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Cis-2-Decenoic Acid Interacts with Bacterial Cell Membranes to Potentiate Additive and Synergistic Responses against Biofilm

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Abstract

Musculoskeletal infection is a major risk in all wounds, especially orthopaedic procedures such as implants or bone grafts. Morbidity due to implant-associated infections has increased and the need for therapeutic strategies to prevent them is growing. Biofilm dispersal agent, cis-2-decenoic Acid (C2DA) may be used in conjunction with a variety of antibiotics to potentiate synergistic, additive and antagonistic responses against orthopaedic pathogens. Results demonstrate that C2DA exhibits additive and synergistic effects with tetracycline, amikacin and ceftazidime against both gram positive and gram negative bacteria. Uptake of a fluorescent probe 1-N-phenylnaphthylamine by *S. aureus* was doubled and uptake by *P. aeruginosa* was increased by over eight fold when exposed to varying concentrations of C2DA. Leakage of intracellular ATP also increased up to three times control values with *P. aeruginosa* and over 280 times control value with S. aureus. This suggests that the mechanism of C2DA additive and synergistic effects with antibiotics is in part due to increasing membrane permeability, allowing increased uptake of additional antimicrobials.

Keywords: Biofilm; Synergy; Infection; Antibiotic; Cis-2-decenoic acid

Abbreviation

C2DA: Cis-2-Decenoic Acid; MRSA: Methicillin-Resistant *Staphylococcus Aureus*; UAMS-1: *Staphylococcus Aureus*; PAO1 and PA ATCC 27317: *Pseudomonas Aeruginosa*; TSB: Tryptic Soy Broth; CFUs: Colony Forming Units; PBS: Phosphate Buffered Saline; FICI: Fractional Inhibitory Concentration Index; MBIC: Minimum Biofilm Inhibitory Concentration; NPN: 1-N-Phenylnaphthylamine; ATP: Adenosine Triphosphate; ONPG: O-Nitrophenyl-B-D-Galactopyranoside

Introduction

The need for innovative therapies that prevent and treat implant-associated infections is becoming more urgent as the mortality due to such infections has increased [1,2]. Musculoskeletal infection is a major risk in all wounds, especially open fractures and surgical or orthopedic procedures such as implants or bone grafts, with a rate of infection as high as 17-21% in open fractures [3]. As this rate grows, so does the number of patients who must undergo revision procedures due to implant failure and infection treatment from otherwise successful orthopedic procedures [4]. Additionally, multi-drug resistant biofilm-based infections can increase morbidity and cost of treatment [4-7]. Biofilm formation occurs when microorganisms attach to a surface and excrete a polymeric matrix in which they can enter a state of reduced metabolic activity [6,8,9]. Specifically, orthopedic implant materials may provide advantageous surfaces for the attachment of bacteria and the formation of biofilm [7,10-12], increasing the risk of wound infection. Causing as much as 80% of infection, biofilm limits the activity of antibiotics or immune cell attack, increasing the severity of infection and making it particularly difficult to treat [13]. Many bacteria and fungi thrive in polymicrobial communities because of mutualistic and symbiotic relationships formed to promote their survival, confer antibiotic resistance, and increase virulence [14-17]. This property makes specific antibiotic therapies ineffective in eliminating the infection. If one specific microorganism is eliminated this may leave a niche for other microorganisms to fill. Methicillin-Resistant *Staphylococcus Aureus* (MRSA) and multidrug-resistant *Pseudomonas aeruginosa* are identified among the most predominant infecting pathogens in musculoskeletal infections [18-20]. The clinical impact of biofilm and musculoskeletal infection has prompted a number of strategies to combat the formation and growth of biofilm. Prophylactic antibiotics can be administered to a patient prior to the implantation of a medical device, however the efficacy and associated risk of these methods, including organ damage and increased bacterial resistance to antibiotics, has put them in question [21,22]. Local drug delivery methods through chitosan coatings have proven to eliminate infection, however these coatings must be prepared and fabricated prior to implantation [23]. A fatty acid, cis-2-decenoic Acid (C2DA), has been shown to both disperse bacterial communities as well as inhibit biofilm growth in polymicrobial communities [13,24]. C2DA is a short chain fatty-acid with a polar head and non-polar tail. Discovered as a chemical messenger in the dispersal of *Pseudomonas aeruginosa*, it has been proven to signal the dispersion of mature biofilms composed of both gram-negative and gram-positive bacteria [24-27]. Additionally, there is evidence that C2DA may have additive or synergistic effects against bacterial growth and biofilm formation when paired with different antibiotics [13,25,28].

