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Computational Design and Lab-Based Investigation of a Novel Small-Molecule Inhibitor That Targets CadA Metal Efflux Pump Activity in Hospital Methicillin-Resistant *Staphylococcus Aureus*: A Research Protocol

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Abstract

Introduction: Heavy metal exposure has been previously reported to decrease bacterial growth. During the growing antibiotic crisis in healthcare settings, metals may reduce bacterial burden in hospital environments. Despite proving successful, this has driven the evolution of metal-resistant strains like methicillin-resistant *Staphylococcus aureus* (MRSA). By expelling cadmium and zinc through surface efflux pumps such as CadA, MRSA is able to thrive in metal-rich environments. This protocol investigates the inhibitory activity of Scinapsin, a novel small-molecule inhibitor designed to bind the catalytic site of CadA. Scinapsin could be applied to clinical settings to combat metal resistance in MRSA populations amidst the COVID-19 pandemic.

Methods: Computational biochemistry is used to characterize the structure of the *S. aureus* CadA protein. Small-molecule library screening generated a hit compound, which after modification to improve binding affinity produced the final Scinapsin structure. Several MSRA strains were screened for the presence of CadA from which the most favourable strain for the experiment is chosen (i.e. prevalence in hospitals, CadA expression levels). For the experimental protocol, MRSA strains with metal resistance are incubated with increasing concentrations of Scinapsin, either in the presence or absence of zinc and cadmium (at 50% of predetermined MIC). Samples are then diluted and plated to allow for CFU counting.

Results: Scinapsin is anticipated to have an inhibitory effect on MRSA growth in the presence of metals at 50% MIC, confirming the successful inactivation of CadA function. A saturation point may also occur at higher concentrations of Scinapsin where no further growth inhibition is achieved.

Discussion: Docking analysis has confirmed the theoretical feasibility for Scinapsin to act as a CadA-specific inhibitor. In an *in vitro* setting like the one presented, Scinapsin should allow for excess zinc and cadmium to accumulate in the cytoplasm and ultimately cause cell death. Further experiments could aim to confirm the proposed biological mechanism of antibacterial activity.

Conclusion: Scinapsin holds promise in reversing metal resistance in hospital MRSA populations and may pave the way for other small-molecule antibacterial drugs. Future research is needed to determine safe levels of Scinapsin exposure for humans and how this inhibitor affects other hospital microbes.

Keywords: *Staphylococcus aureus*; MRSA; CadA; metal resistance; small-molecule inhibition; protein structure analysis; computational biochemistry; microbiology

Introduction

The antimicrobial properties of various metals have been known for many centuries. With advances in the field of metal toxicology, researchers are better able to understand how ions interact with the intracellular and extracellular environments of bacteria to inhibit their growth. This range of inhibitory metals can be broadly classified into two categories: non-essential metals and essential metals. Members of the non-essential classification include metals like silver (Ag) and cadmium (Cd) [1]. These metal ions serve no known biological function and can be toxic even in trace amounts [2]. In contrast, essential metals such as iron (Fe), copper (Cu), and zinc (Zn) are depended upon by organisms to play key biological roles [2]. For instance, iron is required for key bacterial metabolic pathways such as methanogenesis, the trichloroacetic acid (TCA) cycle, and DNA biosynthesis [3]. Despite their importance however, an excess of these essential metals in the cellular environment can result in a range of lethal cytotoxic effects [1].

The Inhibitory Effect of Metals on Bacterial Growth

There are many mechanisms through which both classes of metals interfere with the growth patterns of bacteria. Many bactericidal metals are transition elements, which have incomplete d-orbitals [4]. From a biological

point of view, this allows them to complex with protein scaffolds to form key biomolecules [4]. However, as the concentration of these metal ions is increased, they may begin to exhibit broad-spectrum reactivity, causing the formation of unspecific complex compounds [4]. Certain heavy-metal cations like cadmium are particularly effective in facilitating this non-targeted complex formation, which makes them very cytotoxic when present in the intracellular environment [4]. These metal ions also pose a threat to bacterial survival because of their ability to interfere with existing metabolic processes. By interacting with pathways normally associated with processing non-metals like sulfates and phosphates, certain heavy metal oxyanions (chromate, arsenate) can be reduced to form active radicals [4]. These radicals can go on to interact with other bacterial enzymes and structural proteins, leading to cell wall rupture and death [5]. Finally, due to their ability to participate in oxidation-reduction reactions, metals can drastically alter the pH of their local aqueous environment [5]. This may ultimately produce a suboptimal growth environment for bacteria attempting to replicate in solution.

Use of Cytotoxic Metals to Combat Bacteria in Healthcare Settings

With antibiotic resistance emerging in bacteria around the world, metals have been viewed as a promising alternative for controlling microbe populations. In particular, bactericidal metals find heavy use in many healthcare environments. Alloys of such metals have been incorporated into common hospital fomites like door handles and handrails. A recent 2018 study conducted in long-term care facilities found that copper alloy door handles exhibited an average bacterial burden 59% lower than control door handles, while copper handrails exhibited a 33% average decrease compared to control handrails [6]. In addition, the use of metals like silver in indwelling medical devices has led to lower patient mortality, likely due to a reduction in multidrug-resistant bacterial infections [7].

