synthes) and cut into 6 mm length, autoclaved, and plated in wells along with SH1000 and all clinical strains at 1×10^6 CFU/ml. After plating, fresh MHB media was exchanged at 24 hours. At 48 hours, wire with mature biofilms were either placed into fresh media, or treated with either cefazolin at 3.5 μM (1.6 μg/ml) or WLBU2 at 74 μM (250 μg/ml). At 0.5, 1, 6, and 24 hours Kirschner wire were taken from wells, placed into 1% Tween 20 in PBS and sonicated for 10 minutes. Resulting

sonication media was serial diluted into MHB and CFU were determined on blood agar plates. Sonicated rods were sterilely placed in fresh MHB for 72 hours and assessed for visual turbidity.

6.4.4 Persister cell viability assays

SH1000 at 1×10^6 CFU/ml was pre-treated 90 minutes with carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) diluted to 12.5 μg/ml in MHB[16]. Bacterial cultures were centrifuged, and pellet re-suspended in MHB before antibiotic treatment. Cefazolin treatment was at 3.5 μM (1.6 μg/ml), WLBU2 was at 74 μM (250 μg/ml). Percent survival was calculated from baseline bacterial cultures after pretreatment but before antibiotic addition. After 6 hours of treatment, serial diluted drop assays were performed on samples and plated on blood agar plates for CFU analysis.

6.4.5 Viable bacterial biofilm microscopy

SH1000 was plated at 1×10^6 CFU/ml in 8 chambered slides (Lab-Tek), with wells replaced with fresh MHB 24 hours later. After 48 hours biofilms were treated with cefazolin and WLBU2 at 10xMIC (3.5 μM and 74 μM) and placed into 37 °C incubator. After 10 minutes, LIVE/DEAD BacLight Bacterial Viability Kit fluorescent stain (Invitrogen) was added to well contents and incubated at room temperature protected from light for 20 minutes. Fluorescence microscopy performed using a Nikon Eclipse TE2000 microscope with 20x objective, and a Q Imaging RETIGA EXi camera. Images captured and merged using Northern Eclipse software.

6.4.6 Periprosthetic joint infection animal model

All experiments were performed under approved IACUC animal protocol in University of Pittsburgh Division of Laboratory Animal Resources. Twelve-week-old B57BL/6 J female mice (Jackson) were used for all experiments. Mice where anesthetized by 2% isoflurane, hair was removed from leg and treated with betadine. With a scalpel, a medial parapatellar incision was made, and lateral displacement of the quadriceps-patellar complex allowed for visualization of the femoral intercondylar notch. With a 25-gauge needle, the femoral intramedullary canal was manually reamed. A 0.6 mm wide/6 mm long sterile Kirschner wire (Synthes) was inserted into the canal and was left protruding ~1 mm into the joint. The quadriceps-patellar complex was reduced back to midline and incision was closed using sutures. An inoculation volume of 10 µl with 1×10^6 CFU of SH1000 was injected into the joint space. Treatment group mice received either WLBU2 at 0.01 mg/kg-10 mg/kg, cefazolin at 50 mg/kg, or rifampin at 30 mg/kg) starting 24 hours after surgery and inoculation. Mice received antibiotic dose twice a day for three days. Mice were euthanized and Kirschner wire implant as well as a ~2 mm × 2 mm piece of distal femur were placed in 1% Tween 20 on ice. Implants were sonicated for 10 minutes; distal femur was mechanically homogenized for 30 seconds. Samples were serially diluted into MHB and 10 µl drop assays were performed on blood agar plates.

6.4.7 Statistics

When comparing two groups, a two tailed Mann-Whitney test was performed, with $p < 0.05$ considered statistically significant. When comparing multiple groups, a two tailed Kruskal-

Wallis test with Dunn's multiple comparisons post-test was performed, with $p < 0.05$ considered statistically significant.

6.5 RESULTS

6.5.1 WLBU2 has High Efficacy Against Planktonic *S. Aureus*

We first tested the bactericidal effects of WLBU2 with planktonic bacteria. The MIC of cefazolin was found to be 0.37 ± 0.1 μM (0.17 ± 0.05 μg/ml), and that of WLBU2 was 7.85 ± 2.0 μM (26.7 ± 6.7 μg/ml) (Fig. 1A). At a dose of 10xMIC (3.5 μM), cefazolin yielded a three-log reduction of culture after 24 hours (Fig. 1B). Checkerboard assay demonstrated that WLBU2 activity was not augmented by addition of cefazolin. WLBU2 treated cultures to below the limit of detection for CFUs within 2 hours at all WLBU2 concentrations tested (Fig. 1C). To observe WLBU2 dose response, experiments were repeated at shorter time points of less than 60 minutes. A three-log reduction in culture occurred within 1 minute at 10xMIC (74 μM) of WLBU2 (Fig. 1D).

