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TITLE: sarA as a Target for the Treatment and Prevention of Staphylococcal Biofilm-Associated Infection

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Genetic studies in t	Genetic studies in the PI's laboratory demonstrated that mutation of the staphylococcal accessory regulator (sarA) limits biofilm formation in						
Staphylococcus aureus to a degree that can be correlated with increased antibiotic susceptibility and an improved therapeutic outcome in biofilm-associated							
infections. The goal of this project was to take therapeutic advantage of this observation by identifying small molecule inhibitors of sarA expression and/or							
This was done using of	enetic reporter construct	s proven to accurately re	flect the functional status	s of sarA The seco	and was to then evaluate the therapeutic		
efficacy of the most promising inhibitors using established animal models of biofilm-associated infection. In our screen of >30.000 compounds, we identified 31							
of potential interest, but secondary screens, including those assessing biofilm formation and the production of SarA itself, led us to focus on a single							
compound (ST028355). However, this compound was ultimately found to be unstable, thus greatly delaying and ultimately preventing our ability to carry out							
the intended in vivo studies. To overcome this, we began a collaboration with Dr. Peter Crooks, a medicinal chemist and Chairman of the Department of							
Finantiaceutical Sciences at the University of Alkalisas for ineutical Sciences. This led to the Synthesis of over 200 analogs of this compounds. The nope was to screen these analogs as well as an additional library of compounds that is already available in the Crock's laboratory and consists of compounds with							
favorable pharmacological properties, to both optimize ST028355 and identify additional compounds of interest, thereby putting us in a position to employ							
structure activity relationship (SAR) studies to proceed to in vivo studies an ultimately to fully exploit the proposed therapeutic approach of targeting sarA.							
While we were unable to pursue the in vivo studies proposed in the original application, we did make tremendous progress towards our experimental							
objectives, and this progress forms the basis for a recently submitted application for a PRORP Expansion Award program.							
Staphylococcus, biofilm, staphylococcal accessory regulator, sarA, inhibitor							
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## FINAL REPORT; PRORP OR090571

Biofilm-associated infections, including those associated with bone and indwelling orthopaedic devices, pose a unique and devastating clinical problem (Cierny, 2011). The genesis of our PRORP proposal (OR090571: *sarA* as a target for the prevention and treatment of staphylococcal biofilm associated infection) was our demonstration that mutation of the staphylococcal accessory regulator (*sarA*) limits biofilm formation in diverse strains of *Staphylococcus aureus* to a degree that can be correlated with increased antibiotic susceptibility (Beenken et al., 2003, Weiss et al., 2009a, Weiss et al., 2009b). Subsequent studies not only confirmed this phenotype but also demonstrated that mutation of *sarA* imposes a greater limitation on biofilm formation than any other *S. aureus* regulatory locus we have examined (Atwood et al., in press). Moreover, we demonstrated that mutation of *sarA* also limits the pathological consequences of staphylococcal bacteremia and the development of hematogenous bone infection (Blevins et al., 2002, Blevins et al., 2003, Zielinska et al., 2012) as well as the bone destruction that characterizes post-traumatic osteomyelitis (unpublished observations). These observations emphasize the therapeutic potential of identifying small molecule inhibitors

of *sarA*-mediated regulation, and this was the overall focus of our research effort.

This effort was complicated by the global regulatory roles played by *sarA* (Cassat et al., 2006, Morrison et al., 2012) and the fact that screening for inhibitors based



on the production of SarA itself does not lend itself to high throughput assays. Thus, we proposed to take advantage of our studies confirming that mutation of *sarA* results in a dramatic increase in the production of extracellular proteases and that this accounts for all of the clinically relevant phenotypes cited above (Beenken et al., 2010, Tsang et al. 2008, Zielinska et al., 2012). To this end, we generated green fluorescent protein (*gfp*)

reporters to the promoters of all four S. aureus protease genes/operons and confirmed that fluorescence was in fact enhanced in sarA mutants with all four reporters relative to the fluorescence levels observed in the isogenic parent strains (Fig. 1). Using a luciferase (*lux*) reporter of one of these (sspA::lux), we then developed a high-throughput screen based on



increased bioluminescence in a wild-type strain in the presence of the test compound, thereby allowing us to carry out our screen using a 96-well microtiter plate format (Fig. 2). Using this assay, we explored a number of avenues to identify inhibitors of *sarA* expression and/or function (Hobby et al., 2012, Ordonoz et al., 2011, Quave et al., 2012), some of which took advantage of previous studies investigating the impact of extracts from plants on *S. aureus* biology (Quave et al., 2008), while others focused on more targeted methods aimed at disrupting the interaction between SarA molecules as a means of preventing formation of a functional dimer (Xu et al., 2006). However, our primary effort was focused on screening a commercially available (TimTec)

small molecule library. To this end, we screened over 30,000 compounds and identified 31 that resulted in increased bioluminescence (see previous Progress Report). We then focused on the 3 most active of these compounds, but 2 were problematic owing to solubility issues. Thus, we focused our efforts of the 3<sup>rd</sup> compound

