AWARD NUMBER: W81XWH-15-1-0716

TITLE: Enhancing the Prevention and Treatment of Orthopaedic
Infections Associated with Traumatic Injury

PRINCIPAL INVESTIGATOR: Mark S. Smeltzer, PhD

CONTRACTING ORGANIZATION: University of Arkansas System

Little Rock, AR 72205

REPORT DATE: October 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2018	Annual	30 Sep 2017 – 29 Sep 2018

4. TITLE AND SUBTITLE

Enhancing the Prevention and Treatment of Orthopaedic Infections Associated with Traumatic Injury

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0716

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Mark S. Smeltzer

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

E-Mail: smeltzermarks@uams.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Arkansas System
Sciences, 4301 W. Markham, Little
Rock, AR 72205

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT. The goal of this project is to develop novel methods for the prevention and treatment of orthopaedic infections following traumatic injuries including those incurred on the battlefield. Using *Staphylococcus aureus* as a proof-of-principle pathogen based on its prominence as a cause of such infections and the severity of the infections its causes, we have undertaken studies to identify the most efficacious antibiotics and investigate the ability to conjugate these antibiotics to a novel bone targeting agent derived from a tetracycline moiety that binds hydroxyapatite. We have also demonstrated that the staphylococcal accessory regulator (*sarA*) plays a key role in the pathogenesis of orthopaedic infections owing in part to its impact on biofilm formation and intrinsic antibiotic resistance. Based on this, we have undertaken a search for small molecule inhibitors that could be used alone or in combination with conventional antibiotics and our bone targeting agent. Ultimately, these combinations will be evaluated based on their therapeutic efficacy in established animal models of traumatic bone infection.

15. SUBJECT TERMS
Staphylococcus aureus, biofilm, osteoblast, osteoclast, antibiotic, staphylococcal accessory regulator, *sarA*, SarA, bone targeting agent, daptomycin, ceftaroline, osteomyelitis

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified		

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION

Infection is one of the most devastating complications following traumatic injury to the bone. These infections can be particularly problematic on the battlefield because the injury occurs under adverse conditions in which medical care must often be focused on controlling the life-threatening consequences of the injury without the time or resources required for intensive medical procedures to prevent infection pending transport to a safe, fully equipped hospital. This same concern applies to other forms of traumatic injury in which there is a time lapse between the incident and the availability of adequate medical care, the difference being that this critical time lapse is often much longer in the case of battlefield injuries. Thus, it is imperative to develop point-of-injury methods that can be used to minimize the possibility of infection, particularly bone infection, during this period. It is also imperative that the development of such methods take into account the fact that bone infections are remarkably recalcitrant to conventional antibiotic therapy. Thus, while the overall goal of this project is to optimize point-of-care therapy for the prevention of bone infection, an important secondary goal is to maximize the ability to treat these infections after they occur. We have chosen to focus our efforts on the bacterial pathogen *Staphylococcus aureus* based both on its predominance as a causal agent of such infections and the severity of the resulting infection; indeed, *S. aureus* bone infections often require extensive surgical debridement once the patient reaches a suitably equipped hospital environment. Moreover, even after such intensive medical and surgical intervention, the therapeutic outcome is often suboptimal and many times leads to repetitive debridement surgeries, loss of functional mobility, and even amputation. This comes at great financial and emotional cost and often leads to the loss of a highly trained and highly valued active duty service member. Our goal is to build on our years of experience studying *S. aureus* bone infection to develop novel, improved methods that can be used to treat an established infection and as a point-of-injury therapeutic to prevent infection during the critical period between the injury itself and access to the extensive medical care necessary to more effectively deal with the injury and its potentially adverse consequences including infection.

KEYWORDS

Staphylococcus aureus, biofilm osteoblast, osteoclast, antibiotic, staphylococcal accessory regulator, bone targeting agent, daptomycin, oxacillin, ceftaroline, osteomyelitis

MAJOR GOALS/SPECIFIC AIMS

The overarching focus of our work has two major two aspects, with the ultimate goal being to merge these into a synergistic method that can be used to enhance the prevention and treatment of bone infections following traumatic injury. The first of these is based on the realization that the efficacy of systemic antibiotic therapy is compromised by the inability to deliver effective concentrations of antibiotics to the site of infection, particularly given the compromised wound environment in which they occur. In this respect we are taking advantage of previous work demonstrating the utility of the bone targeting agent BT2-minipeg-2 with respect to maximizing the delivery of conventional antibiotics directly to the bone. The second is based on our extensive work exploring the mechanistic basis for the pathogenesis of *S. aureus* bone infection. This work has led us to focus on the staphylococcal accessory regulator (*sarA*). Specifically, we have demonstrated that mutation of *sarA* limits the ability of *S. aureus* to form a biofilm (Beenken et al., 2014), limits the accumulation of critical surface-associated and extracellular virulence factors (Zielinska et al., 2012), limits cytotoxicity for osteoblasts and osteoclasts (Loughran et al., 2016), and ultimately limits virulence in a murine model of post-traumatic osteomyelitis (Loughran et al., 2016). The scientific premise is that these observations define *sarA* has a valid therapeutic target, with the experimental focus in this application being on identifying effective small molecule inhibitors of *sarA*-mediated regulation. The merger of these two avenues of research into a synergistic whole is based on the realization that the efficacy of such inhibitors would be maximized when used in conjunction with conventional antibiotic therapy. Thus, we will explore the possibility that any inhibitors we identify can also be conjugated to our bone targeting agent to enhance their delivery to bone both alone and in combination with BT2-minipeg-2 conjugated antibiotics. These overall goals are reflected in the specific aims of our Peer Reviewed Orthopaedic Research Program (PRORP) Expansion Award (Award #W81XWH-15-1-0716, Log #OR140356) as detailed below (modified from original application to reflect experimental progress in each aim):

Aim 1: Evaluate existing antibiotics and their bone-targeting (BT2-minipeg-2)-conjugates in the context of a biofilm. Neale et al. (2009) described the use of a hydroxyapatite-binding moiety (BT2-minipeg-2) derived from tetracycline as a targeting agent for the enhanced delivery of estradiol to the bone. In a subsequent study, Karau et al. (2013) examined the efficacy of this approach as a means of delivering vancomycin to enhance therapeutic efficacy. The enhanced targeting of vancomycin to bone was confirmed in these studies, as was an enhanced therapeutic effect in the specific context of bone infection. However, this had the adverse consequence of causing significant pathology in the kidney. In a more detailed pharmacokinetic analysis, we confirmed the bone targeting properties of BT2-minipeg-2 conjugated vancomycin, but also confirmed that this was associated with increased levels of vancomycin in the plasma (Albayati et al., 2015), an observation that likely accounts for the increased nephrotoxicity observed in these earlier studies. During the course of our work we demonstrated that BT2-minipeg-2 in and of itself does not have toxic properties (see below), thus confirming that it was likely the altered pharmacokinetic properties of the vancomycin component of the BT2-minipeg-2 vancomycin conjugate that was responsible for the observed toxicity. These results support the use of the BT2-minipeg-2 as a bone targeting agent when delivered systemically, but they also suggest that vancomycin is not an appropriate antibiotic in that regard owing to its nephrotoxicity. Thus, one of our first goals was to evaluate and prioritize alternative antibiotics for conjugation to BT2-minipeg-2 based on their relative activity in the context of an established biofilm. Having accomplished this task (Meeker et al., 2016), we turned our attention to optimizing methods to conjugate BT2-minipeg-2 to those antibiotics that exhibited the greatest activity in this context. Most notable among these were daptomycin and ceftaroline. We generated an active BT2-minipeg-2 conjugate to daptomycin, thus putting us in a position to pursue *in vivo* studies assessing toxicity and therapeutic efficacy. The results of these studies were disappointing in that they demonstrated that conjugation of daptomycin to BT2-minipeg-2 inhibited, rather than enhanced, the accumulation of daptomycin in bone. Similarly, we encountered difficulty in creating the ceftaroline conjugate, but we have generated a BT2-minipeg-2 conjugate to a ceftaroline analogue. We also successfully generated a BT2-minipeg-2 conjugate to ciprofloxacin. Finally, we are continuing to work on generating a conjugate to a novel linear lipopeptide antibiotic derived from paenipeptin (Huang et al, 2017), but we have prioritized our efforts at present on conjugation of BT2-minipeg-2 to existing antibiotics.

Aim 2: Identify small molecule inhibitors of *S. aureus* biofilm formation and the pathogenesis and therapeutic recalcitrance of orthopaedic infections. We demonstrated that mutation of the staphylococcal accessory regulator (*sarA*) limits the ability of *S. aureus* to form a biofilm to a degree that can be correlated with increased antibiotic susceptibility (Atwood et al., 2016, Weiss et al., 2009) and that it also limits virulence in animal models of *S. aureus* infection including bacteremia (Rom et al., 2017, Zielinska et al., 2012) and acute, post-traumatic osteomyelitis (Loughran et al., 2016). We have also confirmed that mutation of *sarA* limits biofilm formation to a greater extent than mutation of any other *S. aureus* regulatory locus we have examined and that, in those cases in which a mutation results in an enhanced capacity to form a biofilm, concomitant mutation of *sarA* reverses this effect (Atwood et al., 2015). Thus, we believe that small molecule inhibitors of *sarA*-mediated regulation could be used to great therapeutic advantage in the context of diverse forms of *S. aureus* infection including the biofilm-associated bone infections that arise following traumatic injury. This belief accounts for the experimental focus in our previous PRORP proposal (OR090571: *sarA* as a target for the prevention and treatment of staphylococcal biofilm associated infection). Based on this, the focus in this aim is to identify such inhibitors, verify their biological activity, and assess the ability to conjugate these inhibitors to our BT2-minipeg-2 bone targeting agent as a means of enhancing their delivery to bone. Our work has also led us to conclude that the defining factor in the phenotype of *sarA* mutants is the increased production of extracellular proteases (Zielinska et al., 2012). Thus, we generated *gfp* transcriptional reporter constructs using the promoters from all four of the known genes and/operons encoding *S. aureus* proteases, and we are using these reporters, specifically an *scpA::gfp* reporter, in our primary screen. This is being done using an existing small molecule library available in the Crooks laboratory as well as more targeted screens of analogues of promising compounds identified either in our primary screen or based on reports from the literature.

Aim 3: Evaluate the therapeutic efficacy of the agents identified and optimized in Aims 1 and 2 in the context of bone infection. Our overall goal is to develop novel methods that can be used for the

prevention and treatment of bone infection following traumatic injury. Evaluation of these methods will require the use of animal models that accurately reflect the clinical problems that define these infections. In this aim, we will determine the extent to which the BT2-minipeg-2 antibiotic conjugates developed in Aim 1, and the BT2-minipeg-2 *sarA* inhibitor conjugates developed in Aim 2, can be used to prophylactic and/or therapeutic advantage by defining their efficacy, both alone and in combination with each other, in validated animal models that accurately reflect the clinical problem of bone infection (Loughran et al., 2016). As detailed below, we have demonstrated that BT2-minipeg-2 is not toxic, and we have generated BT2-minipeg-2 conjugates to promising antibiotics. As a prelude to studies directly examining the prophylactic and therapeutic efficacy of these conjugates, we are focusing on experiments examining their pharmacological properties as detailed below.