We hypothesized that C2DA increases membrane permeability,

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therefore antimicrobials that act within the bacterial cell, such as those with protein translation mechanisms, will be more likely to portray synergistic responses in conjunction with C2DA. If this hypothesis is true, C2DA will increase the amount of antimicrobial that crosses the bacterial cell membrane and affects the cell. In continuation, antimicrobials that act at the bacterial cell membrane are likely to portray additive responses in conjunction with C2DA as their effects are simply combined at the surface of the cell membrane.

The aim of this study is to investigate the mechanism of action of a biofilm inhibitor, cis-2-decenoic acid, and determine the if the combinatorial effects of C2DA with various antimicrobials could be predicted based off of the mechanism of action of the antimicrobial. This hypothesis was tested using previously developed membrane permeabilization assays [29,30] and combinations of C2DA with the antimicrobials vancomycin, cefazolin, linezolid, tetracycline, chlorhexidine, ceftazidime, daptomycin, amikacin and ciprofloxacin. These antimicrobials have various mechanisms of action, some that act within the bacterial cell body and some that act at the bacterial membrane [31-36]. Synergistic combinations were determined using checkerboard inhibition assays, and the results were studied for trends between antibiotic mechanism of action and synergy. Synergistic combinations of C2DA and antimicrobials, when coupled with an appropriate local drug delivery device, may be able to reduce the rate of orthopedic infection, reducing healthcare costs and improving patient outcome.

Methods

Growth of bacterial stock

The bacterial cultures used in this study were *Staphylococcus aureus* (UAMS-1) and *Pseudomonas aeruginosa* (PAO1 and PA ATCC 27317), which are common gram-positive and gram-negative orthopaedic pathogens respectively. Before each experiment, bacterial culture was diluted from stock. Overnight growth of cultures in tryptic soy broth (TSB) were diluted to obtain 10^5 colony forming units (CFUs)/mL by diluting 1:50 and 1:200 for *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively.

In vitro biofilm inhibition assays

Synergy studies of different antimicrobials combined with C2DA were performed to determine whether C2DA interacts with different antimicrobials to potentiate additive, synergistic, or even antagonistic responses against biofilm and bacterial growth. Interactions between antimicrobials and C2DA were determined using checkerboard assays using varying concentrations of C2DA and the antimicrobials vancomycin, cefazolin, linezolid, tetracycline, chlorhexidine, ceftazidime, daptomycin, amikacin and ciprofloxacin.

An array of sterile tubes containing 1.75mL TSB, 100µL of varying C2DA dilutions, and 100µL of varying antibiotic dilutions were prepared to obtain test concentrations of antimicrobials. Then 100µl of combined mixture was added to wells of 96 well plates in triplicate in a checkerboard array with increasing concentrations of C2DA along one axis and increasing concentrations of antimicrobial on the other axis. Each well was then inoculated with 50µL of diluted bacterial culture for a total inoculum of 10^3 CFUs/mL. Inoculated and non-inoculated TSB only controls were also included on each plate. Final test concentrations of C2DA were 1000, 500, 250, 125, 62.5, 31.3 and 0 µg/ml. Due to the varying minimum biofilm inhibitory concentrations (MBIC) of the antimicrobials used in this study, the testing concentrations for each antimicrobial had to be individually adjusted to obtain a starting concentration of approximately double the MBIC. The starting antimicrobial concentration, two serial two-fold dilutions and a PBS control were tested for each antimicrobial. After incubating overnight at 37°C, the turbidity of each well was measured at 540nm in a plate reader spectrophotometer (Biotek Synergy H1, Winooski VT) to assess initial planktonic bacterial growth. Planktonic bacteria were then removed from the plates by cautiously aspirating all liquid from each well, while leaving the biofilm on the bottom surface undisturbed. The plates were washed gently 3 times with 150µl Phosphate Buffered Saline (PBS) to remove any remaining planktonic bacteria. The remaining biofilm was heat fixed in an oven at 60°C for an hour. Once fixed, the biofilm was stained with 150µl crystal violet. Crystal violet stain was gently rinsed from the plates with water, removing excess stain that had not been taken up by biofilm, before photographing biofilm growth illuminated with a backlight. Finally, 150µL of de-staining solution, composed of 10% methanol and 7.5% acetic acid in water, was added to each well to dissolve the bound crystal violet before measuring the absorbance of each well in a spectrophotometer at 560 nm. Absorbance was proportional to the amount of biofilm present in each well.

