Sustained Delivery of Antimicrobials from 3D-Printed CaP Scaffolds in a Single-Stage Revision of Osteomyelitis with Hardware Exchange Abrogates *S. Aureus* Infections and Promotes Bone Formation in Mice

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Background/Objectives

Implant-associated osteomyelitis is a bacterial infection of the bone that arises from orthopedic procedures (i.e. prosthetic joint infection (PJI) and fracture fixation) and is most commonly associated with the pathogen *Staphylococcus aureus*[1]. The socio-economic costs and relapsing nature of this infection are devastating:

- In 2020, there is a projected 65,000 infections to arise from PJI in the United States[2]
- Once infected, 1 in 2 patients will have a future recurrence of infection[3]
- Total annual costs of PJI expected to exceed $1.62 billion[2]

The current standard of care employs antibiotic-impregnated poly(methyl methacrylate) (PMMA) bone cement spacers to provide local delivery of antibiotics to the infection site. However, this gold standard has many shortcomings including the PMMA spacer requirement of additional removal and reconstruction surgery[4] and the inefficient bolus drug release[5], which render the treatment ineffective against *S. aureus*. Compounding these challenges are *S. aureus*' ability to form biofilm and genetic mutations that allow *S. aureus* to transform into an alternative phenotype known as small colony variants (SCV), thus allowing S. aureus to evade host immune responses and clinical regimens of local and systemic antibiotics[6].
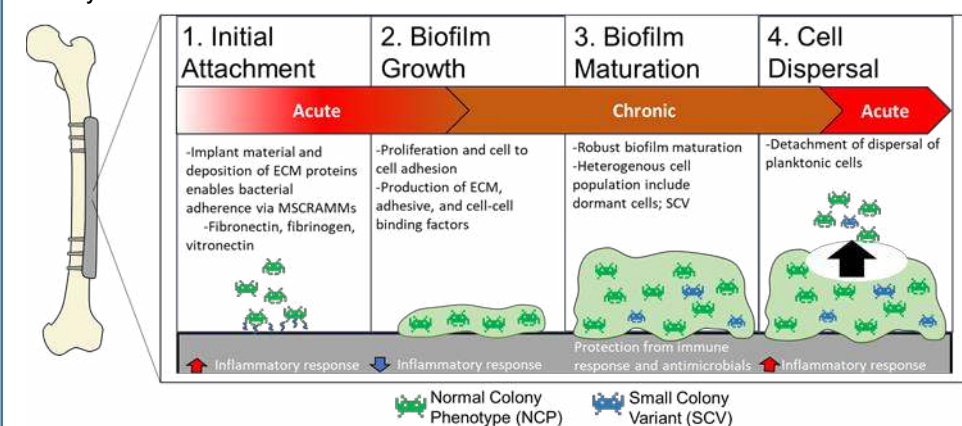

Fig 1. The pathogenesis of *S. aureus* biofilm-related orthopedic infections. Biofilm formation on orthopedic implants (i.e. fixation plate or femoral stem/cup) is a four step process. The formation of biofilm leads to a chronic infection with low inflammation, but dispersal of planktonic bacteria warrants an immune response defining the acute phase of infection. Heterogenous cell population of biofilm contains small colony variants and normal colony phenotypes

Research Objective:

1. To screen a FDA-approved drug library in order to identify new antimicrobials with bactericidal potency against SCV and biofilm
2. Design 3D-printed calcium phosphate scaffold (CaPS) for sustained antimicrobial drugs' release
3. Investigate the efficacy of the antimicrobial-incorporated CaPS in a novel mouse model of femoral implant-associated osteomyelitis

Hypothesis: The osteoconductive CaPS designed for sustained release of incorporated drugs that are bactericidal against both SCV and biofilm will significantly ameliorate outcomes of implant-associated osteomyelitis in comparison to the clinical standard of utilizing antibiotic-impregnated PMMA spacers
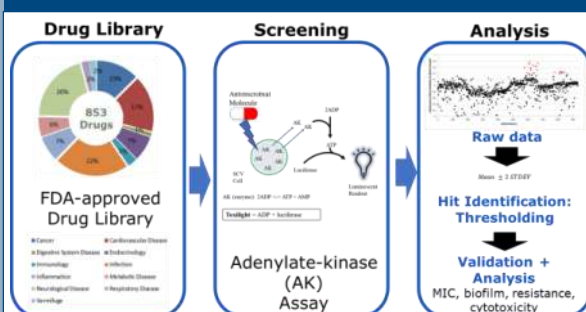
Methods