TASK LIST

Our experimental approach to accomplishing these aims can be summarized in 9 tasks. We have made significant progress in completing these tasks, but as would be expected given the necessity of completing certain tasks before others can be undertaken, most of our work to date has focused on the first 5 of these tasks. However, we have confirmed that our BT2-minipeg-2 bone targeting agent is not toxic in and of itself, and given that we have generated BT2-minipeg-2 conjugates to promising antibiotics, we have now begun to undertake *in vivo* studies to evaluate the toxicity and therapeutic efficacy of alternative BT2-minipeg-2 antibiotic conjugates in the specific context of bone infection (Task 9).

Task 1: Compare antibiotics active against MRSA in the context of a biofilm. This task has been completed as detailed in multiple publications (Atwood et al., 2016, Huang et al., 2017, Meeker et al., 2016). Based on the results of these studies the specific focus in Task 2 has been on daptomycin, ceftaroline, ciprofloxacin, and a novel linear lipopeptide derived from paenipeptin (Huang et al, 2017).

Task 2: Determine whether the most promising antibiotics can be conjugated to BT2-minipeg-2 without compromising efficacy in the context of a biofilm. We have generated BT2-minipeg-2 conjugates to daptomycin and ciprofloxacin without an appreciable loss of antibacterial activity as assessed by standard *in vitro* assays. Efforts to accomplish this goal with ceftaroline and our novel lipopeptide are ongoing.

Task 3: Evaluate *in vivo* pharmacological properties of BT2-minipeg-2 antibiotic conjugates in the context of bone targeting. Our primary efforts in this task to date have focused on the BT2-daptomycin conjugate. This is based on our demonstration that daptomycin has enhanced efficacy in the specific context of an established biofilm by comparison to other existing antibiotics (Meeker et al., 2016). Unfortunately, while conjugation of daptomycin to BT2-minipeg-2 did not limit antibiotic activity *in vitro*, it did limit the delivery of daptomycin to bone. We do not yet have a pharmacological explanation for this, and are currently exploring the possibility that it is related to the methods used to generate the BT2-minipeg-2 daptomycin conjugate, but in the interim we shifted our focus to *in vivo* evaluation of our BT2-ciprofloxacin conjugate as detailed below.

Task 4: Identify small molecule inhibitors of *sarA*-mediated regulation. The focus in this task was to screen a small molecule library generated in the Crooks laboratory and to do so by exploiting the correlation between the phenotype of *sarA* mutants and the demonstrated importance of the increased production of extracellular proteases in defining this phenotype. Specifically, we generated transcriptional reporters using the promoters from all four genes and/or operons encoding known *S. aureus* extracellular proteases and the gene encoding a superfolder green fluorescent protein (*gfp*). Our studies confirmed that transcription was increased in *sarA* mutants with all four reporters and that the *scpA::gfp* reporter exhibited the greatest dynamic range. Thus, we chose this reporter for use in our primary, high-throughput screen. We have now screened 2,493 compounds using this screen, and are currently in the process of validating the most promising compounds using more targeted assays including biofilm formation and the production of SarA itself.

Task 5: Validate compounds previously implicated in *sarA*-mediated regulation and/or biofilm formation. Since funding of our proposal, a number of reports have been published that describe

inhibitors of *S. aureus* biofilm formation, some of which have described as inhibitors of *sarA*. We believe we would be remiss if we did not take these reports into consideration. Thus, in addition to our initial focus on screening our small molecule library, we undertook the task of synthesizing these, as well as closely-related analogues, and tested their activity using our *scpA::gfp* reporter assay. As with compounds identified in our library screen (Task 4), those compounds found to have activity in our assay have now been moved forward to validation in more targeted assays (e.g. biofilm formation).

Task 6: Expand the screen of small molecule inhibitors to include additional staphylococcal strains and species. We have undertaken this task focusing on two genetically and phenotypically diverse clinical isolates of *S. aureus*. These are the methicillin-resistant USA300 strain LAC and the methicillin-sensitive osteomyelitis isolate UAMS-1. As the results of these studies become available, they will be expanded to include additional isolates of *S. aureus* as well as *S. epidermidis*.

Task 7: Evaluate conjugation of the most promising *sarA* inhibitors to BT2-minipeg-2. We have not yet been able to pursue this task pending the outcome of Tasks 4-6.

Task 8: Evaluate *in vivo* pharmacological properties of the most promising *sarA* inhibitors and their BT2-minipeg-2 conjugates. We have also been unable to actively pursue this task pending the outcome of Tasks 4-7.

Task 9: Evaluate the efficacy of the most promising small molecule inhibitor and the most promising antibiotic *in vivo* with and without conjugation to BT2-minipeg-2. With respect to studies focusing on small molecule inhibitors of *sarA*, we cannot pursue these studies until we have identified and prioritized promising inhibitors. Studies to accomplish these tasks and determine whether such an inhibitor can be conjugated to BT2-minipeg-2 without limiting its activity, are ongoing. Our studies focusing on conventional antibiotics have progressed to the point where we have been able to assess *in vivo* pharmacological properties including the efficacy of bone delivery. Once these experiments are completed, we will assess therapeutic efficacy using our animal models.

ACCOMPLISHMENTS

Task 1: Compare antibiotics active against MRSA under standard testing conditions in the context of an established biofilm. We evaluated the relative efficacy of antibiotics that are active against methicillin-resistant *S. aureus* (daptomycin, ceftaroline, vancomycin, telavancin, oritavancin, dalbavancin and tigecycline) in the specific context of an established biofilm under both *in vitro* and *in vivo* conditions. The results demonstrated that daptomycin and ceftaroline have significantly greater efficacy than any other antibiotic tested, including what is widely considered the "last resort" antibiotic vancomycin (Meeker et al., 2016). Thus, we focused our efforts moving forward into Task 2 on these two antibiotics. However, to further assess the versatility of our bone targeting platform, we also explored the relative efficacy of additional antibiotics including ciprofloxacin, oxacillin, and one promising antibiotic that remains under development. Specifically, Dr. En Huang in the College of Public Health at the University of Arkansas for Medical Sciences (UAMS) has identified a novel linear lipopeptide antibiotic derived from paenipeptin (Huang et al, 2017). He also developed a synthetic scheme (paenipeptin C', see below) that greatly facilitates the ability to obtain this antibiotic in large amounts. In addition, Dr. Huang generated a number of analogues of this lipopeptide. One of these (analogue 17) has a minimum inhibitory concentration (MIC) ranging between 0.5 to 8.0 µg per ml across a wide spectrum of bacterial pathogens including *Acinetobacter baumannii* (ATCC 19606), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecium* (ATCC 19434), and two different strains of *S. aureus* (ATCC 29213 and ATCC 43300).
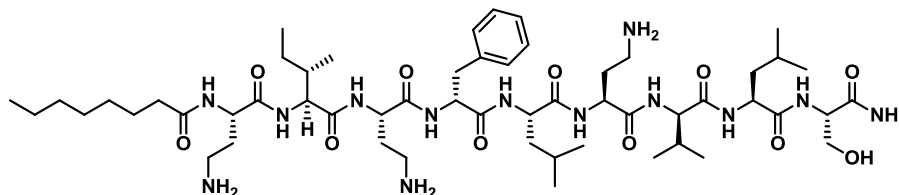

Chemical structure of paenipeptin C'
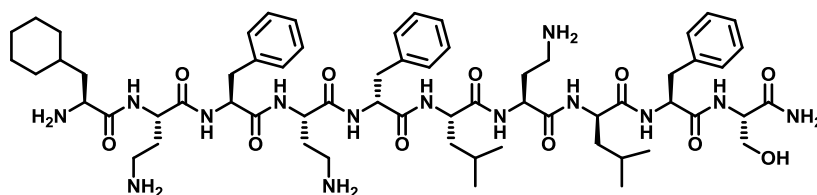
17

Chemical structure of paenipeptin analogue **17**

Although our studies revealed that analogue 17 exhibits a general trend of greater activity against Gram-negative than Gram-positive pathogens, further study demonstrated that, when used at a concentration only 5 times (5X) the MIC for the Gram-positive *S. aureus* as defined under standard planktonic conditions, analogue 17 exhibits significant antibacterial efficacy in the context of an established biofilm formed by the MRSA strain LAC. Moreover, when tested at a concentration corresponding to 20X the MIC for each antibiotic, the activity of analogue 17 in this context was found to be comparable to that of ceftaroline (Cef) and daptomycin (Dap). A manuscript fully describing these results, the methods used to generate these analogues, and the details of their activity against different bacterial species was published in the Journal of Medicinal Chemistry (Moon et al., 2017, Novel linear lipopeptide paenipeptins with potential for eradicating biofilms and sensitizing Gram-negative bacteria to rifampicin and clarithromycin. J. Med. Chem., 60:9630-9640). Based on these studies, efforts are currently underway to conjugate analogue 17 to BT2-minipeg-2 (Task 2). However, given our desire to move forward with *in vivo* pharmacokinetic and ultimately therapeutic efficacy studies, we are prioritizing studies focusing on BT2-minipeg-2 conjugates to antibiotics that have already been approved for use in humans.
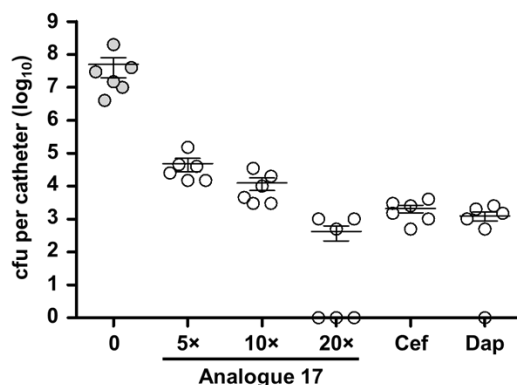
Activity of paenipeptin C' analogue **17** in an established *S. aureus* biofilm. Relative activity was assessed using our established catheter assay (Meeker et al. 2016). Results are shown as colony-forming units (cfu) remaining per catheter after 3 days in the absence of antibiotic exposure (0) or after exposure to the indicated antibiotics at the indicated concentrations. Analogue **17** was assessed at concentrations of 5, 10 and 20x the MIC for the *S. aureus* strain under study. Based on our previous experiments (Meeker et al., 2016), ceftaroline (Cef) and daptomycin (Dap) were tested at 20x the MIC for each antibiotic as defined using the same test strain.