The response of each antimicrobial when combined with C2DA was quantified using the Fractional Inhibitory Concentration Index (FICI). To determine FICI, the ratio of MBIC for the antimicrobial alone/ MBIC in combination with C2DA was added to the ratio of MBIC for C2DA alone/MBIC of C2DA when combined with antimicrobial (Equation 1) [37]. The various MBIC values in these studies were determined as the lowest concentration of antimicrobial to completely inhibit growth of biofilm on the bottom surface of the well plate, as determined by the concentration at which staining values were <10% of positive controls [38]. The effect of synergistic antimicrobials together will be greater than the sum of their parts, causing the FICI to be less than one. Antagonistic combinations will reduce the effectiveness of each antimicrobial and lead to a FICI greater than 2. Those antimicrobials where no effects in improving or reducing effectiveness are considered indifferent and will have FICI values of approximately 2, since MIC concentrations are the same singly or in combination. In accordance with similar studies [39, 40], we considered FICI values below 0.5 to be synergistic responses, values below 1 additive, and values above 2 to be antagonistic. The FICI was calculated separately for each strain of bacteria.

Membrane permeabilization assays

NPN uptake assay: To examine the effects of C2DA as a membrane permeabilizer against common gram-positive and gram-negative pathogens, bacterial membrane permeability was measured using a 1-N-phenylnaphtylamine (NPN) uptake assay [30]. This assay uses hydrophobic probe, NPN, which fluoresces strongly in a phospholipid environment where the membrane is damaged but only weakly in an aqueous environment in the extracellular fluid outside the membrane. In the presence of a membrane permeabilizer, the damaged bacterial membrane will allow more NPN to enter the membrane and become exposed to the phospholipid environment, observed as increased fluorescence. This study used antibiotic polymyxin B as a positive control membrane permeabilizer, as it is

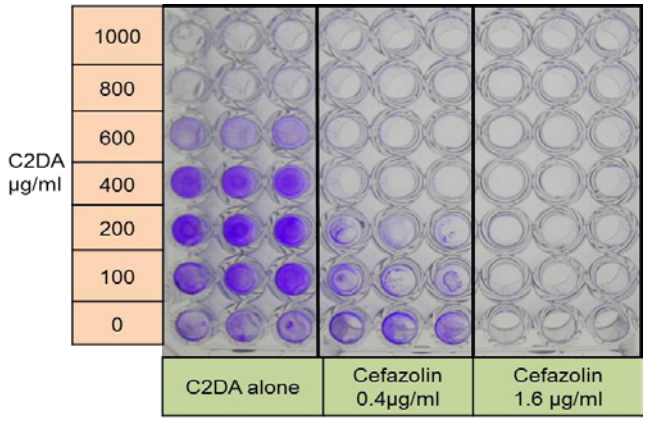

Figure 1: A) Photographic and representation of biofilm formation by clinical isolate of *S. aureus* (UAMS-1) in triplicate wells of microtiter plates in varying concentrations of C2DA with and without the antibiotic cefazolin at inhibitory and sub-inhibitory concentrations.

known to act at by disrupting the bacterial cell membrane [41, 42]. Additionally, the same concentrations of antimicrobials and non-inoculated buffer were used as controls.

