Investigating the Role of the Antimicrobial Peptide TTO-53 in the Disruption of *Pseudomonas aeruginosa* Biofilm

By

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ABSTRACT

Pseudomonas aeruginosa infections are commonly acquired in hospital settings and are oftentimes severe and deadly. *P. aeruginosa* is known for its resistance to a wide range of antibiotics due to the low permeability of its outer membrane, possession of various efflux pumps and modifying enzymes, and its ability to form biofilms. Biofilms are aggregates of bacterial cells enclosed in an extracellular matrix. This extracellular matrix is composed of an assortment of extracellular polymeric substances (EPS's) that can interact with and influence the rate of transport of antimicrobial agents. Previous research using a synthetic antimicrobial peptide made of unnatural amino acids named TTO-53 has shown its ability to disrupt pre-established *P. aeruginosa* biofilm. The goal of this study is to investigate how this disruption is taking place and the influence of this peptide on the efficacy of antibiotics in killing *P. aeruginosa.* Due to the role of quorum sensing, a communication system between cells in biofilm, in the maintenance of biofilms, we hypothesized that exposing *P. aeruginosa* to TTO-53 causes disruption of quorum sensing resulting in dispersion of pre-established biofilm. To test this hypothesis, we used quantitative real-time PCR (qRT-PCR) to determine if there was differential expression in genes within various quorum sensing pathways. Results from qRT-PCR

showed that exposing overnight cultures to TTO-53 did not cause differential expression in genes within the LasR, PQS, and RhlI/RhlR quorum sensing pathways. To investigate the influence of TTO-53 on the efficacy of various antibiotics, we exposed cultures of PAO1 to varying concentrations of each antibiotic and compared the colony-forming units per milliliter (cfu/mL) of those cultures to the cfu/mL of cultures treated with those same concentrations of each antibiotic and TTO-53. These results indicated TTO-53 increases the efficacy of Chloramphenicol and Tazobactam/Piperacillin, while decreasing the efficacy of Tobramycin. This study revealed that an hour-long exposure to TTO-53 does not impact *P. aeruginosa* quorum sensing pathways and the combination treatment of TTO-53 has a synergistic effect with some antibiotics while inhibiting others.

Investigating the Role of the Antimicrobial Peptide TTO-53 in the Disruption of *Pseudomonas aeruginosa* Biofilm

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Pseudomonas aeruginosa

According to the Center for Disease Control (CDC), in 2017 *Pseudomonas aeruginosa* caused approximately 32,600 nosocomial infections and 2,700 estimated deaths in the United States alone[3]. *Pseudomonas aeruginosa* is an opportunistic pathogen commonly acquired in hospital settings. This bacterium thrives in moist environments, such as soil and water, and is easily transmitted between patients through medical equipment or from the hands of healthcare personnel[3]. *P. aeruginosa* is responsible for an assortment of different infections including Urinary Tract Infections (UTIs), Ventilator-associated Pneumonia (VAP), Surgical Site Infections (SSI), and bloodstream infections[4]. *P. aeruginosa* have flagella and type IV pili external appendages allowing it to be motile and aiding in its pathogenicity[1]. Gram-negative bacteria, like *P. aeruginosa,* have an outer membrane containing lipopolysaccharides that is not seen in gram-positive organisms. This outer membrane encourages the adherence of bacteria to surfaces and acts as a barrier protecting bacteria from toxic compounds, such as antibiotics, it encounters. Another important feature of *P. aeruginosa* is its ability to form biofilm.

Biofilms

Biofilms are aggregates of bacterial cells enclosed in an extracellular matrix composed of extracellular polymeric substances (EPS)[25]. Biofilms can be formed by single species or multiple species, and their composition depends on the types of species involved. Approximately 90% of bacterial species can form biofilms[37]. There are many reasons biofilm formation is more favorable than organisms remaining planktonic, one of which being the protection provided by the extracellular matrix. The biofilm extracellular matrix makes up most of the total biofilm biomass. The EPS in this matrix are polysaccharides, extracellular proteins, extracellular DNA

(eDNA), and lipids. These components of the matrix can influence the rate of transport of antimicrobial agents by interacting with the molecule, making it more difficult for the molecule to reach the microbial cells[13]. Along with the extracellular matrix, biofilms have pores and channels allowing for transportation of nutrients, water, signaling molecules, and waste[13]. These pores and channels enhance the survival of microbial cells within biofilm by providing them with the nutrients and water needed to survive.

There are four main stages of biofilm development: initial reversible attachment, irreversible attachment, maturation, and dispersion as shown in figure 1. To begin, microbial cells will reversibly attach to a substratum using adhesins such as flagella or pili[37]. Electrostatic attractions, hydrophobic interactions, Van der Waal forces all play a role in initial attachment of the bacterial cells to the substratum[26]. During the initial reversible attachment phase, bacterial cell attachment can be easily disrupted. After initial reversible attachment, stronger interactions such as hydrogen, ionic, covalent bonds, and adhesion via cell appendages allow for irreversible attachment of bacterial cells to the substratum. Appendages such as flagella, pili, curli, and fimbriae are small enough in size that they can overcome repulsive electrostatic interactions aiding in strong cell attachment[29].

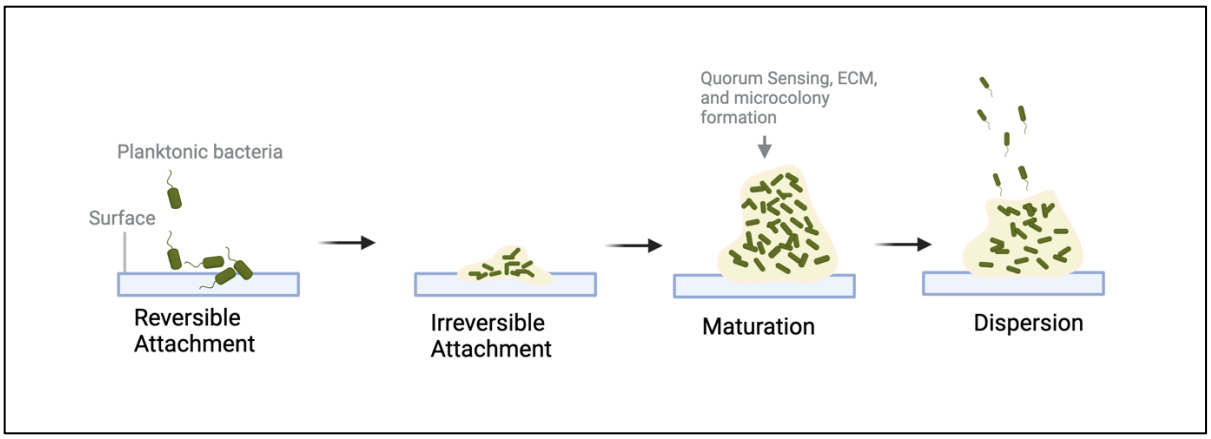

Figure 1: The four main stages of biofilm development: reversible attachment, irreversible attachment, maturation, and dispersion.

The maturation phase of biofilm formation begins with the production of EPS regulated by a cell-to-cell communication process in which cells can sense the number of surrounding microbial cells; this process is referred to as quorum sensing. Following the initial production of EPS, small communities of cells called microcolonies form. As EPS production increases, an extracellular matrix form. The cells begin functioning like a community with changes in gene expression and growth resembling planktonic cell stationary phase growth. The final stage in the biofilm life cycle is dispersion in which cells detach to form more biofilm elsewhere. The different modes of dispersal are erosion, sloughing, and seeding[24]. Erosion involves continuous release of cells that occurs over extended period, seeding involves release of individual cells or small groups of cells at a time, and sloughing is sudden detachment of a large portion of the biofilm[24].

Pseudomonas aeruginosa Biofilm

P. aeruginosa biofilm composition is strain-specific, and the assortment of lipids and proteins varies greatly across strains. The largest component of *Pseudomonas* biofilm is exopolysaccharides, making up 90% of the biofilm biomass[46]. The three main

exopolysaccharides composing these biofilms include Psl, Pel, and alginate[46]. Prior research into each of these exopolysaccharides has shown their involvement in cell adhesion and EPS matrix development. Some exopolysaccharides can also keep antimicrobial agents from reaching cells by binding and inactivating them. Each strain of *P. aeruginosa* uses different ratios of these exopolysaccharides. For example, PA01 primarily utilizes Psl while PA14 is not capable of using Psl and instead uses Pel[6].

The exopolysaccharide Psl is composed of the monosaccharides D-glucose, D-mannose, and L-rhamnose[46]. Studies of *P. aeruginosa* mutants with *psl* knockouts have shown reduced biomass of biofilms and decreased substratum attachment exemplifying its role in surface attachment and biofilm formation[46]. Proteins encoded by the psl operon containing genes pslACDEFGHIJKL are involved in psl synthesis[50]. Expression of the Psl operon is repressed by the flagellar transcriptional regulator FleQ and regulated by the sigma factor RpoS[20]. In most strains of *P. aeruginosa*, the psl exopolysaccharide is the main polysaccharide component of biofilms.

Pel has a net positive charge and is composed of 1→4 partially acetylated galactosamine and glucosamine sugars[23]. Pel is synthesized by the products of genes in the *pel* operon containing *PelA-F*[49]. In most strains of *P. aeruginosa,* Psl is the main exopolysaccharide aiding in biofilm formation and stability. However, as mentioned above, in strains such as PA14 that are incapable of producing Psl, Pel assumes the roles normally held by Psl[23]. Previous studies have shown Pel to be mainly localized within the microcolonies of biofilm and thought to aid in the structural stability of the core of microcolonies[23]. Through ionic bonding, Pel can cross-link eDNA and other negatively charged polymers[23]. Research into the influence of Pel on the

antibiotic resistance of *P. aeruginosa* has displayed that it plays a role in protecting bacterial cells from aminoglycoside antibiotics[5].

Alginate is a linear polysaccharide composed of D-mannuronic acid and L-guluronic acids[14]. Alginate is not as abundant of an exopolysaccharide as pel or psl, and its production is only initiated once *P. aeruginosa* cells attach to a surface. One crucial role of alginate within biofilm is preventing the desiccation of the bacterial cells. This is accomplished by the acetylated uronic acids in alginate keeping cells within the biofilm hydrated. Proteins encoded by the genes *algD, alg8, alg44, algK, algE, algG, algX, algL, algI, algJ, algF*, and *algA* are involved in the synthesis of alginate. Although alginate is important in the maintenance of biofilm architecture, an enzyme produced by the *algL* gene, alginate lyase, is involved in disassembly of biofilm. The alginate lyase enzyme aids in biofilm disassembly by breaking the glycosidic bonds that hold alginate together[51].