Task 2: Determine whether the most promising antibiotics can be conjugated to BT2-minipeg-2 without compromising efficacy in the context of a biofilm. We successfully conjugated daptomycin, ciprofloxacin, and oxacillin to both BT2 itself and BT2-minipeg-2, with the latter including a water soluble linker that separates the BT2 agent from the antimicrobial agent:
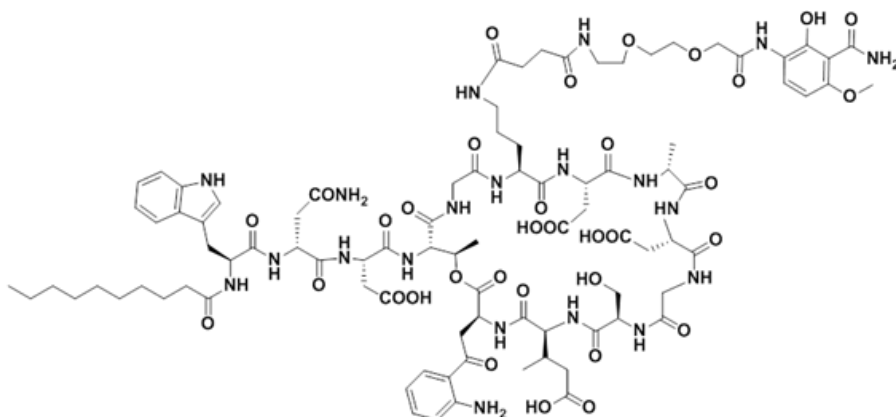

BT2   BT2-minipeg-2

7
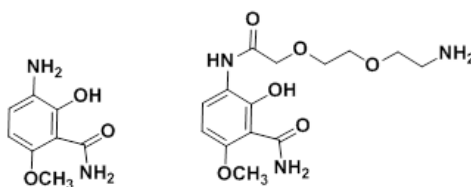
As described in a previous Progress Report, the antibacterial activity of BT2, BT2-minipeg-2, and their antibiotic conjugates was assessed using a standard microtiter plate MIC assay. In order to determine whether conjugation limited antibiotic efficacy, assays were done in comparison to the appropriate freshly-prepared, unconjugated antibiotic. These studies confirmed that neither BT2 nor its BT2-minipeg-2 derivative have antimicrobial activity in and of themselves. As was also emphasized in previous reports, the absence of antibacterial activity is not necessarily desirable, as antibacterial activity of the targeting agent itself could prove useful. However, this does suggest that these agents are less likely to be toxic. As detailed below, this lack of toxicity, specifically with BT2-minipeg-2, has been experimentally confirmed. However, it is relevant to note the lack of antibacterial activity in the context of this task is that it confirms that the antibacterial activity we observed with our antibiotic conjugates is in fact a function of the antibiotic itself. For instance, we generated BT2 and BT2-minipeg-2 conjugates with daptomycin (PNR-10-17 and PNR-10-15, respectively) and tested their impact on antibiotic efficacy. In the case of BT2 itself (PNR-10-17), when tested at equal molarity compared to unconjugated daptomycin, the MIC was increased 8-fold, while in the case of the BT2-minipeg-2 daptomycin conjugate (PNR-10-15) it was increased only 2-fold. This suggests that BT2-minipeg-2 is the preferred targeting agent by comparison to BT2 itself, although it remains to be determined whether this is a generalizable observation or one that is specific for daptomycin. Nevertheless, in our studies examining the BT2-minipeg-2 vancomycin conjugate, uptake in the bone was increased almost 50-fold by comparison to vancomycin alone (Albayati et al. 2015). This suggests that an increase in MIC of 2-fold would not diminish the potential of the BT2-minipeg-2 daptomycin conjugate as a therapeutic agent, as there would still be a net gain in antibiotic accumulation in the bone. We also confirmed that the activity of the BT2-minipeg-2 daptomycin conjugate was evident with both the USA200 methicillin sensitive strain (MSSA) UAMS1 and the USA300 MRSA strain LAC (Task 6).
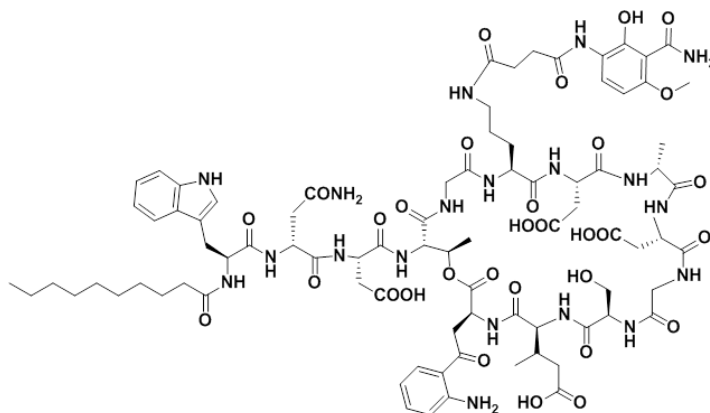
PNR-10-17: Daptomycin BT2-succinic acid conjugate

PNR-10-15: Daptomycin BT2-minipeg-2 conjugate
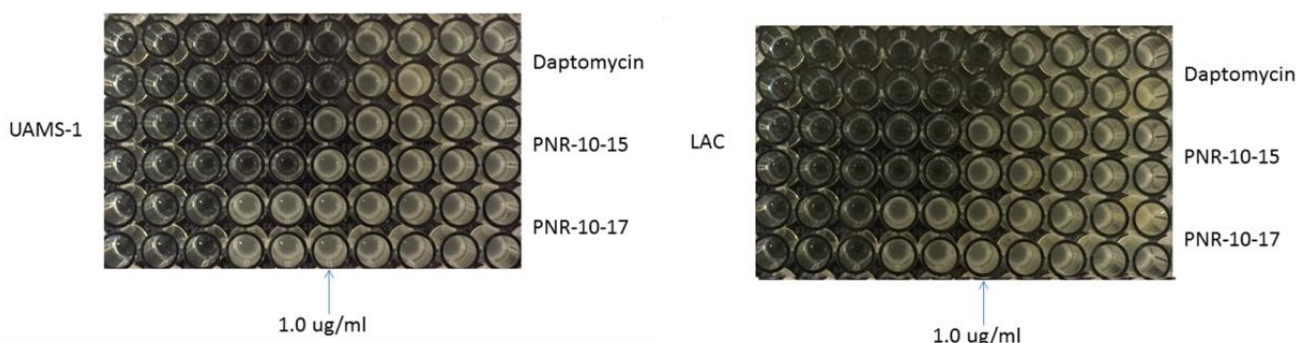
Note: The arrow indicates the CLSI-defined breakpoint MIC for a daptomycin sensitive strain of *S. aureus.* Each BT2 conjugate was tested at the molar equivalent of the concentration of daptomycin itself, which was included as a control in each assay. PNR-10-15 is the BT2-minipeg-2 daptomycin conjugate, while PNR-10-17 is the conjugate to BT2 itself.

Because it is important to limit the use of antibiotics that are active against MRSA to infections caused by MRSA, we also generated several variations of oxacillin conjugated to BT2 (BS-4-125) or BT2-minipeg-2, with the latter including two variants based on the length of the minipeg linker (BS-4-134 and BS-4-142). However, unlike our daptomycin conjugates, antibacterial activity was essentially abolished with all of these conjugates (unlike the daptomycin assays detailed above, these studies were limited to the methicillin-sensitive *S. aureus* (MSSA) strain UAMS-1 because oxacillin is not active against MRSA). Given our results with other antibiotics, most notably daptomycin, and the fact that these other antibiotics are active against both MSSA and MRSA, we have abandoned, at least temporarily, our efforts to generate an active BT2-minipeg-2 oxacillin conjugate in favor of focusing on other antibiotics.
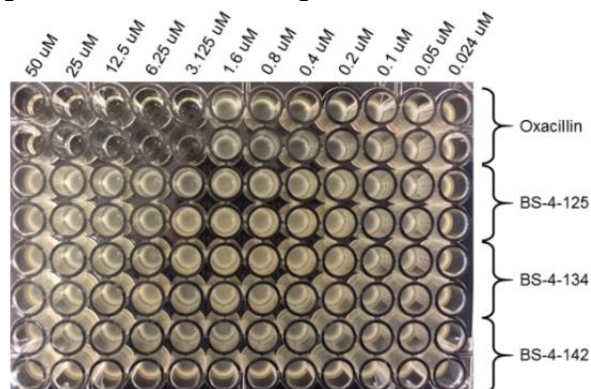

Lack of antimicrobial activity of BT2 (BS-4-125) and BT2-minipeg-2 conjugates (BS-4-134 and BS-4-142) to oxacillin. All three conjugates tested at molar equivalents exhibited a ≥32-fold increase in MIC relative to oxacillin itself.

Unlike daptomycin, we encountered difficulties generating a BT2-minipeg-2 conjugate to ceftaroline. We specifically targeted ceftaroline because it is unique among beta lactam antibiotics in that it is active against methicillin-resistant *S. aureus* (MRSA) and exhibits activity in the context of an established biofilm that is comparable to that of daptomycin (Meeker et al., 2016). However, we have now generated a BT2-minipeg-2 conjugate to a ceftaraline analogue (BS-5-02) that is currently undergoing antibacterial validation.

Ceftaroline fosamil   **BS-5-02**

9
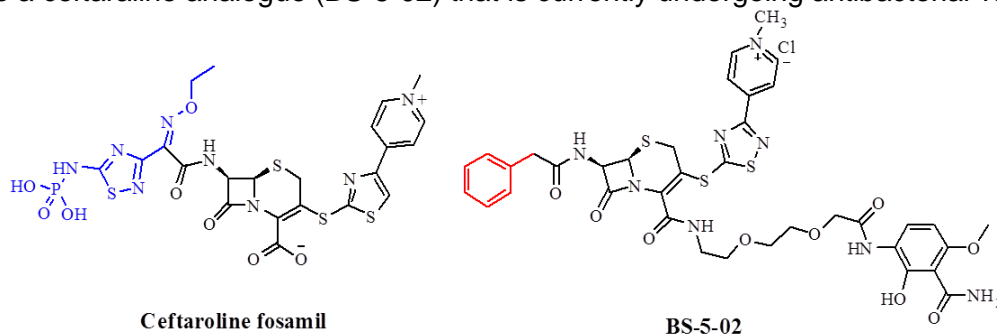
Finally, we generated BT2-minipeg-2 conjugates to moxifloxacin, sparfloxacin, and ciprofloxacin. Conjugation of moxifloxacin and sparfloxacin resulted in an increase in the MIC for UAMS-1 of ≥32-fold. Thus, these conjugates have been excluded from further consideration. In contrast, conjugation to ciprofloxacin (BS-6-68) resulted in an increase in the MIC only 4-fold higher than that observed for ciprofloxacin itself (data not shown). As discussed above in the context of our BT2-minipeg-2 daptomycin conjugate, given the ~50-fold increase in bone targeting, we do not believe this decrease in activity precludes further study of our BT2-minipeg-2 ciprofloxacin conjugate *in vivo*.

BS-6-68
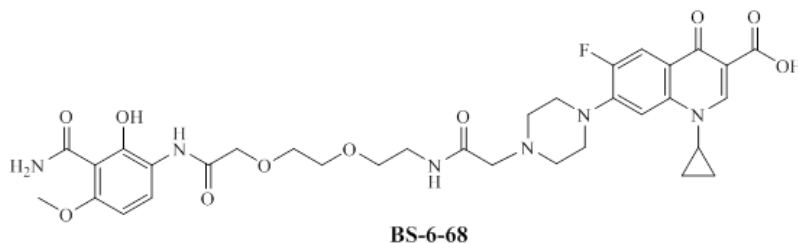
Chemical structure of BT2-minipeg-2 ciprofloxacin.

