Effect of simvastatin on *Staphylococcus aureus* biofilm

An Honors Thesis (BIO 394)

by

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Abstract

*Staphylococcus aureus* biofilm is responsible for two-thirds of infections resulting from surgical implants. Simvastatin is a statin drug used to reduce low-density lipoprotein cholesterol levels, but has been shown to inhibit *S. aureus* invasion into host cells. The use of simvastatin as an inhibitor of *S. aureus* biofilm was investigated, as well as the potential as an adjunct therapeutic drug when used in conjunction with oxacillin. Simvastatin was found to inhibit *S. aureus* biofilm growth at 0.14 mg/ml. Treatment with oxacillin (0.1 µg/ml) and simvastatin (0.035 mg/ml) did not enhance the ability of simvastatin to inhibit biofilm growth. However, it is important to investigate the synergistic effects between simvastatin and oxacillin.

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I would like to thank Dr. Susan McDowell for her guidance in writing my thesis and for her for her never-ending patience and generosity in mentoring me for the past two years.

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Author’s Statement

This project evolved from previous work done by Dr. McDowell, involving the use of simvastatin and *S. aureus* infections, commonly known as staph. Bacterial infections can take the form of biofilm, an adherent colony of bacterial cells that often are resistant to antibiotics and can lead to chronic bacterial infections. In biofilm infections, the treatment process to clear the infection is not only more difficult, but also costly and time consuming. With the emergence of strains of bacteria that develop resistance to certain antibiotics, it is even more important to find alternate ways to treat bacterial infections. For these reasons, it is important that innovations in the treatment of bacterial infections are pursued. The severity of biofilm infections led to the project started with Jacob Henry to test the effectiveness of simvastatin on reducing biofilm growth. He worked hard to establish the conditions in which to culture *S. aureus* biofilm. He established the conditions required for simvastatin to reduce biofilm growth. Lindy Caffo and myself picked up the project. Lindy contributed the doses of simvastatin that were effective at reducing biofilm growth. The work they contributed was very important and made it possible to ask exciting questions about simvastatin’s potential as a drug to help people with biofilm infections, which I pursued when investigating the simvastatin and the antibiotic oxacillin. The following work is the fruit of the hard work of Jacob Henry, Lindy Caffo, myself, and also the never ending patience and ideas of Dr. Susan McDowell.
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Introduction

*Staphylococcus aureus* is a Gram-positive pathogenic bacterium. The bacterium is set apart from other staphylococci by its colony's characteristic golden hue. From the Micrococcaceae family of bacteria, *S. aureus* is found in clusters in the form of coci, sphere shaped bacteria. The bacterium has a cell wall composed in great part of peptidoglycan. *S. aureus'* cell wall contains various surface proteins, some of which are involved in surface adhesion, called microbial surface components recognizing adhesive matrix molecules (MSCRAMM) [1]. *S. aureus'* expression of MSCRAMMs plays an important role in it's pathogenesis through colonization in the human body [2].

*S. aureus* releases several different toxins that cause a variety of diseases. A symptom of infection is inflammation, which is caused by cytotoxins [3]. Other toxins include the exfoliative toxins that, although just produced by approximately 5% of *S. aureus* [4], can cause staphylococcal scalded skin syndrome [1]. Other toxins such as enterotoxins can cause of toxic shock syndrome and food poisoning [5]. Another manifestation of infection is endocarditis, where *S. aureus* is one of the leading causes [6]. Conditions like endocarditis, osteomyilitis, and sepsis can occur as complications after having bacteremia, where the bacteria are found in the bloodstream [7].

People at higher risk for infection with *S. aureus* are those who are already colonized [8]. *S. aureus* is a natural colonizer of the human body, primarily the nasal cavity [9]. However, it can also colonize other places on the human body such as the skin, intestines, and the throat [10]. One study showed that up to 20% of people are permanently colonized with *S. aureus*, while up to 50% are transiently colonized during their lifetime [11]. *S. aureus* bacteria that are carried in the human body naturally are commensal, and do not cause adverse effects on the carrier [10].
However, it is not until the human body's protective barriers of skin and mucus membrane are breached that natural colonization can lead to an infection through soft tissues or the bloodstream [1]. One study found 62% of *S. aureus* bacteremia infections were healthcare acquired [7]. Transient colonization by healthcare providers through contact with an infected individual or through transmission of their own *S. aureus* carriage can spread *S. aureus* to others [1].