Quorum Sensing

The formation and dispersal of biofilm is mediated by a special communication between cells in biofilm referred to as quorum sensing. Quorum sensing involves signaling molecules called autoinducers which bind to various receptors. The main quorum-sensing systems involved in *Pseudomonas aeruginosa* biofilm are the Las, Rhl, and PQS systems. Figure 2 depicts the interconnections of all three quorum sensing systems as well as the products produced by genes within these pathways. The Las quorum-sensing system utilizes the signaling molecule N-3-oxododecanoyl homoserine lactone (3OC12-Hsl). This signaling molecule is synthesized by an acyl-homoserine-lactone synthase protein known as LasI[35]. Once 3OC12-Hsl is synthesized, it binds to the LasR receptor and forms a complex that activates the transcription of genes such as *lasA, lasB, lecA, lecB, aprA, toxA, xcp, etc.* The *lasA* gene produces a protease and appears to

play a role in activation of elastase, produced by *lasB,* however little is known about its function. Elastase is a protease enzyme involved in attachment of bacteria to surfaces and to each other, formation of microcolonies, and production of the extracellular matrix within biofilms. *LecA* and *lecB* produce lectins which are carbohydrate-binding proteins allowing for linkage of exopolysaccharides forming the biofilm matrix structure[48]. Although little is known about the role of the products produced by *aprA* and *toxA,* alkaline metalloproteinase precursor and exotoxin A, in biofilm development it is suggested that they play a role in the pathogenicity of biofilm-producing cultures[47].

The Rhl quorum-sensing system utilizes the signaling molecule C4-HSL which is synthesized by an autoinducer synthesis protein known as RhlI. The signaling molecule C4-HSL binds to a transcriptional regulator known as RhlR creating a complex that activates transcription of the following genes: *apr, rpoS, lecA, lecB, lasA, lasB.* Although the product of the *apr* gene is unknown, the *rpoS* gene encodes a sigma factor referred to as σ^s. This sigma factor has been identified as a central regulator during the stationary phase of *P. aeruginosa*[27].

The final quorum sensing signaling pathway within *Pseudomonas aeruginosa* is the *pseudomonas* quinoline system (PQS). The PQS system has been identified as the signaling pathway involved in pyocyanin production, iron homeostasis, and hydrogen cyanide release[7]. The first key players in this pathway are the *pqsABCDE* genes involved in the production of 4-hydroxy-2-heptylquinoline (HHQ). The product of the *pqsH* gene adds a hydroxyl group onto HHQ, creating 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinoline signal/PQS)[7]. These two signals work together to regulate the transcription of genes such as the *las* and *lec* genes.

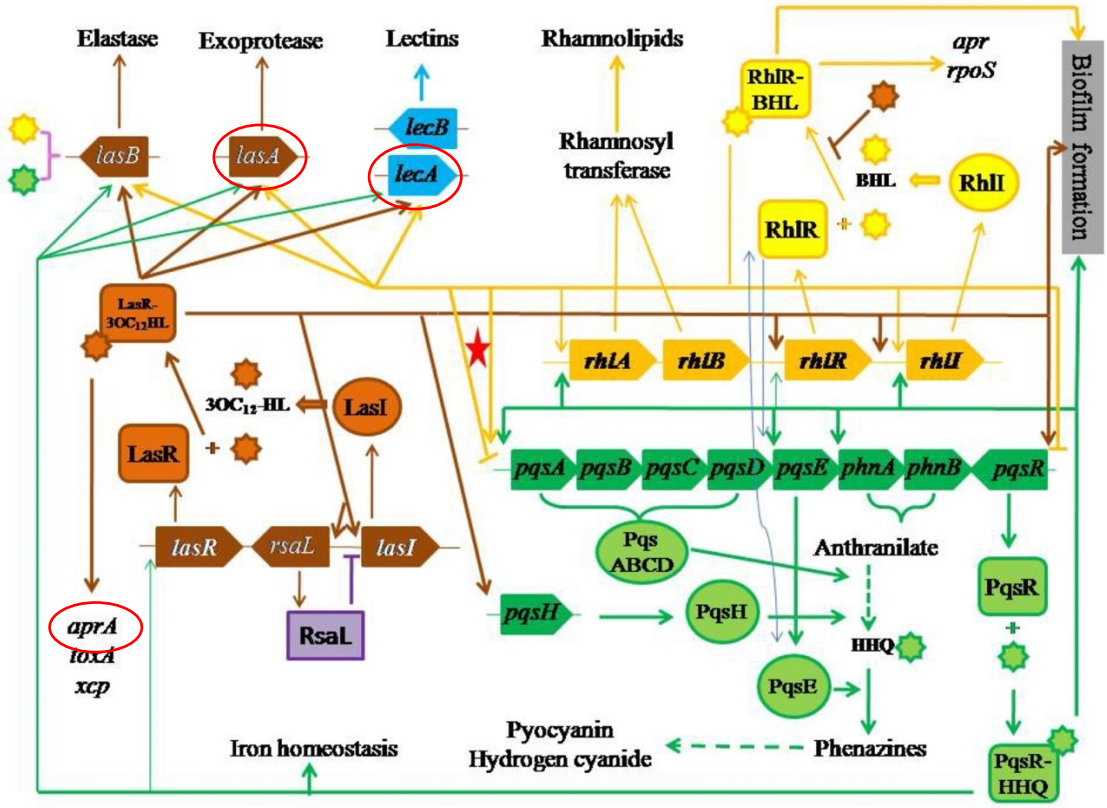

Figure 2: Visual representation of the Las, Rhl, and PQS quorum sensing pathways in P. aeruginosa. The three genes circled in red, *lasA*, *lecA*, and *aprA*, are genes used for qRT-PCR in this study. The *algD, algL*, *bqsS*, and *bqsR* genes are not depicted in this figure but are also used in qRT-PCR. This figure was adapted from https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2019.01582/full.

Antibiotic Resistance

P. aeruginosa is resistant to many antibiotics including aminoglycosides, quinolones, and beta-lactams[34]. Antibiotic resistance seen in *P. aeruginosa* is caused by many different factors including the low permeability of its outer membrane, efflux systems, and possession of modifying enzymes[34]. The outer membrane consists of a phospholipid inner layer and a lipopolysaccharide (LPS) outer layer. Due to the hydrophobic nature of the LPS layer, water-filled channels called porins are necessary for the transportation of hydrophilic molecules or compounds like antibiotics[33]. Many different physical and chemical characteristics of these porins contribute to the low permeability seen in the outer membrane of *P. aeruginosa.* For example, one of the most prevalent of these porins, OprF, can be seen in two confirmations, a

two-domain closed confirmation and a one-domain open channel confirmation, with the closed confirmation being the most predominant conformer found[34].

The outer membrane also contains efflux pumps responsible for removing toxic substances, metabolites, and quorum sensing molecules from the cell[45]. In *P. aeruginosa,* four main efflux pumps have been associated with antimicrobial resistance: MexAB-OprM, MexXY, MexCD-OprJ, and MexEF-OprN[30]. Each of these efflux pumps is composed of three parts: a periplasmic membrane fusion protein (PMFP) located in the periplasm, a resistance-nodulation-cell division transporter (RNDt) located in the inner membrane, and a channel-forming outer membrane factor (OMF) located in the outer membrane[30]. These components work together to transport the toxins through the inner and outer membranes and out of the cell. The MexAB-OprM pump easily exports β-lactams, fluoroquinolones, tetracyclines, tigecycline, novobiocin, thiolactomycin, sulfonamides, macrolides, aminoglycosides out of *P. aeruginosa* cells aiding in its resistance to these antibiotics[36]. The MexXY pump is associated with resistance to chloramphenicol, tetracycline, macrolides, and aminoglycosides[22]. MexCD-OprJ pump allows for resistance to fluoroquinolones such as levofloxacin and ciprofloxacin[30]. Finally, the MexEF-OprN pump is associated with resistance to chloramphenicol, quinolones, and trimethoprim[30].

Another factor contributing to the antibiotic resistant nature of *P. aeruginosa* is its possession of modifying enzymes that can inactivate antimicrobial agents. The most well-known of these modifying enzymes being aminoglycoside-modifying enzymes and β-Lactamases. Some of the classes of aminoglycoside-modifying enzymes include Aminoglycoside phosphotransferase (APH), aminoglycoside acetyltransferase (AAC), or aminoglycoside adenyltransferase (ANT). APHs phosphorylate, or add a phosphate group, to the 3'OH group of aminoglycoside antibiotics resulting in inactivation of the antibiotics[16]. AACs introduce an acetyl

group onto either the 6' or 2' amino groups of common aminoglycosides[16]. While ANTs transfer an adenosine monophosphate (AMP) molecule to a hydroxyl group in the aminoglycoside[28]. The most common ANTs are ANT (2″) and ANT (4′), with ANT (2″) adding a hydroxyl group to the 2″ position of aminoglycoside molecules and ANT (4′) adding to the 4′ position[28].

β-Lactamases are enzymes capable of inactivating β-Lactam antibiotics by breaking open the β-Lactam ring through hydrolysis[15]. There are four classes of β-Lactamases classified by their mechanism of action and amino-acid sequence: class A, B, C, and D[15]. Classes A, C, and D are serine hydrolases while class B contains zinc mellatoenzymes[15]. Some β-Lactamases are innately encoded by the *P. aeruginosa* genome, and some are acquired through horizontal gene transfer[15]. The β-Lactamases encoded by the core genome include: AmpC, PIB-1 (PA5542), and PA5514/poxB[15]. AmpC is a class C β-Lactamase that mediates resistance to cephalothin, cefazolin, cefoxitin, cephalosporins, and most penicillins[21]. PIB-1 (PA5542) is classified as a class A β-Lactamase that can degrade imipenem[15]. PA5514/poxB is in class D and is known for its ability to degrade carbapenems[15]. The most common β-Lactamases acquired through horizontal gene transfer include class A enzyme SHV, class B enzymes VIM and IMP, class C enzyme FOX-4, and class D enzymes OXA-1 and OXA-4.