Fig 2. A FDA-approved drug library consisting of 853 members was screened against a stable strain of SCV (UAMS-1112 ΔhemB) using an adenylate kinase bactericidal reporter[7]. Secondary cell viability assays were employed to validate the bactericidal action of the HTS hits. Identified and validated drugs were then investigated for potency against established biofilm and compared to the effects of the clinical standard, gentamicin. Minimum inhibitory concentrations (MIC) were examined for UAMS-1 and USA300 and therapeutic ratios were determined for each drug using cytotoxicity against human embryonic kidney cells (HEK293T).
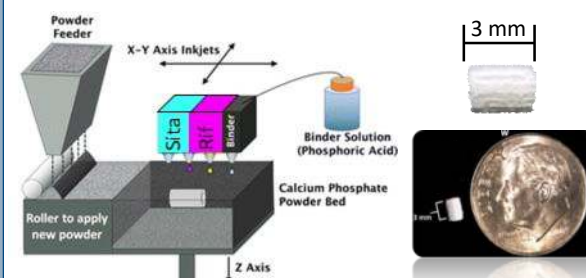
Fig 3. Select drugs were then 3D-printed into a calcium phosphate scaffold (CaPS)[8]. In short, an inkjet based 3D printer (Z Printer 450, Z Corp) was modified to dispense a binder composed of 8.75 wt% phosphoric acid with 0.25 wt% Tween 80 onto a biphasic powder bed consisting of 80 wt% α-tricalcium phosphate and 20 wt% hydroxyapatite. The resulting dissolution-precipitation reaction produced CaP (dicalcium phosphate dihydrate) scaffolds. Select drugs were added to the powder bed at 1 wt% for the incorporation of drug to CaPS. An elution study was performed in PBS and changed daily to characterize release kinetics in vitro with CaPS coated with PLGA (n=5/group)

Fig 4. A titanium screw was contaminated with Xen36, a bioluminescent (BLI) strain of methicillin-susceptible S. aureus (MSSA) and inserted into the mid diaphysis of the right femur of 12-14-week-old Balb/cJ female mice (A). The infection was established for 7 days (B). Then a revision surgery was performed by removing the screw, attaching a PEEK fixation plate with 4 screws, performing a 3 mm ostectomy to debride the infected bone, and then inserting either PMMA cement embedded with gentamicin or a 3D printed CaP scaffold incorporated with sitafloxacin (CaPS-Sita), rifampicin (CaPS-Rif), or both (CaPS-Sita+Rif; n=5-8/group; C-Dc0urage_rootedheart). Mice also received systemic vancomycin (110 mg/kg twice daily subcutaneously) for the remainder of the study post-revision. The infection was monitored for an additional 21 days by bioluminescent (BLI) imaging and μCT were taken directly after the revision and at the end time point. The bone, soft tissue, hardware, and spacers were harvested at the end time point and homogenized or sonicated for CFU counts by enumeration and serial dilutions.
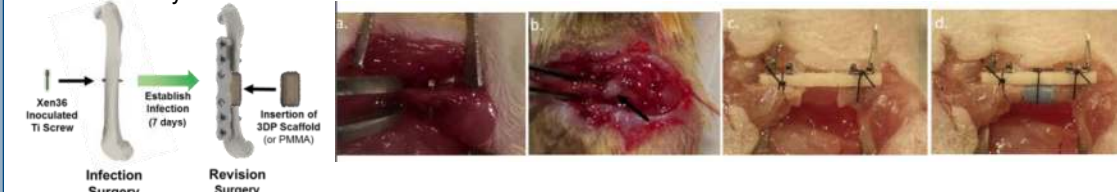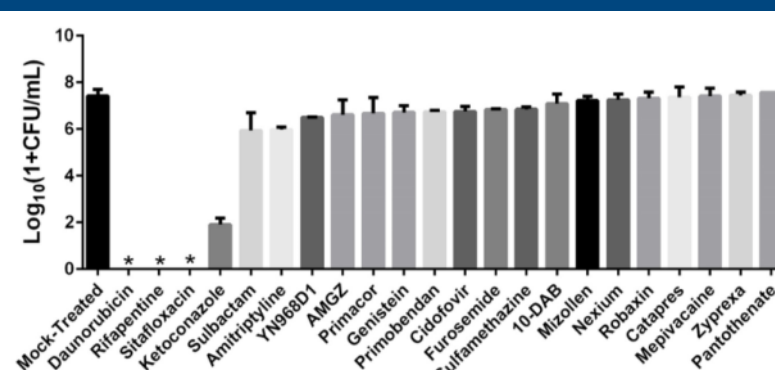