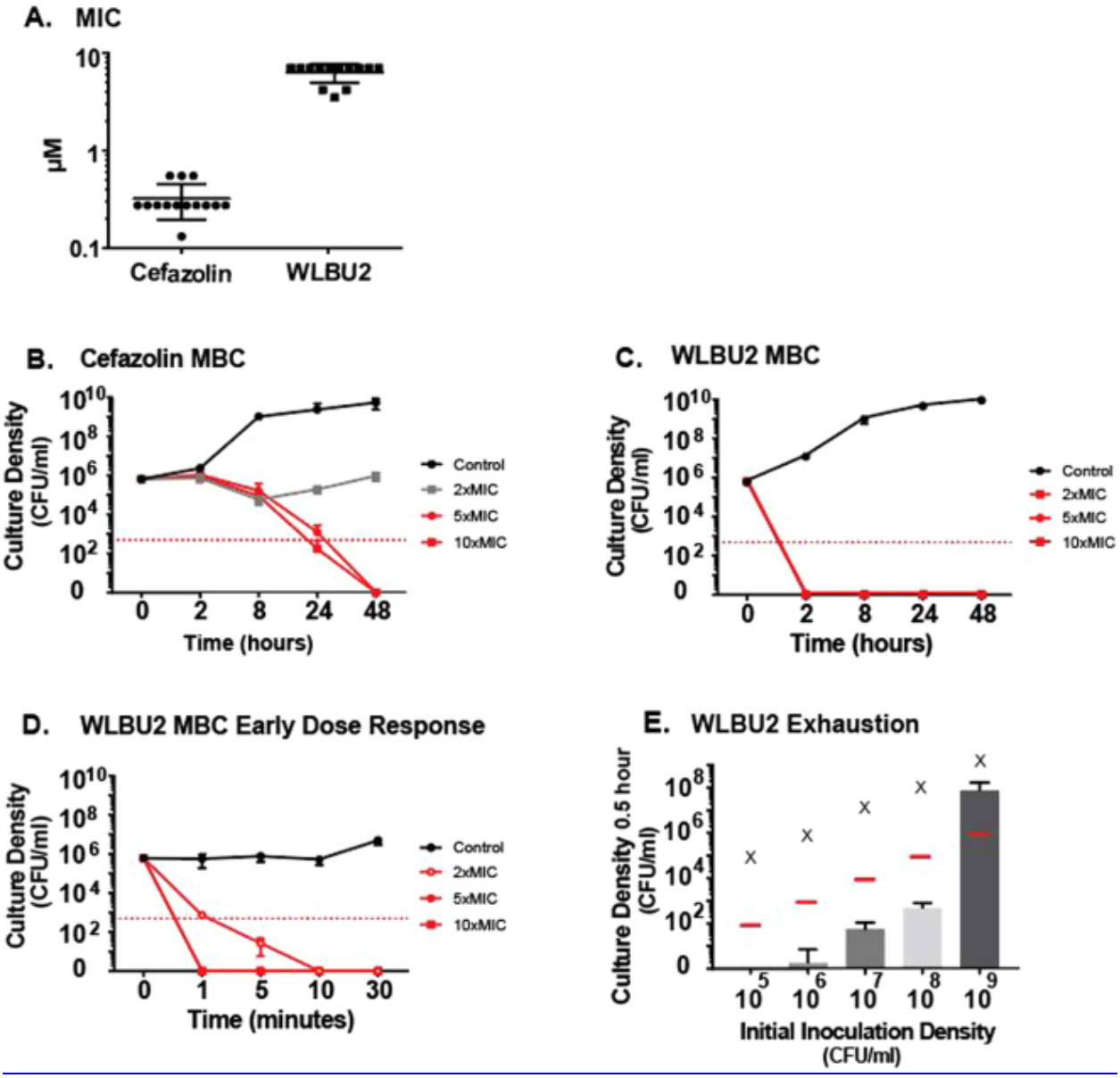

Figure 4. Antimicrobial activity of cefazolin and WLBU2 against planktonic methicillin-sensitive *S. aureus* (SH1000).

(A) Minimum inhibitory concentration (MIC) of cefazolin and WLBU2 determined by serial dilutions of antibiotics added to SH1000 plated at 0.5×10^6 CFU and overnight culture turbidity. (B) Minimum bactericidal concentration (MBC) of cefazolin determined by CFU drop assays at select time points after addition of antibiotic, red dashed line represents 99.9% drop in live bacteria. (C) Initial attempt at WLBU2 MBC quantification based on cefazolin temporal progression, antimicrobial peptide yielded sterile conditions. (D) CFU analysis on WLBU2 treated samples within 30 minutes after treatment, showing dose response of killing. (E) 10xMIC of WLBU2 added to log fold dilutions of SH1000 including overnight stock inoculum (10^9 CFU/ml) and CFU analysis performed after 30 minutes, WLBU2 at this dose kills over 99.9% of SH1000 up to 10^8 CFU/ml.

The bactericidal dose response of WLBU2 at increasing bacterial inoculation densities was next evaluated. We wished to determine WLBU2 antimicrobial efficacy against increasing bacterial burden. WLBU2 at 74 μM (250 μg/ml) to overnight cultures diluted to 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 CFU/ml cultures. Quantitative agar culture (CFU assay) was performed after 30 min of exposure to WLBU2, and revealed a three-log reduction in bacterial colony forming units in all groups except 1×10^9 CFU/ml. At 1×10^9 CFU/ml there was a one log reduction in bacterial density (Fig. 1E).

6.5.2 WLBU2 Eliminates *S. aureus* Implant Biofilms

Mature SH1000 *S. aureus* biofilms were cultured on stainless steel rods (Kirschner wire; K-wire) and treated with 10xMIC cefazolin and WLBU2. At 24 hours, cefazolin did not achieve a three-log reduction while WLBU2 continued to effectively treat biofilms under the limit of detection after 30 minutes (Fig. 2A). These experiments were repeated with WLBU2 at lower doses of 1, 2.5, and 5xMIC. After 24 hours of treatment, CFU assays showed all WLBU2 treated biofilms were under our limit of detection. To further test for complete elimination of biofilms, implant pieces were sterilely re-cultured with fresh MHB for an additional 72 hours and assessed for turbidity. All of the stainless-steel coupons (24/24; 100%) treated with cefazolin were turbid after 24 hours, whereas only 12.5% (3/24) of the coupons treated with WLBU2 for 0.5 hour were turbid. Strikingly, none of the stainless-steel coupons (0/24; 0%) treated with WLBU2 for 24 hours were turbid, and medium remained clear indicating no viable bacteria were present (Fig. 2B). Clear cultures corresponded with quantitative cultures under our limit of detection. WLBU2 eliminated mature implant biofilms on a model strain of *S. aureus*, SH1000.