Current Treatment with Antibiotics

Due to the antibiotic resistant nature of *P. aeruginosa,* patients with these infections are commonly treated with aminoglycosides or a combination of a β-Lactam alongside a β-Lactamase inhibitor. The following antibiotics are most used: Levofloxacin, Tobramycin, Meropenem, and a combination of Piperacillin and Tazobactam.

Levofloxacin is in the fluroquinolone class of antibiotics. The fluroquinolone class targets DNA gyrase and topoisomerase IV and results in inhibited DNA replication[38]. The role of DNA

gyrase and topoisomerase IV is to covalently bind to a supercoiled DNA strand and induce a double-stranded break in the DNA to uncoil the strand to allow replication and transcription to occur. Once the strand is uncoiled, the DNA backbone is ligated back together[41]. When fluoroquinolones are introduced, they bind to either the DNA gyrase or topoisomerase IV enzyme and form a complex that inhibits the enzyme from re-ligating the DNA backbone resulting in "cleaved complexes"[41]. An oral dose of 500mg of Levofloxacin a day for 10-14 days is the recommended dosage for adults.

Tobramycin is an aminoglycoside administered intravenously or through inhalation to treat *P. aeruginosa* infections[42]. Aminoglycosides exhibit bactericidal activity by inhibiting bacterial protein synthesis[42]. Aminoglycosides maintain a net positive charge allowing them to bind to the negative components of a Gram-negative bacterial membrane, the lipopolysaccharides and phospholipids[28]. Once bound to the bacterial membrane, proton motive force (PMF) allows the aminoglycoside to travel to the cytoplasm where ribosomes are located[9]. The aminoglycosides bind to the A-site of the 30s ribosome producing a conformational change resembling the change that occurs when an anticodon successfully binds to the codon during the translation process of protein synthesis causing mistranslation[9]. The proteins with incorrect or shortened sequences that result from mistranslation begin to build up in the cell and wreak havoc, eventually leading to cell death[42]. The dosage for intravenous usage is 5 mg/kg per day for 7 to 10 days[42]. Inhalation is primarily used for cystic fibrosis patients with pneumonia caused by *P. aeruginosa*[42]. During inhalation, 300 mg/5 mL solution is placed in a nebulizer and inhaled for 15 minutes; this treatment is repeated for 28 days[42].

Meropenem is in the Carbapenem class of antibiotics and kills bacteria by inhibiting the synthesis of cell wall components[44]. Meropenem targets proteins called Penicillin-binding-

proteins (PBPs), specifically PBPs 2, 3, and 4 in *P. aeruginosa*[44]. PBPs are involved in the synthesis of a major cell wall component called peptidoglycan. Peptidoglycan is composed of glycan strands and cross-linking peptides allowing it to act as a protective net keeping the cell structurally sound even when exposed to environmental stressors[46]. Once meropenem binds to the PBPs, cross linking of peptidoglycan is inhibited resulting in disruption of the cell wall and eventual cell death. When used to treat *P. aeruginosa* infections, 1g of Meropenem is administered intravenously every 8 hours[44].

The treatment commonly used for critically ill patients suffering from *P. aeruginosa* infections in the combination of the β-Lactam Piperacillin and the β-Lactamase inhibitor Tazobactam. Piperacillin exhibits a similar mechanism of action to Meropenem, as it also binds PBPs in the cell wall disrupting cell wall synthesis and resulting in cell lysis. Due to its ability to resist hydrolysis by many β-Lactamases, this antibiotic is extremely potent compared to other antibiotics in the Penicillin class[10]. Tazobactam is a β-Lactamase inhibitor known as a "suicide inhibitor" as it binds to the active site of β-Lactamase enzymes and triggers unwanted secondary chemical reactions that result in inactivation of the β-Lactamases. This combined treatment is used for nosocomial pneumonia caused by *P. aeruginosa.* For 7-14 days, a total IV dosage of 16g Piperacillin and 2g Tazobactam is utilized[31].

TTO-53

Due to the prevalence of antibiotic resistance amongst ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp*), TTO-53 was developed as an antimicrobial peptide. Antimicrobial peptides (AMPs) are small peptides, typically composed of 10-60 amino acid residues, that display inhibitory activity against various bacteria, fungi, and

viruses[18]. The immune system of most organisms innately produces AMPs as a mode of defense against pathogens they encounter. However, a major issue in using these naturally occurring AMPs is their susceptibility to degradation by the host's immune system. Therefore, researchers have begun developing synthetic AMPs, like TTO-53, that are composed of unnatural amino acids. These unnatural amino acids allow peptides to evade recognition and degradation by the hosts immune system.

TTO-53 was developed in a series of peptides containing three to six Tetrahydroisoquinolinecarboxylic acid (Tic) and Octahydroindolecarboxylic acid (Oic) dipeptide units along with SPACER amino acids[17]. This peptide specifically was synthesized using Tic-Oic dipeptide units and Dab as unnatural amino acids. The Tic acids were designed to take the place of phenylalanine, while the Oic acids took the place of proline. The Tic-Oic dipeptide units allow the peptide to maintain an alpha-helix structure which is important for the interaction of the peptide with bacterial membranes. The small size of TTO-53 was intended to allow the peptide to evade proteolytic degradation and enter the cell to disrupt cell function by interacting with crucial cell components. The structure of TTO-53 is depicted in figure 3 below.

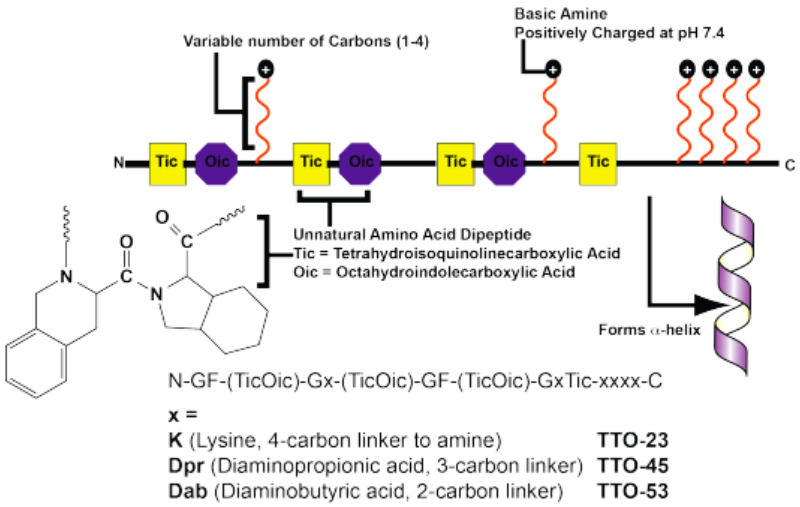

Figure 3: The structure of the AMP's TTO-23, TTO-45, and TTO-53. The yellow square and purple octagon represent the Tic-Oic dipeptide units. Taken from "Electrochemical Detection of Alginate Penetration in Immobilized Layer-by-Layer Films by Unnatural Amino Acid Containing Antimicrobial Peptides," by S. M. Vinogradov, 2015, *Electrochimica Act, 186*, 245-252. 10.1016/j.electacta.2015.10.051.

TTO-53 effects on *P. aeruginosa* viability and biofilm

Although TTO-53 was designed to be an antimicrobial peptide, a series of viability assays utilizing the *P. aeruginosa* strain PA01 displayed no effects on cell viability. These viability assays involved serial dilution and spot plating of cultures of PA01 exposed to 0ug/mL (0uM), 11.3ug/mL (5uM), 22.7ug/mL (10uM), and 56.8ug/mL (25uM) concentrations of TTO-53. Figure 4 shows consistent colony forming units per milliliter (cfu/mL) even as the concentration of TTO-53 increased. These cfu/mL measurements indicated that, regardless of concentration, TTO-53 does not lead to *P. aeruginosa* cell death.

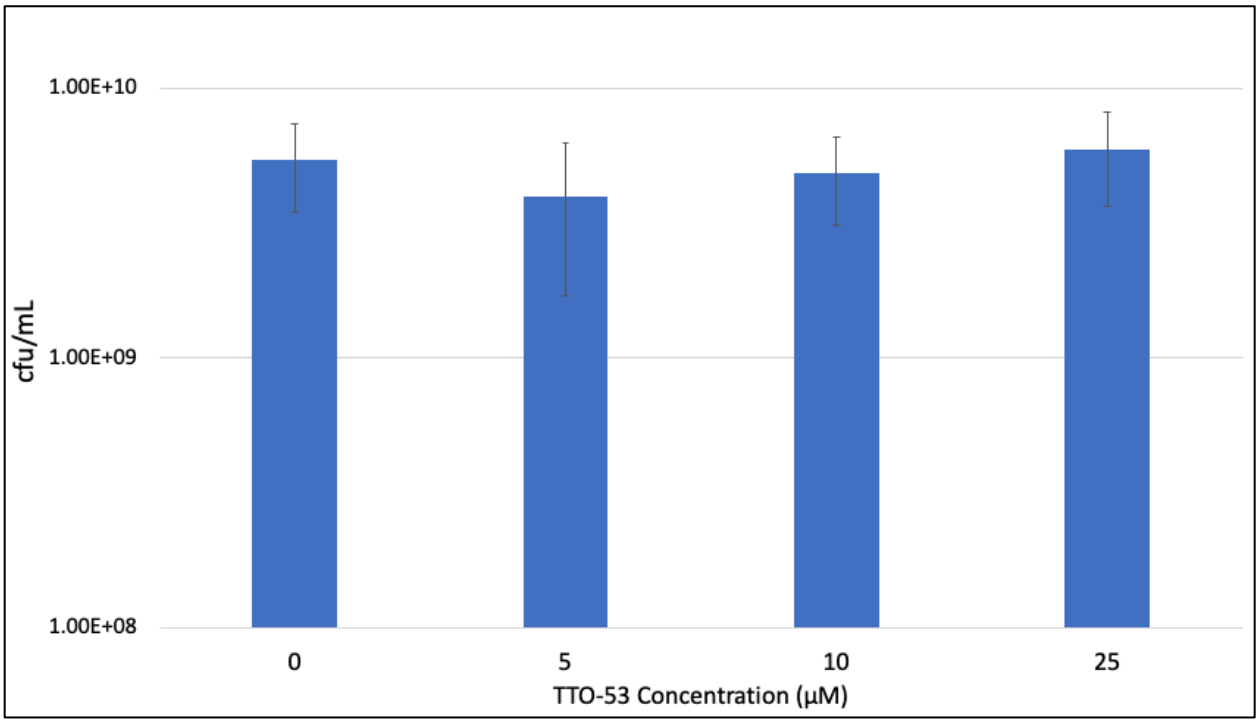

Figure 4: TTO-53 does not affect cell viability. Bacterial cultures were exposed to increasing concentrations of TTO-53 and allowed to grow for 24 hours. Viable bacteria were enumerated by serial dilution and plating. Results indicate there is not significant difference between concentrations.