Results

Fig 5. AK HTS hits were validated by spiking SCVs with 100 μM of each identified hit. Of the 22 hits, 4 drugs; daunorubicin, rifapentine, sitafloxacin and ketoconazole showed bactericidal killing of *S. aureus* SCVs, such that daunorubicin, rifapentine, and sitafloxacin came back culture negative. Ketoconazole had a 5.6-log reduction relative to the mock-treated control. Assay was performed in duplicate. * indicates culture-negative
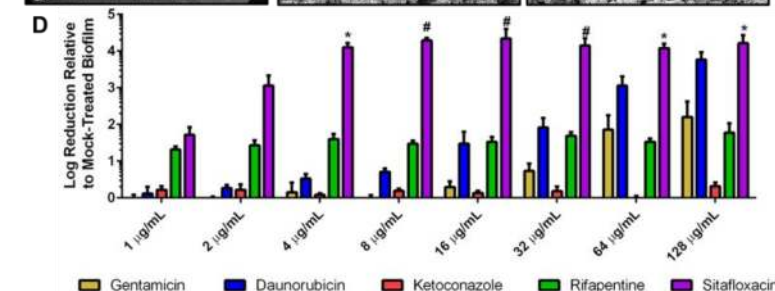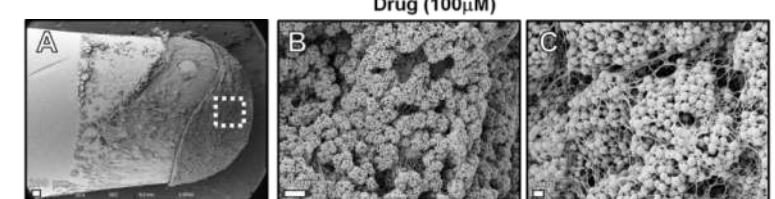
Fig 6. UAMS-1 biofilm was established and representative SEM is shown with magnifications of ×100 (A), ×1000 (B), and ×5000 (C). Biofilm was challenged by 1-128 μg/mL of daunorubicin, ketoconazole, rifapentine, sitafloxacin, and gentamicin (D). Sitafloxacin significantly killed biofilm at concentrations 4-128 μg/mL in comparison to gentamicin at each concentration. * indicates p<0.005; # indicates p<0.001 for the effect of Gentamicin vs. daunorubicin, ketoconazole, rifapentine, or sitafloxacin at each of the respective concentrations by 2-way ANOVA with Sidak's test for post-hoc comparisons. n=3/group. Data presented as mean ± standard deviation.
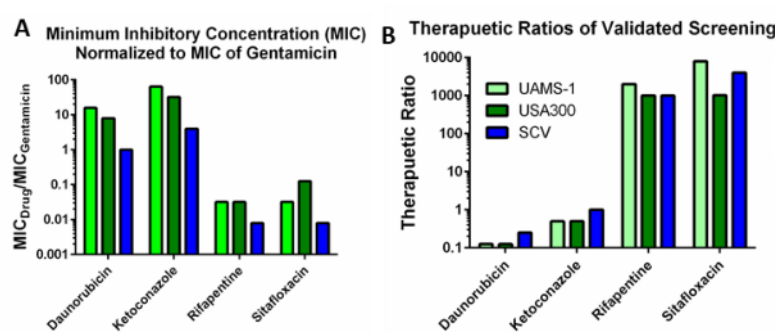
Fig 7. Daunorubicin, ketoconazole, rifapentine, and sitafloxacin were characterized for MIC against various strains of *S. aureus* (UAMS-1; methicillin-susceptible *S. aureus* USA300; methicillin-resistant *S. aureus*, and UAMS-1112; SCV) and normalized to the clinical gold standard; gentamicin (A). Rifapentine and sitafloxacin demonstrated improved MIC relative to gentamicin, daunorubicin, and ketoconazole. Calculated therapeutic ratios* revealed that rifapentine and sitafloxacin were not cytotoxic at effective MICs for each strain (B).
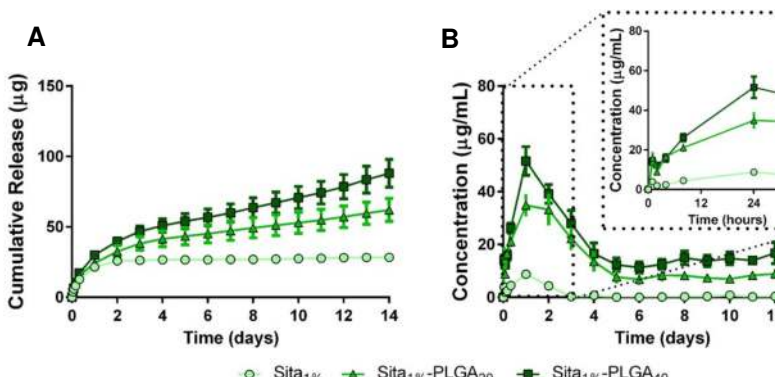
Fig 8 Sitafloxacin and rifampin (data not shown) release profiles were assessed after immersion in PBS for up to 2 weeks (n=5/group). (A-B) Bare scaffolds incorporated with sitafloxacin (Sita[1%]) demonstrated a bolus release within the first 12 h succeeded by no measurable drug concentration by day 3. Coating CaPS with PLGA containing either 20 mg/mL (Sita[1%]-PLGA[20]) or 40 mg/mL