Task 3: Evaluate *in vivo* pharmacological properties of BT2-minipeg-2 antibiotic conjugates in the context of bone targeting. The primary focus of this task is to evaluate BT2-minipeg-2 conjugates to antibiotics prioritized in Task 1 and shown to retain their activity after conjugation as assessed in Task 2. While we have encountered difficulties with certain antibiotics as detailed above, we have also had success with others, most notably ciprofloxacin and daptomycin. Thus, we are now in a position to move forward with studies evaluating the *in vivo* pharmacological properties of these BT2-minipeg-2 antibiotic conjugates (Task 9). However, as noted above, Karau et al. (2013) found that the dosing regimen required to achieve an enhanced therapeutic effect with BT2-minipeg-2 vancomycin was associated with significant nephrotoxicity, thus precluding its prophylactic or therapeutic use at least via systemic routes of administration. Based on this, we first wanted to verify that the nephrotoxicity observed with the BT2-minipeg-2 vancomycin conjugate was a function of altered pharmacokinetic (PK) properties of vancomycin and not a function of BT2-minipeg-2 itself. To this end, we undertook pharmacological studies to assess the targeting efficacy, pharmacokinetic (PK) properties, and toxicity of BT2-minipeg-2.

In these studies, BT2-minipeg-2 was administered by intraperitoneal (IP) injection using a dosing regimen consisting of injections twice daily for 21 days. This regimen was chosen because it was the same regimen used in studies demonstrating that a BT2-minipeg-2 vancomycin conjugate exhibited enhanced therapeutic efficacy in a rat osteomyelitis model but also exhibited severe nephrotoxicity (Karau et al., 2013). Our studies demonstrated that injection of BT2-minipeg-2 at 11 mg/kg, which is the molar equivalent of the amount of BT2-minipeg-2 contained in the BT2-minipeg-2 vancomycin conjugate, resulted in a dramatic accumulation of BT2-minipeg-2 in bone. Specifically, in samples taken 12 hrs after the last injection on day 21, the concentration of BT2-minipeg-2 was 235.0 ± 96.8 ng per gram of bone, while plasma levels were below the limit of detection. Most importantly, we did not observe gross anatomical or histopathological changes in the kidney, and biochemical assays confirmed normal kidney function in all BT2-minipeg-2 treated rats (see previous Progress Report). Similarly, we did not observe any histological changes in the bone, and white blood cell (WBC) counts were not altered indicating normal bone marrow function. Thus, we conclude that BT2-minipeg-2 is not toxic and is likely to be a safe carrier to enhance delivery of antibiotics and ultimately small molecule inhibitors of *sarA*-mediated regulation in *S. aureus* to bone. The results of these studies were presented at the American Chemical Society's Southwest Regional Meeting held in Little Rock, AR on Nov. 7-10, 2018.

Finally, as detailed above the results of our studies with daptomycin suggest that BT2-minipeg-2 is the preferred carrier by comparison to BT2 itself because conjugation of daptomycin to BT2-minipeg-2 has less impact on the antibacterial activity of daptomycin than conjugation to BT2. However, this will not necessarily prove to be the case with other antibiotics. Thus, it was also important to test the toxicity of

BT2 itself. These experiments were done as described above except that rats were given a single oral dose of 500 mg per kg of BT2. Kidneys and the right and left tibia were harvested 7 days after this single oral dose was administered. The results of these studies also demonstrated that BT2 was not associated with significant toxicity in either the kidney or bone (data not shown). However, the more important point is that even 7 days after a single oral dose of BT2, the levels of BT2 in the bone were 326.3 ± 103.3 ng per gram of bone. We have not yet examined the accumulation of BT2-minipeg-2 in the bone after oral administration because we wanted to mimic the route of administration employed by Karau et al. (2013) in our initial studies, but this raises the intriguing possibility that our bone targeting agent could be used in an oral antibiotic formulation, thus greatly enhancing ease of use and potentially patient compliance.

We next extended these studies to examine the *in vivo* pharmacological properties of our BT2-minpeg daptomycin conjugate. To better quantify the degree of accumulation relative to unconjugated daptomycin itself, male and female C57BL/6 mice (20-25 g, n = 2-5) were injected with a single IP dose of 40 mg/kg of daptomycin or 50 mg/kg of BT2-minipeg daptomycin conjugate, or with an intravenous dose of 2 mg/kg of daptomycin or 2.5 mg/kg of BT2-minipeg daptomycin conjugate (the molar equivalent of 2 mg/kg of daptomycin). Mice were euthanized and blood and bone tissues collected at 6 time points (30 min and 1, 2, 4, 7 and 24 h). Tissues were stored at -80ºC for subsequent LC/MS analysis. Significant amounts of daptomycin and BT2-minipeg daptomycin conjugate were detected in the bone after both IV and IP administration (see below). Specifically, after IV administration of daptomycin and BT2-minipeg daptomycin, the area under the curve (AUC) values were 19,193 µg/hr/L and 10,463 µg/hr/L, respectively. This indicates that, following IV administration, the accumulation of daptomycin in the bone was actually 1.4-fold greater than that observed with BT2-minipeg daptomycin. Unfortunately, similar results were observed after IP administration. Specifically, IP injection showed AUC values of 159,201 and 12,955 µg/hr/L, demonstrating a 12.3-fold greater bone exposure of daptomycin compared to BT2-minipeg daptomycin. Preliminary estimates of bone bioavailability values indicate 41.5% and 6.2% for daptomycin and BT2-minipeg daptomycin, respectively, when administered via the IP route.
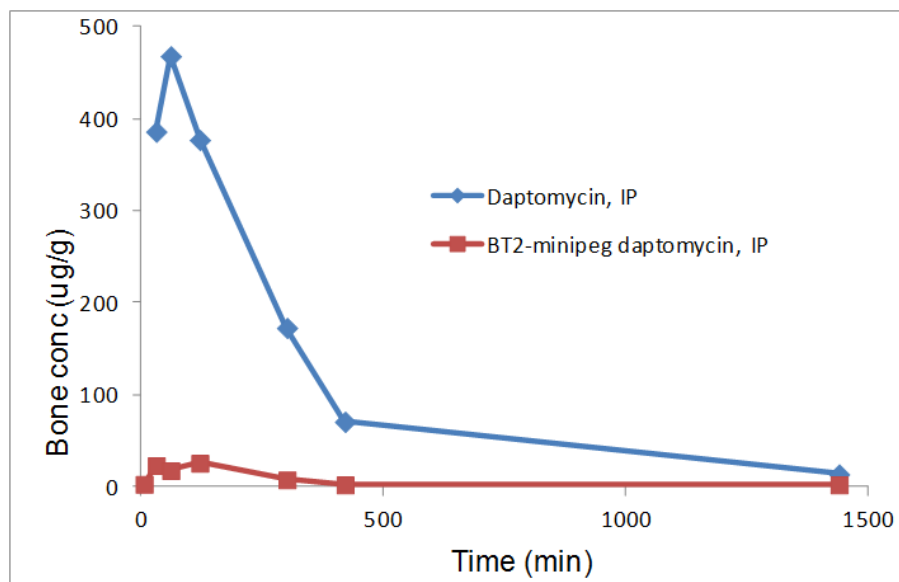

To some degree these results were disappointing in that they clearly indicate that, irrespective of the method of administration, conjugation to BT2-minipeg-2 does not enhance the accumulation of daptomycin in the bone and in fact has the opposite effect. However, they also demonstrate that daptomycin itself exhibits excellent bone availability. That said, it may still prove possible to further enhance the delivery to daptomycin to bone. Specifically, the reduced accumulation observed with the BT2-minipeg-2 daptomycin conjugate could be due to inadequate absorption of the more water-insoluble BT2-minipeg-2 daptomycin formulation, or it could be due to other, as yet undetermined pharmacokinetic issues. Nevertheless, our studies to date demonstrate that, while we generated a BT2-minipeg daptomycin conjugate without

compromising its bioactivity as assessed *in vitro* (Task 2), the specific manner in which we did so (see structure below) compromised the bioavailability of the conjugate.
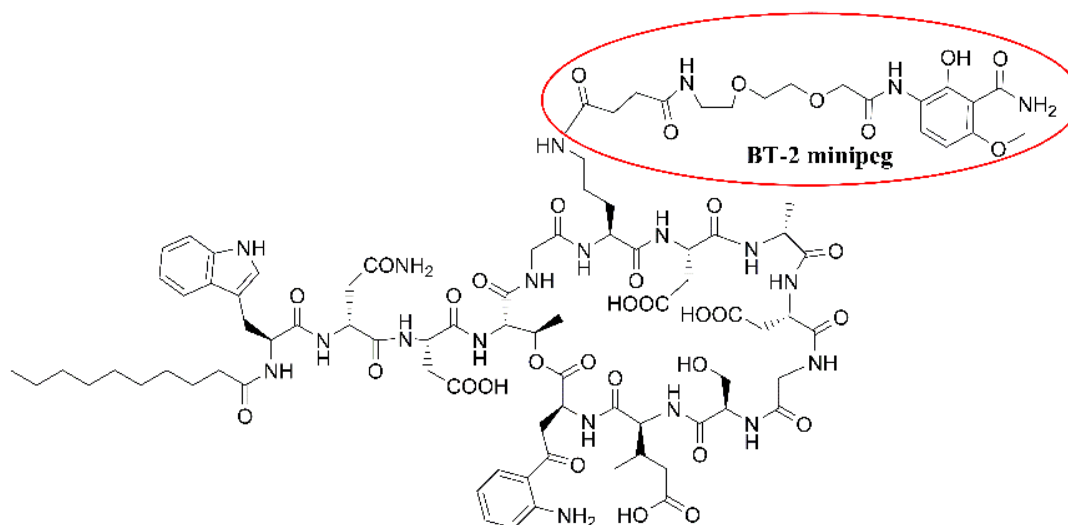