Even though TTO-53 did not display bactericidal activity, crystal violet biofilm assays were performed to determine if it had any effects on pre-established *P. aeruginosa* biofilms. For these assays, samples of PAO1 were cultured for 24 hours and exposed to varying amounts of TTO-53. Twenty-four hours after TTO-53 exposure, samples were stained with crystal violet to assess biofilm concentration. As seen in figure 5, there is a consistent two-fold decrease in biofilm as concentration of this peptide increased, demonstrating disruption of PAO1 biofilm caused by TTO-53. Although this evidence displays the ability of the peptide to disrupt biofilm without killing the bacteria, how is TTO-53 causing this disruption?

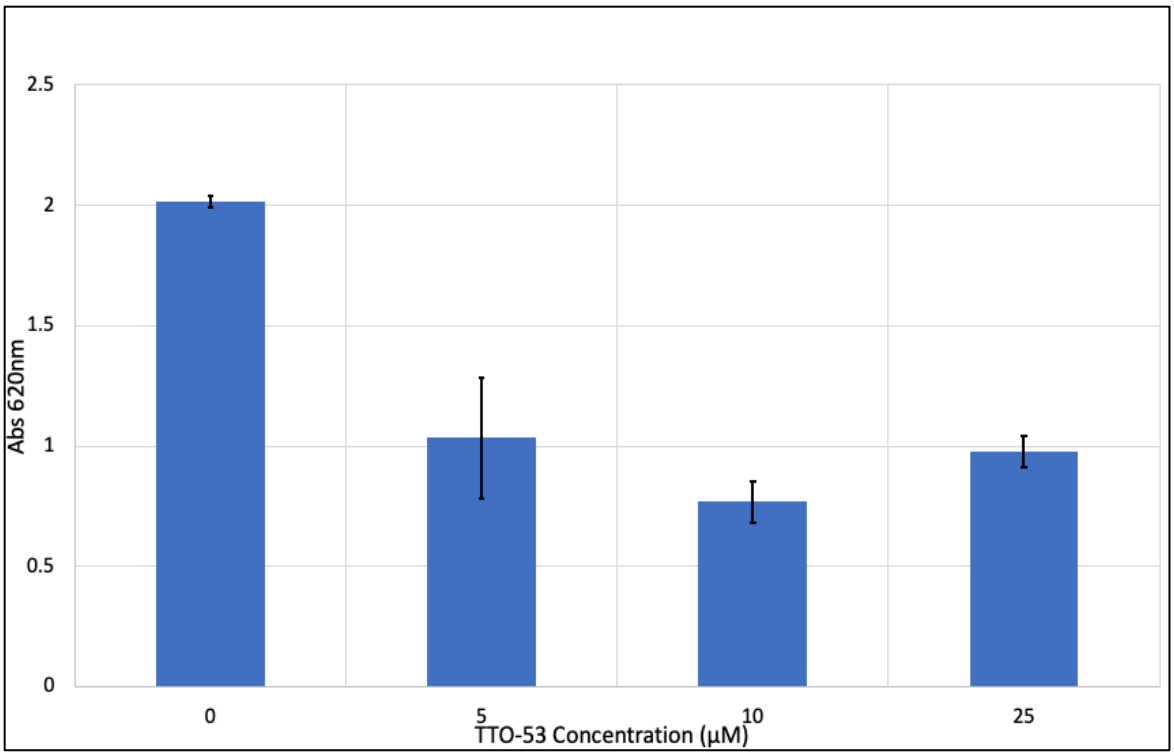

Figure 5: Exposure to TTO-53 disrupts *P. aeruginosa* biofilm. Bacterial cultures were exposed to increasing concentrations of TTO-53 and allowed to grow for 24 hours. Following 24 hours of growth, the wells were stained with crystal violet and their absorbance was measured at a wavelength of 620nm.

The two likely mechanisms of action for the biofilm disruption that occurs after TTO-53 exposure are disruption due to electrostatic interactions and disruption of cell signaling resulting in biofilm disruption. TTO-53 was designed to aggregate onto the bacterial cell membranes allowing for membrane disruption resulting in cell death. For antimicrobial peptides (AMP) to adhere to a bacterial cell membrane, electrostatic interactions between the peptide and membrane must take place[24]. For example, for an AMP to successfully aggregate onto the negatively charged cell membrane of *P. aeruginosa* the AMP must have a net positive charge. Components within the extracellular matrix of biofilm such as exopolysaccharides, DNA, and proteins are charged too. Therefore, it is reasonable to assume that there could be electrostatic interactions between the AMP and components of biofilm resulting in disassembly of the biofilm. Due to the role of quorum sensing in mediating the stages of biofilm development, it is possible that the

peptide could also be interacting with signaling molecules within this pathway leading to biofilm dispersion. Although TTO-53 was designed to interact with bacterial membranes and does have a slight net positive charge, the complex and diverse membrane composition of *P. aeruginosa* makes it unlikely the biofilm disruption is occurring due to electrostatic interactions.

Research Objective

The purpose of this thesis is to understand the role of the antimicrobial peptide TTO-53 in the disruption of *Pseudomonas aeruginosa* biofilm. Understanding how TTO-53 is disrupting *P. aeruginosa* and its effect on antibiotic efficacy could aid in utilizing TTO-53 as a therapeutic for cystic fibrosis patients. This will be done by studying the effects of TTO-53 exposure on quorum sensing in PAO1 and observing effects on viability with combined antibiotic and TTO-53 treatment.

Hypothesis: TTO-53 is disrupting pre-established biofilm by interacting with quorum sensing pathways responsible for biofilm development. Disruption of biofilm caused by TTO-53 will enhance the efficacy of antibiotics in treating *Pseudomonas aeruginosa* infections.

Aim 1: Determine if TTO-53 interacts with the LasR quorum sensing pathway in *Pseudomonas aeruginosa* biofilm.

Aim 2: Determine the effects of TTO-53 administration on antibiotic efficacy for treatment of *P. aeruginosa.*

CHAPTER 2: EXPERIMENTAL

Materials and Methods

Aim 1

RNA extraction

To determine if there are any differences in gene expression amongst cultures of PAO1 exposed to TTO-53 versus cultures of PAO1 not exposed to TTO-53, quantitative real-time PCR (qRT-PCR) was utilized. To prepare for qRT-PCR, one 3mL overnight culture of *P. aeruginosa* was prepared. Approximately 24 hours later, the optical density of the 3mL overnight culture was measured using a spectrophotometer and standardized to 0.5 OD using LB broth. After the culture was adjusted to 0.5 OD, 1mL was used to extract RNA for the control sample and 10uM concentration of TTO-53 was added to 1mL for RNA extraction of the experimental sample. RNA extraction of both samples was then performed using the Qiagen RNeasy Mini Kit with no alterations to kit protocol.

cDNA Synthesis

After RNA extraction, the concentrations of RNA extracted from both samples were quantified using a Nanodrop Spectrophotometer and standardized to equal concentrations for cDNA synthesis. For cDNA synthesis, the iScript cDNA Synthesis Kit was used along with the following parameters on a Bio-Rad PCR thermocycler: 10 minutes at 25°C for annealing, 10 minutes at 50°C for polymerization, and 5 minutes at 85°C for enzyme deactivation. Following cDNA synthesis, the concentrations of cDNA were quantified using the NanoDrop Spectrophotometer previously used.

qRT-PCR

For the qRT-PCR assay, a 384-well Optical Reaction Plate was utilized, with 10µl final volume per well[2]. The reaction consists of 100ng template cDNA, 1X SYBR® Green PCR MasterMix, 100nM each of forward and reverse primer, and nuclease free H_2O[2]. Each sample is run in triplicate for each gene to be assayed[2]. After loading samples, the plate was covered with an optical adhesive film and placed into the Real-Time Thermal Cycler.

Aim 2

Antibiotic Susceptibility Assay

The antibiotics used for these assays were Kanamycin, Chloramphenicol, Tobramycin, Piperacillin/Tazobactam, and Meropenem. The following stock concentrations were used: Kanamycin 45mg/mL, Chloramphenicol 25mg/mL, Tobramycin 6mg/mL, Piperacillin 298mg/mL, Tazobactam 34mg/mL, Meropenem 30mg/mL. The Minimum Inhibitory Concentration (MIC) for each antibiotic was determined using a microtiter plate format. For each antibiotic, twice the normal concentration was added in the first well and 1:2 serial dilutions using LB broth were performed for the remaining wells. Following the dilutions, 1uL of PAO1 was pipetted into each well. After 24-hours of growth, the lowest concentration of the antibiotic in which there was not visible growth of PAO1 was determined as the MIC.

The MIC values obtained were used to determine the concentration of antibiotic that should be used for the antibiotic susceptibility assays. A 96-well plate was used for the susceptibility assays. For each antibiotic, two rows were inoculated: one containing the antibiotic alone and one with the antibiotic and TTO-53. A 2-fold dilution of the antibiotic in LB broth was performed for each row. After the dilution of the antibiotic, 10uM concentration of TTO-53 was

added into each well of one of the rows and then 1uL of overnight culture, standardized to 0.5 OD, was added to all the wells for each row. After 16-hours of growth, seven 1:10 serial dilutions of each well without visible growth and the first well with visible growth were performed. Spot plates containing three 25uL spots for each well were then conducted for each dilution. After approximately 16-hours of growth, the cfu/mL for all plates were calculated using the following formula: cfu/ml = (no. of colonies x dilution factor)/ volume of culture plate.

Biofilm Disruption

The 96-well plate used for the susceptibility assay was rinsed 3x with PBS, and 180uL of PBS was added to each well previously used for dilutions and plating. An ultrasonic homogenizer with a 2mm probe was used to sonicate each well. For each well, there was 10 second sonication to disrupt any biofilm remaining in the wells. The probe was sterilized in between each well using 70% ethanol followed by sonication in PBS. Following sonication, three 1:10 dilution of each well were performed, and the dilutions were spot plated. After approximately 16-hours of growth, the cfu/mL for all plates were calculated as previously described.