PA01 and UAMS-1 bacterial cultures were prepared using previously described methods and then washed 3 times with 5 mM HEPES buffer (pH = 7.4) by spinning down cells at 10,000 rpm for 5 min and re-suspending in buffer. NPN working solution was prepared freshly for each experiment by diluting NPN to a concentration of 0.5mM in acetone. Next, 23µL of HEPES buffer was added to a black 100µL, 96 well plate along with 2µL of NPN solution and increasing concentrations of either C2DA or polymyxin B. The 96 well plate was inoculated with 50µL of either PA01 or UAMS-1 bacterial culture while including the same number of wells with no bacteria added to normalize the NPN fluorescence. Concentrations used for polymyxin B ranged from 0.1µg/mL to 6.4µg/mL using 2X serial dilutions, and 2X dilutions from 15.6-1000 µg/ml for C2DA. PBS and 50% EtOH were used as blank controls for polymyxin B and C2DA, respectively. The permeabilization of membranes was monitored over the course of 30minutes by measuring fluorescence intensity at 460nm with excitation at 355nm (Biotek Synergy H1, Winooski VT). Results are displayed as an NPN uptake ratio, which was calculated as the ratio of highest fluorescence in bacteria and antibiotic samples to the highest fluorescence in bacteria samples in buffer only.

ATP leakage assay: When bacterial membranes become more permeable or when they burst, intracellular contents such as adenosine triphosphate (ATP) will be released into the surrounding medium. Methods to measure ATP in the supernatant after exposure to C2DA were adapted from procedures described by Higgins et al [29]. Overnight cultures of *Staphylococcus aureus* (UAMS-1) and *Pseudomonas aeruginosa* (PA ATCC 27317) were prepared in TSB in 50 ml centrifuge tubes. Bacterial cultures were centrifuged at 3000rpm for 10minutes. TSB was removed and the bacteria were re-suspended in 5mM sterile HEPES buffer at an optical density A600 = 1 for UAMS-1 and A600 = 0.5 for PA ATCC 27317. A 30mg/ml stock of cis-2-decenoic Acid (C2DA) in 50% ethanol was prepared. Two four-fold dilutions of C2DA stock were prepared, including plain 50% ethanol as a non-C2DA control. 1.9ml bacterial suspension and 67µL of the appropriate C2DA test solution were added to 5 ml test tubes (n=3 per group). A control group received 1.9 ml HEPES without bacteria and 67µl of the appropriate test solution. Final C2DA concentrations were 1000, 250, 62.5, and 0µg/ml. The tubes were incubated at 37°C for 30 minutes, then centrifuged at 3000rpm for 5minutes. The supernatant from each tube was plated in triplicate in an opaque white 96 well plate. An equal amount of Cell Titer Glo (Promega, Madison, WI) was added to each sample well. The plate was allowed to sit for 10 minutes at room temperature, then luminescence was read using a Biotek Synergy H1 plate reader.

Statistical analysis

Using SigmaPlot 12.5 (Systat, Chicago, IL, USA), one-way Kruskal-Wallis ANOVA with Dunnett's post-hoc analysis was performed to determine statistical differences between groups and controls for NPN uptake and ATP leakage. Each sample was tested in triplicate.

Results

In vitro biofilm inhibition assays

In brief, tetracycline, linezolid, and chlorhexidine were synergistic against *S. aureus* (Figure 1), while amikacin, ceftazidime, and ciprofloxacin produced synergistic effects against *P. aeruginosa* (Table 1). Vancomycin, daptomycin, cefazolin, amikacin and

Table 1: MBIC values for C2DA and various antibiotics when alone, MBIC values when used in combination, and FICI values for each combination against each strain of bacteria. MBIC for antibiotics not effective against *P. aeruginosa* are marked NE. These antibiotics were not tested for synergy against this strain.

