Repurposing a Serotonin Receptor Antagonist as a Potential Novel Antibiotic

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TABLE OF CONTENTS

INTRODUCTION

Despite living in the golden age of novel technologies and medical advancements, antibiotic resistance continues to be one of the most pertinent challenges in the clinical and public health sector. Increased antibiotic resistance exists worldwide and untreatable infectious diseases are quickly becoming the second highest cause of deadly diseases in developing countries (Martens & Demain 2017). The major causes of antibiotic resistance are increased and improper antibiotic use in the clinical field as well as within the farming and livestock industry. Antibiotics being administered to livestock has contributed greatly to the rise of multi-drug resistant (MDR) bacterial strains, and, in turn, created problems for treating antibiotic resistant infections in humans (Church & McKillip 2021). Additionally, evidence has shown that the crisis is exacerbated by poor education on hygiene and health, inappropriate and overprescription of antibiotics in clinics, and lack of new targeted therapies or diagnostic tools on the market. The World Health Organization has released a report, stressing the urgent need for new and different strategies for managing bacterial infections (Abadi et al., 2019).

An alternative strategy to traditional antimicrobials is the targeting of bacterial virulence factors. Virulence factors are host proteins that enable a bacterial pathogen to replicate and further infect the host in part by eluding the host's immune defenses. This allows for the inhibition of pathogenesis without directly attacking bacterium growth or survival. Stripping pathogens of their virulence properties, thereby taking away their ability to defend themselves in the host, may lead to a reduction in drug-resistant mutations as there is not a direct survival pressure. Another benefit of this strategy is that virulence-specific therapeutics could aid in the protection of the host microbiota, unlike with current antibiotics, which non-specifically kill all bacteria (Cegelski et al., 2008). The use of genomic techniques has led to the identification of new virulence factors that may serve as these new targets for antibiotic and drug therapies (Cross, 2008). Exploration of an even broader framework for new antimicrobial development includes not only the strategy of targeting virulence but also the strategy of targeting bacterial *in vivo* essential gene functions, such as those gene functions that are required for survival within the host yet are distinct from those required for *in vitro* viability (Clatworthy et al., 2007).

The ClpXP protease is a highly conserved intracellular protease that is crucial to the regulation of protein turnover in many bacterial species. Previous studies have shown that loss of *clpX*, the regulatory ATPase subunit of the ClpXP protease, attenuates virulence in the fully virulent *B. anthracis* Ames strain, leading to the survival of the host upon infection (McGillivray et al., 2009). Additionally, further analysis of the *B. anthracis DclpX* mutant uncovered a role for ClpX in resistance to host antimicrobial peptide killing. More specifically, it has been

demonstrated that ClpX is important for maintaining resistance to the antimicrobials that target the cell envelope, including the antimicrobial peptide LL-37 and the antibiotics penicillin and daptomycin (McGillivray et al., 2009; Zou et al., 2021). Therefore, ClpXP inhibition exhibits a novel therapeutic strategy, as inhibiting it would make the bacterium more susceptible to both host defenses as well as clinically relevant antibiotics.

Computational modeling was performed by a graduate student in the lab to identify potential pharmacological inhibitors of ClpXP. Over 10,000 commercially available chemical compounds were screened for predicted activity against the ClpXP protease, and ten compounds were identified that represent viable candidates. Many of these candidate drugs are bioavailable and have had safety testing performed in clinical trials or have been approved as therapeutics for other diseases. Of these inhibitors, ritanserin showed the most promise (Caron unpublished data). Ritanserin was predicted to target the ClpX IGF-loop binding site on ClpP, a critical site for the stabilization of the ClpXP protease. Loss of either portion of the protease results in essentially all loss of protease activity; therefore, disruption of the interaction between ClpX and ClpP would make the protease inactive, causing the dysregulation of many essential bacterial proteins. Ritanserin was originally developed as a serotonin receptor antagonist and has previously underwent clinical trials as a potential treatment for depression, schizophrenia, and substance dependence (Boroda et al., 2017). In this paper, I explore the characterization of ritanserin as a potential inhibitor of the ClpXP protease. I hypothesize that if ritanserin inhibits the ClpXP protease in *B. anthracis* Sterne, then the wild-type bacterium should mimic the phenotypes of the knockout ClpX mutant, *ΔclpX*.