CHAPTER 3: GENE EXPRESSION ANALYSIS

Results

To determine if TTO-53 interacts with the LasR quorum sensing pathway in *P. aeruginosa* biofilm, qRT-PCR was utilized to examine differences in gene expression in cultures of PAO1 with and without exposure to TTO-53. We analyzed expression of the *lasA*, *lecA*, *aprA*, *algD,* and *algL* genes which are directly regulated by the LasR system. We also looked at expression of the *pslA, bqsS,* and *bqsR* genes which are involved in other quorum sensing systems. In table 1, you can see the average cycle threshold (Ct) values for each gene of interest. The relative fold changes listed in the table represent the relative change in gene expression between the control group and treatment group for each gene. In these results, the control group represents a culture inoculated from an overnight culture of PAO1, while the experimental group represents a culture inoculated from the same overnight culture but exposed to TTO-53 for an hour.

	lasA	*lecA*	*algD*	*algL*	*pslA*	*bqsS*	*bqsR*	*aprA*
Average Ct (no TTO-53)	32.01	26.10	28.81	31.43	32.68	30.71	31.59	24.16
Average Ct (with TTO-53)	32.16	25.33	28.50	31.72	31.43	31.05	31.42	24.58
Relative Fold Change	-0.6	-0.2	-0.4	-0.6	0.1	-0.6	-0.5	-0.6

Table 1: There is not a significant fold change in *lasA*, *lecA*, *algD*, *algL*, *pslA*, *bqsS*, *bqsR*, and *aprA* genes between PAO1 cultures with and without one hour exposure to TTO-53.This table shows the average cycle threshold (Ct) for *lasA*, *lecA*, *algD*, *algL*, *pslA*, *bqsS*, *bqsR*, and *aprA* genes determined using cDNA from a normal PAO1 culture, no TTO-53, and using cDNA from a culture exposed to 10uM TTO-53. The relative fold change was calculated based on the average Ct values of control samples versus treatment samples for each gene.

Discussion

To examine if exposure to TTO-53 effects the LasR quorum sensing system in *P. aeruginosa*, qRT-PCR was utilized to examine differences in gene expression in cultures with and without exposure to TTO-53. Results from qRT-PCR, demonstrated there was not a significant fold change in *lasA, lecA, aprA, algD,* and *algL* genes between cultures not exposed to TTO-53 and cultures exposed to TTO-53 for an hour. For a relative fold change to be considered statistically significant, the fold change must be above 1.5 and the p-value, determined by a two-tailed paired t-test, must be above 0.5. Since the transcription of these genes is regulated by the binding of the 3OC12-Ahl signaling molecule to the LasR receptor, we can conclude that after one hour of exposure to TTO-53 there is no impact on the LasR quorum sensing system. There was not a significant fold change difference between control and treatment cultures for the *pslA, bqsS,* and *bqsR* genes either. The *pslA* gene is regulated by the RhlI/RhlR system, while the *bqsS* and *bqsR* genes are regulated by the quinolone signaling (PQS) system. Therefore, these results suggest that one hour exposure to TTO-53 does not impact the RhlI/RhlR or quinolone signaling systems.

Although these results do not demonstrate that exposure of *P. aeruginosa* to TTO-53 for an hour influences quorum sensing systems, more evidence is required to determine if TTO-53 may have an impact at different time points following exposure. Without knowledge of how long after exposure to TTO-53 biofilm disruption occurs, we are unable to determine when changes in signaling might occur following exposure to the peptide.

CHAPTER 4: ANTIBITOIC ASSAYS

Results

A series of antibiotic assays using Chloramphenicol, Meropenem, Tobramycin, Piperacillin-Tazobactam, and Kanamycin were conducted to determine the effects of combining antibiotics with TTO-53 on *Pseudomonas aeruginosa* viability. The amounts of each antibiotic used were determined based on clinically relevant concentrations. Figure 6 shows the colony forming units per milliliter (cfu/mL) of PAO1 cultures exposed to 3.125, 6.25, 12.5, 25, 50, and 100 mg/mL concentrations of Chloramphenicol with and without the addition of 10uM of TTO-53. Figure 6 is representative of three separate repeats. These results indicate that treatment with both Chloramphenicol and TTO-53 is more effective in killing cultures of PAO1 than treatment with only Chloramphenicol.

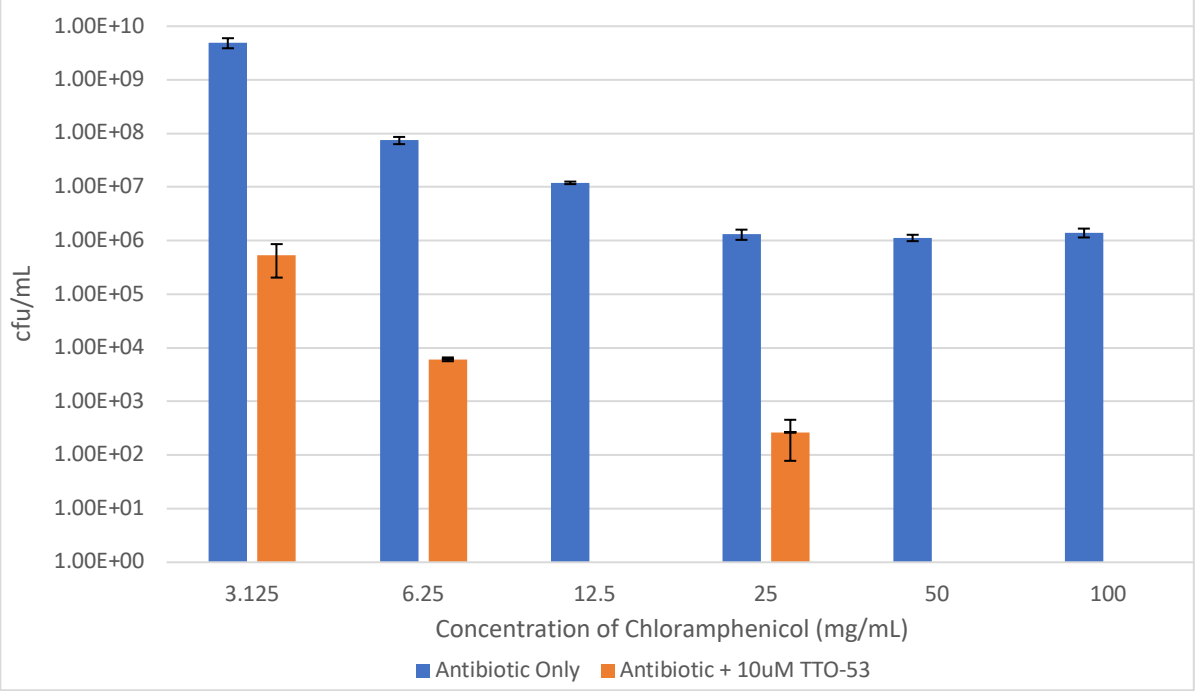

Figure 6: Combining 10uM TTO-53 with Chloramphenicol increases its efficacy. This graph displays the colony forming units per milliliter (cfu/mL) of PAO1 cultures after overnight exposure to various concentrations of Chloramphenicol with and without TTO-53. The blue bars are representative of the cfu/mL of cultures exposed to 3.125, 6.25, 12.5, 25, 50, and 100 mg/mL concentrations of Chloramphenicol alone. The orange bars represent the cfu/mL of cultures exposed to those same concentrations of chloramphenicol with the addition of 10uM TTO-53.

In figure 7, the cfu/mL of PAO1 cultures exposed to 15, 30, and 60 mg/mL concentrations of Meropenem with and without the addition of 10 uM of TTO-53 is displayed. The results of various Meropenem trials were highly variable, resulting in an inability to draw any conclusions regarding the effects of exposing PAO1 to both Meropenem and TTO-53. Figure 8 demonstrates that, when exposed to both TTO-53 and Tobramycin, the number of viable *P. aeruginosa* cells obtained from cfu/mL calculations was higher than when cultures were treated with 6, 3, and 1.5 mg/mL concentrations of Tobramycin combined with TTO-53. The figure below is representative of all three trials using Tobramycin. These results indicate that using Tobramycin alone is more effective in killing PAO1 than using Tobramycin combined with 10uM of TTO-53.

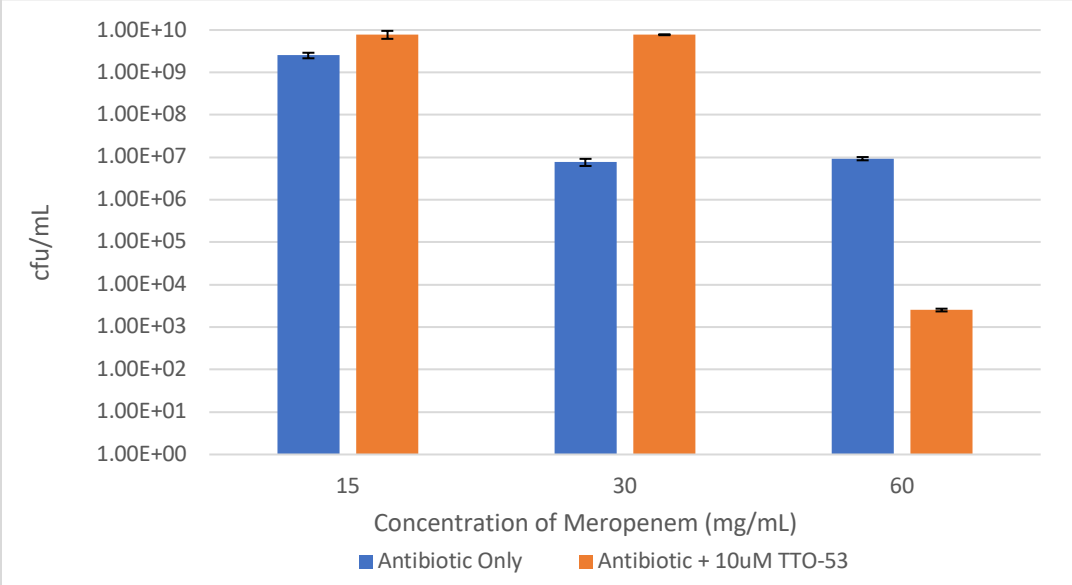

Figure 7: Combining Meropenem with TTO-53 resulted in inconsistent effects on *P. aeruginosa* viability. This graph displays the colony forming units per milliliter (cfu/mL) of PAO1 cultures exposed overnight to various concentrations of Meropenem with and without TTO-53. The blue bars are representative of the cfu/mL of cultures exposed to 15, 30, and 60 mg/mL concentrations of Meropenem alone. The orange bars represent the cfu/mL of cultures exposed to those same concentrations of Meropenem with the addition of 10uM of TTO-53.