Over time, reports have shown an increase in *S. aureus* infections and in the emergence of antibiotic resistant strains, most notably methicillin-resistant *Staphylococcus aureus* (MRSA). In 2005, US hospitals showed MRSA to cause more deaths than other infectious agents such as HIV, or the influenza virus [12], and MRSA has also been shown to be more likely to cause invasive infections [13] than its non-resistant counterpart, MSSA. The presence of MRSA has increased steadily in recent years in hospital settings. This increase has been mirrored by an increase in community associated MRSA [14]. However, MRSA is only one of the multiple *S. aureus* antibiotic resistant strains. Another antibiotic resistant strain is vacomycin-resistant *Staphylococcus aureus* (VRSA), which requires treatment with a higher dose of vacomycin antibiotic to combat infection [15].

Increased disposition in contracting an infection can be caused by a low vitamin A levels [16], obesity [17], and hemodialysis [18]. Persons are also at an increased risk of infection if they naturally carry *S. aureus* [8][17]. Another risk factor lies in invasive surgery, where a medical object is inserted into the body [1]. The presence of objects like catheters [1][18], or prosthetic heart valves [6] increases the risk for *S. aureus* colonization and infection, and is the main cause for bloodstream infections [19].

Colonization of a foreign object by *S. aureus* takes the form of biofilm. Biofilm is a form naturally taken by bacteria that provides protection of the bacteria inside an excreted
extracellular polymeric substance (EPS) and can provide a reservoir for further dissemination [20]. The first stage of biofilm development is called the attachment phase. In the case of an invasive device, such as a catheter, following insertion, the catheter is coated in human matrix proteins for example, fibrinogen [21]. MSCRAMM proteins expressed on the surface of *S. aureus* are able to adhere to the fibrinogen and fibronectin coating the catheter [2]. *S. aureus* attachment to surfaces is also mediated by autolysins [22]. Autolysins are enzyme proteins also expressed on the surface of *S. aureus* that are involved in cell growth through the breakdown of peptidoglycan in the bacterial cell wall [23]. However, they have been found to help in the non-covalent attachment of *S. aureus* to a surface [24].

After the attachment of *S. aureus*, the biofilm begins the maturation phase [25]. The maturation phase of *S. aureus* has been found to be dependent on the excretion of polysaccharide intercellular adhesin (PIA) [26][27]. PIA is a molecule that helps aggregate bacteria through its positive charge that is thought to interact with the negative charge of the teichoic acids found on the surface of *S. aureus* [25][28]. DNA, like PIA, is thought to aid in biofilm formation after being released after autolysis [29].

A mature biofilm has a unique structure that contains fluid filled channels around tower-like structures [25][30]. These channels are thought to be involved in the transport of nutrients to bacteria deeper in the biofilm [30] and to remove metabolic waste [31]. It is believed that *S. aureus* biofilm formation is orchestrated through quorum sensing [25]. Quorum sensing is a form of communication that involves signaling between bacterial cells [32] and in biofilm, involving surfactant peptides to direct biofilm formation [25]. However, details on the formation of biofilm are not well understood, but it is thought that autolysis of bacterial cells also have a role in biofilm formation.
Mature biofilm is maintained through the detachment of single or clusters of cells that causes dispersion of the bacteria [25]. In the case of the catheter, mechanical stress from flow can cause detachment in the biofilm and movement of the bacteria into other areas of the catheter for colonization or into the body. Other causes for detachment also include an over production of surfactant, or enzymes that degrade the EPS. This detachment in biofilm is mediated through the quorum sensing controlled by \textit{agr} [33], which has been shown to down regulate the expression of surface proteins that function in adherence during the initial attachment phase [2]. The \textit{agr} expression in quorum sensing has also been linked to antibiotic resistance [34].