METHODS

Bacterial Strains and Reagents

B. anthracis Sterne wild type (WT) and *ΔclpX* were grown in Brain Heart Infusion (BHI) broth at 37°C under aerobic conditions unless noted otherwise. *B. anthracis* mutant, *ΔclpX*, was previously created (McGillivray et al., 2009). Ritanserin was purchased from Sigma Aldrich, resuspended in DMSO at a stock concentration of 40 mM, aliquoted into single-use aliquots, and stored at -20.

Computational Modeling

The formation of the ClpXP protease is driven by interactions between the IGF-binding loop on ClpX and its binding site on ClpP. An initial *in silico* docking study performed using Vina Wizard via PyRX docked 4,636 compounds from the Zinc database natural product ligand library against the IGF-binding loop binding domain of the *Neisseria meningitidis* ClpP (PDB ID: 6VFS) and identified 10 compounds that showed a binding affinity of -9.0 kcal/mol or less. The *N. meningitidis* ClpP structure, which shows a high level of homology to the *B. anthracis* counterpart, was used in the absence of a suitable structure from *B. anthracis*. The protein sequences of *N. meningitidis* and *B. anthracis* were aligned using BLAST.

Minimal Inhibitory Concentration (MIC) Assays:

Bacterial cultures were grown in BHI until an optical density 600nm (OD600) of 0.4 was reached. The final dilution of bacterial cultures was 1:20 for all assays unless stated otherwise. For the MIC assays using only one antimicrobial (penicillin, ritanserin, or ciprofloxacin), bacteria were added to a 96-well plate with the indicated concentrations diluted in BHI, making a final well volume of 200 µl. The samples were allowed to grow in 37°C under static conditions for 16-20 hours.

For the combination penicillin and ritanserin assays, WT *B. anthracis* and *ΔclpX* were grown in BHI until it reached an OD600 of 0.4. The log phase bacterial samples were diluted 1:10 and 100 µl was added to each well. 50 µl of 5 µg/ml penicillin, 5 µl of 600 µM ritanserin, and 45 µl of BHI were added to each well, creating a final well volume of 200 µl with final concentrations of 1:20 log phase bacteria, 15 µM ritanserin, and 1.25 µg/ml penicillin. Cultures were allowed to grow in 37°C static conditions. The growth at OD600 was measured after 16-20 hours.

For the combination daptomycin and ritanserin assays, Mueller Hinton broth (MHB) was supplemented with 50 µg/ml $CaCl_2$ to make CA-MHB. WT *B. anthracis* and *ΔclpX* were

grown in BHI until it reached an OD600 of 0.4. A 1 ml aliquot was then removed and centrifuged at 16,000 rpm for 5 minutes. The supernatant was removed, and the bacterial pellet was resuspended and washed in 1 ml of PBS. The sample was then centrifuged again at 16,000 rpm for five minutes, the PBS was removed, and the bacterial pellet was resuspended in 1ml of CA-MHB. The bacterial sample was diluted 1:10 in CA-MHB, and then 200 µl was added to 5 ml culture tubes containing 190 µl of 16.84 µg/ml daptomycin and 10 µl of 600 µM ritanserin, creating a final tube volume of 400 µl with final concentrations of 1:20 log phase bacteria, 15 µM ritanserin, and 8 µg/ml daptomycin. As a positive control, *B. anthracis* was grown in CA-MHB without the presence of daptomycin or ritanserin, and as a negative control, 400 µl of CA-MHB was incubated without any bacteria. Cultures were allowed to grow overnight in 37°C shaking conditions. The growth at OD600 value was measured after 16-20 hours.