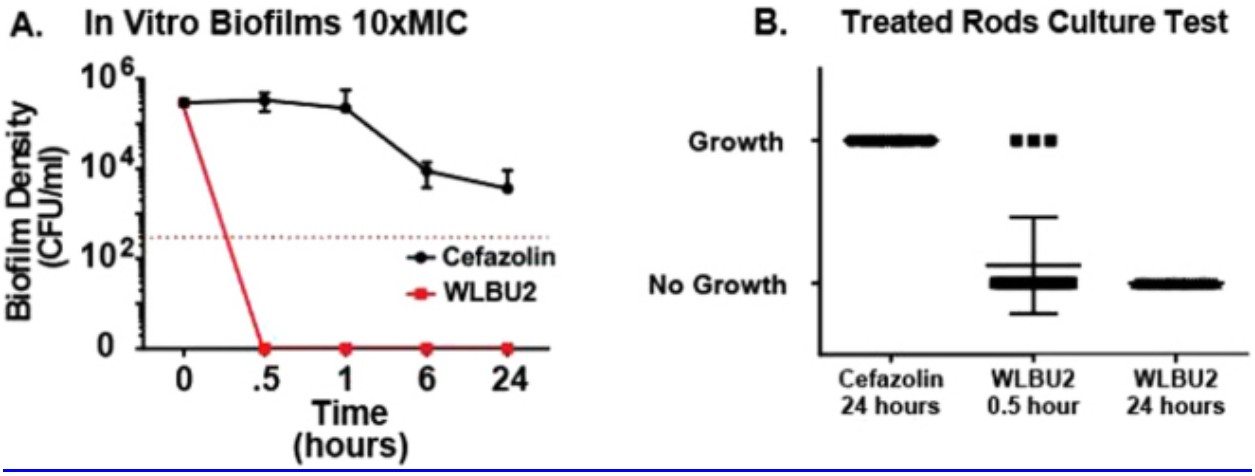

Figure 5. Antimicrobial activity of cefazolin and WLBU2 against *S. aureus* biofilms.

(A) Mature biofilms grown on Kirschner wire treated with cefazolin or WLBU2 at 10xMIC, CFU analysis shows cefazolin failed to clear 99.9% after 24 hours while WLBU2 sterilized Kirschner wire after 30 minutes, red dashed line represents 99.9% drop in live bacteria compared to pretreatment biofilm CFU (**B**) After CFU assay Kirschner wires placed into fresh MHB and turbidity of culture checked every 24 hours for 3 days, sterile cultures seen in 24 hour WLBU2 treated samples.

We next determined whether WLBU2 would demonstrate similar activity against clinical strains of *S aureus*. Biofilms from clinical strains were treated with cefazolin, vancomycin, and WLBU2 at 10xMIC for 24 hours, and then sterility was tested for 72 hours in fresh media. Strains were composed of 5 methicillin-resistant strains and 4 methicillin-susceptible strains. At 24 hours, WLBU2 treated biofilms all showed culture negative tests. Conversely, cefazolin and vancomycin treated clinical strain biofilms all had 100% and 84% positive cultures after 24 hours (Table 1). WLBU2 eliminated MRSA and MSSA clinical strain surgical implant biofilms comparable to SH1000 biofilms.

Table 1. 72-hour culture test from panel of methicillin sensitive and resistant *S. aureus* clinical isolate biofilms

Clinical Isolate	Cefazolin Treated	Vancomycin Treated	WLBU2 Treated
MSSA-1	100% (12/12)	100% (10/10)	0% (0/10)
MSSA-2	100% (9/9)	100% (9/9)	0% (0/9)
MSSA-3	100% (9/9)	100% (9/9)	0% (0/9)
MSSA-4	100% (9/9)	25% (3/12)	0% (0/9)
MRSA-1	100% (9/9)	100% (9/9)	0% (0/9)
MRSA-2	100% (9/9)	100% (9/9)	0% (0/9)
MRSA-3	100% (9/9)	50% (5/10)	0% (0/9)
MRSA-4	100% (9/9)	100% (9/9)	0% (0/9)
MRSA-5	100% (9/9)	100% (9/9)	0% (0/9)

6.5.3 WLBU2 Bactericidal Action is Independent Of Metabolism and Cell Division

To test if the bactericidal capabilities of WLBU2 were dependent on bacterial metabolism, SH1000 was pre-treated with proton-motive-force disrupting agent carbonylcyanide-m-chlorophenylhydrazone (CCCP) before antibiotic was added to decrease metabolism. Serial dilution assays demonstrated that a dose of 12.5 μg/ml of CCCP for 90 minutes suspended growth of SH1000 for 6 hours with minimal loss of viability. After exposure to cefazolin at 10xMIC (3.5 μM) for 6 hours, SH1000 pre-treated with CCCP had a statistically significant

increase in survival compared to cells treated with cefazolin without CCCP with $p < 0.0001$. Pretreatment with CCCP did not alter the bactericidal ability of WLBU2 as compared to untreated controls (Fig. 3A). Not surprisingly, antimicrobial activity of WLBU2 remained unchanged regardless of metabolic activity even after a 30-minute challenge with $p = 0.8867$ (Fig. 3B).