While metals have proven to be a useful tool in the fight against drug resistance, more antibiotic-resistance bacteria are developing virulence factors that make them less susceptible to the antimicrobial metals [8]. Perhaps the most prominent example of this is methicillin-resistant *Staphylococcus aureus* (MRSA), a species of *S. aureus* characteristically known for its resistance to many antibiotics. Despite the growing use of both antibiotics and metals in healthcare settings, MRSA is still widely present in hospitals and select community settings; as of 2005, invasive MRSA infections in the US occurred at a rate of 31.8 per 100,000 members of the population [9]. Recent analysis of hospital MRSA isolates reveals the presence of many antibiotic and metal resistance genes in the plasmids they harbour [10]. With many COVID-19 patients during the pandemic being more susceptible to secondary MRSA infections, a spike in death rates is likely and prompts exploration for a solution [11].

The CadA Metal Efflux Pump, a Mechanism of Metal Resistance Found in Methicillin-Resistant *S. aureus*

One way MRSA can resist high intracellular metal concentrations is through the expression of CadA, a membrane transport protein. CadA is a P-type ATPase efflux pump which is normally repressed by the DNA-binding repressor protein CadC [12]. When intracellular metal concentrations increase, CadC is inactivated, allowing for subsequent transcription and translation of CadC. After localization to the plasma membrane, CadA facilitates the ATP-driven expulsion of excess cadmium (Cd^{2+}) and zinc (Zn^{2+}) ions from the cytoplasm (Figure 1) [13]. This transport takes place at a central binding pocket surrounding a critical catalytic aspartic acid 414 (Asp-414) amino acid residue [13]. Interestingly, the genes for both CadA and its transcriptional regulator CadC are carried by plasmids like pI258, allowing for potential horizontal transfer of metal resistance genes within populations of microbes [12]. With this in mind, the significance and transferable nature of the CadA gene make it a prominent obstacle that must be addressed to reverse metal resistance in hospital MRSA populations.

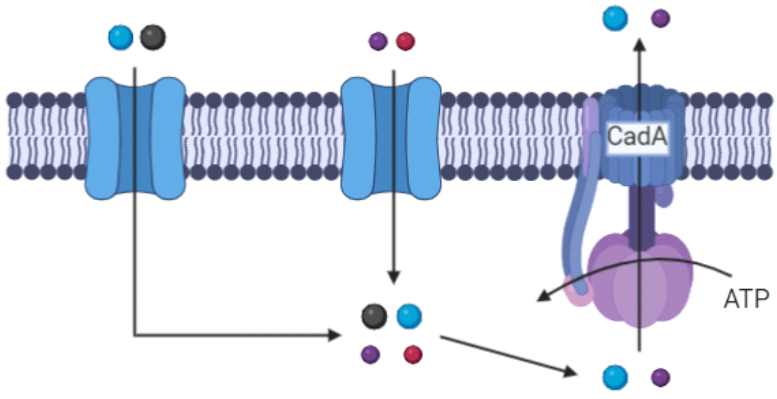

Figure 1. Heavy metal effluxing ability of CadA P-type ATPase transporter under high zinc (purple) and cadmium (blue) concentration conditions. Figure was generated using BioRender.

Investigation of Small-Molecule Inhibition as a Means to Reverse Metal Resistance in MRSA

Small-molecule inhibition has become an increasingly prominent method of targeting the activity of desired proteins. Through analyzing a specific binding interface (such as the binding pocket of CadA), a custom molecule can be designed to have favourable interactions with local hydrophobic and hydrophilic/ionic regions such that it is able to display a high affinity for the interface and displace the native substrate/ligand [14]. This approach can lead to narrow-spectrum inhibition and minimize the potential for off-target effects. Investigation of a small-molecule inhibitor that binds to the metal binding pocket of CadA and sequesters its efflux activity can be used to promote the restoration of MRSA's susceptibility to $Cd^{2+}$and Zn^{2+} ions.

The present protocol investigates the computational design of Scinapsin, a novel small-molecule inhibitor that specifically targets *S. aureus* (and particularly MRSA) CadA metal efflux pump activity. A lab-based approach to verifying Scinapsin's effectiveness on MRSA isolates is also proposed. If successful, Scinapsin could promote the sterilization of metal hospital surfaces and medical devices against characteristically resistant MRSA strains, serving as a preventative measure for drastically lowering cases of hospital-acquired infections.

Methods

Characterization of CadA Binding Pocket and Constructing the Final Structure of Scinapsin

In the absence of any Protein Data Bank (PDB) structural data for *S. aureus* CadA, predictive modeling was used to obtain a three-dimensional view of the protein. The primary amino acid sequence for *S. aureus* CadA (FASTA file, NCBI Accession: WP_003378396.1) was obtained from the National Center for Biotechnology Information (NCBI) online database. This sequence was then inputted into Phyre2, which was able to use the primary sequence to generate a predicted structure for the CadA protein [15]. The "normal modelling" mode was used for structure prediction.