(Compound 28, TimTec ST028355), which was subsequently shown to limit biofilm formation in a dose dependent manner to a degree comparable to mutation of *sarA* (Fig. 3) and, as assessed by western blot, to limit the accumulation of SarA itself (Fig. 4).

This primary screen was done using very limited amounts of each compound kindly





provided by Dr. Paul Dunman at the University of Rochester Medical Center. We obtained additional Compound 28 from TimTec, but unfortunately we were unable to reproduce these promising results. Subsequent analysis by <sup>1</sup>H- and <sup>13</sup>C-nuclear magnetic resonance (NMR) spectroscopy confirmed that the structure of the compound we were provided was not consistent with the reported structure (data not shown), and based on we initiated a collaboration with Dr. Peter A. Crooks, Ph.D., D.Sc. and Chair of the Pharmaceutical Sciences Department at the University of Arkansas for Medical Sciences, who has a

distinguished history in drug discovery and development and was in fact recruited to UAMS specifically to promote such efforts. Indeed, Dr. Crooks is the author of over 1,000 papers that focus on the development of therapeutic compounds in many different clinical contexts. Specifically, the Crook's laboratory carried out a de novo synthesis of this compound. This newly synthesized compound did



demonstrating its impact on biofilm formation (left) and the accumulation of SarA as assessed by western blot (right). Impact of mutating sarA in the USA300 strain LAC is also shown for comparison.

increase bioluminescence with our *sspA::lux* reporter, but it was also found to be unstable. Thus, a key component of our efforts moving forward was to address this issue by synthesizing more stable and potentially more active derivatives of Compound 28, and we have successfully synthesized ~200 such compounds (Fig. 5). In addition, we had hoped to expand our screen to include a well-defined library of ~3,000 compounds, all of which have been developed by the Crooks' laboratory, and all of which have been shown to be non-toxic to mammalian cells and have drug-like properties favorable for clinical development, but unfortunately we were unable to screen any of these compounds within the context of our PRORP grant. We have, however,

submitted a proposal as part of the PRORP Expansion Award program that is pending review and will hopefully allow us to complete these studies.



Fig. 5. Structures of biofilm-inhibitory molecules. Top: Structures of indole-based analogs found to inhibit biofilm formation in various bacterial species shown relative to that of Compound 28. Bottom: Novel structures illustrating analogs of Compound 28 predicted to have greater structural stability and potency.

### KEY RESEARCH ACCOMPLISHMENTS

- 1. Identified one compound from the original library that we believe, with further optimization of its pharmacological properties, will ultimately prove to be therapeutically relevant.
- 2. Synthesized analogs of this compound that hopefully will prove to have greater stability and improved pharmacological properties without diminishing its biological properties.
- Began to investigate novel methods for the targeting delivery of conventional antibiotics as well as small molecule inhibitors identified in our studies directly to bone.

### **REPORTABLE OUTCOMES**

The experiments being carried out under the auspices of this project do not involve human subjects and therefore do not have reportable outcomes.

#### CONCLUSION

By comparison to the original 3 year timetable of this project, the experiments were delayed owing to the lack of stability of our most promising compound, thus precluding our ability to proceed to *in vivo* experiments. However, we did identify one compound of tremendous promise, the issue being that it lacked the necessary chemical stability. Thus, we shifted our efforts to build on the results of our primary screen by using this compound as a chemical starting point for the synthesis of more stable and pharmacologically active compounds. Screening these compounds will put us in a position to move forward with animal studies focusing on those that offer the greatest therapeutic promise. Thus, we are confident we will ultimately

accomplish the objectives of this project, and we are equally confident that the results we ultimately obtain will have a significant impact on the clinical approach and, more importantly, the therapeutic outcome in biofilm-associated infections arising from traumatic injury including those directly associated with military service.