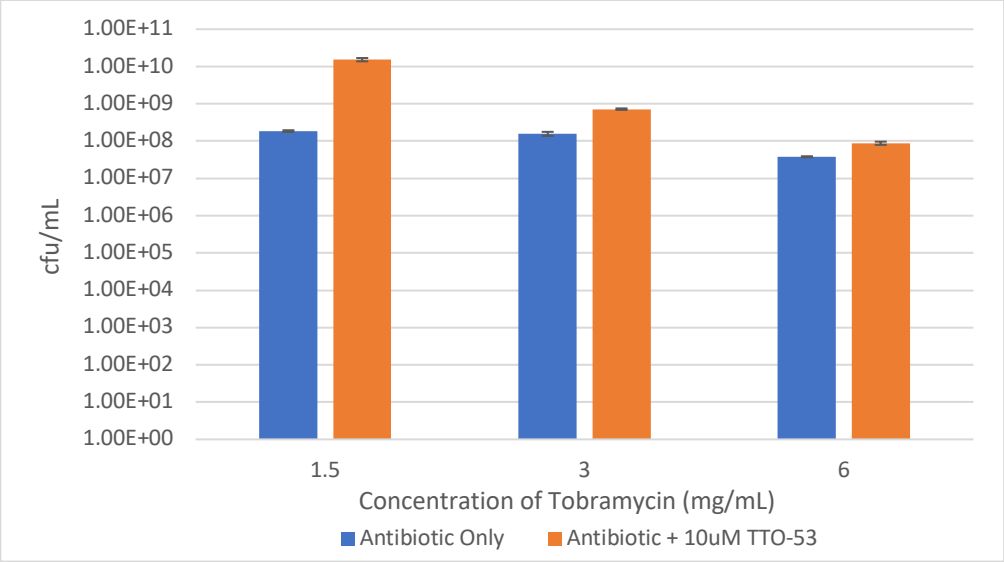

Figure 8: Utilizing Tobramycin alone is more effective in killing *P. aeruginosa* than combining Tobramycin with TTO-53. This graph displays the colony forming units per milliliter (cfu/mL) of PAO1 cultures after overnight exposure to various concentrations of Tobramycin with and without TTO-53. The blue bars are representative of the cfu/mL of cultures exposed to 1.5, 3, and 6 mg/mL concentrations of Tobramycin alone. The orange bars represent the cfu/mL of cultures exposed to those same concentrations of Tobramycin with the addition of 10uM TTO-53.

Figure 9 displays the cfu/mL of PAO1 cultures exposed to only Tazobactam/Piperacillin and Tazobactam/Piperacillin combined with 10uM of TTO-53. The following concentrations of Tazobactam/Piperacillin are shown: 1.87 mg/mL PP + 0.213 mg/mL Tz, 3.73 mg/mL PP + 0.43 mg/mL Tz, 7.45 mg/mL PP + 0.85 mg/mL Tz, 14.9 mg/mL PP + 1.7 mg/mL Tz, 29.8 mg/mL PP + 3.4 mg/mL Tz , and 59.6 mg/mL Piperacillin (PP) + 6.8 mg/mL Tazobactam (Tz). In all six different concentrations, you can see a significantly lower cfu/mL when utilizing Tazobactam/Piperacillin in conjunction with TTO-53. Figure 9 is representative of all three assays utilizing Tazobactam/Piperacillin.

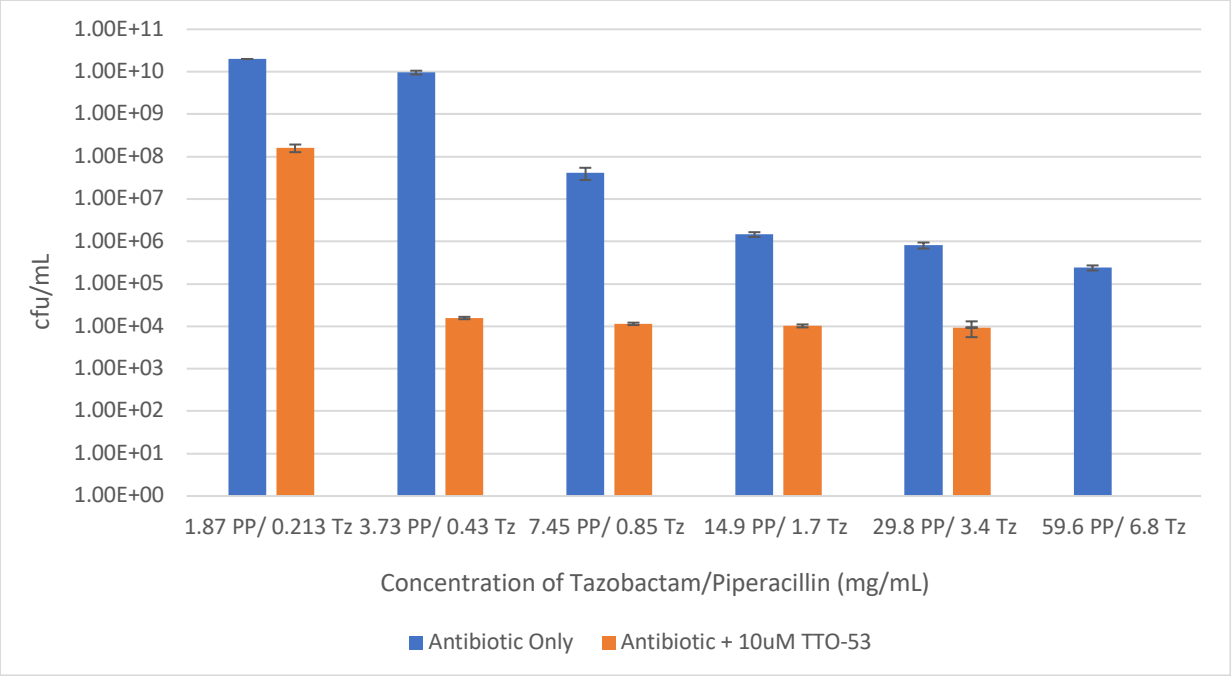

Figure 9: The combination treatment of Tazobactam/Piperacillin with TTO-53 is more effective in killing *P. aeruginosa* than using Tazobactam/Piperacillin alone. This graph displays the colony forming units per milliliter (cfu/mL) of cultures of PAO1 after overnight exposure to different concentrations of Tazobactam/Piperacillin with and without 10uM of TTO-53. The blue bars are representative of the cfu/mL of cultures exposed to only Tazobactam/Piperacillin. The orange bars represent the cfu/mL of cultures exposed to those same concentrations of Tazobactam/Piperacillin with the addition of 10uM of TTO-53.

Figure 10 shows the cfu/mL for PAO1 cultures exposed to 22.5, 45, and 90 mg/mL concentrations of Kanamycin with and without the addition of TTO-53. Like Meropenem, the cfu/mL counts of cultures exposed to 10uM of TTO-53 were highly variable for each concentration making it difficult to draw a conclusion regarding the effects of the combined treatment of Kanamycin and TTO-53.

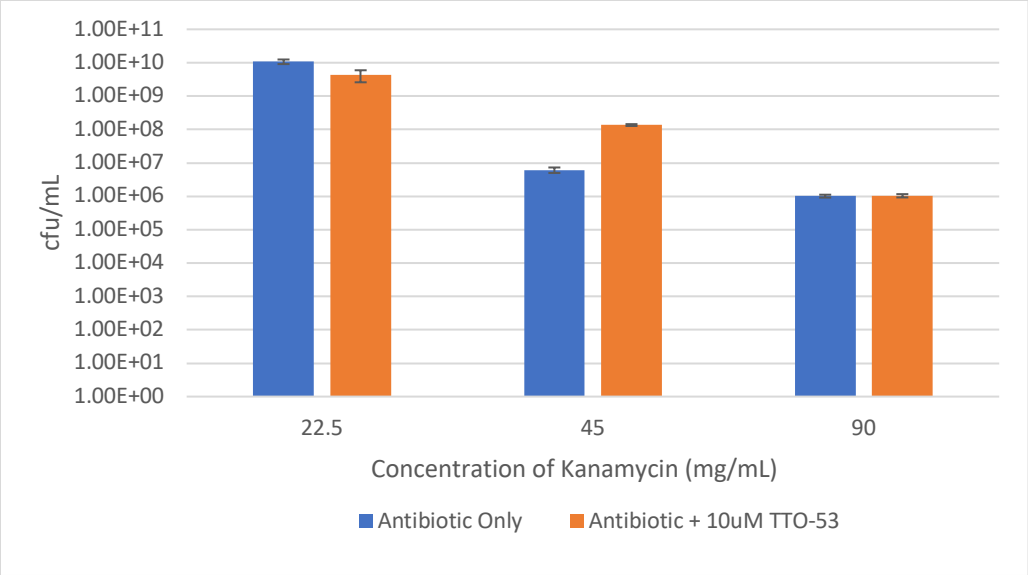

Figure 10: Combining Kanamycin with TTO-53 resulted in inconsistent effects on *P. aeruginosa* viability This graph displays the colony forming units per milliliter (cfu/mL) of cultures of PAO1 after overnight exposure to different concentrations of Kanamycin with and without 10uM of TTO-53. The blue bars are representative of the cfu/mL of cultures exposed to only Kanamycin. The orange bars represent the cfu/mL of cultures exposed to those same concentrations of Kanamycin with the addition of 10uM of TTO-53.

Effects of Combining TTO-53 and Antibiotics on Biofilm Degradation

Following the serial dilution and plating of each of the samples, the protocol listed above for disrupting biofilm using sonication was followed. However, the results of this assay were inconclusive. To supplement the cfu/mL data, we decided to perform a crystal violet biofilm assay to determine if there were differences in biofilm abundance when using antibiotics alone versus with TTO-53. For the crystal violet biofilm assays Chloramphenicol, Tobramycin, Meropenem, Tazobactam/Piperacillin were utilized. After diluting and plating the samples to obtain viability results, the wells were washed with PBS, stained with crystal violet, and resuspended in acetic acid to obtain absorbance values. Since we have previously seen that TTO-53 effectively disrupts biofilm, we would expect the wells exposed to only antibiotic to have more biofilm than the wells with antibiotic and TTO-53 combined.