We have also undertaken similar *in vivo* studies with our BT2-minipeg ciprofloxacin conjugate. This will not only allow us to assess the therapeutic efficacy of this conjugate but also to determine whether the results we observed with the BT2-minipeg daptomycin conjugate are in fact due to structural considerations related to the methods used for antibiotic conjugation or are an antibiotic-specific effect. As a prerequisite to these studies, we developed an LC/MS/MS analytical method to quantify ciprofloxacin and its conjugate BT2-minipeg-2 ciprofloxacin in plasma and bone. The chromatograms shown below illustrate the specificity of this method and the fact that we can readily distinguish between the BT2-minipeg-2 ciprofloxacin conjugate (top panel) and unconjugated ciprofloxacin itself (bottom panel).
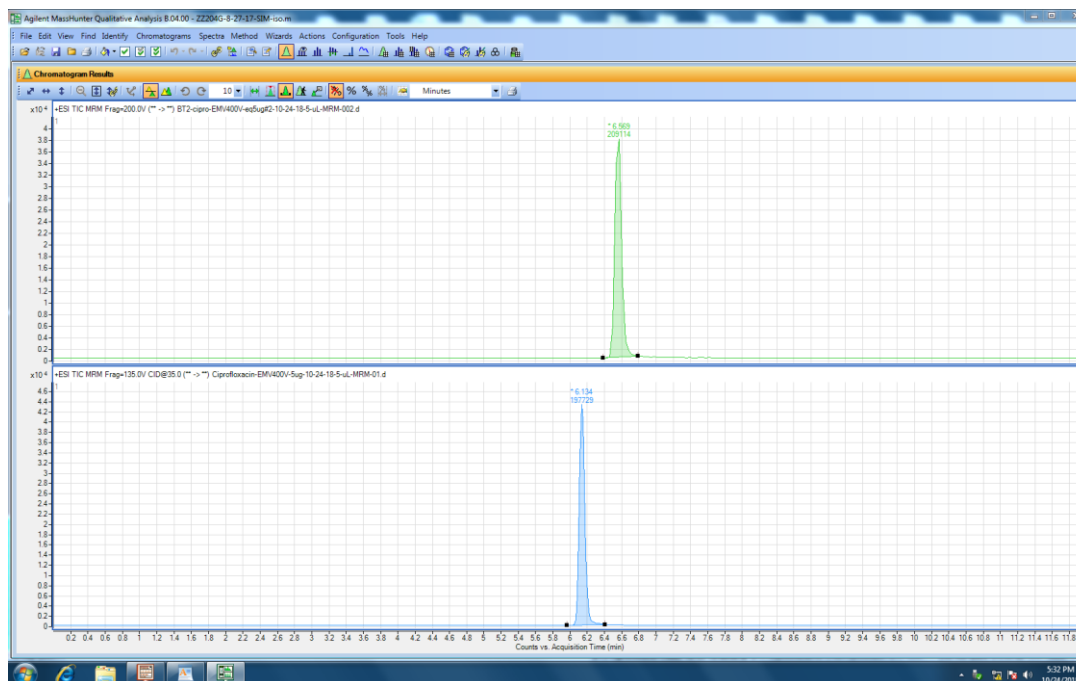
To assess our analytical ability to accomplish this task with samples obtained *in vivo*, a single female C57BL/6 mouse weighting 20-25 gms was treated IP with a single dose of BT2-minipeg-2 ciprofloxacin at 22.2 mg/kg (the molar eq. to 10 mg/kg ciprofloxacin, a simulated human dose based on Jimenez-Valera M, Sampedro A, Moreno E, Ruiz-Bravo A. 1995. Modification of immune response in mice by ciprofloxacin.

Antimicrob Agents Chemother. 39:150-154). Plasma and bone tissues were collected at 5, 10, 20, 45 and 120 min. Plasma was analyzed for BT2-minipeg-2 ciprofloxacin. The results indicate absorption of BT2-minipeg-2 ciprofloxacin into plasma was fast and peaked at 5 min and reached maximum at 45 min and then started to decrease after 120 min. The next step was to apply the LC/MS/MS method of analysis to bone tissues. Based on the results from plasma, a sample of bone tissue was taken 1 h after treatment and analyzed to determine the level of BT2-minipeg-2 ciprofloxacin. The results showed a broad chromatogram for BT2-minipeg-2 ciprofloxacin in bone, suggesting interference from endogenous substances from bone tissue.
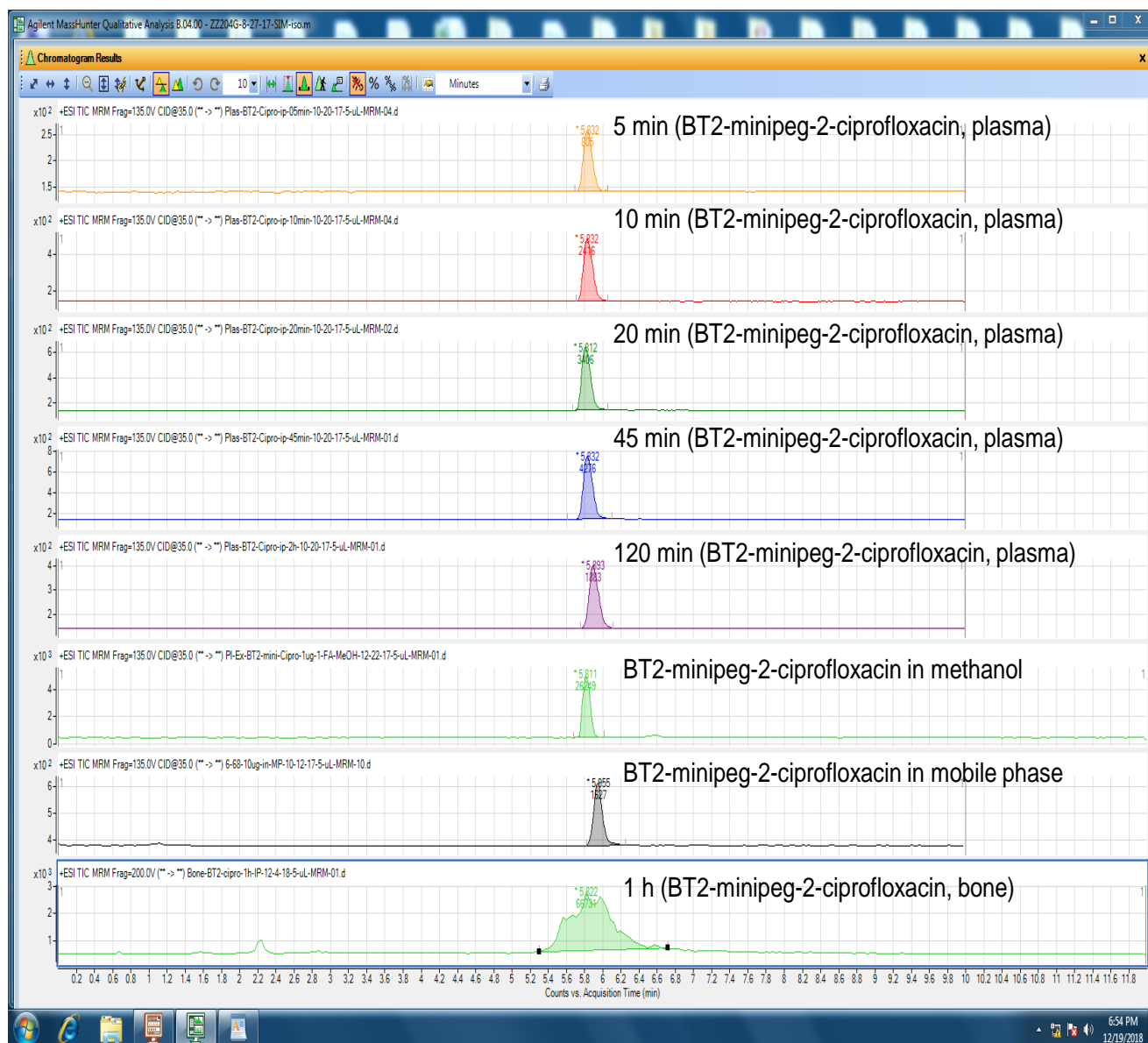
A primary focus of the limited studies discussed above was to optimize our analysis methods, and we are currently working with the manufacturer of our LC/MS/MS instrument to optimize these methods. Pending resolution of these issues, we have not yet completed the analysis of all samples, but we have extended these experiments to include an additional 14 mice, each of which was injected either IV or IP with ciprofloxacin itself or the BT2-minipeg-2 ciprofloxacin conjugate. Samples were collected at various intervals between 2 and 24 hrs after treatment. The goal is to determine the PK properties based on plasma and bone concentrations of ciprofloxacin and its conjugate BT2-minipeg-2 ciprofloxacin as a function of

time. These studies have been delayed pending optimization of our analysis methods. That said, all samples have been collected and are currently stored at -80⁰C pending analysis.

Task 4: Identify small molecule inhibitors of *sarA*-mediated regulation. In addition to our studies focusing on conventional antibiotics and the ability to optimize their conjugation to our BT2-minipeg-2, we have continued to carry out our screen for inhibitors of *sarA*-mediated expression and/or function. This is based on our demonstration that mutation of *sarA* limits biofilm formation to a degree that can be correlated with increased antibiotic susceptibility (Weiss et al., 2009, Atwood et al., 2016), limits cytotoxicity for both osteoblasts and osteoclasts (Loughran et al., 2016), and limits overall virulence in animal models of both bacteremia (Rom et al., 2017) and osteomyelitis (Loughran et al., 2016). We have also demonstrated that this is due in large part to the increased production of extracellular proteases in *sarA* mutants and the resulting decrease in the accumulation of multiple surface-associated and extracellular virulence factors (Beenken et al., 2014, Loughran et al., 2014, Tsang et al., 2008, Zielinska et al., 2012). *S. aureus* encodes 10 known extracellular proteases encoded by individual genes (aureolysin; *aur*) or organized into each of 3 operons (*scpAB*, *splA-F*, and *sspABC*), We generated reporter constructs consisting of the promoters from these genes/operons fused to superfolder green fluorescent protein (*gfp*). Subsequent studies confirmed that, relative to levels observed in the isogenic parents strains, fluorescence was significantly increased with all four reporters when they were present in *sarA* mutants generated in both the methicillin-sensitive strain UAMS-1 (U1) and the methicillin-resistant strain LAC.
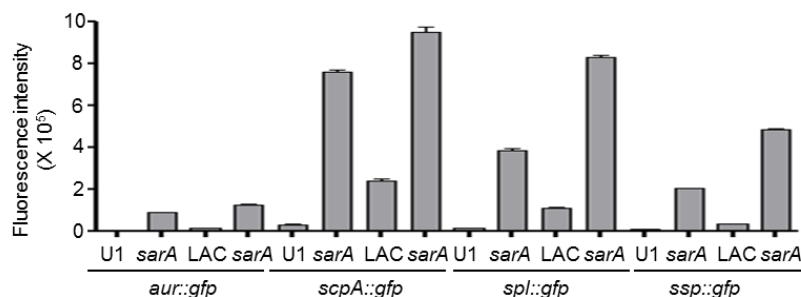

Fluorescence intensity is shown in each of two *S. aureus* strains (LAC and UAMS-1, designated here as U1) and their isogenic *sarA* mutants. The specific protease gene/operon promoter fused to the gfp reporter is shown below the graph. While expression from all 4 promoters was increased in the *sarA* mutant generated in both strains, we chose to use the *scpA::gfp* reporter in our primary screen based on the fact that fluorescence was highest in both strain with this reporter, thus providing us with an extended dynamic range.