LITERATURE CITED

- 1. Atwood, D.N., Loughran, A.J., Courtney, A., Anthony, A.C., Meeker, D.G., Gupta, R.K., Lee, C.Y., Beenken, K.E., and Smeltzer, M.S. Comparative impact of diverse regulatory loci on *Staphylococcus aureus* biofilm formation. *MicrobiologyOpen*, in press.
- 2. Beenken, K.E., Blevins, J.S., Smeltzer, M.S. 2003. Mutation of *sarA* impairs biofilm formation in *Staphylococcus aureus*. Infect. Immun., 71:4206-4211
- 3. Blevins, J.S., Beenkeen, K.E., Elasri, M., Hurlburt, B., Smeltzer, M.S. 2002. Strain-dependent differences in regulatory roles of *sarA* and *agr* in *Staphylococcus aureus*. Infect. Immun., 70:470-480.
- 4. Blevins, J.S., Elasri, M.O., Skinner, R.A., Thomas, J.R., and Smeltzer. 2003. Role of *sarA* in the pathogenesis of *Staphylococcus aureus* musculoskeletal infection. *Infection and Immunity*. 71:516-523.
- Cassat, J.E., Dunman, P.M., Murphy, E.J., Projan, S.J., Beenken, K.E., Palm, K.J., Yang, S-J., Rice, K.C., Bayles, K.W., Smeltzer, M.S., 2006. Transcriptional profiling of a *Staphylococcus aureus* clinical isolate and its isogenic *agr* and *sarA* mutants reveals global differences by comparison to the laboratory strain RN6390. *Microbiology*. 152:3075-3090.
- 6. Cierny, G. 2011. Surgical treatment of osteomyelitis. Plast. Reconstr. Surg. 127:190S-204S.
- 7. Hobby, G.H., Quave, C.L., Nelson, K., Compardre, C.M., Beenken, K.E., Smeltzer, M.S. 2012. Quercus cerris extracts limit *Staphylococcus aureus* biofilm formation. J. Ethnopharmcol., 144:812-815.
- Morrison JM, Anderson KL, Beenken KE, Smeltzer MS, Dunman PM. 2012. The staphylococcal accessory regulator, SarA, is an RNA-binding protein that modulates the mRNA turnover properties of late-exponential and stationary phase *Staphylococcus aureus* Cells. *Frontiers Cell Infection Microbiology*. 2:26.
- Olson, P.D., Kuechnmeister, L.J., Anderson, K.L., Daily, S.J., Beenken, K.E., Roux, C.M., Reniere, M.L., Lewis, T.L., Weiss, W.J., Pulse, M., Nguyen, P., Simecka, J.W., Morrison, J.M., Sayood, K., Asojo, O.A., Smeltzer, M.S., Skaar, E.P., Dunman, P.M. 2011. Small molecule inhibitors of *Staphylococcus aureus* RnpA alter cellular mRNA turnover, exhibit antimicrobial properties, and attenuate pathogenesis. PLoS Pathogens. 7:e1001287.
- Ordonoz, P.E., Quave, C.L., Reynolds, W.F., Varughese, K.I., Berry, B., Malagon, O., Smeltzer, M.S., Compadre, C.M. 2011. Sesquiterpene lactons from Gynoxys verrucosa and their anti-MRSA activity. *Journal of Ethnopharmacology*, **137**:1055-1059.
- 11. Quave, C.L., Plano, L.R., Pantuso, T., Bennett, B.C. 2008. Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation, and adherence of methicillin-resistant *Staphylococcus aureus*. J. Ethnopharmacol., 118:418-428.
- 12. Quave CL, Estévez-Carmona M, Compadre CM, Hobby G, Hendrickson H, Beenken KE, Smeltzer MS. 2012. Ellagic acid derivatives from *Rubus ulmifolius* inhibit *Staphylococcus aureus* biofilm formation and improve response to antibiotics. *PLoS One*. **7**:e28737.
- 13. Weiss, E.C., Spencer, H.J., Daily, S.J., Weiss, B.D, and Smeltzer, M.S. 2009a. Impact of *sarA* on the antibiotic susceptibility of *Staphylococcus aureus in vitro* in a catheter-associated biofilm model. Antimicrob. Agents Chemother., 53:2475-2482.
- 14. Weiss, E.C., Zielinska, A., Beenken, K.E., Spencer, H.J., Daily, S.J. Smeltzer, M.S. 2009b. Mutation of *sarA* increases the susceptibility of *Staphylococcus aureus* biofilms to daptomycin *in vivo*. Antimicrob. Agents Chemother., 53:4096-4102.
- 15. Xu, Y., Shi, J., Yamamoto, N., Moss, J.A., Vogt, P.K., Janda, K.D. 2006. A credit-card library approach for disrupting protein-protein interactions. Bioorg. Med. Chem. 14:2660-2673.
- 16. 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. Mol. Micro., ePub ahead of print.