

REPURPOSING A SEROTONIN RECEPTOR ANTAGONIST
AS A POTENTIAL NOVEL ANTIBIOTIC

by

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AS A POTENTIAL NOVEL ANTIBIOTIC

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ABSTRACT

With the surge of multidrug resistant bacteria and increasing antibiotic resistance, there is a critical need for the development of new drug therapies. A new antimicrobial technique revolves around targeting virulence factors, which enable the bacterial pathogen to evade host immune defenses. Inhibitors that target pathogenicity hinder the capacity of the bacterium to cause an infection, thus allowing the host immune system to better clear the infection. In this study, we aim to inhibit the ClpXP protease, a highly conserved intracellular protease involved in the virulence in different bacterial pathogens. Previous studies have shown that loss of ClpX completely attenuates virulence in *Bacillus anthracis*, rendering the pathogen more susceptible to cell envelope targeting antibiotics such as penicillin, daptomycin and LL-37. Computational modeling was performed and ten commercially available inhibitors with predicted activity against the ClpXP protease were identified, with ritanserin showing the most promise. In this study we explore the antimicrobial effects of ritanserin, a previously identified serotonin 2A receptor antagonist that underwent clinical trials as a potential treatment for depression, schizophrenia, and substance dependence. We hypothesized that if ritanserin inhibits ClpXP formation in *B. anthracis* Sterne it should mimic the phenotype of the knockout *clpX* mutant, $\Delta clpX$. We found that ritanserin increased WT *Bacillus anthracis* susceptibility to the cell envelope targeting antibiotics penicillin and daptomycin. Future studies showed increased susceptibility to host defenses, such as antimicrobial peptides, including LL-37. This demonstrates that ritanserin could be potentially repurposed as an antibacterial drug with the potential to be used by itself or in combination with antibiotics.

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INTRODUCTION

Despite living in the golden age of novel technologies and medical advancements, antibiotic resistance continues to be one of the most pertinent challenges of 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 and 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 and McKillip 2021). Additionally, evidence has shown that the crisis is exacerbated by poor education on hygiene and health, inappropriate and over prescription 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, Rizvanov 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 host 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 its ability to defend itself 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, Marshall et al. 2008). The use of genomic techniques have 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 that are distinct from those required for *in vitro* viability (Clatworthy, Pierson and Hung 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, Ebrahimi et al. 2009). Additionally, further analysis of the *B. anthracis* $\Delta clpX$ 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, Ebrahimi et al. 2009, Zou, Evans 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 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, Niccum 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 wildtype bacterium should mimic the phenotypes of the knockout ClpX mutant, $\Delta clpX$.

METHODS

Bacterial Strains and Reagents

B. anthracis Sterne wild type (WT) and $\Delta clpX$ was grown in Brain Heart Infusion (BHI) broth at 37°C under aerobic conditions unless noted otherwise. *B. anthracis* mutant, $\Delta clpX$, was previously created (McGillivray, Ebrahimi 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 μ l. 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 $\Delta clpX$ were grown in BHI until it reached an OD600 of 0.4. The log phase bacterial samples were diluted 1:10 and 100 μ l was added to each well. 50 μ l of 5 μ g/ml penicillin, 5 μ l of 600 μ M ritanserin, and 45 μ l of BHI were added to each well, creating a final well volume of 200 μ l with final concentrations of 1:20 log phase bacteria, 15 μ M ritanserin and 1.25 μ g/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 μ g/ml CaCl₂ to make CA-MHB. WT *B. anthracis* and $\Delta clpX$ 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 μ l was added to 5 ml culture tubes containing 190 μ l of 16.84 μ g/ml daptomycin and 10 μ l of 600 μ M ritanserin, creating a final tube volume of 400 μ l with final concentrations of 1:20 log phase bacteria, 15 μ M ritanserin, and 8 μ g/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 μ l 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 $\Delta clpX$ 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 100 μ l was added to a 96-well plate. 50 μ l of 4 μ M LL-37, 5 μ l of 600 μ M ritanserin, and 45 μ l of RPMI + 5% LB were added to each well, creating a final well volume of 200 μ l with 1:20 log phase bacteria, 1 μ M LL-37, and 15 μ M 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 $\Delta clpX$ *B. anthracis* were grown to log phase at 37°C shaking, until an OD600 of 0.4. 100 μ l 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 $\Delta clpX$ 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, Evans 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 $\Delta clpX$ mutant. To do so, I performed a minimum inhibitory concentration (MIC) assay of *B. anthracis* Sterne WT and $\Delta clpX$ 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 $\Delta clpX$ was 1.25 µg/ml of penicillin, thus concluding that the mutant is much more susceptible to this antibiotic.