Figure 11 shows the absorbances measured at 620nm for each of the wells that were exposed to twelve different concentrations (in mg/mL) of Chloramphenicol with and without TTO-53. The abundance of biofilm, determined by the absorbance, was consistent for the 3.13, 6.25, 12.5, 25, 50, and 100 mg/mL concentrations of Chloramphenicol in the wells that were and were not exposed to 10uM TTO-53. At 0.049, 0.39, 0.781, and 1.56 mg/mL concentrations of Chloramphenicol there are peaks in absorbance in wells exposed to Chloramphenicol only compared with 10uM peptide added. These peaks are indicative of higher biofilm levels in cultures exposed to the same concentration of Chloramphenicol without TTO-53 added.

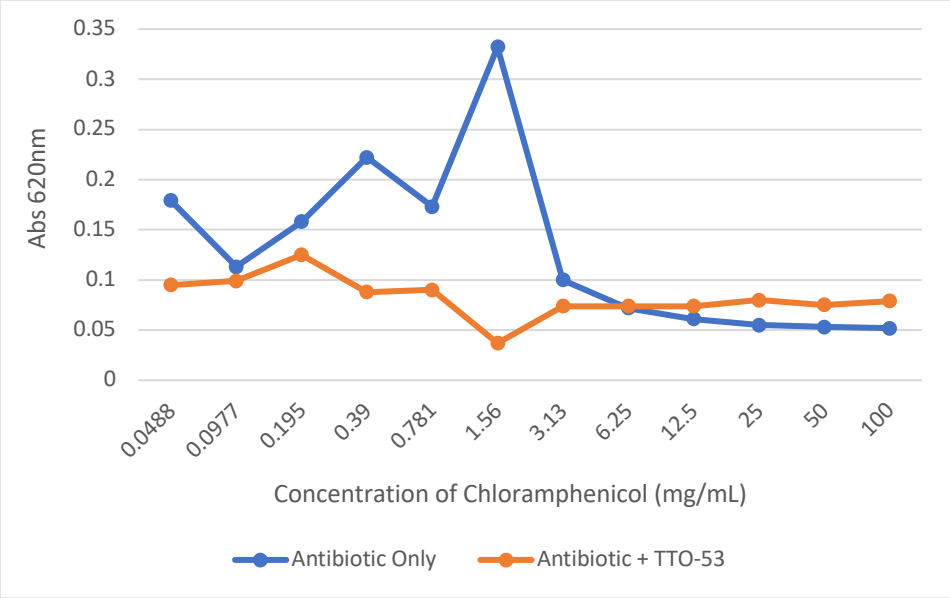

Figure 11: Biofilm degradation is in a dose-dependent manner when utilizing Chloramphenicol with and without TTO-53. Biofilm biomass assessment by crystal violet staining (at OD 620nm) of cultures of PAO1 exposed to varying concentrations of Chloramphenicol with and without the addition of TTO-53. The blue line is representative of the absorbances measured from cultures exposed to only Chloramphenicol, while the orange line represents absorbances measured from cultures exposed to Chloramphenicol with 10uM TTO-53.

Figure 12 shows the absorbances measured at 620nm of each of the wells that were exposed to twelve different concentrations (in mg/mL) of Meropenem with and without TTO-53. At the 7.5, 15, 30, and 60 mg/mL concentrations of Meropenem, the absorbances of cultures exposed to Meropenem alone and Meropenem with TTO-53 were equivalent. However, when

looking at the rest of the absorbance measurements for concentrations of Meropenem below 7.5, the wells of PAO1 treated with Meropenem only had higher absorbances than the wells with Meropenem and TTO-53.

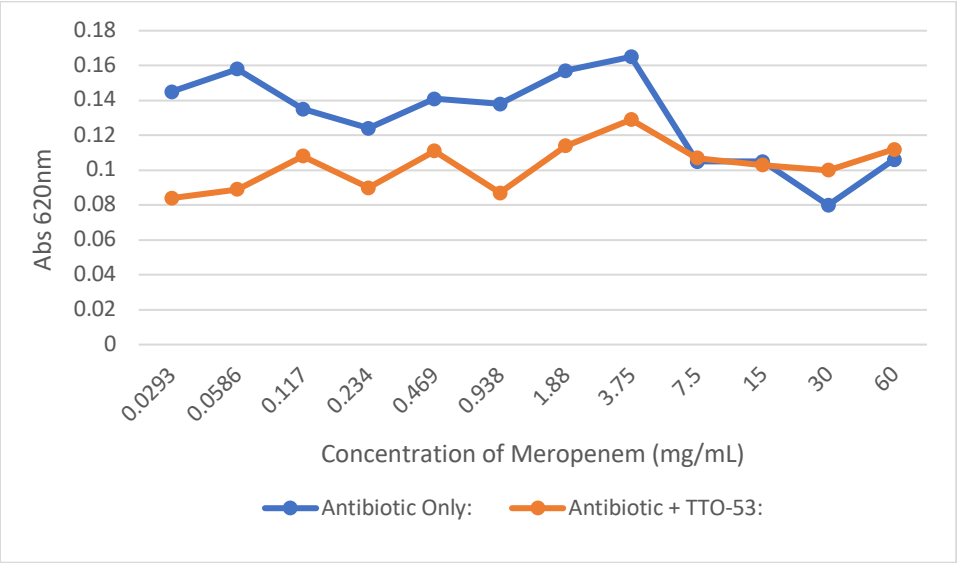

Figure 12: Biofilm degradation is in a dose-dependent manner when utilizing Meropenem with and without TTO-53. Biofilm biomass assessment by crystal violet staining (at OD 620nm) of cultures of PAO1 exposed to varying concentrations of Meropenem with and without the addition of TTO-53. The blue line is representative of the absorbances measured from cultures exposed to only Meropenem, while the orange line represents absorbances measured from cultures exposed to Meropenem with 10uM TTO-53.

In figure 13, we can see the difference in biofilm abundance, determined by the crystal violet biofilm assay, of cultures of PAO1 exposed to various concentrations of Tobramycin with and without the peptide TTO-53. With this assay, the wells containing cultures exposed to 3, 6, and 12 mg/mL of Tobramycin with 10uM TTO-53 have a higher absorbance than the wells with only Tobramycin. In this figure, there is a spike in absorbance for cultures exposed to a 1.5 mg/mL concentration alone compared to a culture exposed to the same concentration with peptide added. There also appears to be a dip in absorbance in the well that contained 0.188 mg/mL of

Tobramycin and 10uM of TTO-53, where the absorbance is half the absorbance obtained from the well containing Tobramycin only.

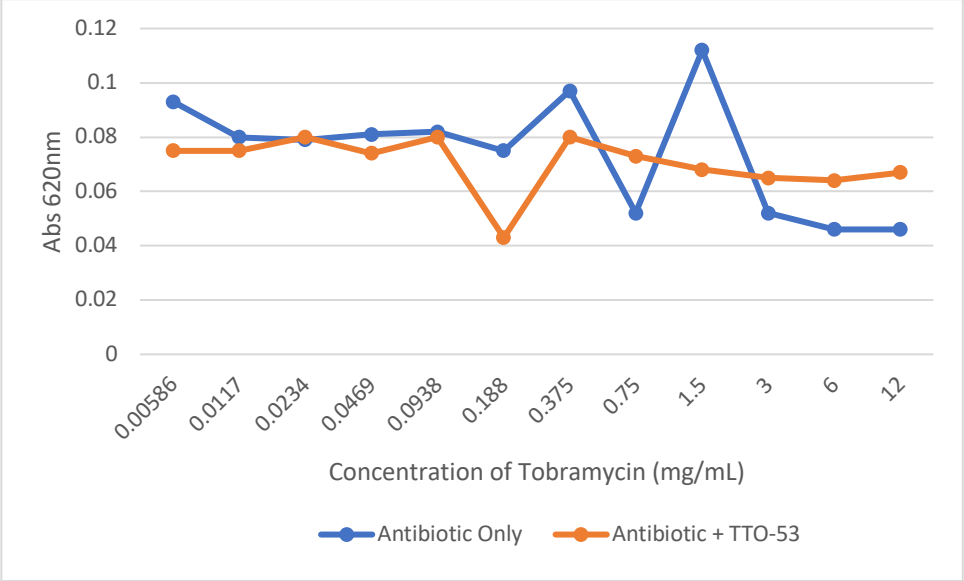

Figure 13: Biofilm degradation is in a dose-dependent manner when utilizing Tobramycin with and without TTO-53. Biofilm biomass assessment by crystal violet staining (at OD 620nm) of cultures of PAO1 exposed to varying concentrations of Tobramycin with and without the addition of TTO-53. The blue line is representative of the absorbances measured from cultures exposed to only Tobramycin, while the orange line represents absorbances measured from cultures exposed to Tobramycin with 10uM TTO-53.

Figure 14 depicts the absorbance values obtained using the crystal violet biofilm assay in wells exposed to varying concentrations of Tazobactam/Piperacillin versus wells containing those same concentrations but with the addition of 10uM TTO-53. Piperacillin alone was ineffective due to its susceptibility to degradation by *P. aeruginosa* β- lactamases. Therefore, it had to be used alongside the β- lactamase inhibitor Tazobactam. There is only a large difference in absorbance values observed in wells that once contained cultures exposed to 0.106 mg/mL of Tazobactam and 0.94 mg/mL Piperacillin with and without peptide. In these wells, the absorbance is twice as high for wells that once contained cultures exposed to only Tazobactam/Piperacillin.

