Office of Research and Sponsored Programs (ORSP)
2022 Summer Research Fellowship Program

Project title: Development of non-beta-lactam allosteric covalent inhibitor against methicillin-resistant *Staphylococcus aureus*.
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PROJECT SUMMARY

MRSA infections remain a serious threat to public health, resulting in tens of thousands of deaths, hundreds of thousands of infection, and over 1 billion USD annually in attributable healthcare costs. Still, only a handful of antibiotics have been approved to combat these infections over the past 15 years. While this seems promising, these drugs act on the same targets using the same mechanisms (competitive inhibition of PBP, D-ala-D-ala terminal binding, etc.) as previous antibiotics against which bacteria are already developing resistance. Therefore, in order to better combat antibiotic resistance, new agents utilizing new mechanisms or pathways are required. The discovery of such agents, used alone or in combination with existing antibiotics, would significantly benefit clinicians in combatting current infections and retarding increased resistance.

We have therefore developed a lead compound that acts by irreversibly allosterically modulating the activity of penicillin binding protein 2a (PBP2a), a critical cell-wall synthesis enzyme responsible for β-lactam resistance in MRSA. This is accomplished by covalent binding of our agent to this site, thus preventing the activation of the key protein. This new mode of action is supported by our preliminary data, comprising LC-MS/MS and computational docking data. Furthermore, the efficacy and non-toxicity of this agent were also demonstrated both in vitro and in vivo using a murine skin and soft tissue infection (SSTI) model with topical treatment.

In this project, we will use our current lead compound as a core structure for further derivitization and structural exploration. In doing so, we aim to (i) improve efficacy by optimizing covalent, allosteric docking and (ii) improve aqueous solubility. These properties will be measured against a variety of MRSA clinical isolates, which also demonstrate differing degrees of multi-drug resistance, and they will be used to select a new lead-compound, exhibiting improved efficacy and solubility. Its ability to treat infections will then be validated in murine models of both an SSTI and systemic infection, wherein our drug candidate will be administered IV. In these experiments, the efficacy of our inhibitors will also be compared to vancomycin, a common first choice in acute settings.

Successfully completing this project will demonstrate the pre-clinical efficacy of a new non-β-lactam antibiotic that acts through a new mode of action, namely allosteric modulation of PBP2a. We believe that this approach may be used either alone to block PBP2a activation, or it may also potentially sensitize MRSA to conventional β-lactam antibiotics allowing for these first-line drugs to be effectively recycled. By completing this project, we will also gain crucial information that will allow us to proceed towards our goal of bringing one of our derivatives to clinical trial.

BACKGROUND

A.1. The urgent need for new antimicrobials to combat multidrug-resistant Staphylococcus aureus. Antimicrobial resistance (AMR) poses a significant threat to global public health. MRSA, in particular, has become resistant to many antibiotics used for ordinary staph infections, complicating the treatment of skin and

soft-tissue infections and increasing the risk of life-threatening diseases such as endocarditis, toxic shock syndrome, and necrotizing pneumonia (1). The continuous evolution of MRSA strains to develop resistance to multiple antibiotics makes its treatment an urgent and formidable challenge (2, 3). Central to MRSA's resistance is the mecA gene, which encodes the β-lactam-insensitive penicillin-binding protein 2a (PBP2a). PBPs catalyze the final step of peptidoglycan cell wall synthesis, allowing for unhindered cell wall formation (4, 5).

Unlike the PBPs produced by drug-sensitive Staphylococcus aureus, the active site of PBP2a in MRSA is closed, necessitating conformational changes in the β3-β4 loop for activation (6, 7). These changes are mediated by allosteric regulation through peptides or peptidoglycan molecules located 60 Å away from the active site (6, 8). This same mechanism has been used to explain how two fifth-generation cephalosporins are active against MRSA; namely, by binding at both the allosteric site and the active site once it has been opened (6, 9). However, these studies also indicate that these drugs interact non-covalently with the allosteric site, and mutations around this site can lead to resistance. By designing new covalent inhibitors targeting this site, we aim to overcome resistance caused by non-specific mutations at the allosteric site. These drugs could potentially be used alone or in combination therapy. The long-term goal of our study is to rationally design synthetic antimicrobial agents that are (i) therapeutically effective, (ii) highly selective, and (iii) exhibit improved resistance profiles against multidrug-resistant S. aureus.