Biofilm related infections pose a significant challenge to healthcare providers, due to the development of antibiotic resistance [31]. Biofilms provide protection to the bacterial cells inside that cannot be overcome by macrophage phagocytosis [35] of the immune system. Additionally, the EPS of biofilms makes the diffusion of some antibiotics difficult, rendering them ineffective at reaching their targets [36]. Cells deep within the biofilm also have a lowered metabolism, which contributes to the ineffectiveness of antibiotics [37].

Treatment of \textit{S. aureus} infections is approached through the drainage of local abscesses and therapy through the administration of varying antibiotics depending on the type of infection. Methicillin-susceptible \textit{Staphylococcus aureus} (MSSA) is treated with semi-synthetic antibiotics such as oxacillin, dicloxacillin, or nafcillin for persons not allergic to penicillin. Soft tissue infections can be treated with linezolid for less severe infections [38]. When infections are catheter related, the tubing can be locked and filled with antibiotics to kill the bacteria, followed by antibiotic therapy for the patient. However, if the infection persists, catheters are removed and replaced [39].
Studies on the effects of combined treatments are important, as different antibiotics could have antagonistic effects or whose synergy could work to the advantage in treatment. One study looked at the combined effectiveness of oxacillin and vancomycin and found they were not antagonistic towards one another when treating MSSA [40]. However, another study found that oxacillin combined with alkyl gallate molecules had a positive synergistic effect against MRSA [41].

Use of vancomycin is reserved for complicated infections that include more severe infections such as severe soft tissue infections, osteomyelitis, bacteremia, people allergic to penicillin, catheter related infections, and MRSA infections [38]. Previous over use of vancomycin has led to the emergence of vancomycin resistant strains of *Staphylococcus aureus* [15]. More importantly, vacomycin resistance has also been reported in MRSA strains, further complicating treatment for infections [42]. In biofilm-associated infections, there is an increase in multiple-drug resistance, adding to the challenge in patient treatment [43].

Due to bacterial ability to develop antibiotic resistance and the ineffectiveness of high doses of antibiotics on biofilm [44], it is important to be proactive in finding treatment therapies that are less dependent on the use of antibiotics. *S. aureus* hospital acquired infections have increased over time, to become one of the most common infectious agents involved in hospital acquired infections [45]. This makes it an important target for alternative therapies. One possible alternative may lie in the use of drugs that are not antimicrobial in nature, but rather are effective in inhibiting bacterial growth.

Simvastatin is a drug used to lower LDL cholesterol levels [46]. Patients taking statin drugs were found to have more favorable outcomes after *S. aureus* bacteremia infection [47]. Additionally, simvastatin was also found to protect against inflammation caused by *S. aureus'* a-
toxin [48]. It was also found that simvastatin inhibits S. aureus invasion of host cells [49]. Due to simvastatin's effects on S. aureus, we set out to investigate its effect on S. aureus growth in the biofilm form. Patients undergoing invasive surgery are at an increased risk for infection [1], therefore, we proposed that simvastatin might function as a preventative treatment during periods of high risk. Additionally, simvastatin was also studied as a potential adjunct therapeutic drug to be used in conjunction with an antibiotic.

Hypothesis

Simvastatin can be used as a preventative treatment for S. aureus infections during periods of high risk by inhibiting bacterial growth, with the potential of being used as an effective adjunct therapeutic drug.

Materials and Methods

Chemicals

Fetal bovine serum, FBS (Atlanta Biologicals, Lawrenceville, GA, S111S0); Staphylococcus aureus (American Type Culture Collection, Manassas, VA, 29213); tryptic soy broth, TSB (22092); ethanol (E7148); 2-propanol (I9516); oxacillin (28221-1G), 0.5% crystal violet (HT901-8Foz) (Sigma, St. Louis, MO); simvastatin (EMD Millipore, Billerica, MA, 567021); XTT (Biotium, Hayward, CA, 100600); phosphate buffered saline, PBS (Life Technologies, Carlsbad, CA, 20012-043); menadione (VWR, Radnor, PA, 95034-382); 37% solution formaldehyde (Fisher, Waltham, MA, BP531-500);.