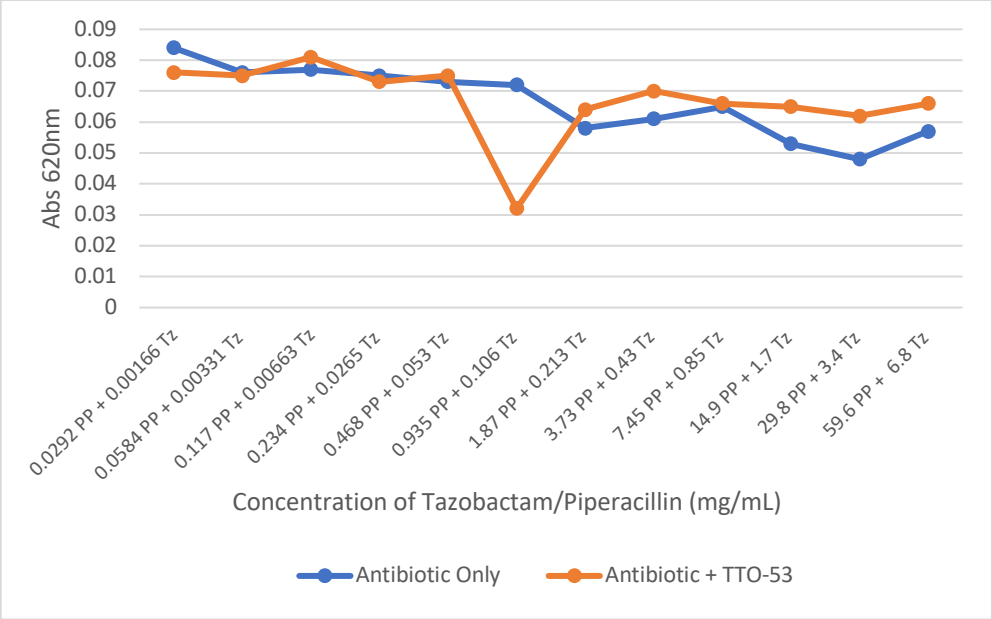

Figure 14: Biofilm degradation is in a dose-dependent manner when utilizing Tazobactam/Piperacillin with and without TTO-53. Biofilm biomass assessment by crystal violet staining (at OD 620nm) of cultures of PAO1 exposed to varying concentrations of Tazobactam/Piperacillin with and without the addition of TTO-53. The blue line is representative of the absorbances measured from cultures exposed to only Tazobactam/Piperacillin, while the orange line represents absorbances measured from cultures exposed to Tazobactam/Piperacillin with 10uM TTO-53.

Discussion

The results of the assays examining viability of PAO1 when exposed to Chloramphenicol, Meropenem, Tobramycin, Piperacillin-Tazobactam, and Kanamycin alone versus with TTO-53 were highly variable and dependent on the antibiotic. The results displayed that combination treatment of TTO-53 and the antibiotics Chloramphenicol and Tazobactam-Piperacillin is more effective in eradicating *P. aeruginosa* compared to using the antibiotics alone. However, the combination treatment of TTO-53 and Tobramycin is less effective in eradicating *P. aeruginosa* compared to utilizing these antibiotics alone. The other antibiotics, Meropenem and Kanamycin, yielded results that were too variable to draw conclusions from.

Without knowing the mechanism of action of TTO-53, we can only hypothesize why it has a synergistic effect when combined with certain antibiotics. It is possible that this synergistic

effect is occurring simply because TTO-53 is disrupting biofilm giving the antibiotics better access to the cells. However, if this were the case, we would assume TTO-53 would increase the efficacy of all the antibiotics utilized but this was not the case.

Another hypothesis is that TTO-53 creates pores in the bacterial membrane allowing antibiotics to enter the cell more easily. TTO-53 was one of a series of AMP's synthesized using Tic-Oic dipeptide units designed to interact with and disrupt bacterial cell membranes. Although not much research has been done with TTO-53, Dr. Hicks and his team have investigated the interactions between a few of the other TTO AMPs and lipid models mimicking bacterial membranes. Utilizing Isothermal Calorimetry (ITC) and calcein fluorescence leakage experiments, Dr. Hick's and his team have investigated the interactions between the AMPs with small (SUV) and large unilamellar vesicles (LUVs) with different lipid composition[43]. Dr. Hick's team stated that some of the complex interactions they observed were indicative of pore formation[43]. Although this data was not collected specifically on TTO-53, it can be hypothesized that it may interact similarly to these amp's due to the similarity of their structures. Other research examining synergistic effects of amps and antibiotics against *Clostridium difficile* have suggested that pore formation on the bacterial cell membrane can allow for the increased uptake of antibiotics[32].

Another important consideration when examining the differing effects of TTO-53 on various antibiotics with similar modes of killing is the properties of the antibiotic compounds. If pores in the bacterial membrane are being formed allowing an increased uptake of antibiotics, why would TTO-53 not increase the efficacy of all antibiotics being tested? Our hypothesis is that TTO-53 may be creating small pores in the membrane, making it difficult for larger antibiotic compounds to pass through the membrane. This hypothesis would explain why the

combination treatment of the peptide with Chloramphenicol increased the efficacy of Chloramphenicol, but combining the peptide with Tobramycin made it less effective in bacterial killing and inhibition. Although both compounds function by disrupting protein synthesis, there is a large size difference between them. As seen in figure 15, the structure of Chloramphenicol has only one aromatic ring, while Tobramycin contains three.

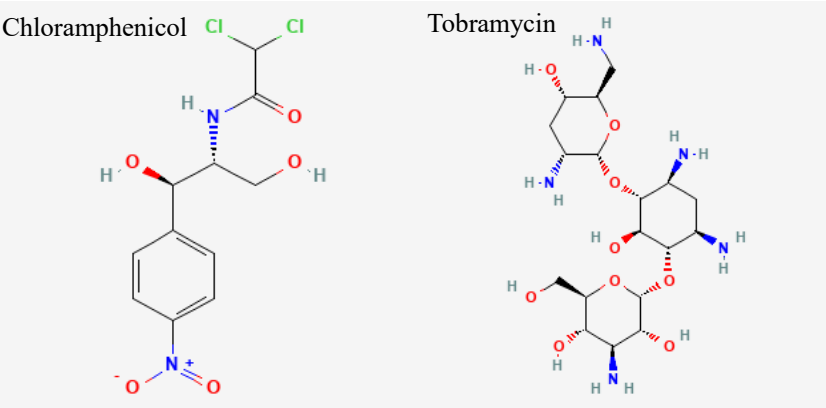

Figure 15: Structures of Chloramphenicol and Tobramycin. The left side displays the structure of Chloramphenicol, while the right side displays the structure of Tobramycin. The images for the structure of Chloramphenicol: https://pubchem.ncbi.nlm.nih.gov/compound/Chloramphenicol#section=Structures. The image for the structure of Tobramycin: https://pubchem.ncbi.nlm.nih.gov/compound/Tobramycin#section=Structures.

Due to charges associated with antibiotics and TTO-53, it is also possible that TTO-53 could be directly interacting with the antibiotics or their binding sites affecting their efficacy. However, more information regarding interactions between TTO-53 and other compounds will need to be investigated to provide insight into this hypothesis.

After seeing the effects of combining antibiotics with TTO-53 on PAO1 viability, we used the crystal violet biofilm assay to determine the effects of the antibiotic and TTO-53 combination on the abundance of biofilm. Since TTO-53 breaks down *P. aeruginosa* biofilm, it would be expected that cultures exposed to antibiotics combined with TTO-53 would have lower levels of biofilm than those exposed to antibiotics alone. The assays performed using

Chloramphenicol, Meropenem, and Tazobactam/Piperacillin displayed the results we were expecting. For these tests, most of the absorbance values obtained from wells containing cultures with antibiotics and TTO-53 were lower than those with only antibiotics. The peaks seen in the assays with Chloramphenicol, Tobramycin, and Tazobactam/Piperacillin could be indicative that the ability of TTO-53 to aid the antibiotics in breaking down biofilm is dependent upon the concentration of antibiotic being used. However, the absorbance values we obtained were much lower than anticipated which could explain the peaks and dips seen in these assays.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

This study aimed to investigate the role of the synthetic peptide TTO-53 in disrupting *Pseudomonas aeruginosa* biofilm as well as examine the effects of TTO-53 on antibiotic efficacy. Due to the role of quorum sensing in the maintenance of biofilm, we investigated the effects of this peptide on the LasR quorum sensing signaling pathway as well as genes from other quorum sensing pathways using qRT-PCR. Our results indicated that there was not differential expression in *lasA*, *lecA*, *aprA*, *algD, algL, bqsS,* or *bqsR* genes in cultures exposed to TTO-53 for an hour compared with cultures without exposure to TTO-53. Therefore, TTO-53 does not influence the LasR, PQS, or RhlI/RhlR quorum sensing signaling pathways.

Since TTO-53 disrupts *P. aeruginosa* biofilm, we observed its effects on the efficacy of Chloramphenicol, Meropenem, Tobramycin, Tazobactam/Piperacillin, and Kanamycin. Our results demonstrated that combining TTO-53 with Chloramphenicol and Tazobactam/Piperacillin increased their efficacy, while combining TTO-53 with Tobramycin decreased its efficacy. We hypothesize that TTO-53 is creating pores in the membrane of *P. aeruginosa* allowing for increased uptake of smaller antibiotics, but not aiding in the uptake of larger ones. Another hypothesis is that TTO-53 is interacting directly with the antibiotics affecting their efficacy.

Although there are still a lot of questions regarding how TTO-53 is disrupting *P. aeruginosa* biofilm and how long after exposure this disruption takes place, it could be a promising new therapeutic option for treating patients suffering from *P. aeruginosa* infections. In immunocompromised individuals, such as ones with cystic fibrosis, contracting these infections oftentimes results in their death. With TTO-53's ability to disrupt biofilm and increase the efficacy of some antibiotics, it could aid in treating these infections and ultimately saving lives.

Future Directions

In this study, we sought to investigate the role of TTO-53 in disrupting *P. aeruginosa* biofilm. Using qRT-PCR, we provided evidence that exposing cultures of *P. aeruginosa* to TTO-53 for one hour does not influence the LasR, PQS, and RhlI/RhlR *P. aeruginosa* quorum sensing pathways. This knowledge provides compelling evidence that biofilm disruption is not likely occurring due to disruption *P. aeruginosa* quorum sensing signaling pathways by TTO-53. This study also provided evidence that TTO-53 influences the efficacy of various antibiotics. Although these findings contributed to our knowledge of TTO-53, there are still questions to be answered regarding how it is disrupting biofilm and interacting with the membrane of *P. aeruginosa.*

Using a flow cell biofilm system combined with confocal laser screening microscopy (CLSM), could be useful for determining how long after TTO-53 exposure the biofilm disruption begins and how long it lasts. This technique could also provide insight on the type of dispersion caused by TTO-53 exposure. Utilizing the results from the flow cell biofilm system experiment, one could examine differences in the expression of various genes within a quorum sensing system at the appropriate time following TTO-53 exposure. To further investigate how TTO-53 is interacting with the membrane of *P. aeruginosa,* one could perform the Isothermal Calorimetry (ITC) and calcein fluorescence leakage experiments designed by Dr. Hick's team using TTO-53 specifically.

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