For the combination LL-37 and ritanserin assays, Roswell Park Mark Memorial Institute (RPMI) media supplemented with 5% Luria-Bertani (LB) broth was used. WT *B. anthracis* and *ΔclpX* were grown in BHI until it reached an OD600 of 0.4. A 1 ml aliquot was then removed and centrifuged at 16,000 rpm for 5 minutes. The supernatant was removed, and the bacterial pellet was resuspended and washed in 1ml of PBS. The sample was then centrifuged again at 16,000 rpm for five minutes, the PBS was removed, and the bacterial pellet was resuspended in 1ml of RPMI + 5% LB. The bacterial sample was diluted 1:10 in RPMI + 5% LB, and 100µl was added to a 96-well plate. 50 µl of 4 µM LL-37, 5µl of 600 µM ritanserin, and 45 µl of RPMI + 5% LB were added to each well, creating a final well volume of 200 µl with 1:20 log phase bacteria, 1 µM LL-37, and 15 µM ritanserin. Cultures were allowed to grow in 37°C static conditions. The growth at OD600 was measured after 16-20 hours.

Checkerboard Assay

Ritanserin and penicillin were serially diluted to create the different concentrations. Stock solutions and dilutions of ritanserin were prepared in DMSO. Overnight cultures of WT and *ΔclpX B. anthracis* were grown to log phase at 37°C shaking until an OD600 of 0.4. 100µl of 1:10 diluted log phase bacteria, 5µl of 4x the final indicated concentrations ritanserin, and 50 µl of 4x the indicated final concentrations of penicillin were added to a 96-well plate, making a final well volume of 200µl. 5µl of DMSO was added for the column labeled with 0 µM of ritanserin to control for the presence of the vehicle control. The samples were allowed to grow in 37°C aerobic, static conditions for 16-20 hours.

RESULTS

Establishing MIC conditions

The goal of this project is to determine whether ritanserin can inhibit the ClpXP protease in *B. anthracis* Sterne. We hypothesize that if ritanserin can inhibit the protease, then wild-type *B. anthracis* Sterne will behave in a similar manner as our genetic mutant *ΔclpX* which lacks the ClpX gene and, therefore, cannot form the ClpXP protease. Loss of *clpX* has previously been shown to increase sensitivity to penicillin (Zou et al., 2021). My first step was to confirm this finding and establish the lowest concentration of penicillin that would result in the growth inhibition of *B. anthracis* wild type (WT) and the *ΔclpX* mutant. To do so, I performed a minimum inhibitory concentration (MIC) assay of *B. anthracis* Sterne WT and *ΔclpX* in the presence of the cell wall targeting antibiotic, penicillin (Figure 1). The MIC of WT was found to be 80 μg/ml, while the MIC of *ΔclpX* was 1.25 μg/ml of penicillin, thus concluding that the mutant is much more susceptible to this antibiotic.

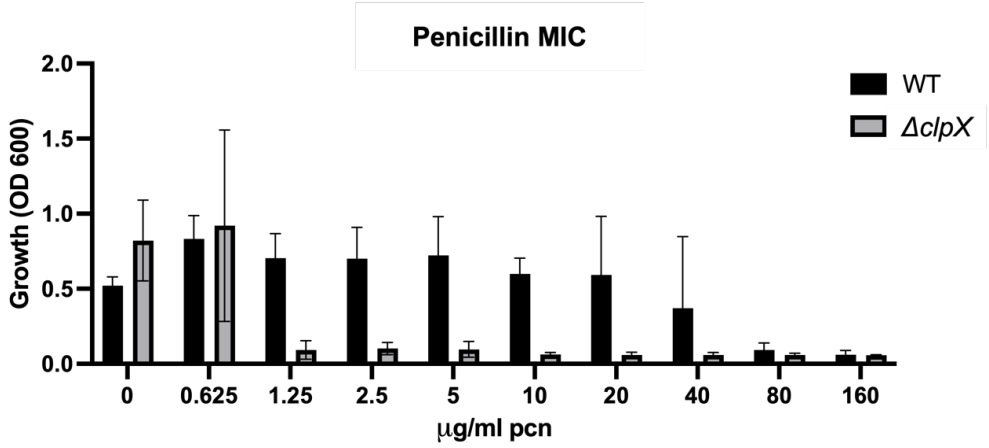

Figure 1

Growth of wildtype *B. anthracis* Sterne and *ΔclpX* in penicillin. Growth of wildtype (WT) and *ΔclpX* in increasing concentrations of penicillin. Data is presented as the mean +/- standard deviation of at least 3 independent experiments.