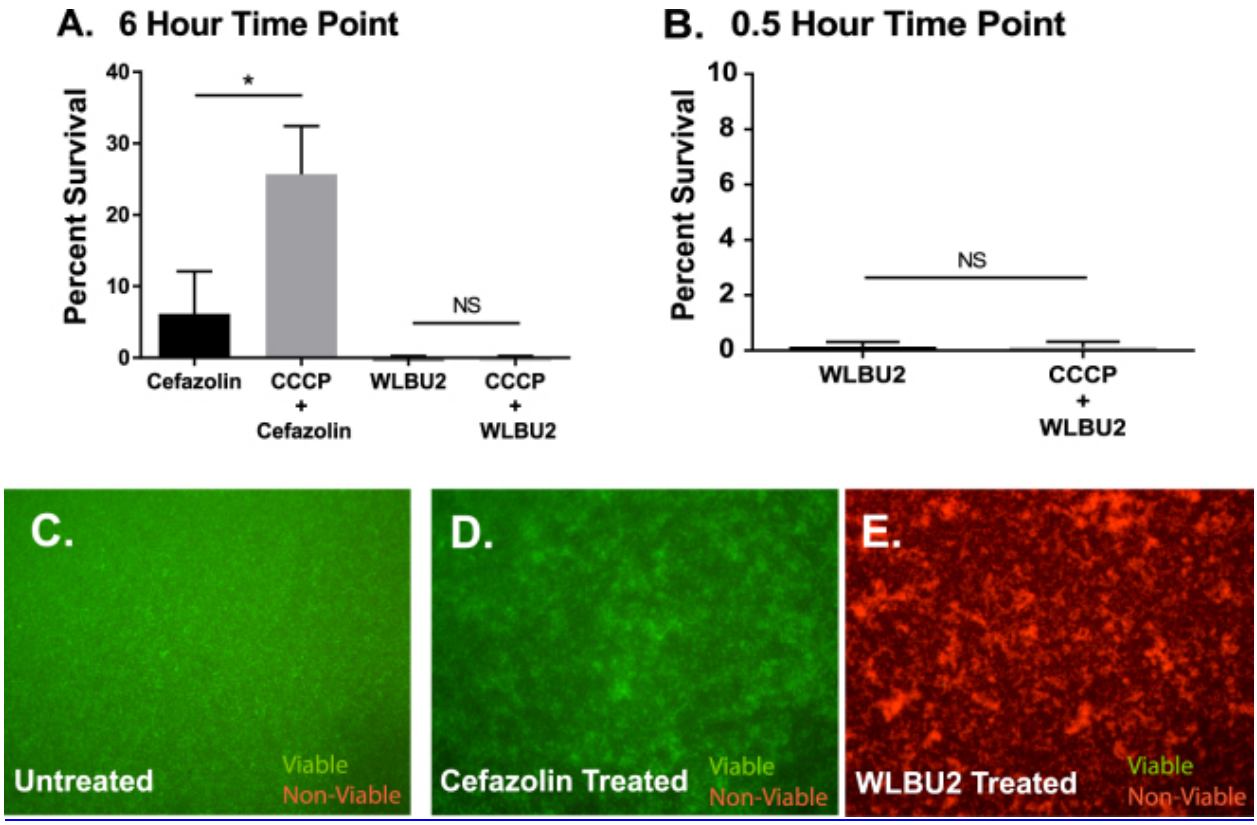

Figure 6. Further evaluation of differences in cefazolin and WLBU2 bactericidal action against SH1000.
(**A**) Planktonic SH1000 treated with cefazolin or WLBU2 at 10xMIC after pretreatment with 12.5 µg/ml CCCP, significant increase in percent survival in cefazolin group but not in WLBU2 group. (**B**) CFU assay performed at earlier 30-minute time point after WLBU2 treatment to pretreated and control showed no difference in bactericidal efficacy. Mature biofilms grown on chamber slides were stained with LIVE/DEAD bacterial viability kit and fluorescent microscopy performed after no treatment (**C**) 30-minute cefazolin treatment (**D**), or 30-minute Cefazolin treated (**E**).

The above experiments indicated that bacteria in biofilms were being efficiently killed by WLBU2; however, it was not clear if WLBU2 was also disrupting the biofilms structure. To analyze biofilms structure following WLBU2 challenge and as a second measure of bacterial viability, fluorescent microscopy with LIVE/DEAD staining was performed. Untreated SH1000 biofilms showed mostly viable cells present in the FITC (green) channel with minimal dead cells present in Cy3 (red) channel (Fig. 3C). Treatment of biofilms with cefazolin at 3.5 μM for 30 minutes showed minimal change from untreated biofilms staining and in biofilms structure (Fig. 3D). Treatment of biofilms with WLBU2 at 74 μM for 30 minutes showed a clear drop in viable signal and increase in dead signal and a clear disruption in the biofilms structure (Fig. 3E).

6.5.4 WLBU2 has Comparable Efficacy to Cefazolin and Rifampin in a Periprosthetic Joint Infection Murine Model

Periprosthetic joint infection was modeled in a mouse by placing an intra-articular K-wire through the medullary canal of the proximal femur followed by intra-articular inoculation with *S. aureus*. Animals were treated systemically with an intraperitoneal delivery of cefazolin, rifampin, or WLBU2. Intraperitoneal delivery was chosen due to technical difficulties associated with intravenous delivery. Implant sonication and proximal femur homogenate were used to quantify viable bacteria. We observed a dose response for WLBU2 in reduction of biofilm CFU burden with doses between 0.01 and 10 mg/kg. (Fig. 4A). Quantitative agar culture of implant biofilms sonicates showed a statistical significant reduction in 0.1–10 mg/kg WLBU2 treated mice compared to untreated control mice. Cefazolin showed a one log reduction in viable bacteria as compared to untreated controls, but this reduction was not statistically significant. Rifampin had a comparable reduction in biofilms sonicate as compared to WLBU2 (Fig. 4A).

Distal femur homogenate of mice showed similar results to paired K-wire implants, with WLBU2 treated samples showing significant reduction compared to untreated controls (Fig. 4B). This was a localized infection model that is not expected to result in life threatening sepsis, therefore no survival study was performed. Due to need for quantification of local tissue around infected implant, histologic analysis of joint was not performed. No significant drop in body weight was observed in mice among treatment groups.