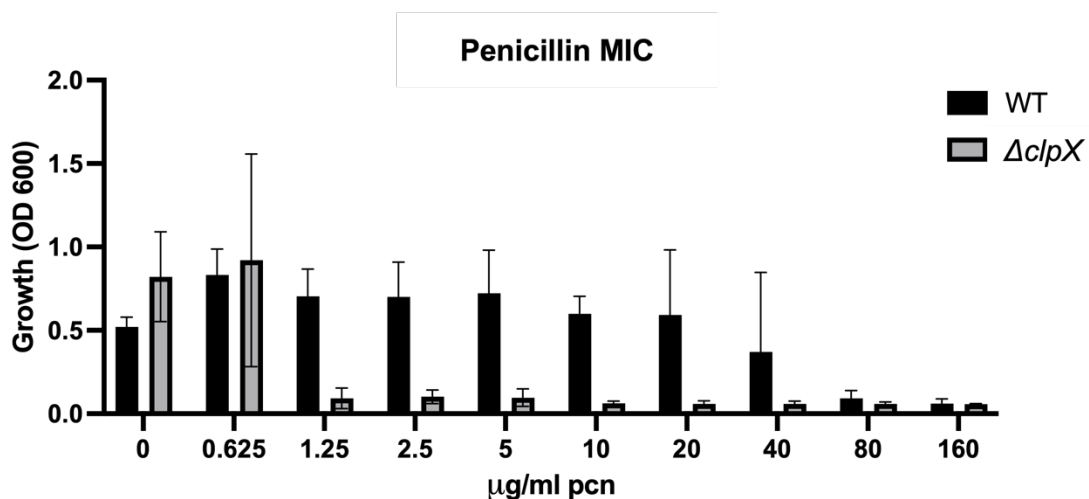


Figure 1. Growth of wildtype *B. anthracis* Sterne and $\Delta clpX$ in penicillin. Growth of wildtype (WT) and $\Delta clpX$ in increasing concentrations of penicillin. Data is presented as the mean +/- standard deviation of at least 3 independent experiments.

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 30 μ M and had an MIC of 60 μ M. This shows that ritanserin can inhibit growth on its own and demonstrated that we would need to use a dose less than 30 μ M in our assays.

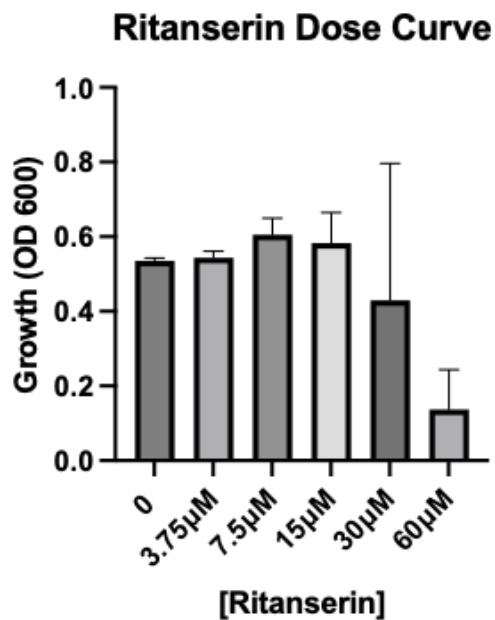


Figure 2. Growth of WT Ba in ritanserin. Growth presented as the mean +/- standard deviation of at least 3 independent experiments.

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 $\Delta clpX$ treated with penicillin. According to our hypothesis, when treated with ritanserin and penicillin, WT *B. anthracis* Sterne would mimic the susceptibility phenotype of the $\Delta clpX$ 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 $\mu\text{g/ml}$ of penicillin in combination with 15 μM of ritanserin showed complete inhibition of WT growth. This mimicked the MIC pattern of $\Delta clpX$ which also exhibited complete inhibition at 1.25 $\mu\text{g/ml}$ of penicillin.