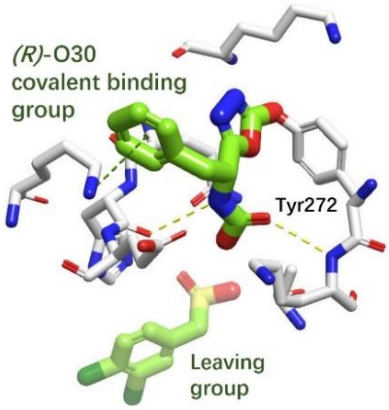

Figure 1. Graphical representation of (*R*)-O30 covalent allosteric binding to Tyr272 on PBP2a.

A.2. Scientific Premise; Sulfonyl Oxadiazole as antibiotic lead candidate core.

Based on our preliminary data we have identified (R)-(2-Methyl-2-propanyl (1-{5-[(3,4-dichlorobenzyl)sulfonyl]-1,3,4-oxadiazol-2-yl}-2-phenylethyl)carbamate, henceforth referred to as (R)-O30, as our lead compound based on our in vitro, in vivo, and in silico data (Data shown in section C). From this data, we have designed Aim 1 to improve efficacy (Aim 1a) and improve solubility (Aim 1b).

Our structure-activity and mechanistic analyses indicate the R2 group is crucial for directing the compound into the PBP2a allosteric site. This is supported by our observation that the removal of the R2 group (or R2=H) significantly reduces antibacterial activity. Therefore, Aim 1a will retain the R1 and NHBoc groups while screening benzyl group modifications, following our established computational-to-in vitro pipeline. Additionally, literature reports on the docking of ceftobiprole and ceftaroline at the Lobe-2 allosteric site of PBP2a further support modifying this group to enhance docking as some of these residues also interact with (R)-O30 (6, 9).

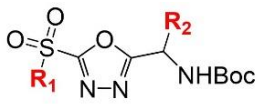

Figure 2. The 5-sulfonyl-3,4-oxadiazole core.

Aim 1b aims to enhance the aqueous solubility of the most active candidate compounds identified in Aim 1a. Poor aqueous solubility is a significant barrier to achieving high systemic drug concentration. Despite the anti-MRSA potency of (R)-O30, it is currently limited by its lipophilicity. Our strategy involves synthesizing structural analogs of Aim 1 candidate compounds with polar and/or charged functional groups, such as carboxylic acids, basic amines, and heterocyclic rings. These functionalities are common in current antibiotics as solubility-enhancing components, enabling the formation of salts and improving aqueous solubility. In (R)-O30, the carbamate (NHBoc) provides a suitable handle for building these analogs (10), as our data suggests, the tert-butyl group is not essential for activity. The primary objective of this proposal is, therefore, to develop stable and potent sulfonyl oxadiazole-based antibiotics with improved solubility and bioavailability by enhancing selectivity for PBP2a and validating their efficacy using in vivo infection models.

IMPACT

Overall, the successful completion of this project has the potential to result in the development of an effective new therapeutic strategy targeted at combatting MRSA infections. By targeting allosteric regulatory

mechanisms and employing covalent inhibition, our (R)-O30 derivatives offer a new approach to overcoming antibiotic resistance and improving patient outcomes in the treatment of MRSA.

GOALS AND OBJECTIVES FOR SUMMER RESEARCH PROJECT

MRSA strains continue to evolve and develop resistance to other antibiotics, the treatment of such infections is becoming increasingly challenging. Consequently, the development of new antibiotics to effectively overcome this resistance has become a top priority. An emerging approach is the development of allosteric modulators and inhibitors. Although at least two fifth-generation cephalosporins, ceftaroline, and ceftobiprole, have been reported to utilize this approach in combatting MRSA, they interact with the allosteric site of penicillin-binding protein 2a (PBP2a) non-covalently. As a result, resistance has been correlated with mutations around these sites.