Based on the results of these assays, we chose the *scpA::gfp* reporter for our primary screen because it exhibited the greatest increase in fluorescence in *sarA* mutants generated in both UAMS-1 and LAC relative to the appropriate isogenic parent strain. This task was focused on screening an existing library that is available in the Crooks lab and consists of small molecules previously shown to exhibit drug-like properties and to lack toxicity for mammalian cells. To date, we have screened 2,493 compounds in triplicate. Of these, we have identified 21 compounds in which fluorescence is increased in the parent strain in the presence of the compound to a level at least 25% of that observed in the isogenic *sarA* mutant. Because these 21 compounds were identified in independent experiments done over an extended period of time, we are now re-screening these compounds in order to both verify their activity and make direct comparisons. This is being done with all 21 compounds simultaneously using 4 independent biological replicates, each of which includes 3 experimental replicates. This will allow us to definitively prioritize compounds for further analysis based on the degree to which they enhance fluorescence, and the degree to which they do so reproducibly, as assessed using the *scpA::gfp* reporter relative. The highest priority compounds will be validated using other protease reporters (as detailed above, mutation of *sarA* results in increased fluorescence with all 4 reporters, and it would therefore be anticipated that this would also be true for a small molecular inhibitor of *sarA*-mediated regulation). We are also using more direct secondary assays including biofilm formation, the production of SarA, and relative antibiotic susceptibility in the context of an established biofilm.

14

Task 5: Validate compounds previously implicated in *sarA*-mediated regulation and/or biofilm formation. In addition to focusing more closely on the 21 library compounds identified to date, we have also comprehensively reviewed the literature to identify putative inhibitors of *S. aureus* biofilm formation, some of which have been directly implicated as inhibitors of *sarA*-mediated regulation. For example, Balamurugan et al. (2017) described the synthesis of 2-[(methylamino)methyl]phenol and reported that they found this compound specifically targeted SarA based on *in silico* studies. They also concluded that "the SarA targeted inhibitor showed negligible antimicrobial activity and markedly reduced the minimum inhibitory concentration of conventional antibiotics when used in combination". Based on this, we synthesized this compound (PNR-10-117) and a closely related analogue (2-methoxy-5-((methylamino)methyl)phenol; PNR-10-118A):



PNR-10-117   **PNR-10-118A**
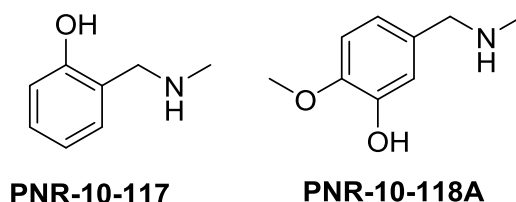
Because the number of compounds included in these studies is much smaller and therefore more manageable, we initially chose to proceed directly to biofilm assays. Unfortunately, as illustrated below using PNR-10-117, we found that this compound did not inhibit biofilm formation as assessed using our *in vitro* biofilm assay. We would note in this regard that our collective studies have validated this assay as defined by the consistent results we have observed as we have moved forward to *in vivo* studies with *S. aureus* mutants including *sarA* mutants. Thus, we are confident that PNR-10-117 does not offer the therapeutic promise suggested by Balamurugan et al. (2017).
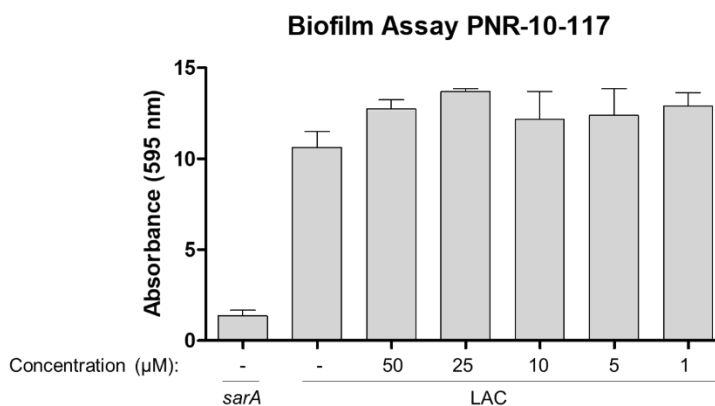

As another example, we synthesized compounds based on the report of Sambanthamoorthy et al. (2011), who reported the synthesis of novel benzimidazole molecules that prevent biofilm formation in multiple Gram-negative and Gram-positive pathogens including *S. aureus*. We synthesized these compounds (PNR-10-126A and PNR-10-126B, respectively), but as with PNR-10-117 we were unable to verify that either inhibited biofilm formation to a significant degree in our assay system.

Another report described a biofilm inhibitor (SarABI[M]) that was specifically reported to be effective against multidrug resistant *S. aureus* associated with gestational urinary tract infections (Balamurugan et al., 2015). Based on this report, we synthesized this compound and several closely-related analogues to confirm and perhaps even enhance their inhibitory activity against SarA and biofilm formation. At the time of this report, studies examining the ability of these compounds to inhibit biofilm formation are underway.

PNR-10-103B   PNR-10-104B   PNR-10-105B   PNR-10-112
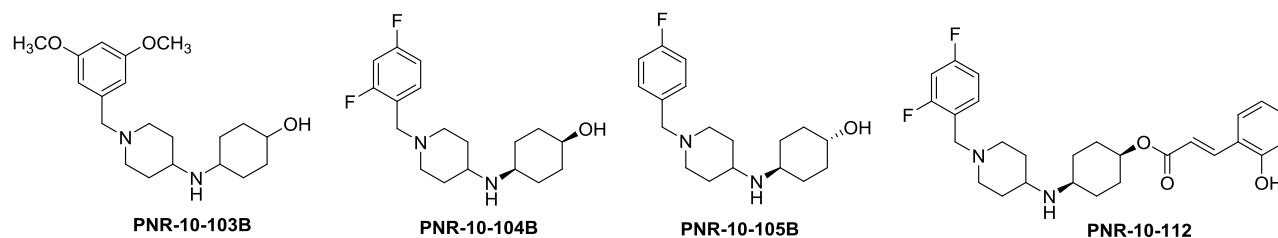
To date, we have synthesized 12 compounds that we identified in our literature search, as well as 37 analogues of these compounds. We will ultimately test all of these, as well as additional compounds developed as our studies progress, based on the inhibition of biofilm formation. However, to prioritize these studies, we first made direct comparisons using our *scpA::gfp* reporter. These studies have led us to focus on the 10 compounds identified by arrows in the figure below:
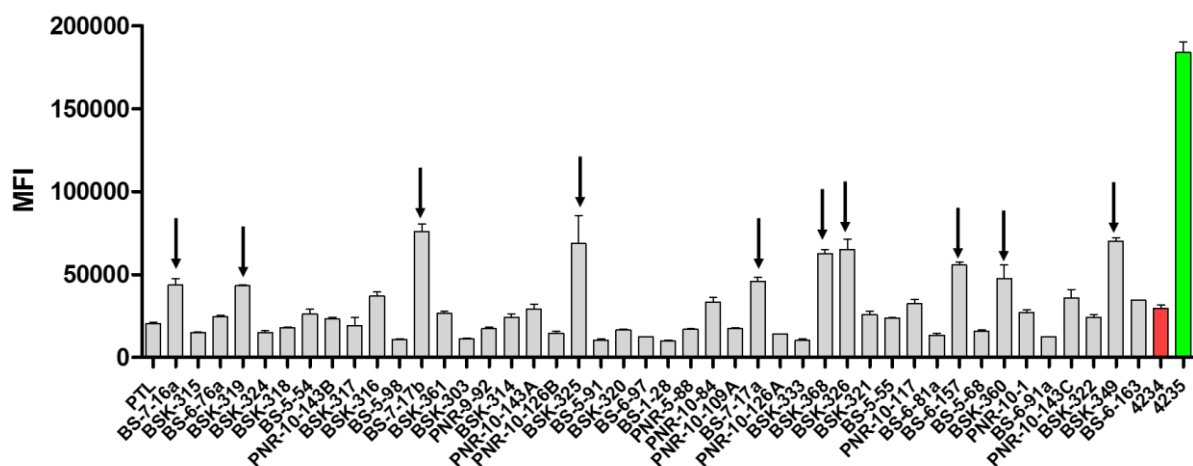
We are also continuing to synthesize additional compounds, examples of which are shown below, and we will evaluate these as sufficient quantities become available:

BSK-352   BSK-353   BSK-355

BSK-356   BSK-357   BSK-358

BSK-359   BSK-360   BSK-362

BSK-364   BSK-363   BSK-372

BSK-366   BSK-367   BSK-370

16
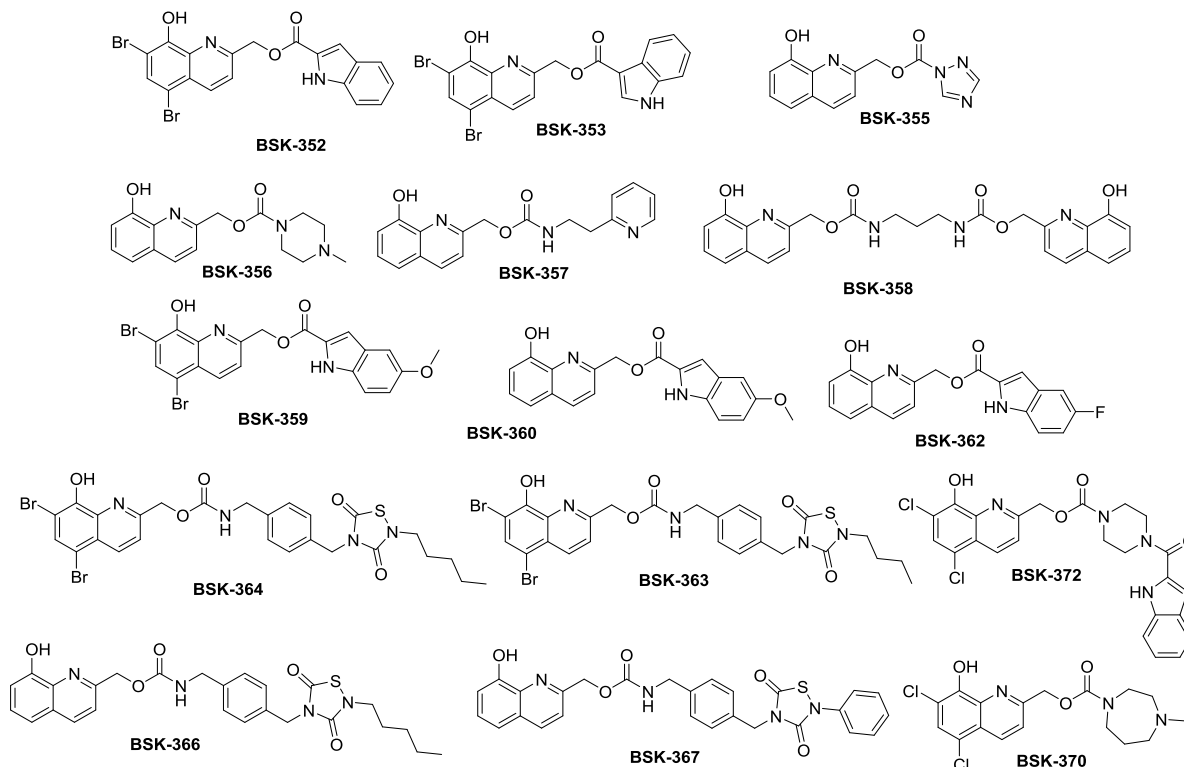
At the same time, we have moved forward with more targeted assays with those compounds already prioritized as discussed above. The results of these studies have proven promising in that we have confirmed that several of these do inhibit biofilm formation in *S. aureus*. The most promising of these identified to date are BSK-7-17b, BSK-349, BSK-326, BSK-324, PNR-10-91B, BS-1-28, and BS-5-91:
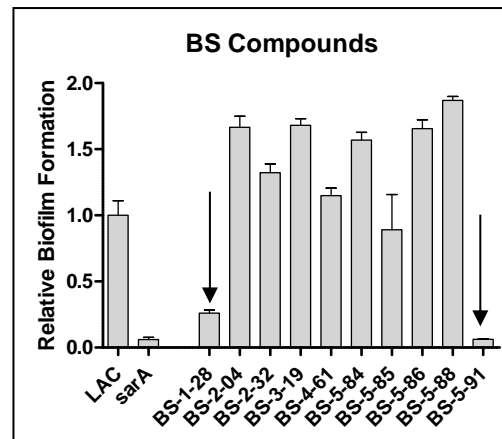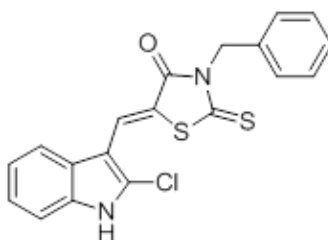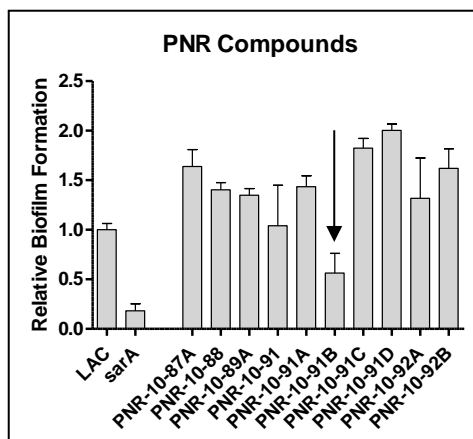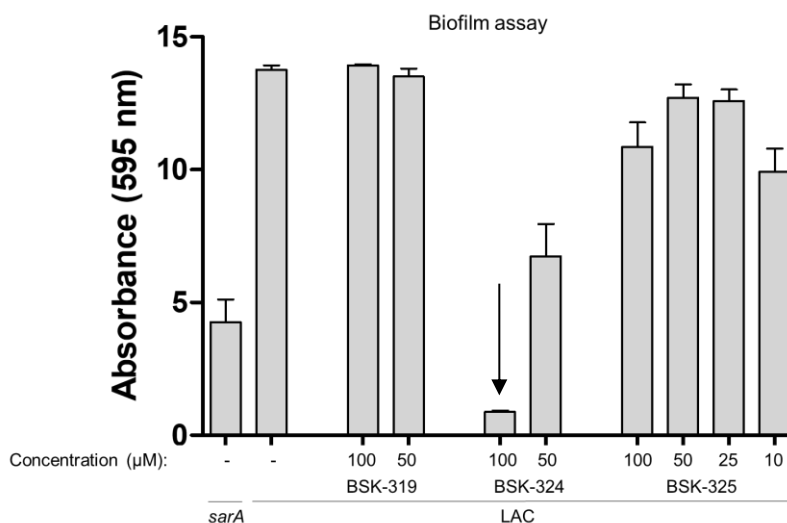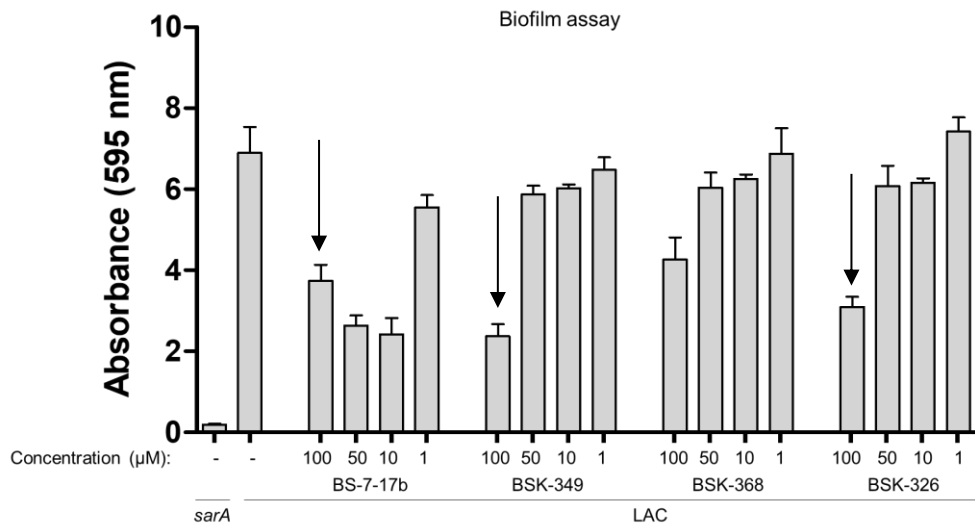
Chemical structure of PNR-10-91B

17

Task 6: Expand the screen of small molecule inhibitors to include additional staphylococcal strains and species. We included the MRSA strain LAC and the MSSA strain UAMS-1 in our initial experiments because they clinical isolates of *S. aureus* that are genetically and phenotypically distinct by comparison to each other including in their methicillin-resistance status. Based on this, we believe that identifying *sarA* inhibitors that are effective against both of these strains will greatly increase the likelihood that the results we observe will have broad utility in the context of diverse strains of *S. aureus*. As these inhibitors are identified and then verified in our secondary assays, we will extend our studies to include additional strains of *S. aureus* as well as representative coagulase-negative species beginning with *S. epidermidis*.

Task 7: Evaluate conjugation of the most promising *sarA* inhibitor to BT-2-minipeg-2. To date, we have not yet identified a *sarA* inhibitor that meets all of our selection criteria, but as noted above we have identified a number of promising compounds. This puts us in a position to ask two critical questions. First, can these inhibitors be conjugated to BT2-minipeg-2 without compromising their biological activity? Second, is the degree of biofilm inhibition observed with these compounds therapeutically relevant with respect to enhancing antibiotic susceptibility in the context of an established biofilm?

Task 8: Evaluate *in vivo* pharmacological properties of the most promising *sarA* inhibitor and its BT-2-minipeg-2 conjugate. We have not yet undertaken these studies pending completion of the studies outlined in the context of Task 7.

Task 9: Evaluate the efficacy of the most promising small molecule inhibitor and the most promising antibiotic *in vivo* with and without conjugation to BT-2-minipeg-2. As detailed above, we have completed our analysis of our BT2-minipeg-2 daptomycin conjugate, albeit with disappointing results, but are continuing to explore alternative means of generating this conjugate as a means of overcoming the pharmacological problems we encountered. We have also generated a BT2-minipeg-2 ciprofloxacin conjugate and developed the analytical methods required to examine this conjugate *in vivo* in the context of both its pharmacological properties and, assuming we find that these properties are favorable with respect to bone targeting, therapeutic efficacy in the specific context of bone infection. As was also detailed above, we are making significant progress with respect to identifying and prioritizing small molecule inhibitors of *S. aureus* biofilm formation that can be used in conjunction with these antibiotic conjugates.

OPPORTUNITIES FOR PROFESSIONAL DEVELOPMENT

This is an interdisciplinary collaborative project between the microbiologists in the Smeltzer research group and the medicinal chemists/pharmaceutical scientists in the Crooks research group. Both have a great deal to learn from the other, and thus are benefitting greatly from their collaborative effort. This is significantly enhanced by combined lab meetings that include members of both groups. The Smeltzer research group also includes technicians who are participating directly in various aspects of the project and thereby learning directly from members of both research groups. Every effort is also being made to publish the results of our studies in respected, peer-reviewed, open-access journals and to present these results at relevant scientific meetings. We believe this will increase our exposure on a national and international level and has the potential to lead to additional collaborative efforts that may ultimately further expand the clinical relevance and impact of our work.

DISSEMINATION OF RESULTS TO COMMUNITIES OF INTEREST

Results are being disseminated through publication in respected, peer-reviewed, open-access journals and participation at relevant scientific meetings.

PLANS TO ACCOMPLISH OUR GOALS

We will continue to screen for inhibitors of *sarA*, optimize the chemistry and function of such inhibitors, optimize conjugation of these inhibitors and previously prioritized antibiotics to BT2-minipeg-2, and ultimately undertake *in vivo* studies examining pharmacological properties and therapeutic efficacy.

IMPACT ON PRINCIPAL DISCIPLINE(S) OF THE PROJECT

The experiments we describe have not yet progressed to the point where we can carry out the studies necessary to accomplish three primary tasks: 1) confirm the pharmacokinetic and bone targeting properties

of alternative BT2-minipeg-2 antibiotics and/or *sarA* inhibitor conjugates; 2) demonstrate a lack of mammalian cell and host systemic toxicity and 3) evaluate the prophylactic and therapeutic efficacy of our BT2-minipeg-2 conjugates. However, we have made tremendous strides toward these goals, and we are confident that we will ultimately be successful in accomplishing all of these tasks. Moreover, we are equally confident that this will have a tremendous impact on the standard of care in the specific context of enhancing the prevention and treatment of orthopaedic infections associated with traumatic injury.

IMPACT ON OTHER DISCIPLINES

Our studies aimed at optimizing the use of BT2-minipeg-2 as a bone targeting agent in the context of antibiotics could have broad applicability to the delivery of other types of bioactive agents to the bone, thereby opening up the possibility of developing combination therapies that exploit our bone targeting agent not only to prevent and/or treat infection but also to enhance bone physiology and potentially even promote bone healing and regeneration.

IMPACT ON TECHNOLOGY TRANSFER

Nothing to report.

IMPACT ON SOCIETY BEYOND SCIENCE AND TECHNOLOGY

Nothing to report.

CHANGES IN APPROACH AND REASONS FOR CHANGE

Nothing to report.

ACTUAL OR ANTICIPATED PROBLEMS

Nothing to report.

CHANGES THAT IMPACTED EXPENDITURES

Nothing to report.

SIGNIFICANT CHANGES IN HUMAN SUBJECTS, VERTEBRATE ANIMALS, BIOHAZARDS, AND/OR SELECT AGENTS

Human subjects are not applicable. There have been no changes with respect to biohazards, select agents, or vertebrate animals since the previous progress report. As detailed in the previous report, all changes involving vertebrate animals received prior approval from both the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences (UAMS) and the Animal Care and Use Review Office (ACURO) of the U.S. Army Medical Research and Materiel Command (USAMRMC).

PUBLICATIONS, CONFERENCE PAPERS, PRESENTATIONS

1. **Meeker, D.G., Loughran, A.J., Beenken, K.E., Spencer, H.J., Lynn, W.B., Mills, W.B., and Smeltzer, M.S.** 2016. Evaluation of antibiotics effective against methicillin-resistant *Staphylococcus aureus* based on efficacy in the context of an established biofilm. Antimicrobial Agents and Chemotherapy, 60:5688-5694. PMCID: PMC5038242.