Simvastatin Biofilm Inhibition Assay

A culture of Staphylococcus aureus was grown in an overnight, shaking incubation (37°C, 225 rpm) for 18-24 h. A 2% FBS in TSB solution was prepared and was used to dilute the overnight bacteria in a 3-fold 1:2 serial dilution. In a 96-well plate (35-3072, BD Biosciences,
San Jose, CA), wells containing 2% FBS/TSB were treated with 95% ethanol or simvastatin (0.14 mg/ml). Bacteria were added to the treated samples. The controls were: diluted stock, 2% FBS/TSB and TSB. After an initial plate reading was taken at 595 nm using the Bio-Rad plate reader 680, the plate was incubated at 37°C at 5% CO₂ for 18 h. After an 18 h incubation, a plate reading was taken at 595 nm. Supernatant containing planktonic bacteria was removed from the sample wells to a separate 96 well plate and 10% formaldehyde was added to fix the remaining biofilm. The biofilm was incubated for 10 min at room temperature. After incubation, the 10% formaldehyde solution was dumped. 0.5% crystal violet in water was added to stain the biofilm and was incubated for 30 min at room temperature. After incubation, the biofilm was washed with PBS, followed by extraction of the crystal violet using 2-propanol for 1 h at room temperature. After the extraction, each well was mixed with a pipette and the well contents were placed in a new 96 well plate. The plate was read at 490 nm in the plate reader with a 5 s mix setting.

Simvastatin Dose Response Assay

A culture of *Staphylococcus aureus* was grown as described. In a 96-well plate, wells containing 2% FBS/TSB were treated with a 5-fold 1:2 serial dilution of simvastatin starting at 0.14 mg/ml. A serial dilution of ethanol was performed as the control. The concentrations of simvastatin used were 0.14 mg/ml, 0.07 mg/ml, 0.035 mg/ml, 0.0175 mg/ml, and 0.00875 mg/ml. The bacteria were added to the wells. The controls were 2% FBS/TSB, overnight culture, and TSB. A plate reading was taken at at 595 nm and the plate incubated at 37°C at 5% CO₂. After an 18 h incubation, a plate reading of the biofilm and planktonic was taken at 595 nm using the Bio-Rad plate reader 680.
Oxacillin Minimum Inhibitory Concentration Assay

A culture of *Staphylococcus aureus* was grown as described in a 96-well plate. Wells containing 2% FBS/TSB were treated with a 5-fold 1:2 serial dilution of oxacillin starting at 0.1 μg/ml. Control wells were treated with a 5-fold 1:2 serial dilution of sterile water. The concentrations of oxacillin used were 0.1 μg/ml, 0.05 μg/ml, 0.025 μg/ml, 0.0125 μg/ml, and 0.00625 μg/ml. The bacteria were added to the wells and biofilm was grown overnight at 37°C, 5% CO₂ for 18 h. A plate reading at 18 h was taken at 595 nm using the Bio-Rad plate reader 680 of the biofilm and planktonic bacteria. Following the 18 h plate reading, the planktonic bacteria were discarded. The biofilm was washed with 1X PBS. 0.01% menadione/XTT was added to the biofilm and incubated for 2-3 h. Supernatant was removed to a new 96-well plate. The color change resulting from metabolic activity was measured using Bio-Rad plate reader 680 at 490 nm.

Oxacillin and Simvastatin Inhibition Assay

A culture of *Staphylococcus aureus* was grown as above. A 2% FBS in TSB solution was prepared and was used to dilute the overnight bacteria in a 3-fold 1:2 serial dilution. In a 96-well plate, wells containing 2% FBS/TSB were treated with the water control, oxacillin at 0.1 μg/ml, simvastatin at 0.035 mg/ml, or both in the same concentration. Bacteria were added to the wells and the biofilm was allowed to grow overnight at 37°C, 5% CO₂ for 18-24 h. Following incubation, the planktonic bacteria were discarded and the biofilm was washed with 1X PBS. 0.01% menadione/XTT was added to the biofilm and incubated for 2-3 h. Supernatant was removed to a new 96-well plate. The color change resulting from metabolic activity was measured using Bio-Rad plate reader 680 at 490 nm.
Statistical Analyses

For analysis of the data, a Student’s t-test was performed to compare 2 treatment groups. A one way analysis of variance followed by a Student Newman-Keuls post hoc analysis was performed to compare 3 or more groups (SigmaStat; Systat Software, Inc., Point Richmond, CA). Groups were considered statistically different at \( p < 0.05 \).