	S. aureus					*P. aeruginosa*				
	MBIC for C2DA (alone)	MBIC for C2DA (combined)	MBIC for antibiotic (alone)	MBIC for antibiotic (combined)	FICI	MBIC for C2DA (alone)	MBIC for C2DA (combined)	MBIC for antibiotic (alone)	MBIC for antibiotic (combined)	FICI
Vancomycin	1000	1000	2	2	1	2000	2000	NE	NA	2
Daptomycin	1000	1000	2	2	1	2000	2000	NE	NA	2
Linezolid	1000	31.25	2	0.5	0.3	2000	2000	NE	NA	2
tetracycline	800	100	1.25	0.31	0.4	1000	250	12.5	6.25	0.75
cefazolin	1000	400	1.6	0.4	0.6	2000	2000	NE	NA	2
chlorhexidine	1000	31.12	6.25	3.13	0.5	1000	31.25	3.13	12.5	4
amikacin	200	50	4	3	1	1000	125	6.25	1.56	0.38
ceftazidime	2000	1000	1	0.5	1	2000	125	4	1	0.31
ciprofloxacin	1000	1000	0.5	0.5	2	2000	31.25	1	0.5	0.5

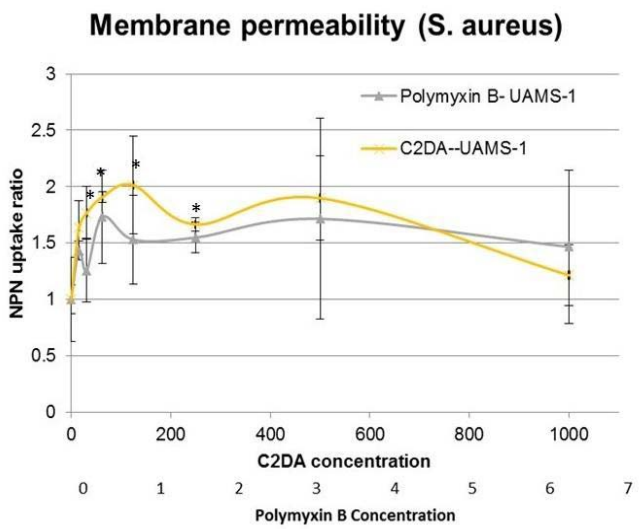

Figure 2: Graph of NPN uptake ratio in UAMS-1 exposed to varying concentrations of C2DA (μg/ml) and Polymyxin B controls. Data is mean ± standard deviation. Asterisks represent statistical differences between C2DA groups and buffer only controls.

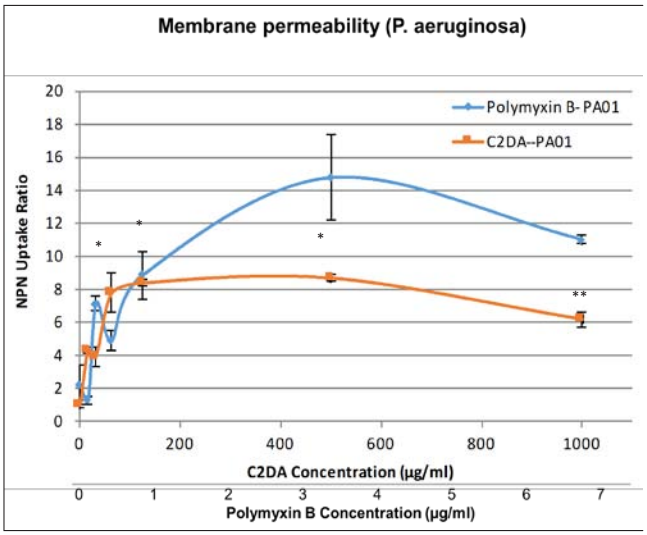

Figure 3: Graph of NPN uptake ratio in PA01 exposed to varying concentrations of C2DA (μg/ml) and Polymyxin B controls. Data is mean ± standard deviation. Asterisks represent statistical differences between C2DA groups and buffer only controls.

ceftazidime were additive with *S. aureus*, while tetracycline was additive with *P. aeruginosa*. Chlorhexidine is the only antimicrobial with a mechanism of action at the cell membrane to produce a synergistic response with C2DA. Additionally, chlorhexidine is the only antimicrobial to potentiate an antagonistic response against bacterial growth, producing a FICI of 4.0 against *P. aeruginosa*. All antibiotics not active against gram-negative microorganisms, produced indifferent interactions with C2DA against gram-negative bacteria.