Our next step was to determine the effects of ritanserin alone on *B. anthracis*. The goal is not to directly kill the bacteria with ritanserin, but rather to inhibit the bacterial defense to the antibiotic. Therefore, we wanted to determine whether ritanserin would cause growth inhibition of the WT bacteria by itself (Figure 2). As can be seen in figure 2, ritanserin showed no inhibition of WT growth until a concentration of 30 μM and had an MIC of 60 μM. This shows that ritanserin can inhibit growth on its own and demonstrated that we would need to use a dose less than 30 μM in our assays.

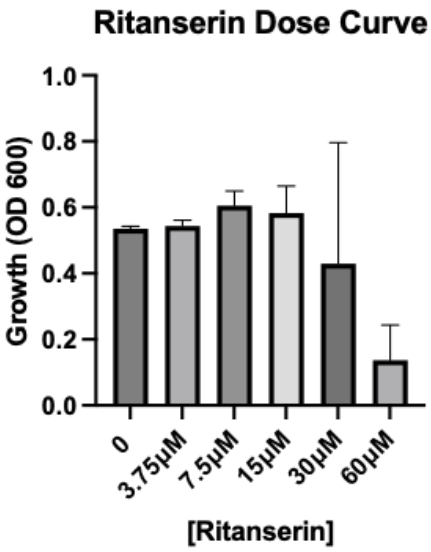

Figure 2
Growth of WT Ba in ritanserin. Growth presented as the mean +/- standard deviation of at least 3 independent experiments.

We then wanted to investigate the phenotype of WT treated with a combination of the cell envelope targeting antibiotic, penicillin, together with ritanserin. As a positive control, we also included *ΔclpX* treated with penicillin. According to our hypothesis, when treated with ritanserin and penicillin, WT *B.* anthracis Sterne would mimic the susceptibility phenotype of the *ΔclpX* mutant. A checkerboard assay was performed with a series of increasing ritanserin and penicillin concentrations performed in combination, which allowed us to determine the smallest concentrations of each that would inhibit the growth of WT (Figure 3). It was found that as little as 1.25 μg/ml of penicillin in combination with 15 μM of ritanserin showed complete inhibition of WT growth. This mimicked the MIC pattern of *ΔclpX,* which also exhibited complete inhibition at 1.25 μg/ml of penicillin.

[Ritanserin μM]

[Penicillin μg/ml]	WT 0	3.75	7.5	15	30	60
0	0.54	0.54	0.61	0.58	0.43	0.14
1.25	0.48	0.45	0.39	0.06	0.06	0.09
2.5	0.61	0.42	0.53	0.06	0.06	0.09
5	0.55	0.51	0.66	0.06	0.06	0.08
10	0.52	0.57	0.38	0.07	0.06	0.09
20	0.17	0.16	0.28	0.06	0.07	0.08
40	0.07	0.07	0.10	0.06	0.07	0.09

ΔclpX	
0	0.63
1.25	0.06
2.5	0.06
5	0.06
10	0.06
20	0.07
40	0.07

Figure 3
Ritanserin and penicillin checkerboard assay. Bacteria was grown in BHI with increasing concentrations of ritanserin and penicillin. Growth presented as the mean of at least 3 independent experiments.

Ritanserin demonstrates specificity when combined with antibiotics

Now that we had found that 15 µM of ritanserin is an effective dose, we next repeated the penicillin assay using the lowest effective dose of penicillin, 1.25 µg/ml. There was no statistical difference in the growth of untreated WT and *ΔclpX*, WT treated with penicillin alone, and WT treated with ritanserin (Figure 4). In contrast, there was significant inhibition of the WT treated with penicillin and ritanserin, and this inhibition was no different than *ΔclpX* treated with penicillin by itself. Therefore, in agreement with our hypothesis, WT mimicked the penicillin susceptibility of *ΔclpX* when treated with ritanserin.