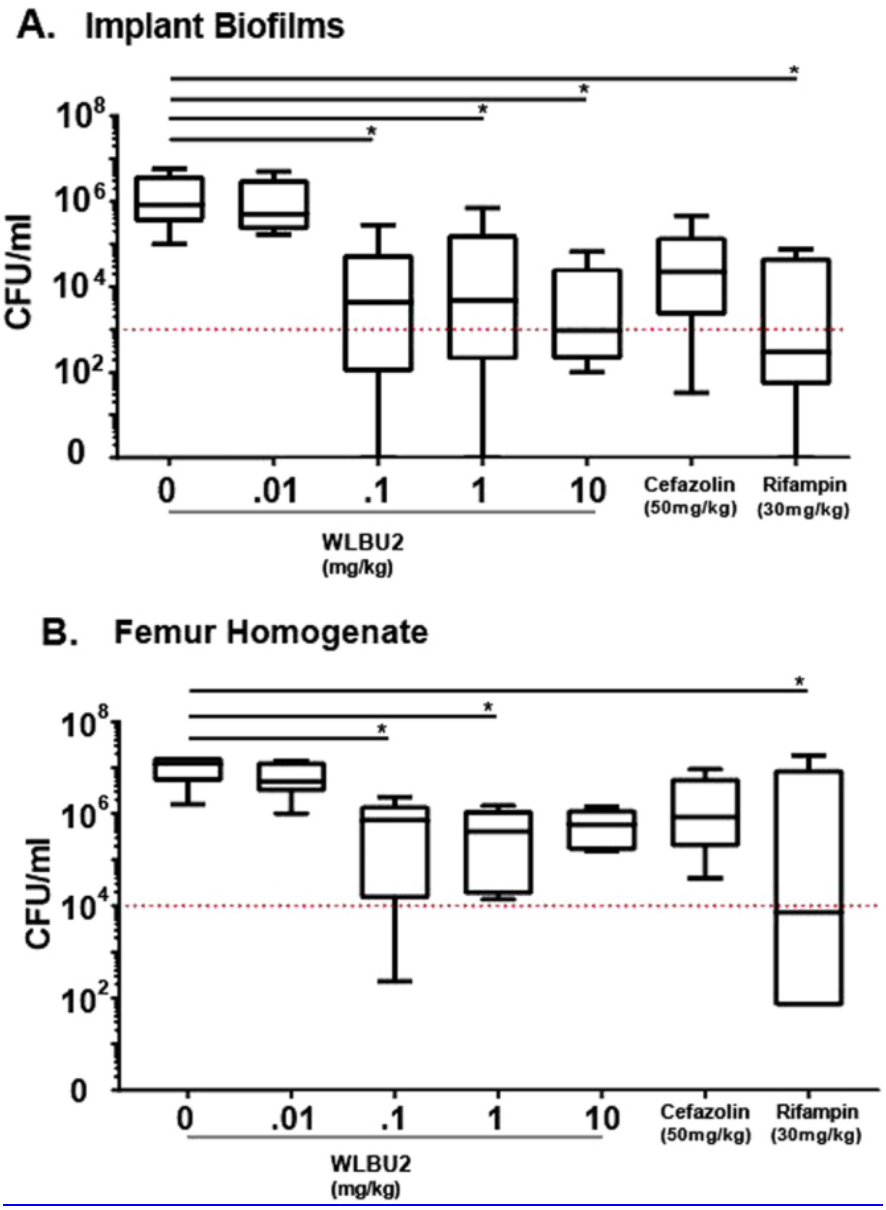

Figure 7. Periprosthetic joint infection (PJI) murine model testing bactericidal efficacy of WLBU2 *in vivo*.
Mice received Kirschner wire implant up femoral canal and 1×10^6 CFUs of SH1000 injected into knee joint. Groups received log increases in WLBU2 intraperitoneally twice a day for 3 days and compared to untreated as well as traditional antibiotic treated groups (cefazolin and rifampin). (A) Kirscher wire implant placed into 1% Tween 20 and sonicated 10 minutes, drop assays on blood agar plates shows significant reduction of implant biofilm in WLBU2 groups (0.1–10 mg/kg), red dashed line represents 99.9% drop in live bacteria compared to untreated. (B) Proximal femur piece cut placed into 1% Tween 20 and homogenized for 60 seconds, shows significant reduction in local bacterial tissue burden in WLBU2 groups, red dashed line represents 99.9% drop in live bacteria compared to untreated

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6.6 DISCUSSION

The high tolerance of biofilms to antibiotics makes it difficult to eliminate medical device infections. In total knee arthroplasty, treatment of chronic infection requires removal of the implant followed by an extended course of antibiotics before a new, final implant can be inserted. In this study, we investigated the activity of the eCAP, WLBU2, against *S. aureus* planktonic and biofilm cells as compared to cefazolin. We demonstrate that in the killing of planktonic *S. aureus* cells, WLBU2 and cefazolin had similar activity, but WLBU2 time to kill was approximately three orders of magnitude faster (WLBU2 5 minutes; cefazolin 2 days). Even for notoriously antimicrobial tolerant *S. aureus* biofilms, WLBU2 maintained its activity, disrupted biofilms, and effectively treated biofilms made by strain SH1000 and clinical strains to under the limit of detection. Cefazolin had a large reduction of exhibited bactericidal activity in biofilms as compared to planktonic cells. The mechanism behind WLBU2 activity appeared to be cell lysis and this activity was independent of metabolism. In a periprosthetic joint infection animal model, WLBU2 maintained a superior level of efficacy as compared to cefazolin and no obvious toxicity.

WLBU2 maintained comparable activity between *S. aureus* planktonic and biofilm cells. This is in sharp contrast to other antibiotics where there is a large loss of activity between biofilms as compared to planktonic cells[12,26-28]. WLBU2 maintained its bactericidal action against SH1000 biofilms, as well as established biofilms of MSSA and MRSA clinical strains after less than 1 hour. There have been few other antimicrobial chemotherapeutic agents that have demonstrated an ability to eliminate persistent biofilms. A novel antibiotic, ADEP, can activate the bacterial protease, ClpP, independent of ATP in bacterial persisters, inducing metabolic activity and allowing for total clearance of infection in combination with a traditional antibiotic, rifampin[29]. Optimal ADEP activity requires the addition of a secondary antibiotic, rifampin. A second approach includes using a chemotherapeutic agents, Mitomycin C and cisplatin. Cisplatin is found to eradicate persister cells in clinical strains of *S. aureus* [30]. It is unclear if the dosing necessary for these chemotherapeutic agents to eradicate biofilms falls within the range of systemic toxicity associated with systemic dosing for oncologic disease.