To identify a "hit" molecule that exhibits high affinity for the CadA binding pocket, a structure-based virtual screen was performed using MCULE [16]. Vina docking simulations were performed using the Asp-414 OD1 atom as a binding target. The small-molecule in the MCULE library with the highest affinity for the CadA catalytic pocket is shown in Figure 2a (ΔG = -9.5 kcal/mol). An additional moiety was added to the structure of the molecule to yield the final structure of the Scinapsin inhibitor, improving binding affinity to ΔG = -9.8 kcal/mol as well as facilitating hydrogen bond interactions with the side chain of Asp-414 (Figure 2b). The PDB structure file of Scinapsin within the CadA binding pocket was downloaded and imported into Protein Ligand Interaction Profiler (PLIP). Analysis using PLIP confirmed the hydrogen bond interactions between Scinapsin and Asp-414, as well as characterizing Scinapsin's strong interactions with other residues in the binding pocket (Figure 3).

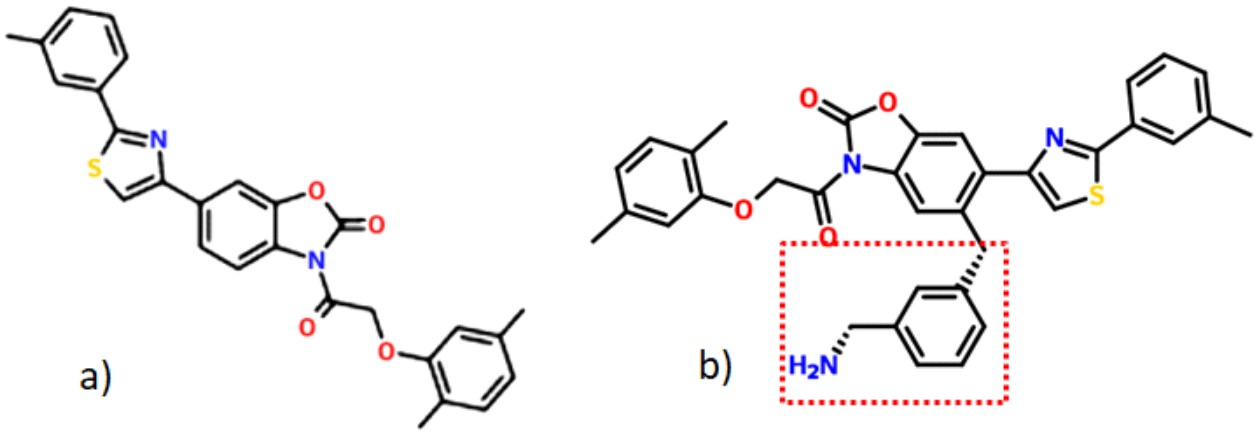

Figure 2. a) Initial small-molecule with the best putative binding affinity (-9.5 kcal/mol) for the CadA catalytic pocket of all hits from the MCULE library of molecules. b) Final Scinapsin inhibitor design with improved binding score of -9.8 kcal/mol. Red box indicates additions to the original small-molecule in a), which promote interaction with the Asp-414 catalytic residue of CadA as verified by PLIP analysis. Figure was generated using MCULE.