Through this summer project, we aim to establish a foundational platform for demonstrating the in vitro and in vivo efficacy of a novel set of non-β-lactam covalent allosteric inhibitors targeting PBP2a, based on the lead compound (R)-O30.

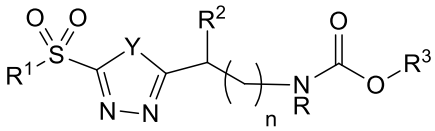

Figure 3. Structure of sulfonyl oxadiazole compound.

AIM 1. Synthesis and characterization of *(R)*-O30-derivatives. Here, we will synthesize structural analogs of our *(R)*-O30 lead compound by altering the R_2 benzyl group to improve efficacy (**Aim 1a**). Following this, we will introduce ionizable functional groups (carboxylic acids, amines) at various positions on compounds that exhibit equal or improved efficacy compared to *(R)*-O30 in order to improve solubility (**Aim 1b**).

Aim 2: Evaluation of the *(R)*-O30-derivatives for both localized and systemic infections using clinical isolates. Here, we will first determine the most active *(R)*-O30 derivative at the *in vitro* level using clinical MRSA isolates, establish its resistance profile, and determine the optimal concentration for *in vivo* application (**Aim 2a.1**). We will also prepare bioluminescent forms of some clinical isolates (**Aim 2a.2**).

CONTRIBUTION OF SUMMER RESEARCH FELLOW

The new potential lead compound-based analog structure findings with its structural activity relationships (SAR) from the summer research fellow will contribute to elucidate the exact mechanism of inhibition and the specific protein-ligand interactions using state-of-the-art computer-aided drug design techniques.

And we will further demonstrate the therapeutic potential of this new potent metallo β-lactamase inhibitor with broad-spectrum activity and identify the stability with pharmacokinetics (pK) against *in vitro* system.

This short-term summer project that uses computing power to rapidly find lead compounds and validate the *in-silico* data with simple *in-vitro* experiments are exactly aligned with our laboratory's goal of drug design and will provide a great research experience to our professional level students.

STUDENT FELLOW TRAINING / MENTORING PLAN

The research timeline for summer students will be divided into computer-aided drug design and wet lab biology research sessions according to their respective research interests and background expertise. The students will learn the principles of drug design starting with basic training, participating in the subscale study, research poster presentation, writing research manuscript, and prepare of the grant proposal. The students will rotate in different research areas within a period of two to three weeks, learn various research approaches, and participate in the project. The topics of each research field that students will learn and participate in are as follows.

Computer-aided Drug Design

- Bioinformatics (ClastalW, Schrodinger)
- Molecular modeling and visualization (Maestro, VMD, SYBYL, Pymol)
- 3D Structure motif-based virtual screening (Phase)
- Protein-ligand docking and binding free energy calculation (Glide, AutoDock)
- Molecular dynamic simulation and analysis (NAMD, VMD)
- Prediction of protein structure (Prime, SWISS-Model)
- Pharmacophore modeling (PHASE)
- Huge scale multi-sequence data analysis (ClastalW, Schrodinger)

Biological Experiment

- Basic cell culture (neuroblastoma, biosensor cell line)
- Cell Lysis and protein extraction
- SDS/Native Gel PAGE (Western Blot / Coomassie blue staining)
- Human cell proliferation assay and toxicity testing
- Toxicity testing
- Protein crystallization study
- Enzyme kinetics
- Protein concentration measurement (BCA/ELIZA)
- Skin wound model in vivo test

Available Resource and Laboratory location

- Computer-aided Drug Design (RGE #401 write-up area) – Schrodinger CADD modeling package
- BSL 1 area (RGE #400) – Enzyme kinetics assay / inhibitor preparation / Proteomics study / SDS & Native Gel Page / Protein crystallization
- BSL 2 area (RGE #407 / RGE #416) – Human & bacteria culture environment / Toxicity test / Cell proliferation assay
- Mouse surgery (RGE#414) – Skin wound model in vivo test

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