Bacterial metabolism had no effect on WLBU2 activity. The decreased metabolic activity of bacterial persister cells has been proposed as a mechanism behind biofilms antibiotic tolerance. Chronic infections are facilitated by the survival of dormant persister cells[31]. In *S. aureus*, metabolically dormant stationary bacteria with depleted ATP levels are associated with wide-spectrum antibiotic tolerance[32]. Based on these findings, we reasoned that if the antimicrobial peptide WLBU2 could eliminate *S. aureus* biofilms, then the mechanism was likely independent of metabolic state of the bacterial cells. When CCCP, a chemical inhibitor of oxidative phosphorylation and proton-motive force, was used to decrease metabolic activity in *S. aureus* [16], WLBU2 activity was unchanged as compared to a statistically significant decrease in the activity of cefazolin.

Host defense peptides have two possible limitations that include systemic toxicity and labile activity related to proteases, pH, and ionic strength. These major limitations were tested in the periprosthetic joint infection animal model. Our group did not observe systemic toxicity with WLBU2 in the initial range of therapeutic efficacy. This agrees with previously published data that demonstrate minimal eukaryotic cytotoxicity *in vitro* and *in vivo* [18-22]. Further, WLBU2 had greater efficacy compared to cefazolin with systemic delivery demonstrating the ability to maintain a stable level of activity. Other groups have demonstrated that WLBU2 maintains its activity under diverse physiologic conditions, which supports our results[19-22]. Although WLBU2 demonstrated a high level of efficacy in our animal model, the effect was not as robust as inferred from the *in vitro* results. This attenuation in efficacy suggests that WLBU2 is still inhibited to a limited degree by these or other factors not accounted for in our *in vitro* studies. Changes in bactericidal action of antimicrobial peptides have been shown in other animal models, with peptide bioavailability reduced due to protease activity *in vivo* [17]. This shortcoming can be overcome by carefully designed D-enatiomers of WLBU2 as shown by previous studies of other cationic peptides[33,34].

S. aureus biofilms are highly tolerant of traditional antibiotics making surgical implant infections an enormous clinical challenge. Our data add to growing evidence that the eCAP WLBU2 has high efficacy *in vitro* and *in vivo* with minimal systemic toxicity. WLBU2 could eliminate *S. aureus* biofilms regardless of their methicillin resistance status, which has not been demonstrated by other clinically available chemotherapeutic agents. CCCP treatment experimental results were consistent with WLBU2 activity being independent of bacterial metabolism and cell division, which has not been previously demonstrated with other antimicrobial peptides. WLBU2 shows promise as a novel therapeutic in the treatment of *S.*

aureus infections in the challenging setting of surgical implants such as periprosthetic joint infection. Considering the previously demonstrated antibacterial activity of WLBU2 against diverse multidrug-resistant bacterial strains[23], WLBU2 may offer a novel effective treatment for periprosthetic joint infections involving antibiotic-resistant bacterial biofilms, including those associated with knee replacement surgeries.

6.7 ACKNOWLEDGEMENTS

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6.9 FURTHER OPTIMIZATION OF WLBU2 ANTI-BIOFILM ACTIVITY IN VITRO AND EFFICACY IN VIVO

6.10 INTRODUCTION

Poor outcomes seen in PJI is due to the presence of established *S. aureus* bacterial biofilms. Biofilms are adherent communities of bacteria with a dense extracellular matrix. Additionally, biofilms have higher proportions of "persister" cells which significantly reduce cellular metabolic output. These factors result in biofilms being extremely tolerant to traditional antibiotics, necessitating the search for other anti-biofilm compounds. WLBU2 is an engineered antimicrobial peptide designed to optimize helical structure, maximize antimicrobial efficacy, and minimize mammalian cell toxicity. WLBU2 previously used in our PJI animal model did significantly reduce bacterial burden, but efficacy in vivo was greatly reduced compared to the impressive activity *in vitro*. We have obtained the more recently made D-isomer of WLBU2 (D8), designed to address the labile nature of the peptides in vivo. We also will test efficacy of WLBU2 in a neutropenic mouse model.

6.11 METHODS

Staphylococcus aureus (SH1000) was the model organism. Minimum biofilm bactericidal concentration for WLBU2 was determined in MHB and dPBS. WLBU2 was tested in varying concentrations of human serum and altered pH. Mature biofilm was cultured on Kirschner wire

implant pieces for 48 hours prior to exposure to antibiotic for the in vitro PJI model. Following antibiotic treatment in MHB or dPBS, the K-wires were sonicated and a quantitative culture (Colony forming unit assay; CFU) assay was performed to determine bacterial reduction. The K-wire pieces were then placed into media to determine culture viability in a qualitative fashion. For neutropenic PJI mouse model, twelve-week-old B57BL/6 J female mice (Jackson) were used for all experiments. Mice were rendered neutropenic by two 100 ul intra peritoneal injections of cyclophosphamide (150 mg/kg three days pre-infection and 100 mg/kg one day pre-infection). PJI as previously described was then performed.