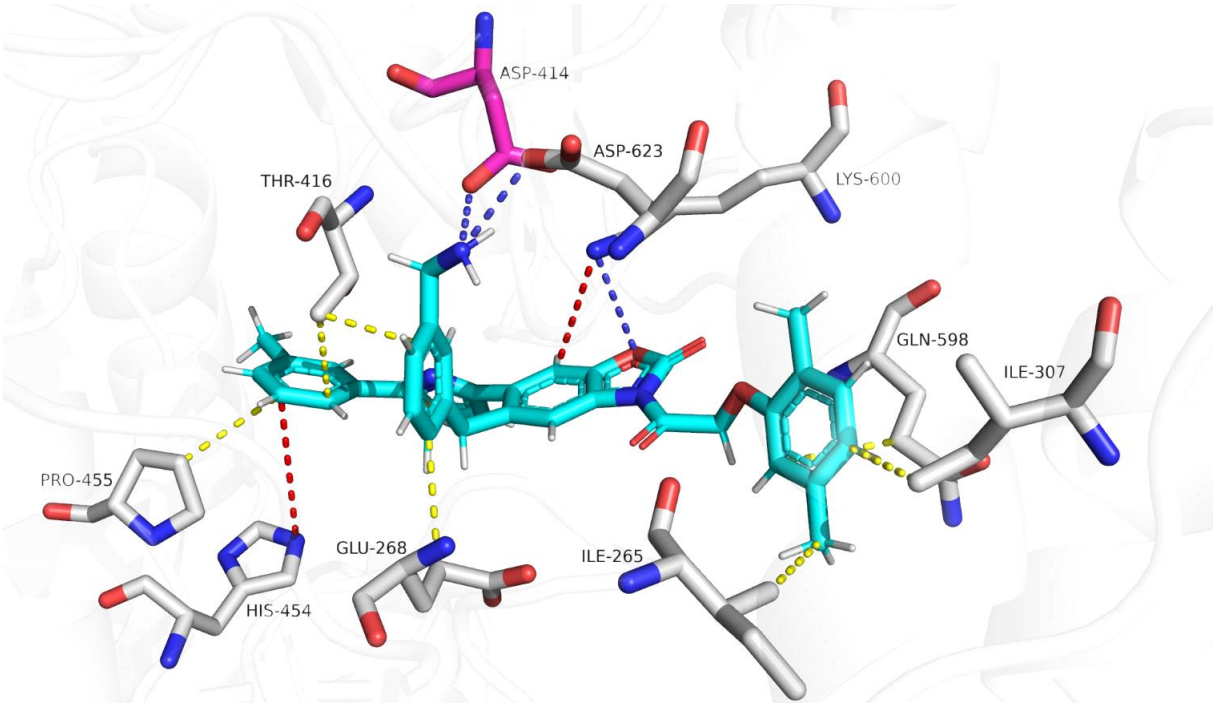

Figure 3. Interactions between catalytic binding site of MRSA CadA metal efflux pump and Scinapsin small-molecule inhibitor. Structure file was visualized using PyMOL software. Blue dashed lines represent hydrogen bond interactions between Scinapsin and active site residues. Hydrogen bond interaction parameters included a maximum bond distance of 4.1 Å and a minimum bond angle of 100°. Yellow lines represent hydrophobic interactions, while red lines indicate pi-cation interactions.

Obtaining Metal-Resistant MRSA Strain Isolates and Cultural Preparation

MRSA isolates can be obtained from the American Type Culture Collection [17]. The following steps draw reference from a protocol presented in a previous study by A. Lima de Silva et al. [18]. Experimental conditions were modified based on MRSA BAA-2313 data sheets provided by the American Type Culture Collection and simplified for preliminary investigation [17].

The metal resistant MRSA is stored as 2 mL spore suspensions at -20°C in sterile capped tubes. This procedure can be performed to ensure that stock cultures are not altered by successive sub-culturing. Experimental tools must be leached with nitric acid to prevent heavy metal contamination. For use, cultures are defrosted at room temperature and inoculated with 8 mL of Tryptic soy broth (TSB) to produce a 10 mL bacterial strain culture.

Determination of Minimum Inhibitory Concentrations of $Zn^{2+}$and Cd^{2+} Against Metal-Resistant MRSA

Sterile sulfate salt solutions of Zn^{2+} ($ZnSO_4*5H_2O$) and Cd^{2+} ($CdSO_4*5H_2O$) can be used to evaluate metal resistance. Stock solutions are prepared in distilled water and autoclaved to prevent contamination. The minimum inhibitory concentration (MIC) for Zn^{2+} and Cd^{2+} has not been determined in previous literature for some MRSA strains: as such, successive dilutions of these metals can be prepared as 10 mL tubes and cultured with the 10 mL suspensions of several MRSA strains to gauge their basal resistance. Previous literature has revealed that for some strains of MRSA, this tends to be in the low millimolar range [19]. Experimental concentrations of Zn^{2+} and Cd^{2+} used for determining the MIC of each divalent metal may be set up as shown in Table 1. After incubation at 37°C for 24 hours, the cultures will be assessed for visible colony formation to determine the experimental values that will be used in the trials with Scinapsin. The MIC will be defined as the lowest concentration required to prevent visible colony formation.

Table 1. Successive dilutions of Zn^{2+} and Cd^{2+} cultured with metal-resistant MRSA cultures, for the purposes of determining the MIC for each divalent metal.

Metal cation in solution	Concentration of metal cultured with metal-resistant MRSA cultures (mM)				
Zn^{2+}	0	0.10	0.50	1.00	10 .00
Cd^{2+}	0	0.10	0.50	1.00	10 .00

Serial Dilution of Scinapsin and Incubation with Metal-Resistant MRSA

Successive serial dilutions can be performed to produce five initial concentrations of Scinapsin inhibitor (0: deionized water control, 1.0 mM, 1.0 x 10^1 mM, 1.0 x 10^2 mM, and 1.0 x 10^3 mM) in separate 10 mL tubes. A wide range of concentrations of the inhibitor are selected to broadly explore its potential inhibitory activity, given it has not been previously studied or characterized. Further studies can use the data obtained from carrying out the protocol proposed to fine-tune the suggested concentrations above.

9mL of MRSA cultures are added to 1mL of serially diluted Scinapsin tubes, yielding a total volume of 10 mL. These solutions are homogenized before being incubated at 37°C for 48 hours in the presence of divalent heavy metal ions (Zn^{2+} or Cd^{2+}) at 50% of their respective MICs. A schematic of the experimental conditions for each tube is shown in Table 2. Five or more replicates for each experimental condition are suggested to provide a larger sample for data analysis.

Table 2. Experimental concentrations of Scinapsin inhibitor cultured with the metal-resistant MRSA strain in either the presence or absence of heavy metal cations. Five or more replicates of each experimental condition should be carried out.

Metal cation in solution	Final concentrations of Scinapsin inhibitor for each trial (mM)˜				
Zn^{2+}*	0^+	1.0 x 10^{-1}	1.0	1.0 x 10^1	1.0 x 10^2
Cd^{2+}*	0^+	1.0 x 10^{-1}	1.0	1.0 x 10^1	1.0 x 10^2
No metal	0^-	1.0 x 10^{-1} ^{Exp}	1.0	1.0 x 10^1 ^{Exp}	1.0 x 10^2

* = at 5 0% of MIC concentration. $^+$ = positive control. $^-$ = negative control. ^{Exp} = experimental control (to ensure the inhibitor alone is not affecting MRSA growth).
˜Final concentrations result from a 10x dilution when 1 mL Scinapsin tubes are added to 9 mL of metal-resistant MRSA cultures.