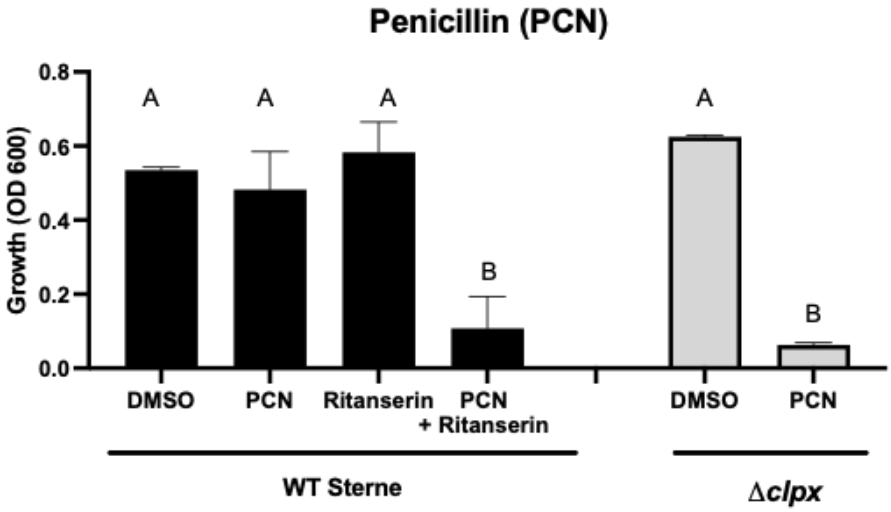

Figure 4

WT Ba showed susceptibility to penicillin in the presence of ritanserin. Growth of WT and *ΔclpX* in BHI, 1.25 µg/ml penicillin and/or 15 µM ritanserin under static conditions. OD600 reading taken at 16-20 hours. Growth is presented as the mean +/- standard deviation of at least 3 independent experiments. Statistically significant differences represented by different letters as determined by one-way ANOVA followed by Tukey's *post-hoc* test.

We then tested another cell envelope targeting antibiotic, daptomycin, to further solidify our hypothesis. It was found that WT treated with 8 µg/ml of daptomycin and 15 µM of ritanserin mimicked the growth inhibition found in *ΔclpX* treated with 8 µg/ml of daptomycin (Figure 5). In contrast, there was no statistical difference in the growth of untreated WT and *ΔclpX*, WT treated with daptomycin alone, and WT treated with ritanserin alone. This further solidifies that loss of *clpX* results in increased susceptibility to cell envelope targeting antibiotics specifically.

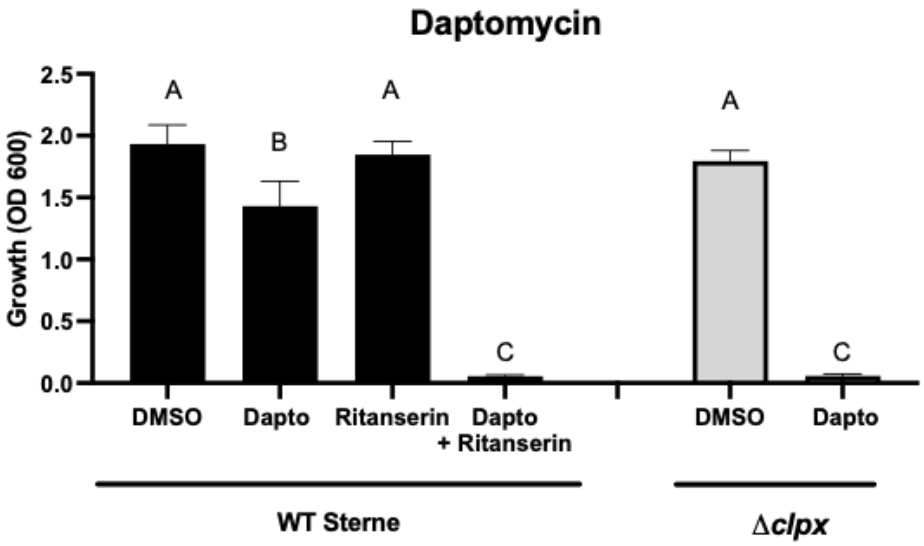

Figure 5
WT Ba showed susceptibility to daptomycin in the presence of ritanserin. Growth of WT and *ΔclpX* in CA-MHB, 8 µg/ml daptomycin and/or 15 µM ritanserin. Growth is presented as the mean +/- standard deviation of at least 3 independent experiments. Statistically significant differences represented by different letters as determined by one-way ANOVA followed by Tukey's *post-hoc* test.

