Elucidating The Role Of *S. aureus* Proteases In Fibrin Ring Degradation During Sitafloxacin Eradication Of Staphylococcal Abscess Communities

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Introduction: *Staphylococcus aureus*, the primary pathogen in bone infections, possesses unique capabilities to evade antibiotic therapy. For example, *S. aureus* forms abscesses within bone marrow or surrounding soft tissue, known as Staphylococcal abscess communities (SACs), which are recalcitrant to antibiotics and host immunity via a fibrin pseudocapsule that protects encased bacteria[1]. While vancomycin, the standard of care (SOC) antibiotic for methicillin-resistant *S. aureus* bone infections, is ineffective against SACs, previous studies demonstrate that sitafloxacin, a fourth-generation fluoroquinolone clinically used in Japan, can kill *S. aureus* inside SACs in vivo with degradation of the encasing fibrin ring[2]. Sitafloxacin's superior efficacy and ability to degrade the fibrin ring were also reproducible in an in vitro SAC model. However, the underlying mechanism of sitafloxacin-induced fibrin disintegration remains unclear and potentially represents a novel SAC dispersal program. Since *S. aureus* proteases are established mediators of emigration from biofilm communities, we hypothesize that they contribute to sitafloxacin's effects on fibrin ring architecture. To test this, we leveraged our in vitro SAC model to perform longitudinal bulk RNA sequencing of sitafloxacin-treated SACs, and to directly assess the proteolytic activity of identified targets against fibrinogen.

Methods: In vitro SACs were grown using methicillin-sensitive *S. aureus* JAR 06.01.31 as previously described[3]. Briefly, overnight cultures were diluted to ~14 colony-forming units (CFUs) in 25 µL of tryptic soy broth (TSB) and incubated between 2 layers of 1.78 mg/mL polymerized rat tail collagen diluted in TSB in a 48-well plate. Gels were then overlaid with plasma and incubated at 37 °C. After 24 hours, plasma overlying gels with mature SACs was replaced with sitafloxacin or vancomycin at 1,000 times their respective minimum inhibitory concentrations, along with phosphate-buffered saline (PBS) as a vehicle control. SACs were then incubated for 5, 15, or 30 minutes, and RNA was extracted using the Direct-zol RNA Miniprep kit. Libraries were prepared using the Illumina Stranded Total RNA Prep kit and sequenced on an Illumina NovaSeq X to generate paired-end 150-base pair reads, with an average depth of 30 million reads per sample. Adapter sequences were trimmed with fastp, transcript abundances were quantified against the JAR reference transcriptome using Kallisto, and differential expression analysis was performed with the limma-voom pipeline with Benjamini-Hochberg correction (FDR < 0.05). The log 2 counts per million of genes of interest were reanalyzed across different treatment conditions and times by one-way ANOVA with Tukey's multiple comparisons test (n = 3; *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001). Recombinant staphopain A was purified from *E. coli* BL21 as previously described, requiring solubilization of inclusion bodies, Ni-affinity chromatography, and refolding to yield active protease[4]. Activity of plasmin, recombinant staphopain A, and V8 protease against human fibrinogen was semi-quantified by incubating the respective proteases with fibrinogen at 37 °C for 2 hours, and visualizing cleavage products with a Coomassie-stained SDS-PAGE gel. The relative band intensity of the α, β, and γ chains compared to the no-protease negative control was determined using Image Lab software. For quantitative activity, azo-labeled casein was incubated with 2-fold increasing concentrations of recombinant staphopain A for 90 minutes at 37 °C. Undigested azocasein substrate was then precipitated with trichloroacetic acid, and the absorbance of soluble cleavage products was measured at 440 nm. Data were fitted with a logistic regression model.

A. Sitafloxacin vs. PBS (30 min)

Gene	log_2FC	Adjusted p-value	Significance
scpA	1.3	3.76×10^{-6}	****
splD	1.1	1.39×10^{-2}	*
sspA	1.0	2.19×10^{-4}	***
mecA	1.0	3.04×10^{-13}	****
JAR_01510	0.9	6.03×10^{-9}	****
sspB	0.8	5.34×10^{-6}	****
JAR_00456	0.7	3.11×10^{-8}	****
clpX	0.2	9.48×10^{-3}	**
clpP	0.2	1.53×10^{-2}	*
yjbH	0.2	3.56×10^{-2}	*

B. Sitafloxacin vs. Vancomycin (30 min)

Gene	log_2FC	Adjusted p-value	Significance
scpA	1.6	3.66×10^{-7}	****
aur	1.2	4.77×10^{-5}	****
sspA	1.0	1.59×10^{-4}	***
sspB	0.7	1.47×10^{-5}	****
JAR_01510	0.7	3.37×10^{-7}	****
JAR_00456	0.4	1.70×10^{-4}	***
JAR_01579	0.2	3.58×10^{-3}	**
mecA	0.2	2.27×10^{-3}	**
yjbH	−0.3	1.19×10^{-4}	***
tsaB	−0.6	8.52×10^{-4}	***

Figure 1. Sitafloxacin induces *S. aureus* proteases within in vitro SACs.