Analysis of Metal-Resistant MRSA Growth After Incubation with Scinapsin

After 48 hours of growth, the cultures are serially diluted and pipetted onto a solid nutrient agar (Figure 4). These plates are incubated, then used to identify the colony forming units per mL (CFU/mL) of each liquid tube (Figure 5).

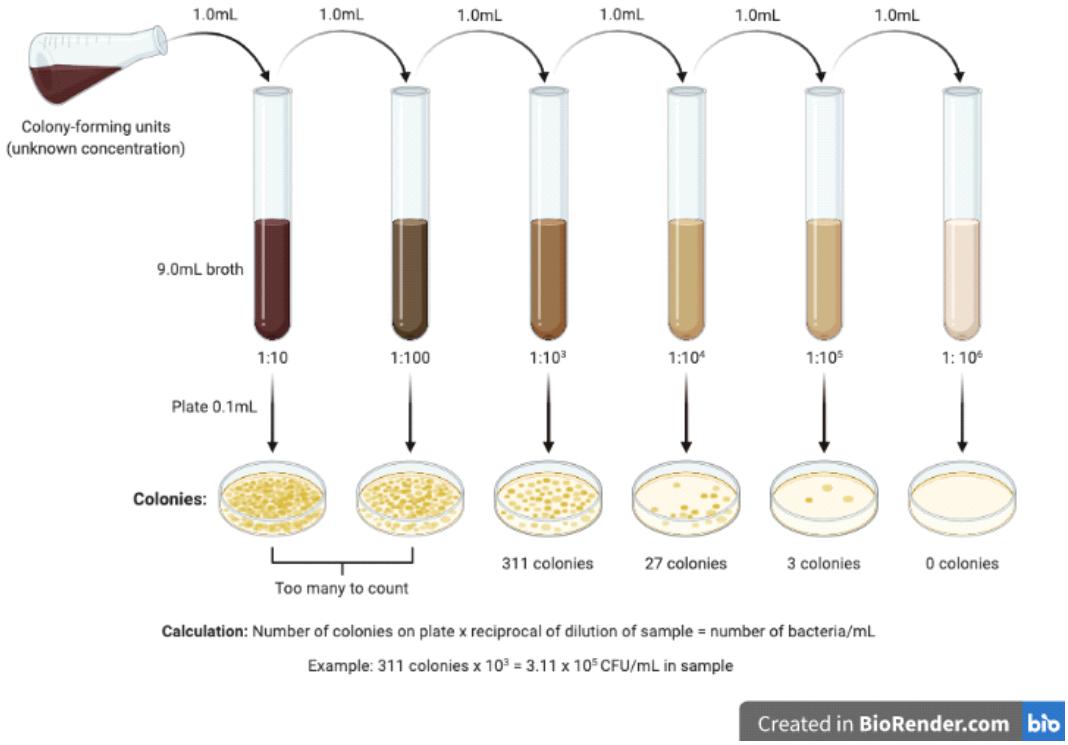

Figure 4. Example protocol for successive 10-fold serial dilutions of MRSA culture solutions. Figure was generated using BioRender