(Sita[1%]-PLGA[40]) of drug enabled a biphasic release profile. Initial burst peaking in concentration at 24 h followed by sustained release exceeding 2 weeks. Drug eluted PBS samples were taken at day 14 to qualitatively and quantitatively measure bioactivity after 2 weeks elution (data not shown). Eluate was adsorbed overnight by filter paper disks and then placed on a bacterial lawn of Xen36. All PLGA coated groups had a distinct zone of inhibition while bare scaffolds had no observed zones.
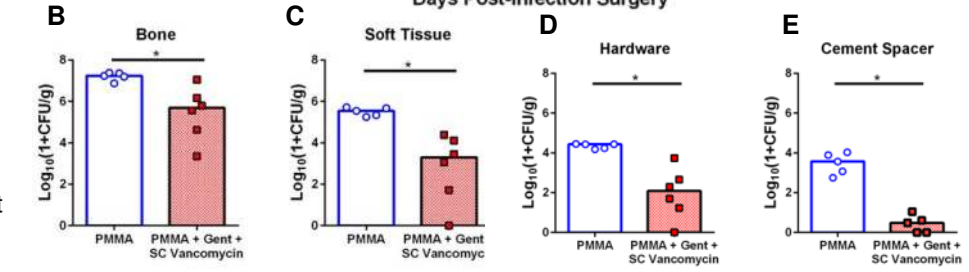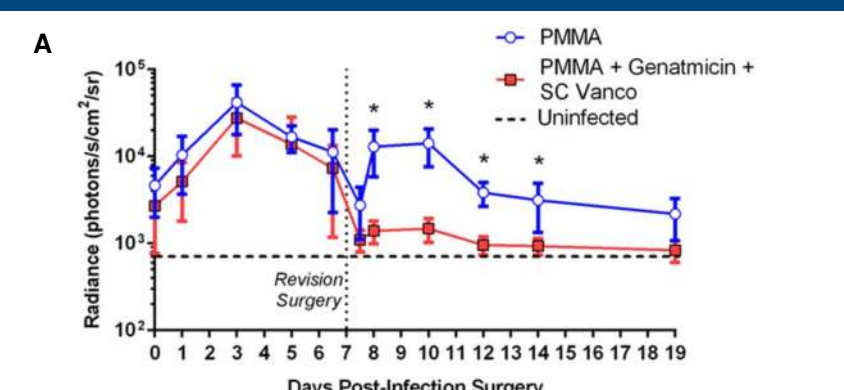

Fig 9. Validation of the mouse model of implant-associated osteomyelitis using a 1-stage revealed that after establishing the infection using a contaminated screw, hardware exchange utilizing the clinical standard of PMMA-impregnated with gentamicin and systemic vancomycin enable a significant reduction in bacterial burden in comparison to a negative control of PMMA only (A). CFU analysis revealed reduction of colonized bacteria for bone, soft tissue, hardware, and cement spacer, however substantial bacteria still persisted (B-E).
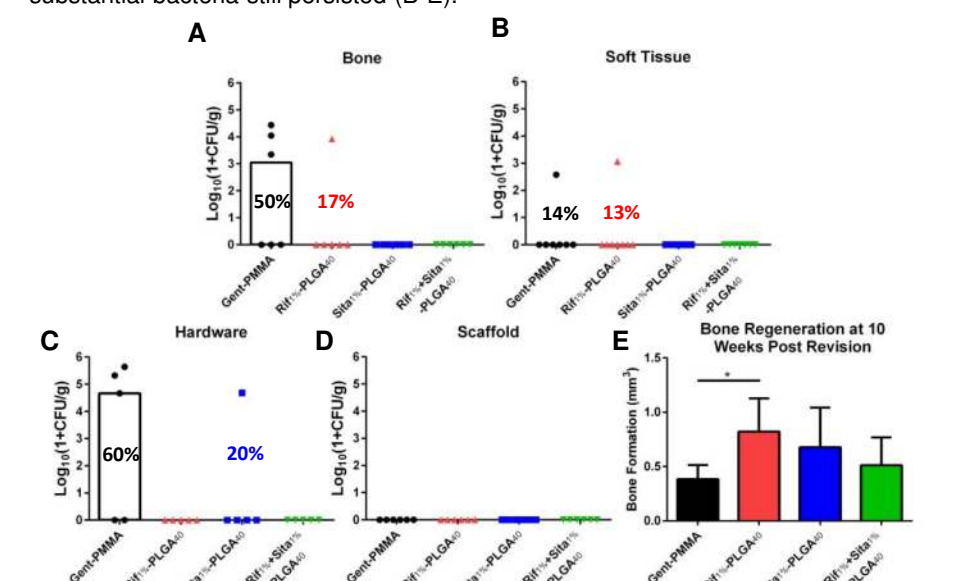