As a negative control, we wanted to explore the effects of the inhibition of ClpXP when treated with non-cell envelope targeting antibiotics, such as ciprofloxacin. Previous studies from our lab have demonstrated that the role of ClpXP in antimicrobial resistance is specific to cell envelope targeting antibiotics and no change in susceptibility was seen in the *ΔclpX* mutant with ciprofloxacin (McGillivray et al., 2012). If ritanserin is disrupting the interaction between ClpX and ClpP, then WT treated with the combination of ritanserin and ciprofloxacin should show no statistical difference in growth to untreated WT and WT treated with ciprofloxacin or ritanserin alone. Additionally, in theory, there should be no statistical difference between the growth of untreated *ΔclpX* and *ΔclpX* treated with ciprofloxacin. We assayed bacterial growth at three different concentrations of ciprofloxacin, and as hypothesized, found no statistical difference in the growth of the three test groups at any concentration (Figure 6). This demonstrates that ritanserin is mimicking the phenotypes of the *ΔclpX* mutant.

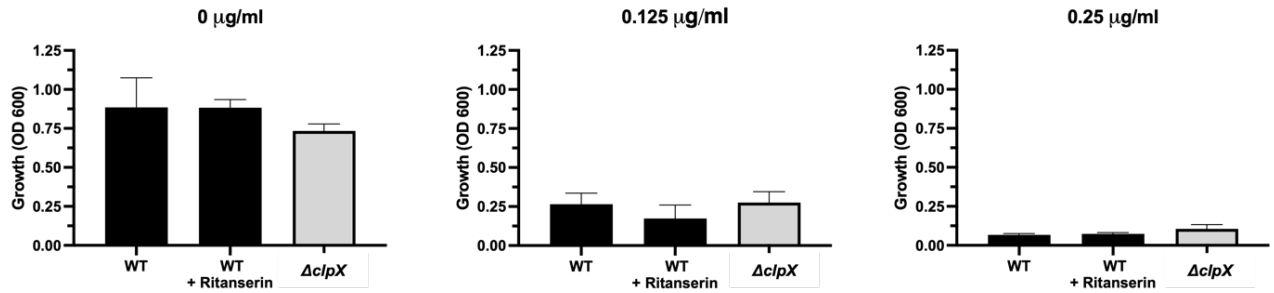

Figure 6

No difference in susceptibility is seen with ciprofloxacin. Growth of WT and *ΔclpX* in BHI, increasing concentrations of ciprofloxacin (0, 0.125, and 0.25 μg/ml) and 15μM ritanserin. Growth is presented as the mean +/- standard deviation of at least 3 independent experiments.

Inhibition of ClpXP protease results in susceptibility to host defenses.

Loss of ClpX has also been previously found to increase bacteria susceptibility to host defenses, such as antimicrobial peptides LL-37 (Zou et al., 2021). These are naturally made in the host as a defense mechanism, aiding in the killing of bacteria; therefore, if WT treated with ritanserin mimics the susceptibility of *ΔclpX* to LL-37, this would provide evidence that ritanserin could work as an antivirulence drug in an *in vivo* model. We assayed this using 1 μM of LL-37 and 15 μM of ritanserin, and we found that WT treated with the combination of LL-37 and ritanserin showed susceptibility and inhibition of growth similarly to *ΔclpX* treated with LL-37 (Figure 7).