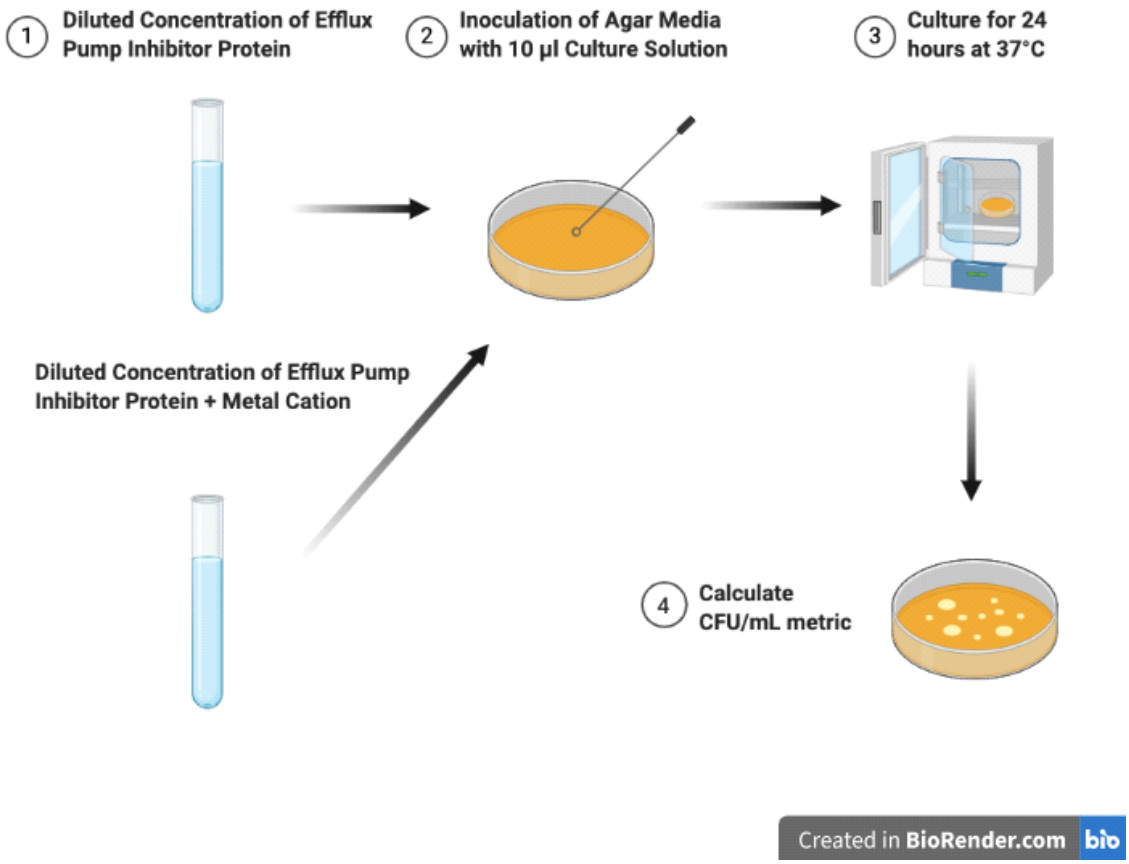

Figure 5. Theoretical protocol for inoculating culture solution onto agar plates after 48 hours of growth in liquid media in order to determine viable bacterial cell counts using CFU/mL metric. Figure was generated using BioRender.

The number of bacteria colonies on each plate is recorded after 24 hours, and the average CFU/mL is calculated for each treatment (Figure 6). The average CFU values can then be plotted for each metal at different inhibitor concentrations (Figure 7). The CFU values are analyzed using a one-way analysis of variance (ANOVA) test ($\alpha = p<0.05$), followed by a Post Hoc test to examine differences between the experimental conditions.

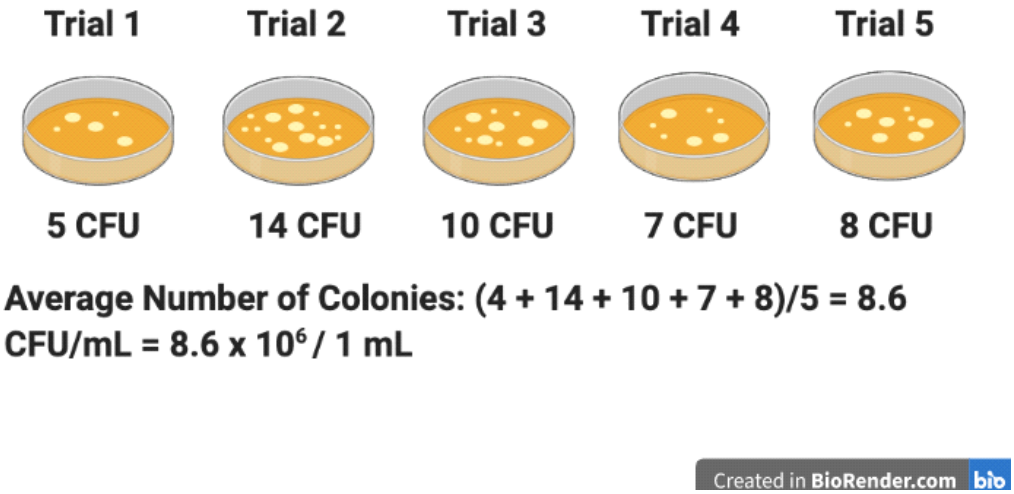

Figure 6. Example calculation for a MRSA culture data set. Figure was generated using BioRender.