Results

*Staphylococcus aureus* was treated with simvastatin at 0.14 mg/ml or ethanol and grown overnight to form biofilm. Analysis of the planktonic bacteria suspended in the supernatant showed a 56% decrease in biofilm growth in the simvastatin treated group compared to the ethanol control (Fig. 1A). The biofilm also showed a significant decrease in growth of 60%, as indicated by the uptake of crystal violet by the cells in the biofilm (Fig. 1B). An XTT assay was then performed to evaluate the metabolic activity of the biofilm when treated with simvastatin at the same concentration. When treated with simvastatin, *S. aureus* biofilm metabolic activity decreased by 67% when compared to the ethanol control (Fig. 2).

A dose response using simvastatin was done to further investigate lower concentrations with inhibitory capabilities on biofilm and planktonic growth of *S. aureus*. *S. aureus* growth decreased by 42% when pre-treated with simvastatin at 0.14 mg/ml. At 0.07 mg/ml there was a decrease of 38% and at 0.035 mg/ml growth decreased by 17%. Simvastatin at 0.0175 mg/ml and 0.00875 mg/ml did not show significant inhibition of growth.

In order to test if the effectiveness of simvastatin was increased when used in conjunction with oxacillin, a minimally inhibitory concentration of oxacillin was determined with a dose response. Oxacillin inhibited *S. aureus* growth at 0.025 mg/ml by 21% and 30% at 0.05 mg/ml compared to the water control. The concentration of 0.1 \( \mu \)g/ml was used when treating with...
simvastatin to increase the probability of seeing an increase in effectiveness when using both treatments.

Treatment with oxacillin at 0.1 μg/ml showed a 49% decrease in *S. aureus* growth compared to the water control. Simvastatin at 0.035 mg/ml did not show a significant difference when compared to the water control. When *S. aureus* was treated with both oxacillin at 0.01 μg/ml and simvastatin at 0.035 mg/ml, there was a 32% decrease in growth. This was not statistically different than oxacillin alone.