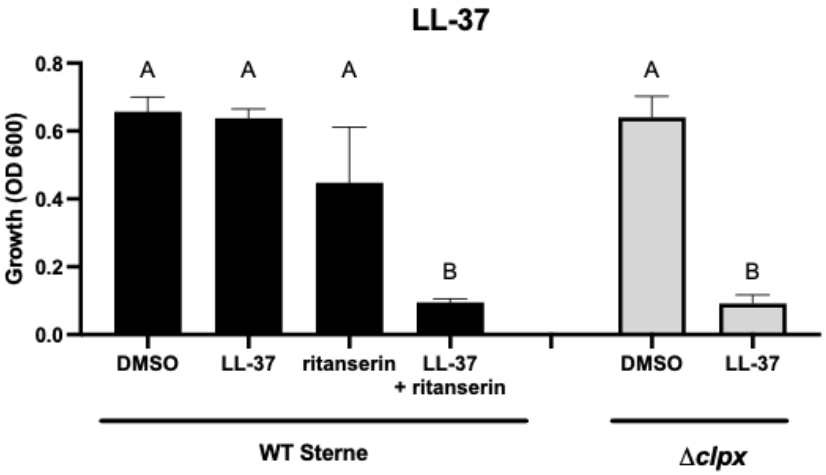

Figure 7.

Ba WT showed susceptibility to antimicrobial peptide LL-37 in the presence of ritanserin. Growth of WT and *ΔclpX* in RPMI+5% LB, 1 μM LL-37 and/or 15 μM ritanserin. Growth is presented as the mean +/- standard deviation of at least 3 independent experiments. Statistically significant differences represented by different letters as determined by one-way ANOVA followed by Tukey's *post-hoc* test.

DISCUSSION

Our objective was to find a drug that would inhibit the ClpXP protease, by disrupting the interaction between ClpX and ClpP, thus rendering the bacteria more susceptible to host defenses and antibiotics. In doing so, we did not want to use a concentration of ritanserin that would actively kill or inhibit the growth of the bacteria when administered by itself. Instead, we are using an anti-virulence strategy that involves targeting bacterial virulence factors, thus taking away the bacteria's ability to defend itself within the host or when treated in combination with antibiotics (Cegelski et al., 2008). While antibiotics that target essential bacterial functions have proven effective in the past, these modes of action create a selective pressure, fostering the development of antibiotic-resistant mutations. Therefore, if we simply disarm the pathogen, this may relieve that selective pressure and reduce the incidences of drug-resistant strains. Additionally, anti-virulence therapies could reduce the impact on the normal host microbiota, which are also being targeted and killed by antimicrobials (Clatworthy et al., 2007). Our results are consistent with those we would expect if ritanserin is inhibiting ClpXP formation.

While we believe that ritanserin is binding to its intended target, ClpXP, it could potentially have off-target effects as well. This is best portrayed by the results found in our ritanserin dose curve, notably that ritanserin administered at a concentration of 60 μM by itself inhibited the growth of WT bacteria. This is contrary to what we see with loss of ClpXP, which is not detrimental unless cell envelope targeting antibiotics are present. Therefore, ritanserin might be exhibiting off-target effects, specifically at higher doses. Future directions will include testing some additional *ΔclpX* associated phenotypes, including examining other stress-associated deficits and changes in expression of genes known to be regulated by *ΔclpX*. However, to see the extent of off-target effects, one possibility would be to investigate changes in global gene expression. Specifically, we would examine the overlap of expression patterns between WT treated with ritanserin and *ΔclpX*. If ritanserin is inhibiting primarily ClpXP formation, the changes in gene expression between the two should be similar. If there are significant changes seen only in the ritanserin treated cells but not *ΔclpX,* that could indicate that other pathways are being targeted. As long as the dose we are using is significantly less than the ritanserin toxicity threshold, it may not matter if there are some off-target effects as the primary objective is to eliminate the pathogen. The ability to use ritanserin at lower concentrations, especially in combination with other antibiotics, should minimize any potential toxicity, particularly when the patient would likely not be taking this treatment for longer than about a two-week period.