6.12 RESULTS

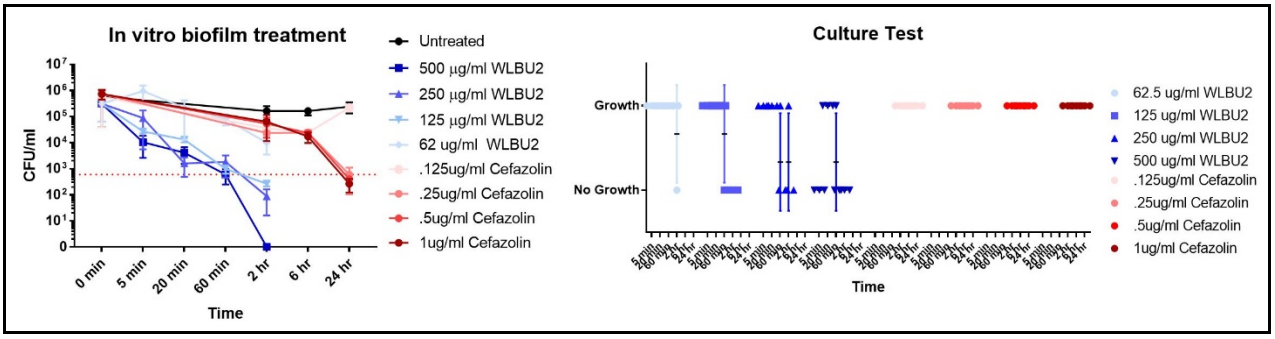

Figure 8. WLBU2 displays better *S. aureus* biofilm killing and implant sterility compared to cefazolin.
SH1000 mature biofilms were grown on stainless steel Kirschner wire implant pieces over 48 hours in MHB. Biofilms were washed with dPBS and placed into MHB with fold dilutions of WLBU2 or cefazolin. Biofilm pieces were treated with WLBU2 for 5-120 minutes, and treated with cefazolin for 2, 6, and 24 hours. Treated biofilms were washed with dPBS, placed into 1% Tween 20 in dPBS sonication solution and sonicated for 10 minutes. Colony forming unit (CFU) quantification on blood agar plates was performed to determine biofilm burden present after treatment (Fig 3A). Implant pieces were additionally placed in fresh MHB overnight and culture turbidity was determined as a check for complete sterilization of the implant material (Fig 3B).

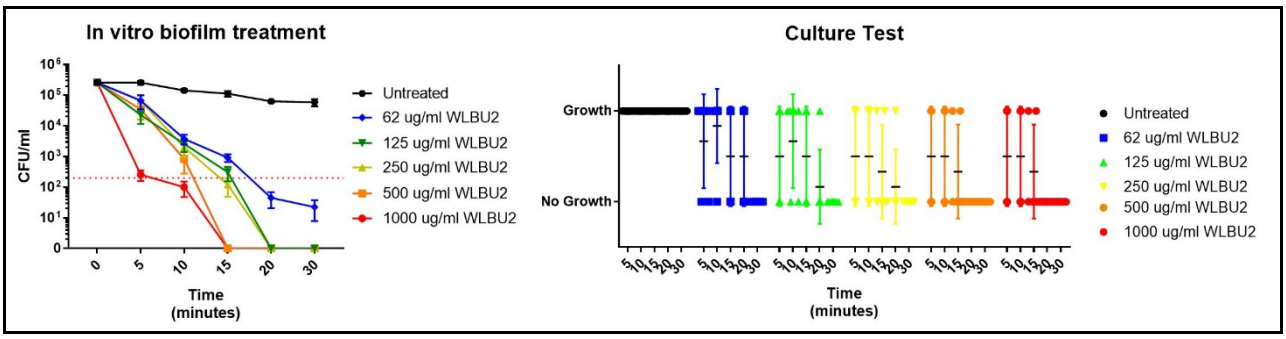

Figure 9. WLBU2 displays enhanced *S. aureus* biofilm killing and implant sterility when treated in buffered dPBS.

SH1000 biofilms were grown on Kirscher wire for 48 hours. Implant pieces were placed in dPBS with fold dilutions of WLBU2. Biofilm implant pieces were treated for at very early time points of 5-30 minutes. Biofilms were washed with dPBS, and processed for CFU analysis. Treatment using WLBU2 in dPBS resulted in increased speed and magnitude of biofilm killing, red line represents 99.9% decrease in untreated biofilm bacterial burden (Fig 4A). Compared to treatment in MHB, treatment in dPBS resulted in increased sterile implant pieces with lower doses of WLBU2 (Fig 4B).

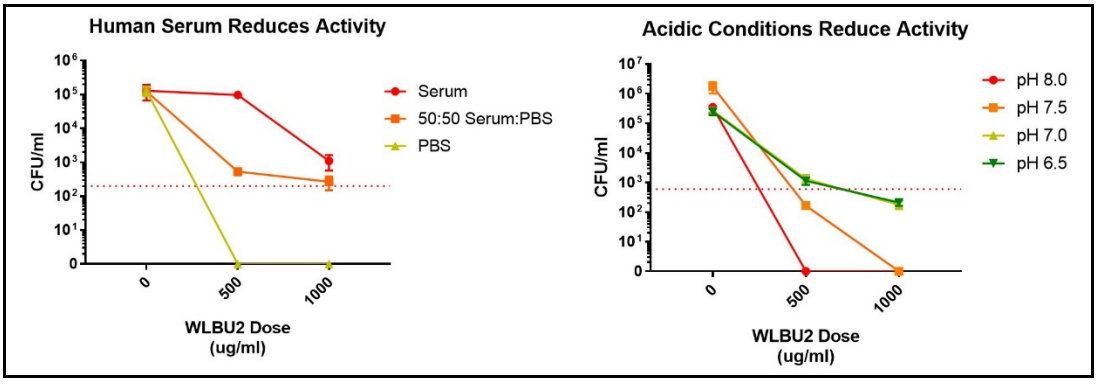

Figure 10. WLBU2 displays decreased *S. aureus* biofilm killing at 10 minutes in dPBS with human serum supplementation and lowered pH.

SH1000 biofilm implant pieces were treated with 500 and 1000 ug/ml WLBU2 in dPBS alone, 50% human serum in dPBS, and 100% human serum for 15 minutes. CFU analysis on sonicated implant material was performed, showing human serum clearly reducing WLBU2 activity in vitro (Fig 5A). Biofilms were similarly treated at WLBU2 doses in dPBS with pH using acetic acid and sodium hydroxide. WLBU2 activity is reduced at lower pH values.