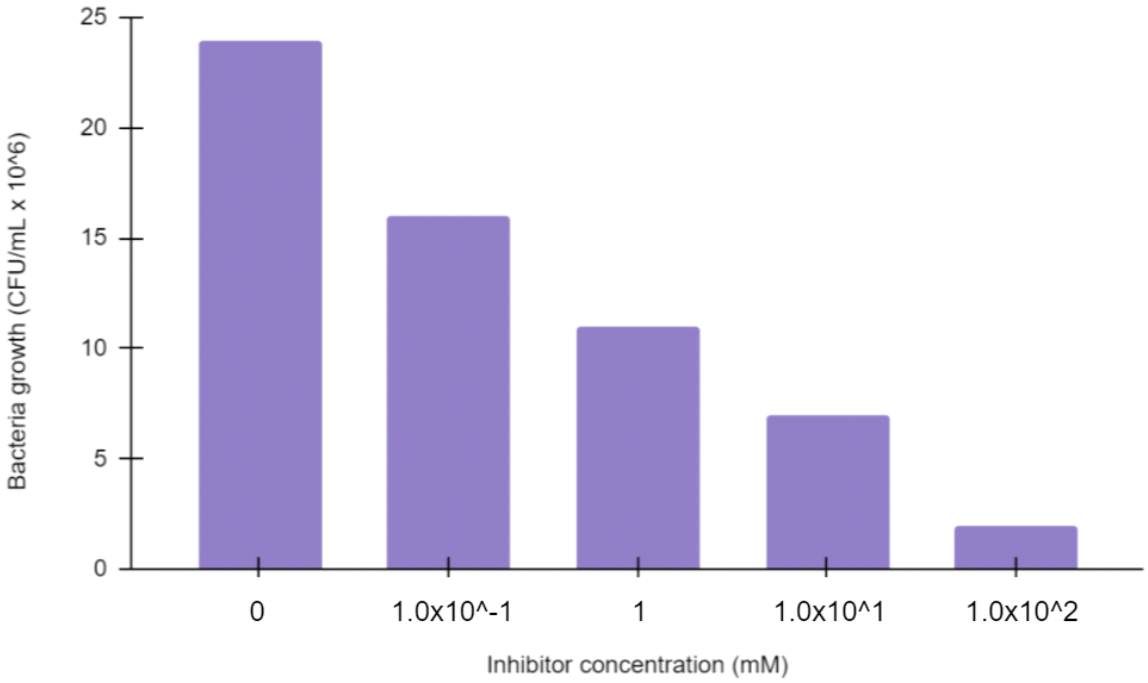

Figure 7. Theoretical mean growth of metal-resistant MRSA strain under exposure to Zn^{2+} with various concentrations of Scinapsin inhibitor over 48 hours. A similar graph would exist for the samples grown under exposure to Cd^{2+}. Figure was generated using Microsoft Excel.

Results

It is expected that Scinapsin will be successful in binding to the catalytic pocket of CadA as a small-molecule inhibitor. This would result in the inactivation of CadA-mediated efflux of zinc and cadmium, leading to cell death in metal-resistant MRSA cultures incubated at 5 0% MIC of the metals. The degree of cell death would be observed in the form of reduced mean CFU/mL counts in samples incubated at higher concentrations of Scinapsin compared to lower concentrations and the no metal controls. Depending on the inhibitory potency of Scinapsin, further increasing the concentration beyond a certain point may result in no further inhibition of metal-resistant MRSA growth (no further mean CFU/mL reduction).

According to previous literature, cadmium and zinc have been demonstrated to be similarly effective in limiting MRSA growth [20]. However, the addition of Scinapsin may restore differing degrees of MRSA's susceptibility to each of the metals. This may be unpredictable, but can be analyzed by comparing the cultures incubated with cadmium against ones incubated with zinc at identical Scinapsin inhibitor concentrations.

Discussion

If Scinapsin proves to be effective in inhibiting the growth of hospital metal-resistant MRSA strains, then this likely means that the CadA efflux pump has been successfully inactivated. As mentioned, this will appear in the form of a significant reduction in the mean CFU/mL count in MRSA samples incubated with higher concentrations of Scinapsin, compared to lower concentrations and no metal controls. By preventing zinc and cadmium from exiting the cell cytoplasm, this leads to the accumulation of cytotoxic metals that ultimately lead to the death of the bacteria.

The *in silico* docking simulation of Scinapsin to the catalytic pocket of CadA proves the theoretical efficacy of the protocol. However, it is important to note that only the relative growth of the MRSA samples was measured after exposure to Scinapsin. Further steps need to be taken to directly measure the efflux activity of CadA, such as quantifying the intracellular zinc and cadmium metal concentration or measuring the rate of metal transport. The relative binding affinities of zinc and cadmium to CadA would also need to be experimentally elucidated for comparison against Scinapsin since the MCULE software cannot predict metal ion binding affinity and no literature value could be found.

Confirming the biological mechanism of Scinapsin's inhibitory activity would support the idea that the molecule has docked correctly, rather than indirectly inhibiting growth through an unintended off-target interaction. An *in vivo* study would also account for many of the biological aspects of Scinapsin as an inhibitor that were not able to be

accounted for reliability using *in silico* analysis, such as how the open/closed conformation of the CadA ATPase may affect Scinapsin binding affinity. Given the homologies that exist between CadA and other well-characterized metal efflux ATPases, a comparison could be drawn to establish a putative "open" conformation, as well as a "closed" conformation using the structure file of the protein. Despite Phyre2's relatively accurate protein structure predictions, steps could also be taken to simulate the molecular dynamics interactions when Scinapsin binds lipid bilayer-embedded CadA. This would allow for experimental verification of the predicted structure for CadA in its native state (as opposed to structural modeling) interacting with Scinapsin.

Scinapsin has been designed to specifically bind to *S. aureus* CadA and therefore inhibit the efflux of zinc and cadmium ions. However, there are many other bactericidal metals that are used in hospitals as well. For example, silver has been used to coat the surface of endotracheal tubes during intubation procedures, which reduces the incidence of ventilator-associated pneumonia in patients [21]. Despite being an effective measure, metal resistant species of *S. aureus* were still found in patients' lungs [21]. While it is unlikely that Scinapsin could facilitate the reversal of silver resistance in these strains, the present protocol provides a framework for addressing other forms of resistance. Small-molecule inhibitors serve as a brand-new technology for interfering with important protein-ligand interactions. These inhibitors are able to exhibit narrow-spectrum, high affinity binding, minimizing the risk for off-target interactions. This can be used to target a variety of common biological targets, including ion pumps, channels, and receptors. While this is a sound premise in principle, even *in vitro* experimentation could reveal additional factors which may prevent small-molecule drugs from working effectively. For instance, bacteria in a cell culture may harbour virulence factors that allow them to enzymatically or chemically degrade compounds like Scinapsin, as they often do with many antibiotics [22]. Other confounding factors may include off-target interactions with other cell membrane proteins, as well as any unpredictable conformational changes that could occur that disrupt binding activity [23]. This will be apparent if the results of the present protocol reveal no significant reduction in MRSA BAA-2313 growth after the administration of Scinapsin in high concentrations. These limitations can be overcome with enough experimentation and structural modification of the small-molecule to strike a balance between binding affinity and *in vitro* efficacy.