Although not all anti-virulence strategies are used in combination with antibiotics, this might be particularly useful with ritanserin. The results of our penicillin and daptomycin assays have shown that WT bacteria treated with a combination of ritanserin and cell envelope antibiotics increases the susceptibility of the bacteria to the antibiotic, thus allowing for growth inhibition at lower concentrations of both drugs. A combination treatment therapy may also reduce instances and rates of antibiotic resistance, as it has already been proven that synergy and drug combinations are a winning strategy in fighting multi-drug resistant bacteria (Annunziato, 2019). Additionally, using antibiotic adjuvants might help in protecting the already existing drugs from gaining further resistance. Antibiotic adjuvants are molecules with weak or absent antimicrobial activity but possess the ability to enhance the activity of antibiotics (Annunziato, 2019). Antibiotic adjuvants can also suppress intrinsic resistance, leading to an expansion of the activity spectrum of antibiotics. As discussed, antibiotic adjuvants that increase bacterial susceptibility to antibiotics also allow them to be efficacious at lower doses, thereby mitigating potential side effects (Annunziato, 2019). Therefore, we believe that ritanserin could be used therapeutically in tandem with other antibiotics.

There is much more research to be done with ritanserin before it reaches the point of becoming an antimicrobial used in the clinical setting. However, the fact that ritanserin has made it through phase III of clinical trials means that its overall toxicity is low. In addition, the success seen when used in combination with LL-37, a host antimicrobial peptide, provides some evidence that ritanserin could be effective in an *in vivo* model. Our next step will be to test the efficacy of ritanserin in our invertebrate model, *Galleria mellonella,* which uses the wax worm as an animal infection model. We have previously shown that *ΔclpX* is attenuated for virulence relative to the WT *B. anthracis* (Malmquist & McGillivray, 2019). Based upon those results, we hypothesize that *G. mellonella* infected with WT *B. anthracis* pretreated with ritanserin would have a similar survival as those *G. mellonella* infected with *ΔclpX*. If our hypothesis holds, this would provide more definitive evidence that ritanserin could be successfully used in an anti-virulence strategy.

The results of this study also support the idea that we could potentially repurpose current commercially available chemical compounds or drugs, specifically those that have undergone extensive clinical trial testing, as potentially novel antimicrobials. Not only would this save valuable time and money in the drug development and deployment process, it could also ensure that these therapies have the ability to be therapeutically significant in humans. A previously identified pharmacological inhibitor of the ClpXP protease, F2, exhibited synergistic antimicrobial activity with cell wall and membrane targeting antimicrobial peptides and

antibiotics, such as penicillin and daptomycin, in *B. anthracis* Sterne (McGillivray et al., 2012). However, the addition of human serum completely inactivated F2. Therefore, while F2 showed promise for ClpXP inhibition *in vitro*, it could never be applied *in vivo* (McGillivray et al., 2012). In contrast, ritanserin has previously undergone stage III clinical trials and exhibits an ideal half-life activity *in vivo*; therefore, ritanserin has already cleared some of the hurdles necessary for drug development. Screening and analyzing chemical compounds that have undergone extensive clinical testing could potentially offer a better chance of having therapeutic significance *in vivo*, as well as alleviate some of the expensive requirements and red tape that a new antibiotic must go through, such as clinical trials and FDA approval.

This study also demonstrates the potential applications of structure-based virtual screening (SBVS), or molecular docking. This technique has been applied in the discovery of small-molecule ligands based upon specific protein structures and is increasingly being utilized to find new targets of interest in the early stages of drug discovery. Particularly, molecular docking identifies small molecules that are complementary to the target's ligand-binding pocket with a known 3D structure from a large chemical library. The most significant advantage of molecular docking is its ability to identify novel chemotypes effectively and efficiently from a large chemical library against a target of interest, and in the case of this study, against the ClpXP protease. The discovery of novel chemotypes that were previously unrelated to the known target ligands might offer new biological consequences (Zhu et al., 2022). Through this study, we have shown the success of SBVS and displayed the potential for repurposing already existing chemical compounds for anti-virulence or antimicrobial therapies. Moreover, rather than starting from the very beginning of the drug design process, we could save a lot of time and money by redirecting compounds to a different target at later stages in the deployment process. Overall, this study demonstrates the potential success of repurposing commercially available chemical compounds for antimicrobial use, a concept that is particularly critical in this time of antibiotic crisis.

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