Membrane permeabilization assays

NPN permeability assays indicated that C2DA potentiated an increase in *S. aureus* outer membrane permeability comparable to that of polymyxin B, with uptake ratios up to two fold higher than controls for concentrations ranging from 62.5-125μg/ml (Figure 2). Concentrations of C2DA from 62.5-500μg/ml led to up to 8times the uptake ratio for *P. aeruginosa* (Figure 3).

Concentrations of C2DA ranging from 31μg/ml to 250 resulted in 100-200 fold changes in ATP concentration in the supernatant from UAMS-1 samples compared to cells with 2.5% ethanol only (Figure 4). Concentrations from 250 to 1000μg/ml increased ATP leakage up to 3-fold in PA ATCC 27317 samples, in a dose-responsive manner.

Discussion

This study first explored the different interactions between C2DA and various antimicrobials to potentiate additive, synergistic or antagonistic responses against biofilm and bacterial growth. We were able to successfully show that different antimicrobials potentiated different responses in conjunction with C2DA. We had also hypothesized that the antimicrobial's mechanism of action can be used as a tool to predict how the antibiotic will respond in conjunction with C2DA. This was shown by a tendency for antimicrobials with mechanisms of action within the bacterial cell

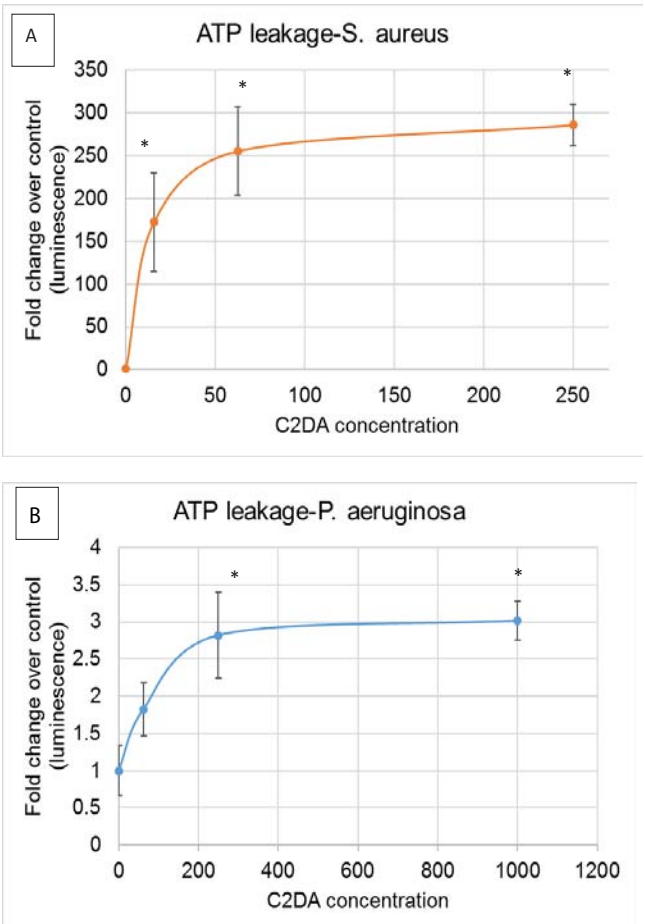

Figure 4: Graphs of ATP leakage vs. C2DA concentration for (A) *S. aureus* and (B) *P. aeruginosa*. Data is expressed as average of fold change over luminescence value for corresponding non-C2DA controls. Data is mean ± standard deviation. Asterisks represent statistical differences between C2DA concentrations and no-additive controls.

to respond synergistically and antimicrobials that act at the cell's surface to respond additively. In continuation, bacterial membrane permeability was studied to validate activity of C2DA as a membrane permeabilizer and resultant ability to allow additional antibiotic to pass through the membrane.