Fig 10. Implant-associated osteomyelitis yielded no significant differences between any of the four treatment groups regarding infection management, however a significant increase in bone formation was observed after 10 weeks of healing. Quantification of bacterial colonization in bone, soft tissue, hardware, and antibiotic spacer 10 weeks post-revision surgery yielded no differences, however 3/6 and 1/6 bone samples were culture positive for the clinical control and Rif[1%]-PLGA[40] respectively (A). 1/7 soft tissues samples were culture positive for both Gent-PMMA and Rif[1%]-PLGA[40] (B). 3/5 and 1/5 hardware samples were culture positive for Gent-PMMA and Sita[1%]-PLGA[40] respectively (C). No bacteria were recovered from any of the implanted spacers (D). Lastly, significant bone healing was observed in Rif[1%]-PLGA[40] in comparison to the clinical control (E). * indicates p<0.05 by 1- and 2-way ANOVA. Bacterial quantification represented as median, all other data was represented as mean ± standard deviation. n=5-8 samples/group for CFU and μCT-quantification

Discussion

Implant-associated osteomyelitis is the bane of all orthopedic procedures due to its highly recalcitrant and recurrent nature. This is attributed to the ability of *S. aureus* to form biofilm and subpopulations of SCV, which are less susceptible to conventional antibiotics[4]. In this study, we initially identified 22 drugs that demonstrated AK release of SCV and validated 4 drugs to show bactericidal activity against a stable strain of SCV. Furthermore, sitafloxacin was identified as the best performing drug due to its low concentration of bactericidal efficacy against various *S. aureus* strains, significant potency against established *S. aureus* biofilm and its high therapeutic ratio*. This drug was then incorporated into 3D-printed CaPS and coated with PLGA. A biphasic release profile was observed in which an initial burst release was followed by sustained release, which was 900× the minimum inhibitory concentration . The efficacy of this scaffold was then tested in a clinically-relevant and validated model of implant-associated osteomyelitis utilizing a 1-stage revision.

Implantation into the murine infection model resulted in decreased bacterial colonization rates at 3-and 10-weeks post revision for the 3D printed CaPS in comparison to gentamicin-laden PMMA. In comparison to revision models that retain hardware[9], this surgical approach had superior outcomes in both longitudinal BLI and end-point CFUs exemplifying the true advantage of exchanging hardware in order to prevent bacterial colonization, biofilm formation, and recurrence of infection. Furthermore, a significant increase in bone formation was observed for 3D printed CaPS incorporated with rifampin at 3- and 10-weeks. The results of this study demonstrate that osteoconductive 3D printed CaPS incorporated with rifampin and sitafloxacin demonstrate more efficacious bacterial colonization outcomes and bone growth in a single-stage revision in comparison to gentamicin-laden PMMA requiring a two-stage revision.

*Therapeutic ratio defined as the ratio of the lowest concentration causing cytotoxicity over the MIC of each tested *S. aureus* strain

Conclusion/Significance

This study showcases the therapeutic efficacy of performing a single-stage revision with complete hardware exchange in a mouse, enabling the infection to be successfully controlled using a degradable and osteoconductive 3D-printed CaP scaffolds designed to serve as an effective vehicle for drug delivery of sitafloxacin and rifampin to eliminate the need for additional revision surgeries and possibly support bone regeneration in the long term. Results of this work will potentially develop the next generation of new CaP spacers that will replace the disadvantageous PMMA cements that are the clinical standard of today.

Acknowledgements/References

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