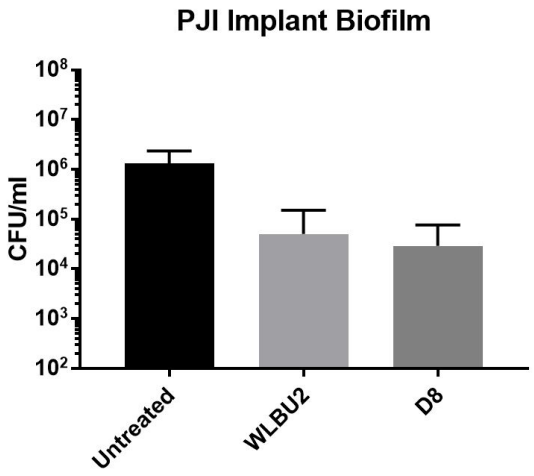

Figure 11. D-enantiomer D8 failed to increase PJI treatment efficacy compared to WLBU2 *in vivo*.

Mice were treated for PJI with either L-enantiomer WLBU2 or D-enantiomer D8 to determine of this would reduce possible protease activity against the compound and increase biofilm clearance. While D8 did reduce bacterial biofilm burden in mice with PJI, this reduction was not significantly different compared to WLBU2.

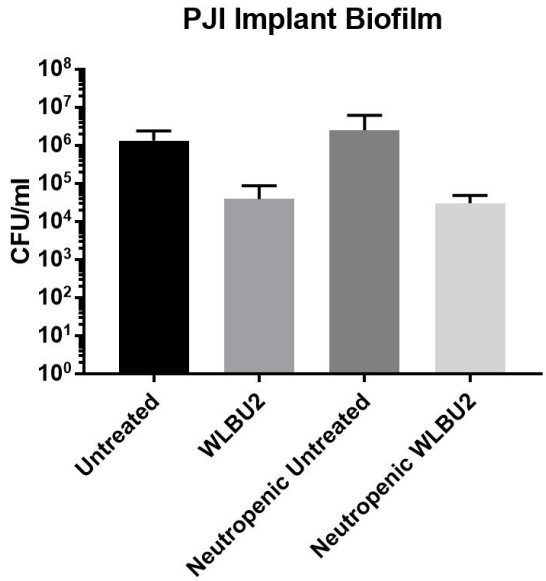

Figure 12. WLBU2 treated PJI effectively in neutropenic mice.

Mice were rendered neutropenic by two 100 ul intra peritoneal injections of cyclophosphamide (200 mg/kg three days pre-infection and 100 mg/kg one day pre-infection). While D8 did reduce bacterial biofilm burden in mice with PJI, this reduction was not significantly different compared to WLBU2.

6.13 CONCLUSIONS

WLBU2 effectively killed and cleared 99.9% of biofilms grown on implant metal material within hours *in vitro*, while it took cefazolin over 24 hours to kill biofilms equivalently. Importantly, all cefazolin treated biofilm implant pieces were culture positive when placed back into fresh MHB while WLBU2 treated biofilm implant pieces were culture negative. WLBU2 anti-biofilm activity magnitude and speed was increased when biofilms were treated in dPBS. Culture negative treated implant pieces were achievable at lower doses of WLBU2 when used in dPBS. Addition of human serum decreased activity of WLBU2 against biofilms. Acidic conditions reduced WLBU2 activity, while alkaline conditions increased WLBU2 activity.

D- enantiomer of WLBU2 (D8) shows efficacy in reducing biofilms in mice with PJI. WLBU2 shows efficacy in reducing biofilms in neutropenic mice with PJI. Our data suggests that WLBU2 best potential would be local application of the peptide to established biofilms. Also, conditions like presence of human serum and acidic pH will be factors which will reduce WLBU2 anti-biofilm activity. Additionally, the D-enantiomer of WLBU2 (D8) showed significant efficacy in treating biofilms *in vivo* compared to untreated controls, it did not show significant improved efficacy *in vivo* compared to WLBU2.

7.0 DISCUSSION

The formation of bacterial biofilms is increasingly recognized as the primary reason for the chronic nature of periprosthetic joint infections despite aggressive antibiotic therapy. To date, there has been little comprehensive characterization of the degree and variability of traditional antibiotic tolerance in PJI *S. aureus* clinical isolate biofilms. Additionally, it is vital to continue to display that the engineered antimicrobial peptide WLBU2 has improved bactericidal activity in the context of the failure of clinically used antibiotics. This is specifically the failure to effectively kill and clear PJI *S. aureus* bacterial biofilms.

Antibiotic tolerance was increased in our PJI *S. aureus* clinical isolates when grown as mature biofilms compared to planktonic cells across all drugs tested. Importantly, many clinically used antibiotics were unable to effectively kill PJI *S. aureus* biofilms including cefazolin and vancomycin. The best traditional antibiotics at killing mature biofilms were rifampin and doxycycline. Alternatively, WLBU2 showed superior killing *S. aureus* biofilms *in vitro*, and was able to kill 100% of clinical isolates tested. Further testing revealed that WLBU2 activity in dPBS increased speed and magnitude of WLBU2 anti-biofilm activity *in vitro.*

In terms of public health significance, understanding how to better kill and clear established bacterial biofilms in PJI is important considering the increasing rate of TKA procedures. Improving PJI treatment success by using antimicrobial peptides could greatly reduce future morbidity and mortality in this patient population. More broadly, bacterial biofilms

are responsible for a significant number of HAIs such as surgical site infections. An effective anti-biofilm compound would undoubtedly greatly reduce morbidity and mortality in this country and potentially globally. WLBU2 and other engineered antimicrobial peptides could serve to expand therapeutic interventions and improve treatment success in treatment of PJI and all chronic HAIs.

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