It should also be noted that CadA is one of the many ways through which bacteria resist metals. As such, other antiseptic measures should still be enforced even if Scinapsin proves to be an effective inhibitor of CadA activity. In addition, it is unpredictable how a hospital MRSA population will respond to excessive amounts of efflux pump inhibitors. If misused, Scinapsin could act as a selection pressure that directs the evolution of MRSA (or other strains of similar bacteria through horizontal gene transfer) to become even more metal-resistant [24]. This can include mutations that induce the upregulated expression of alternative metal efflux pumps. For instance, there are also other P-type ATPase membrane proteins involved in MRSA metal resistance [11]. Some of these proteins like the P_{1B}-ATPase would be extremely difficult to target, as their homologues are also found in humans as a mechanism of regulating intracellular metal concentrations [25].

While not an encompassing solution to metal resistance in hospital MRSA populations, it is anticipated that Scinapsin will be effective in significantly disrupting cadmium and zinc efflux. The buildup of these metals in the cytoplasm will be sufficient in reducing proliferation and MRSA-associated infections. Scinapsin could be applied in many healthcare settings as an antiseptic chemical used to sterilize metal surfaces. By exposing metal-resistant bacteria like MRSA to both bactericidal metals as well as this efflux pump inhibitor, it is our hope that this will significantly lower the bacterial burden found on metal surfaces and medical devices. Sensitizing MRSA in this way may also allow for less heavy metal usage in hospital settings, since characteristically resistant strains would be more susceptible to them. This could resolve potential toxicity concerns that arise from having too much of these metals in the environment.

Conclusion

Given that its usage in a hospital environment has not been characterized, the efficacy and safety of Scinapsin as a method to reverse bacterial metal resistance remains in its early stages. Further investigation should aim to validate its efficacy against MRSA when administered to real hospital surfaces and medical devices, as well as its safety profile for humans. Scinapsin highlights the promising future of small-molecule inhibitors in targeting specific protein-ligand interactions, such as the one between zinc/cadmium and CadA. The core structure of Scinapsin could serve as a scaffold for the development of future metal efflux pump inhibitors that exhibit more fine-tuned affinity for particular MRSA strains, as well as even entirely different species of metal-resistant hospital microbes. Our hope is that the exploration of Scinapsin prompts further investigation into novel solutions for bacterial metal resistance before the problem worsens in the healthcare sector.

List of Abbreviations Used

Ag: silver
ANOVA: analysis of variance
Asp-414: aspartic acid at residue number 414 (of *S. aureus*/MRSA CadA)
Cd: cadmium
CFU/mL: colony forming units per millilitre
COVID-19: coronavirus disease of 2019
Cu: copper

DNA: deoxyribonucleic acid
Fe: iron
MIC: minimum inhibitory concentration
MRSA: methicillin-resistant *Staphylococcus aureus*
NCBI: National Center for Biotechnology Information
PDB: Protein Data Bank
PLIP: protein ligand interaction profiler
qPCR: quantitative polymerase chain reaction
S. aureus: *Staphylococcus aureus*
TCA cycle: trichloroacetic acid cycle

Conflicts of Interest
The author(s) declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent
This study did not require ethics approval or participant consent since it is a proposed research protocol and did not involve humans, animals, or tissues in its completion.

Authors' Contributions
DC: Significant contribution to the literature review, protocol design, drafting the manuscript, and gave final approval of the version to be published.
JW: Significant contribution to the literature review, protocol design, drafting the manuscript, and gave final approval of the version to be published.
LW: Significant contribution to the literature review, protocol design, drafting the manuscript, and gave final approval of the version to be published.
EC: Significant contribution to the literature review, protocol design, drafting the manuscript, and gave final approval of the version to be published.

Acknowledgements
We would like to thank the organizers of the 2020-2021 Scinapse Undergraduate Case Competition for providing us with the opportunity to submit our preliminary proposal and present our research.

Funding
This study was not funded.

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Article Information
Managing Editor: Jeremy Y. Ng
Peer Reviewers: Daniel Tarade, Siobhan O'Brien
Article Dates: Received May 31 21; Accepted Sep 14 21; Published Oct 5 21

Citation
Please cite this article as follows:
Wang J, Chen D, Wong L, Chung E. Computational design and lab-based investigation of a novel small-molecule inhibitor that targets CadA metal efflux pump activity in hospital methicillin-resistant *Staphylococcus aureus*: A research protocol. URNCST Journal. 2021 Oct 5: 5(10). https://urncst.com/index.php/urncst/article/view/288
DOI Link: https://doi.org/10.26685/urncst.288