When compared to positive control membrane permeabilizer, polymyxin B, we found that C2DA had a similar membrane permeability curve with relative peak NPN ratio values in an NPN permeabilization assay [30,43,44]. The structure of C2DA may contribute to its mechanism for incorporating into the bacterial cell membrane and increasing membrane permeability. C2DA is a short chain fatty acid with a cis bond, thus creating a large "kink" in the chain. This bent chain structure, along with the molecule's amphipathic properties, may allow interaction with the phospholipid membrane of bacterial cells. It has been proposed that this interaction may be able to permeabilize the cell membrane [45]. Additionally, we found that there was a greater increase in NPN uptake ratio in PAO1 studies compared to UAMS-1. This can be explained because PAO1 is a gram-negative bacteria strain whereas UAMS-1 is a gram-positive bacteria strain, which have double and single membranes respectively. The NPN assay allows for the monitoring of membrane permeability by measuring a relative increase in fluorescence, caused by the NPN molecule interacting with a phospholipid environment. Therefore, a damaged double membrane like that of PAO1 will expose NPN to a greater amount of lipophilic structures and will fluoresce more strongly, as opposed to a single damaged UAMS-1 membrane. Although the NPN membrane permeability assay is designed for outer membrane permeabilization, our results indicate that it could also indicate permeability for gram-positive bacteria, which only have a single membrane. Membrane permeability found with C2DA is similar to some antimicrobial peptides, antibiotics, and antimicrobial materials [46-49]. Membrane permeabilization as measured through leakage of ATP was found to be significantly higher in *S. aureus* than *P. aeruginosa*. ATP leakage also approaches the maximum luminescence value at lower C2DA concentrations when using UAMS-1 than PA ATCC 27317, which is in agreement with previous research showing free fatty acids show greater inhibition of gram positive bacteria [45]. It is likely that the C2DA causes the *S. aureus* cell membrane to lyse at these concentrations, but is unable to lyse gram negative *P. aeruginosa*. Since PA-ATCC has another membrane for the C2DA to lyse, it naturally follows that more C2DA would be needed for complete cell rupture. The results of this study indicate that there is significantly increased permeability of the outer and inner membranes, but that inner membrane permeability only increases by up to a factor of 3 times normal permeability. Further studies may be performed to test inner membrane permeability for gram-negative bacteria such as an ONPG assay, which utilizes o-nitrophenyl-b-D-galactopyranoside as a substrate [50].

Evidence supports our hypothesis that the antimicrobial mechanism of action may be used as a response predictor for interaction between C2DA and antimicrobials. Additive effects are observed when both antimicrobial agents act at the site of the membrane simply by combining their effects to combat bacterial growth. This was observed with antibiotics vancomycin, daptomycin, cefazolin and ceftazidime [31,33,36]. On the other hand, synergistic effects are observed when C2DA allows internally acting antibiotic to cross bacterial membrane and exert maximal effects inside the cell, shown with antibiotics tetracycline, linezolid, ciprofloxacin and amikacin [32,34,35,51,52]. The use of C2DA in conjunction with chosen synergistic antibiotics may provide an advantageous therapeutic approach to combating biofilm. Studies by other groups have also confirmed significant improvements in antibiotic activity when C2DA is combined with traditional antibiotic or antimicrobial molecules [25,28], though the primary mechanism was postulated to be reversion of cells from biofilm to planktonic phenotypes. Other synergistic combinations of antimicrobials have been studied, such as rifampin being used in combination with other antibiotics. Rifampin has been proven to interact synergistically with various antibiotics against common orthopaedic pathogens [53,54]. Yoon et al. found synergistic activity between polymyxin B, imipenem and rifampin, and concluded that imipenem and rifampin worked synergistically in this combination because polymyxin B, a known membrane permeabilizer, allowed more antibiotic to enter the cell, similarly to conclusions on the effect of C2DA in the present study. However, there are no current synergistic combinations of antimicrobials in clinical use that specifically target biofilms. Farnesol, a biofilm quorum sensing chemical messenger, has also been evaluated as a potential antibiofilm agent. Initial studies have shown that farnesol also exhibits synergistic activity with common antibiotics [55-57], however active concentrations of farnesol exhibit cytotoxicity [58] and results against biofilm cells are mixed [56]. D-amino acids have been investigated for biofilm dispersal and inhibition, and in a study by Sanchez et al., while there were indifferent effects of combinations of antibiotics and D-amino acids against planktonic bacteria, additive or synergistic effects of the combination against pre-formed biofilms were observed in a minimum biofilm eradication assay [59]. Other strain-specific biofilm inhibitors such as lysostaphin, hamamelitannin, and others have been shown to increase activity of antibiotics [60-62]. C2DA is advantageous in that this naturally-derived biofilm inhibitor can be produced synthetically or harvested from microorganisms, which reduces some of the biocompatibility concerns and expense of recombinant protein production and isolation.