2. **Albayati, Z.A.F., Sunkara, M., Schimdt-Malan, S.M., Karau, M.J., Morris, A.J., Steckelberg, J.M, Patel, R., Breen, P., Smeltzer, M.S., Taylor, K.G., Merten, K.E., Pierce, W.M., and Crooks, P.A.** 2015. Use of a novel bone-targeting agent for the enhanced delivery of vancomycin to bone. Antimicrobial Agents and Chemotherapy. 60:1865-1868. PMCID: PMC4776008.

3. **Moon, S.H., Zhang, X., Zheng, G., Meeker, D.G., Smeltzer, M.S., and Huang, E.** 2017. Novel Linear Lipopeptide Paenipeptins with Potential for Eradicating Biofilms and Sensitizing Gram-Negative Bacteria to Rifampicin and Clarithromycin. Journal of Medicincal Chemistry. 60:9630-9640. PMID: 29136469.

4. **Albayati, Z.A.F., Bommaganni, S., Penthala, N., Post, G.R., Smeltzer, M.S., Crooks, P.A.** BT2-minipeg-2 is a safe carrier for the targeted systemic delivery of antibiotics in the treatment of bone infections, submitted to American Association of Pharmaceutical Sciences Journal.

5. **American Association of Pharmaceutical Sciences (AAPS), 2017, San Diego, CA.** BT2-minipeg-2 is a safe carrier for the targeted systemic delivery of antibiotics in the treatment of bone infections.

6. **Military Health Science Research Symposium (MHSRS) 2016, Orlando, FL.** Enhancing the prevention and treatment of orthopaedic infections associated with traumatic injury.

7. **American Chemical Society Southwest Regional Meeting. 2018. Little Rock, AR.** Evaluation of bone and kidney toxicity of BT2-minipeg-2, a carrier for the targeted delivery of antibiotics to bone.

8. **Albayati, A.F., Penthala, N.R., Bommagani, S., Post, G.R., Smeltzer, M.S. and Crooks, P.A.** Evaluation of bone and kidney toxicity of BT2-minipeg-2, a potential carrier for the targeted delivery of antibiotics to bone. Submitted to Drug Development Research, currently in revision.

WEBSITE/OTHER INTERNET SITES

Nothing to report.

TECHOLOGIES OR TECHNIQUES

Nothing to report.

INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSE

Huang E., Moon S.H., Zheng G., Zhang X., Smeltzer M.S., Meeker D.G. 2017. Linear Polypeptide Paenipeptins and Methods of Using the Same. U.S. Provisional Application No. 62/541,200

OTHER PRODUCTS

Nothing to report.

INDIVIDUALS WHO HAVE WORKED ON THE PROJECT

Personnel	Role	Percent Effort
Mark Smeltzer	PI	25%
Peter Crooks	Co-Investigator	0%
Albayati Zaineb	Post-doctoral Research Associate	10%
Narsimha R. Penthala	Research Instructor	10%
Karen Beenken	Research Associate Professor	15%
Christopher Walker	Laboratory Technician	50%
Sonja Daily	Laboratory Technician	50%
Shobanbabu Bommagani	Post-doctoral Research Associate	25%

CHANGES IN ACTIVE SUPPORT OF PD/PI

Nothing to report since the previous annual progress report.

OTHER ORGANIZATIONS INVOLVED

Not applicable.

LITERATURE CITED

Neale JR, Richter NB, Merten KE, Taylor KG, Singh S, Waite LC, Emery NK, Smith NB, Cai J, Pierce WM Jr. 2009. Bone selective effect of an estradiol conjugate with a novel tetracycline-derived bone-targeting agent. Bioorg Med Chem Lett. 19:680-683.

Karau MJ, Schmidt-Malan SM, Greenwood-Quaintance KE, Mandrekar J, Cai J, Pierce WM Jr, Merten K, Patel R. 2013. Treatment of Methicillin-resistant *Staphylococcus aureus* experimental osteomyelitis with bone-targeted vancomycin. Springerplus. 2:329.

Albayati, Z.A.F., Sunkara, M., Schimdt-Malan, S.M., Karau, M.J., Morris, A.J., Steckelberg, J.M, Patel, R., Breen, P., Smeltzer, M.S., Taylor, K.G., Merten, K.E., Pierce, W.M., and Crooks, P.A. 2015. Use of a novel bone-targeting agent for the enhanced delivery of vancomycin to bone. Antimicrobial Agents and Chemotherapy. 60:1865-1868.

Albayati, Z.A.F., Bommaganni, S., Penthala, N., Post, G.R., Smeltzer, M.S., Crooks, P.A. BT2-minipeg-2 is a safe carrier for the targeted systemic delivery of antibiotics in the treatment of bone infections, submitted to American Association of Pharmaceutical Sciences Journal (see appendix).

Meeker, D.G., Loughran, A.J., Beenken, K.E., Spencer, H.J., Lynn, W.B., Mills, W.B., and Smeltzer, M.S. 2016. Evaluation of antibiotics effective against methicillin-resistant *Staphylococcus aureus* based on efficacy in the context of an established biofilm. Antimicrobial Agents and Chemotherapy, 60:5688-5694.

Huang E., Yang X., Zhang L., Yousef A.E. 2017. New *Paenibacillus* strain produces a family of linear and cyclic antimicrobial lipopeptides: Cyclization is not essential for their antimicrobial activity. FEMS Microbiology Letters 364 (8), fnx049.

Atwood, D.N., Beenken, K.E., Lantz, T.L., Meeker, D.G., Lynn, W.B., Mills, W.B., Spencer, H.J., and Smeltzer, M.S. 2016. Regulatory mutations impacting antibiotic susceptibility in an established *Staphylococcus aureus* biofilm. Antimicrobial Agents and Chemotherapy, 2016 60:1826-182.

Weiss, E.C., Spencer, H.J., Daily, S.J., Weiss, B.D, and Smeltzer, M.S. 2009. Impact of *sarA* on the antibiotic susceptibility of *Staphylococcus aureus in vitro* in a catheter-associated biofilm model. Antimicrobial Agents and Chemotherapy, 53:2475-2482.

Rom, J.S., Atwood, D.N., Beenken, K.E., Meeker, D.G., Loughran, A.J., Spencer, H.J., and Smeltzer, M.S. Impact of *Staphylococcus aureus* regulatory mutations that modulate biofilm formation in the USA300 LAC on virulence in a murine bacteremia model. Virulence, in press (published online: 14 Sep 2017).

Zielinska, A.K., Beenken, K.E., Mrak, L.N., Spencer, H.J., Post, G.R., Skinner, R.A., Tackett, A.J., Horswill, A.R., Smeltzer, M.S. 2012. *sarA*-mediated repression of protease production plays a key role in the pathogenesis of *Staphylococcus aureus* USA300 isolates. Molecular Microbiology, 86:1183-1196.

Loughran, A.J., Gaddy, D., Beenken, K.E., Meeker, D.G., Morello, R., Zhao, H., Byrum, S.D., Tackett, A.J., Cassat, J.E., and Smeltzer, M.S. 2016. Impact of *sarA* and phenol-soluble modulins in the pathogenesis of osteomyelitis in diverse clinical isolates of *Staphylococcus aureus*. Infection and Immunity, 84:2586-2594.

Atwood, D.N., Loughran, A.J., Courtney, A.P., Anthony, A.C., Meeker, D.G., Spencer, H.J., Gupta, R.K., Lee, C.Y., Beenken, K.E., Smeltzer, M.S. 2015. Comparative impact of diverse regulatory loci on *Staphylococcus aureus* biofilm formation. MicrobiologyOpen. 4:436-451.

Moon, S.H., Zhang, X., Zheng, G., Meeker, D.G., Smeltzer, M.S., and Huang, E. Structure-activity relationship studies of linear lipopeptide paenipeptins with potential for eradicating biofilms and sensitizing Gram-negative bacteria to rifampicin, submitted to Journal of Medicinal Chemistry (see appendix).

Yarlagadda V., Sarkar P., Samaddar S., and Haldar J.A. 2016. A vancomycin derivative with a pyrophosphate-binding group: A strategy to combat vancomycin-resistant bacteria. Angewandte Chemie. 55:7836-7840.

Beenken, K.E., Mrak L.N., Zielinska, A.K., Atwood, D.N., Loughran, A.J., Griffin, L.M., Matthews, K.A., Anthony, A.C., Spencer, H.J., Post, G.R., Lee, C.Y., and Smeltzer, M.S. 2014. Impact of the functional status of *saeRS* on *in vivo* phenotypes of *sarA* mutants in *Staphylococcus aureus*. Molecular Microbiology, 92:1299-1312.

Loughran, A.J., Atwood, D.N., Anthony, A.C., Harik, N.S., Spencer, H.J., Beenken, K.E., and Smeltzer, M.S. 2014. Impact of individual extracellular proteases on *Staphylococcus aureus* biofilm formation in diverse clinical isolates and their isogenic *sarA* mutants. MicrobiologyOpen, 3:897-909.

Tsang, L.H., Cassat, J.E., Shaw, L.N., Beenken, K.E. and Smeltzer, M.S. 2008. Factors contributing to the

biofilm-deficient phenotype of *Staphylococcus aureus sarA* mutants. PLoS ONE, 3:e3361.

Balamurugan, P., Krishna, V.P., Bharath, D., Lavanya, R., Vairaprakash, P. and Princy, A.S. 2017. *Staphylococcus aureus* quorum regulator SarA targeted compound, 2-[(Methylamino)methyl]phenol inhibits biofilm and down-regulates virulence genes. Frontiers in Microbiology. 8:1290.

Arya, R., Ravikumar, R., Santhosh, R.S., and Princy, S.A. 2015. SarA based novel therapeutic candidate against *Staphylococcus aureus* associated with vascular graft infections. Frontiers in Microbiology. 6:416.

Sambanthamoorthy, K., Gokhale, A.A., Lao, W., Parashar, V., Neiditch, M.B., Semmelhack, M.F., Lee, I., and Waters, C.M. 2011. Identification of a novel benzimidazole that inhibits bacterial biofilm formation in a broad-spectrum manner. Antimicrobial Agents Chemotherapy. 55:4369-4378.

Balamurugan, P., Hema, M., Kaur, G., Sridharan, V., Prabu, P.C., Sumana, M.N., and Princy, S.A. 2015. Development of a biofilm inhibitor molecule against multidrug resistant *Staphylococcus aureus* associated with gestational urinary tract infections. Frontiers in Microbiology. 6:832.

Rechtin, T.M., Gillaspy, A.F., Schumacher, M., Brennan, R.G., Smeltzer, M.S. and Hurlburt, B.K. 1999. Characterization of SarA virulence gene regulator of *Staphylococcus aureus*. Molecular Microbiology. 33:307-316.

Beenken, K.E. Dunman, P.M., McAleese, F., Macapagal, D., Murphy, E., Projan, S.J., Blevins, J.S. Smeltzer, M.S., 2004. Global gene expression in *Staphylococcus aureus* biofilms, Journal of Bacteriology, 186:4665-4684.

Beenken K.E., Spencer H., Griffin L.M., and Smeltzer M.S. 2012. Impact of extracellular nuclease production on the biofilm phenotype of *Staphylococcus aureus* under *in vitro* and *in vivo* conditions. Infection and Immunity, 80:1634-1638.