		[Ritanserin μM]							
WT		0	3.75	7.5	15	30	60	$\Delta clpX$	
[Penicillin $\mu\text{g/ml}$]	0	0.54	0.54	0.61	0.58	0.43	0.14	0	0.63
	1.25	0.48	0.45	0.39	0.06	0.06	0.09	1.25	0.06
	2.5	0.61	0.42	0.53	0.06	0.06	0.09	2.5	0.06
	5	0.55	0.51	0.66	0.06	0.06	0.08	5	0.06
	10	0.52	0.57	0.38	0.07	0.06	0.09	10	0.06
	20	0.17	0.16	0.28	0.06	0.07	0.08	20	0.07
	40	0.07	0.07	0.10	0.06	0.07	0.09	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 $\mu\text{g/ml}$. There was no statistical difference in the growth of untreated WT and $\Delta clpX$, 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 $\Delta clpX$ treated with penicillin by itself. Therefore, in agreement with our hypothesis, WT mimicked the penicillin susceptibility of $\Delta clpX$ when treated with ritanserin.

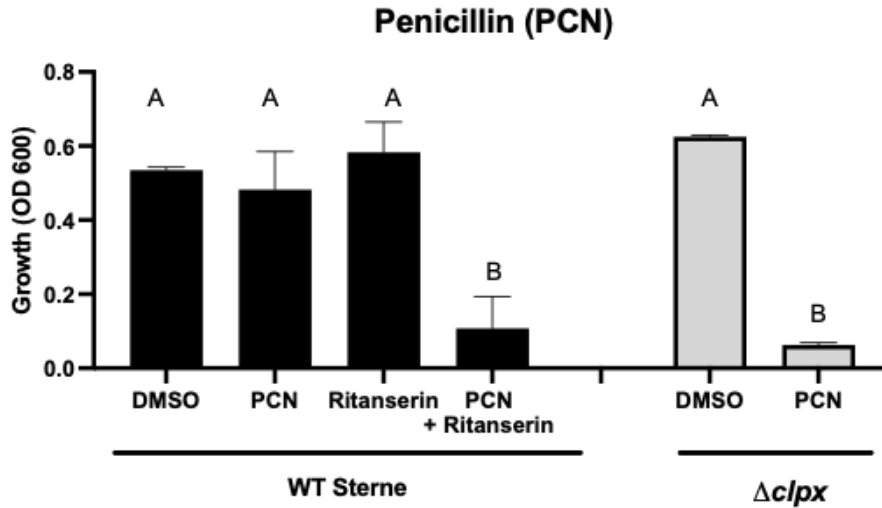


Figure 4. WT Ba showed susceptibility to penicillin in the presence of ritanserin. Growth of WT and $\Delta clpX$ in BHI, 1.25 $\mu\text{g/ml}$ penicillin and/or 15 μM ritanserin under static conditions. OD600 reading taken at 16-20 hours. Growth is presented as the mean \pm standard deviation of at 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 $\mu\text{g/ml}$ of daptomycin and 15 μM of ritanserin mimicked the growth inhibition found in $\Delta clpX$ treated with 8 $\mu\text{g/ml}$ of daptomycin (Figure 5). In contrast, there was no statistical difference in the growth of untreated WT and $\Delta clpX$, 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.

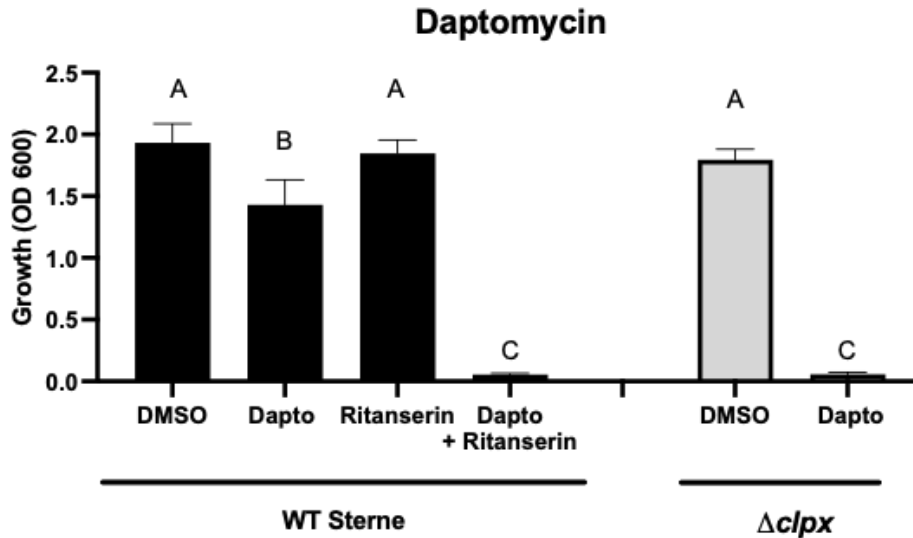


Figure 5. WT Ba showed susceptibility to daptomycin in the presence of ritanserin. Growth of WT and $\Delta clpX$ in CA-MHB, 8 $\mu\text{g/ml}$ daptomycin and/or 15 μM ritanserin. Growth is presented as the mean \pm standard deviation of at least 3 independent experiments. Statistically significant differences 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 $\Delta clpX$ 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 $\Delta clpX$ and $\Delta clpX$ 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 $\Delta clpX$ mutant.