**Figures**

**Figure 1a.**
Figure 1b.

Figure 1. Simvastatin reduces growth of Staphylococcus aureus biofilm. *S. aureus* was pretreated with simvastatin (0.14 mg/ml) or the vehicle control (EtOH) in 2% fetal bovine serum in tryptic soy broth and grown as biofilm in a 96 well-plate (16 h, 37°C, 5% CO₂). Following incubation, planktonic bacteria were removed to a new 96 well-plate and read at 595 nm. 10% formaldehyde was added to fix the biofilm (room temperature, 10 min) and removed. Biofilm was stained with 0.5% crystal violet (room temperature, 30 min) and washed with sterile saline. Crystal violet uptake was extracted using 2-propanol (room temperature, 1 h) and well contents were transferred to a new 96 well-plate. The plate was read at 490 nm and data were analyzed with Student's t-test. Figure 1a shows the planktonic bacteria suspended in the supernatant (*, less than control p ≤ 0.05). Figure 1b shows biofilm (*, less than control p ≤ 0.05).
Figure 2. *Staphylococcus aureus* metabolic activity is reduced by simvastatin. *S. aureus* was pretreated with simvastatin (0.14 mg/ml) or the vehicle control (EtOH) in 2% fetal bovine serum in tryptic soy broth and grown as biofilm in a 96 well-plate (18 h, 37°C, 5% CO₂). Planktonic bacteria were removed after incubation and the remaining biofilm was washed with phosphate buffered saline. Biofilm was incubated in the dark with 0.01% menadione in XTT (37°C at 5% CO₂, 1 h). After incubation, the supernatant was placed into a new 96 well-plate and the color change was measured with a plate reading at 490 nm. Data were analyzed with a Student’s t-test (*, less than control p ≤ 0.05).
**Figure 3.** Simvastatin inhibits *Staphylococcus aureus* growth. *S. aureus* was pretreated with simvastatin at the concentrations indicated or the vehicle control (EtOH) in 2% fetal bovine serum in tryptic soy broth and grown as biofilm in a 96 well-plate (18 h, 37°C, 5% CO₂). After incubation, a plate reading was taken at 595 nm. Data were analyzed with a one-way analysis of variance followed by Student-Newman-Keul post-hoc analysis (*, less than control p ≤ 0.05).
Figure 4. *Staphylococcus aureus* biofilm growth is inhibited by oxacillin at 0.025 μg/ml. *S. aureus* was pretreated with oxacillin at the concentrations indicated or the vehicle control (EtOH) in 2% fetal bovine serum in tryptic soy broth and grown as biofilm in a 96 well-plate (18 h, 37°C, 5% CO₂). Planktonic and biofilm bacteria growth was read using a microplate reader at an absorbance of 595 nm. Data were analyzed with a one-way analysis of variance followed by Student-Newman-Keul post-hoc analysis (*, less than control p ≤ 0.05).
**Figure 5.** Treatment with oxacillin and simvastatin does not further inhibit biofilm growth than oxacillin. *S. aureus* was pretreated with simvastatin (0.035 mg/ml), oxacillin (0.1 μg/ml), simvastatin (0.035 mg/ml) and oxacillin (0.1 μg/ml), or a water control in 2% FBS/TSB and grown as biofilm in a 96 well-plate (18 h, 37°C, 5% CO₂). Planktonic bacteria were discarded and biofilm was washed with phosphate buffered saline. Biofilm was incubated with 0.01% menadione in XTT (2-3 h, 37°C, 5% CO₂). After incubation, the supernatant was placed into a new 96 well-plate and the color change was measured with a plate reading at 490 nm. Data were analyzed with a one-way analysis of variance followed by Student-Newman-Keul post-hoc analysis (*, different than control; +, different than simvastatin p ≤ 0.05).
Discussion

*S. aureus* is a pathogenic bacterium that can form biofilm and is responsible for two-thirds of infections resulting from surgical implants [50]. Biofilm has a tendency to form on invasive devices, such as surgical pins, catheters, or pacemakers, and often leads to difficulty during treatment. The ability of *S. aureus* to colonize as a biofilm, coupled with biofilm’s reduced sensitivity to chemotherapeutic treatments compared to the planktonic, non-adherent form [51], has led to the increase in combination of antibiotics for effective control [52].

However, with increased antibiotic use, the emergence of antibiotic resistant strains, such as MRSA and VRSA, is risked [31][14]. Antibiotic resistant bacterial infections can lead to higher health care related costs, compared to an infection with susceptible bacteria [53]. Bacterial pathogens capable of forming biofilms are also likely to develop multi-drug resistance, making it more difficult to treat infections [43]. Biofilm formation can add to the difficulty of treatment and cost in treatment of the infection [35][36][37]. Evidence that infections stemming from biofilm formation are connected to chronic bacterial infections has been found [43].

Antibiotic alternatives, such as drugs, may be effective therapies against hard to treat biofilm related infection. It is important to investigate alternative treatments that may stem the occurrence of multi-drug resistant bacterial strains and are more effective in preventing chronic infections related to biofilm formation.

Simvastatin is a statin drug developed to lower low-density lipoprotein (LDL) cholesterol levels [46]. Simvastatin inhibits host cell invasion of *S. aureus* [49]. The inhibitory capabilities of simvastatin of *S. aureus* cell invasion has led to the investigation of possible inhibitory effects on *S. aureus* biofilm growth. It was proposed that simvastatin could function as a preventative treatment of *S. aureus* infections by inhibiting biofilm formation during periods of high risk for
infection. In addition, the effectiveness of simvastatin as an adjunct therapeutic drug was investigated by using the drug in conjunction with the antibiotic oxacillin.