The outlying antimicrobial in these studies was chlorhexidine as it responded antagonistically with PAO1 but synergistically with UAMS-1. Chlorhexidine has a mechanism of action that acts at the bacterial cell membrane [63], which according to our hypothesis would result in an additive response. Chlorhexidine is a charged molecule and may form a salt with the fatty acid, thereby reducing its bactericidal activity against PAO1, but the mechanism for this response has not been verified. However, it remains unclear as to why it may respond synergistically in the presence of *S. aureus*.

Although we have shown repeatable results, there are limitations to static culture evaluations of anti-biofilm activity. Bioreactor systems with simulated physiological conditions are available and may be beneficial to improve the accuracy of our biofilm inhibition studies. For example, there may be differences in bacterial adhesion to surfaces under shear conditions like those in a physiological environment. However, our model for *in vitro* biofilm is still effective because orthopaedic wounds are typically avascular with little fluid flow. Further studies can expand on synergistic eradication of existing biofilm through MBEC assays, especially since clinically effective removal of existing biofilm from implants or tissue is elusive.

Additionally, our design is limited in this number of pathogens tested. In reality, biofilm can be a polymicrobial community and can be significantly more resistant to antibiotic treatment. Only PAO1 and UAMS-1 were used in individual cultures to serve as an initial study of C2DA synergism against representative pathogenic gram-negative and gram-positive bacteria. These pathogens were chosen because they are most likely to infect implant sites and to form biofilm on an implant surface. Further studies may be conducted to incorporate additional bacterial strains, as well as testing with polymicrobial biofilms. Finally, we have not yet confirmed that synergism or additivism occurs *in vivo* as our studies were performed fully *in vivo*. In order to show evidence of clinical efficacy, we must expand our studies to an *in vivo* model of biofilm inhibition with C2DA and antimicrobials.

Biofilm inhibitors are usually not sufficient to completely inhibit bacteria; antimicrobials must be selected to work with these biofilm-specific molecules for optimal efficacy. This information is important in understanding if the mechanism affects the interaction of antibiotics with C2DA. While it is known that C2DA acts in biofilm dispersal, evidence in this study suggests that this fatty acid, similar in structure to the outer membrane of bacteria, may incorporate into the membrane and increase antimicrobial action, particularly for those antibiotics that have internal mechanisms of action, including amikacin, tetracycline, linezolid, and ciprofloxacin. Synergistic effects could be caused by increased membrane permeability allowing more antibiotic to cross the membrane and exert its effects whereas additive effects may occur when both antimicrobial agents act at the site of the membrane. Incorporating C2DA and specifically chosen antibiotics that act within the bacterial cell into a drug delivery system could prevent biofilm growth and decrease the risk of implant-associated musculoskeletal infection.

Conclusion and Future Work

These studies have shown that synergistic responses between C2DA and antimicrobials can be predicted by the mechanism of action of the antimicrobials. Evidence suggests that C2DA is capable of increasing the permeability of bacterial cell membranes, thus allowing the more antibiotic to cross the cell membrane and exert its effects inside the cells. Particularly those antimicrobials with mechanisms that act within the cell membrane may respond synergistically. Incorporating C2DA and specifically chosen antimicrobials that act within the bacterial cell into a drug delivery system could prevent biofilm growth and decrease the risk of implant-associated musculoskeletal infection.

Ongoing and future studies are being performed to develop local drug delivery systems for the delivery of C2DA in conjunction with antibiotic to the implant surface. This will allow a medical professional to select which antibiotic to include in the local delivery scheme in order to customize the therapy to patient history, implant site or other relevant factors. Ultimately, studies relating to the action of anti-biofilm agent C2DA with various antibiotics are needed to develop a potential clinical therapy, effective in completely inhibiting biofilm growth at an implant surface.

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