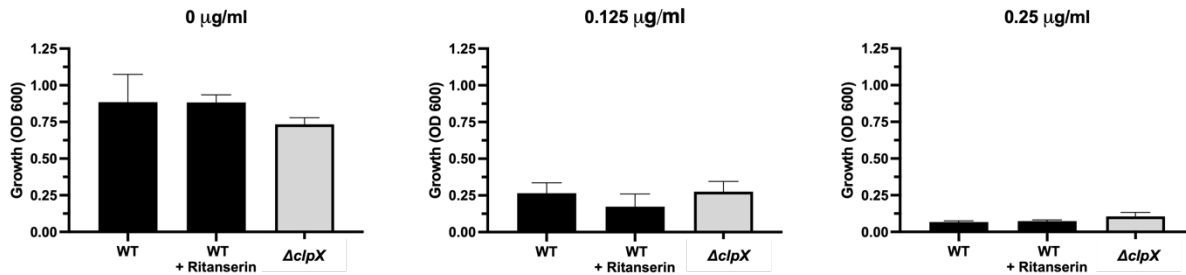


Figure 6. No difference in susceptibility is seen with ciprofloxacin. Growth of WT and $\Delta clpX$ in BHI, increasing concentrations of ciprofloxacin (0, 0.125, and 0.25 $\mu\text{g/ml}$) and 15 μM ritanserin. Growth is presented as the mean \pm standard deviation of at least 3 independent experiments.

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, Evans et al. 2021). These are naturally made in the host as a defense mechanism, aiding in the killing bacteria; therefore, if WT treated with ritanserin mimics the susceptibility of $\Delta clpX$ 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 $\Delta clpX$ treated with LL-37 (Figure 7).

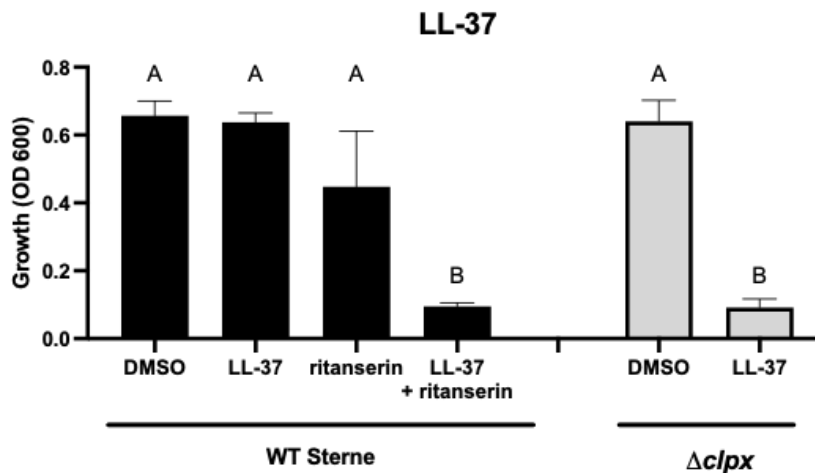


Figure 7. WT showed susceptibility to antimicrobial peptide LL-37 in the presence of ritanserin. Growth of WT and $\Delta clpX$ in RPMI+5% LB, 1 μM LL-37 and/or 15 μM ritanserin. Growth is presented as the mean \pm standard deviation of at least 3 independent experiments. Statistically significant differences 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, Marshall 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, Pierson and Hung 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 μ M 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 *Δ clpX* associated phenotypes including examining other stress-associated deficits and changes in expression of genes known to be regulated by *Δ clpX*. 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 $\Delta clpX$. 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 $\Delta clpX$, 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 antibiotic 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 $\Delta clpX$ is attenuated for virulence relative to the WT *B. anthracis* (Malmquist, Rogan and 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 $\Delta clpX$. 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, Tran 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, Tran 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, Zhang 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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