We have shown that simvastatin is effective as a pre-treatment to reduce *S. aureus* planktonic growth and biofilm formation. However, our results have also shown that use of an antibiotic in conjunction with simvastatin, does not enhance its effect compared to an antibiotic alone.

To detect whether *S. aureus* growth could be inhibited with simvastatin, *S. aureus* bacteria were pre-treated with simvastatin. Pre-treatment of *S. aureus* with simvastatin at 0.14 mg/ml resulted in the inhibition of planktonic and biofilm growth compared to the ethanol control. A 57% decrease in planktonic bacteria suspended in the supernatant above the biofilm was seen compared to the ethanol control. This finding suggests that pre-treating *S. aureus* with simvastatin is effective at inhibiting *S. aureus* growth in the planktonic form.

To analyze biofilm growth, crystal violet was used to stain *S. aureus* cells in the biofilm. The stain was then extracted as a measure of the amount of bacterial cells present in the biofilm, which corresponded to biofilm growth. Analysis of *S. aureus* biofilm formed after bacteria were pre-treated with simvastatin at 0.14 mg/ml showed growth inhibition compared to the ethanol control. Crystal violet staining of the biofilm showed a 60% inhibition of biofilm formation compared to the ethanol control. The inhibition of biofilm growth treated with simvastatin correlated with the previous findings that simvastatin inhibits planktonic growth. Together, these findings support that simvastatin may be effective as a preventative treatment for biofilm infection. However, crystal violet staining did not distinguish between viable and non-viable bacterial cells.
Further investigation was necessary to assess the effect of simvastatin on the viability of *S. aureus* biofilm. To assess bacterial viability, a menadione/XTT solution was used to measure the metabolic activity in biofilm pre-treated with simvastatin at 0.14 mg/ml. Increased metabolic activity was indicated by a color change that corresponded to increased bacterial viability. Simvastatin treated biofilm showed a 67% decrease in metabolic activity compared to the ethanol control. This decrease in *S. aureus* viability correlates with the previous finding of inhibition of *S. aureus* biofilm formation when treated with simvastatin. After establishing simvastatin as an inhibitor of *S. aureus* growth in both planktonic and biofilm forms, it was important to investigate the range of concentrations where simvastatin treatment produced a significant effect.

To determine the lowest effective dose of simvastatin that inhibited *S. aureus* growth, *S. aureus* were pre-treated with simvastatin at concentrations of 0.14 mg/ml, 0.07 mg/ml, 0.035 mg/ml, 0.0175 mg/ml, and 0.000875 mg/ml. Simvastatin was effective at inhibiting combined biofilm and planktonic growth at 0.14 mg/ml, 0.07 mg/ml and 0.035 mg/ml compared to the ethanol control. Simvastatin at 0.14 mg/ml was used for the previous assays and inhibited combined growth by 42%. These data were comparable to the inhibition of planktonic growth of 56% and biofilm growth of 60% in the previous results. Simvastatin at 0.07 mg/ml inhibited growth to 38% and 0.035 mg/ml to 17%. The lowest effective dose was 0.035 mg/ml and was used in conjunction with oxacillin at an inhibitory concentration to investigate the potential of simvastatin as an adjunct therapeutic drug.

An inhibitory concentration of oxacillin was determined using a dose response. The inhibitory concentration of oxacillin was used with simvastatin to determine if the combination would enhance the effect of simvastatin compared to oxacillin. Oxacillin concentrations of 0.05
μg/ml, 0.025 μg/ml, 0.0125 μg/ml, and 0.00625 μg/ml were used to treat *S. aureus* and biofilm growth was measured with a menadione/XTT solution. The oxacillin concentrations of 0.05 μg/ml and 0.025 μg/ml significantly inhibited *S. aureus* biofilm growth compared to the water vehicle control. At the concentration of 0.05 μg/ml, *S. aureus* biofilm growth decreased by 30% compared to the water vehicle control. When *S. aureus* was treated with oxacillin at 0.025 μg/ml, biofilm growth decreased by 21% compared to the control. However, the concentration of 0.1 μg/ml of oxacillin was used as the inhibitory concentration to better observe the combined effect with simvastatin.