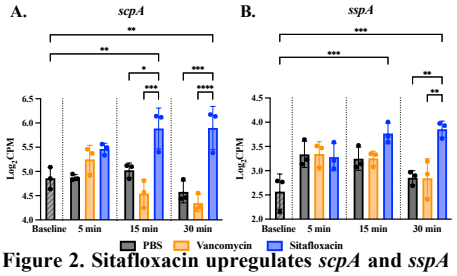
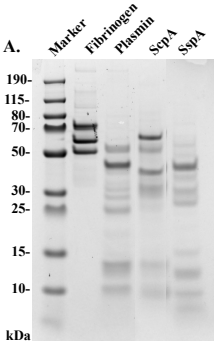


Figure 2. Sitafloxacin upregulates *scpA* and *sspA* in a time-dependent manner within in vitro SACs.

Results: When sitafloxacin-treated SACs were compared to those challenged with PBS (**Fig. 1A**) and vancomycin (**Fig. 1B**) controls at the 30-minute timepoint, several protease-encoding genes were upregulated. Of the genes with at least a log 2-fold-change of 1.0, two proteases had a significant increase in expression with sitafloxacin treatment when compared to both PBS and vancomycin: staphopain A (*scpA*) and V8 protease (*sspA*). Moreover, sitafloxacin upregulated *scpA* (**Fig. 2A**) and *sspA* (**Fig. 2B**) in a time-dependent manner, with a significant increase in expression compared to both controls beginning at 15 and 30 minutes, respectively. To determine the activity of these proteases against human fibrinogen, recombinant ScpA and SspA were incubated with fibrinogen, and the cleavage products were visualized by Coomassie-stained SDS-PAGE. Human plasmin, a host protease known to degrade fibrinogen efficiently, served as a positive control. Both ScpA and SspA displayed activity against all three chains of fibrinogen, with both proteases efficiently cleaving the α chain (0.1% and 1.3%, respectively) (**Fig. 3A-B**). SspA cleaved the β (2.2%) and γ chains (4.9%) to a greater extent than ScpA (68.0% and 31.5%, respectively), and interestingly possessed greater activity against the γ chain (48.1%) than host plasmin. Since we purified recombinant ScpA, which required solubilization and refolding from an insoluble fraction, we sought to further validate the quantitative activity of the protease using an azocasein cleavage assay. Increasing concentrations of ScpA caused a dose-dependent increase in absorbance at 440 nm ($R^2 = 0.9825$, $p = 0.00879$), producing a characteristic logarithmic enzyme response curve (**Fig. 3C**). These findings suggest that sitafloxacin degrades the fibrin ring of SACs through the induction of *scpA* and *sspA*, which encode for proteases that have specific and potent proteolytic activity against human fibrinogen.



B. % Relative Intensity to Fbg

Band	Fbg	Plm	ScpA	SspA
Total	100.0%	15.4%	31.7%	2.7%
α	100.0%	0.3%	0.1%	1.3%
β	100.0%	1.0%	68.0%	2.2%
γ	100.0%	48.1%	31.5%	4.9%

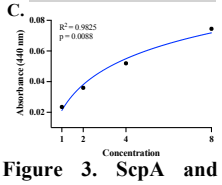


Figure 3. ScpA and SspA efficiently cleave human fibrinogen.

Discussion: Understanding the underlying mechanism of sitafloxacin-mediated fibrin capsule degradation can reveal strategies for improving therapy for orthopaedic infection. Here, we demonstrate that sitafloxacin upregulates *scpA* and *sspA* within in vitro SACs in a time-dependent manner. Moreover, we display that the proteases encoded by these two genes have comparable activity to plasmin against human fibrinogen. Therefore, we are positing that sitafloxacin degrades the fibrin ring of SACs indirectly by inducing ScpA and SspA. This, in turn, compromises the integrity and protective function of the fibrin ring, allowing for penetration of antimicrobial and immune stressors into the SAC core, and thus, killing of encased bacteria. Enhancing ScpA and SspA expression, whether by promoting upstream activators, inhibiting repressors, or delivering exogenous protease to sites of infection, represents a novel approach for dispersing SACs and increasing the efficacy of current SOC antibiotics. In future studies, we will inject in vitro SACs with recombinant ScpA and SspA to determine their sufficiency for fibrin ring cleavage and characterize their influence on the antimicrobial susceptibility of SACs. This will be supplemented with *scpA* and *sspA* deletion mutants, which will illustrate whether these proteases are necessary for sitafloxacin to destabilize the fibrin ring. Additionally, exploring sitafloxacin's effects on the direct upstream regulators of *scpA* and *sspA*, such as the *agr* and *sarA* operons, or alternative stress-response pathways, like the *recA-lexA* axis, could clarify its regulation of protease expression and identify additional targets for therapeutic intervention.

Significance/Clinical Relevance: Defining how sitafloxacin disrupts the fibrin pseudocapsule of SACs by increasing production of *S. aureus* proteases will corroborate their targeting to disperse SACs and enhance bacterial susceptibility to SOC antimicrobials and host immunity.

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References: 1) Cheng *FASEB J* 2009, 2) Ren *Bone Res* 2023, 3) Hofstee *Infect Immun* 2020, 4) Wladyka *Biochem* 2005