The effectiveness of simvastatin as a possible adjunct therapeutic drug to inhibit biofilm growth was explored when used in conjunction with oxacillin. Biofilm growth was measured using a menadione/XTT solution. Combined pre-treatment of simvastatin and oxacillin at an inhibitory concentration did not result in simvastatin inhibiting biofilm growth greater than when *S. aureus* was pre-treated with oxacillin alone. *S. aureus* treated with oxacillin at 0.1 μg/ml, inhibited biofilm formation by 49% compared to the water control. However, pre-treatment with simvastatin at 0.035 mg/ml did not inhibit biofilm formation compared to the water control. Pre-treatment of *S. aureus* with both oxacillin at 0.1 μg/ml and simvastatin at 0.035 mg/ml resulted in a 32% decrease in biofilm growth compared to the water control. The combined pre-treatment of oxacillin and simvastatin did not result in a statistically significant difference in inhibition of biofilm growth compared to oxacillin treatment. However, the combined oxacillin and simvastatin treatment did decrease biofilm growth compared to simvastatin treatment.

Although both the oxacillin and combined pre-treatment groups significantly inhibited biofilm growth compared to water control, the simvastatin pre-treatment did not. A previous simvastatin dose response showed simvastatin at 0.035 mg/ml to inhibit biofilm growth by 17%
and was the lowest effective dose found to have inhibited biofilm growth. The inhibition at 0.035 mg/ml was low compared to the other two effective doses of 0.07 mg/ml (42%) and 0.14 mg/ml (38%) found earlier in the simvastatin dose response. It is possible that variability in the low replicate number diminished the effect of simvastatin at a concentration of 0.035 mg/ml, which then affected the mean absorbance value for the simvastatin treatment group. Data analysis suggests that the mean for the simvastatin group could have been driven up by a single replicate. The low replicate number (n = 3) could have increased the mean absorbance value for the simvastatin treated group.

Using an antibiotic with simvastatin increases its effectiveness in inhibiting S. aureus biofilm formation. The effectiveness of the combination of oxacillin and simvastatin compared to simvastatin alone decreased biofilm growth by 51%. However, this was probably affected by variability in the mean absorbance of the simvastatin treatment group. When comparing the combined simvastatin and oxacillin treatment to the oxacillin treatment group, we found that there was no difference between the two treatments. Although there was no significant difference when simvastatin was used in combination with oxacillin compared to oxacillin alone, we can further investigate the synergistic effects of simvastatin with oxacillin at a higher concentration. It might be beneficial to use simvastatin at a concentration of 0.14 mg/ml instead of 0.035 mg/ml, which has shown greater inhibition of 60% compared to 17%. It might also be of interest to replicate treatment with simvastatin and oxacillin combined with a higher number of replicates to observe a more accurate inhibitory effect. Other future replicates include the simvastatin dose response assay, to distinguish the planktonic and biofilm growth at the different effective concentrations found.
We have shown simvastatin at the concentrations of 0.14 mg/ml, 0.07 mg/ml, and 0.035 mg/ml to inhibit planktonic and biofilm growth. The results of pre-treatment of *S. aureus* suggest that simvastatin has the potential to act as a preventative treatment to reduce biofilm formation. Simvastatin could perhaps be used to reduce the risk of infection of invasive devices such as pacemakers that could cause a biofilm related infection after surgery.

We have also shown that simvastatin when used in conjunction with oxacillin does not further inhibit biofilm growth compared to treatment with oxacillin. This result suggests that simvastatin with oxacillin at an inhibitory concentration does not enhance the ability of simvastatin to inhibit biofilm formation. These results also point out that there might not be a benefit to using simvastatin with an antibiotic as an adjunctive therapeutic drug. However, it is important to investigate the interactions between simvastatin and oxacillin to shed further light on the possible synergy occurring when used together. Investigation of synergistic effects is important in the possibility that a person on a simvastatin regimen is also treated with oxacillin for an infection.


