THE IDENTIFICATION OF NOVEL AMINOGLYCOSIDE ADJUVANTS FOR THE ERADICATION OF *PSEUDOMONAS AERUGINOSA* BIOFILMS

By

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ABSTRACT

THE IDENTIFICATION OF NOVEL AMINOGLYCOSIDE ADJUVANTS FOR THE ERADICATION OF *PSEUDOMONAS AERUGINOSA* BIOFILMS

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The Infectious Disease Society of America has named antimicrobial resistance the greatest global threat to human health. More than half of all infections are due to bacteria growing as biofilms, which are a community of cells enmeshed in a self-made matrix that can be up to 1000x more resistant conventional antimicrobials. *Pseudomonas aeruginosa* in particular, due to its numerous resistance mechanisms is a formable threat that often forms biofilms. Few new therapies have been developed to combat *P. aeruginosa*, and our antibacterial arsenal continues to decline. One solution to this daunting problem are anti-resistance compounds or adjuvants, which enhance conventional antimicrobials, extending and improving their utility. Here, we describe three adjuvants, triclosan, oxyclozanide and melittin. We demonstrate that each synergizes with tobramycin against mature *P. aeruginosa* biofilms. We also define the mechanism of action of triclosan and oxyclozanide, as protonophores that inhibit efflux pump activity, rendering cells susceptible to tobramycin killing. These adjuvants could be used in conjunction with current therapies to both improve their effectiveness, extend their lifespan, and target cells in biofilms

For my Mom, Dad and Kennedy.

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KEY TO ABBREVIATIONS

ABC the Adenosine triphosphate binding cassette family

AMPs Antimicrobial peptides

APC allophycocyanin

ATP adenosine triphosphate levels

Bcc *Burkholderia cenocepacia complex*

BONCAT bioorthogonal noncanonical amino acid tagging

CBC Complete blood count

CCG Center for Chemical Genomics

CF Cystic Fibrosis

CFU colony forming unit

CI confidence interval

D/E Dey-Engley media

DEA Dey-Engley neutralizing agar plates

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

DPBS Dulbecco's Phosphate Buffered Saline with magnesium and calcium

DRCs Dose response curves

EC50 effective concentration 50

EDPI Energy dependent phase I

EDPII Energy dependent phase II

EDTA Ethylenediaminetetraacetic acid

EF-G elongation factor G

EFG1A elongation factor G I A

EG-G1B elongation factor G I B

EPI Efflux pump inhibitors

EPS Extra polymeric substance

ESKAPE *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter species*

FDA Food and Drug Administration

FITC fluorescein isothiocyanate

gDNA Genomic deoxyribonucleic acid

GRAS Generally Recognized as Safe

GTP guanosine triphosphate

H&E hematoxylin and eosin

HTS high-throughput screen

IMP inner membrane protein

IVIS In Vivo Imaging System

LC-MS/MS Liquid chromatography mass spectroscopy/mass spectroscopy

LPS lipopolysaccharide

MATE the multidrug and toxic compound extrusion family

MDR Multidrug-resistant

MFP membrane fusion proteins

MHB Müeller-Hinton Broth II

MIC minimum inhibitory concentration

mL milliliter

MSF the major facilitator superfamily

NCE new chemical entity

OD optical density

OM outer membrane

OMP outer membrane protein

PBS phosphate buffered solution

PE phycoerythrin channel

PMF proton motive force

RND resistance-nodulation-division

RPM revolutions per minute

SD Standard error deviation

SEM Standard error mean

SMR the small multidrug resistance family

SNPs single nucleotide polymorphisms

TAE Tris-acetate-Ethylenediaminetetraacetic acid

TSA tryptic soy agar plates

UM University of Michigan

v/v volume/volume

ΔpH protein gradient

Δψ Memrane potential

CHAPTER 1

INTRODUCTION

1.1 *Pseudomonas aeruginosa* and Cystic Fibrosis

Cystic fibrosis (CF) is the most common life-shortening genetic disease in Caucasians. It affects 70,000 people worldwide and 30,000 people in the United States.[1] A mutation in the cystic fibrosis transmembrane conductance regulator gene and subsequent loss of a chloride channel and bicarbonate transport throughout the body causes CF. In the lungs, the loss of coordinated chloride and bicarbonate transport results in the airway mucus becoming thick and dry, hindering the clearance of bacteria and debris.[2,3] This immunological defect makes CF patients prone to recurrent lung infections, including several members of the multidrug-resistant (MDR) "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species)*.[4] By the mid-to-late teens, the dominant bacterial pathogen and leading cause of death in CF patients is *P. aeruginosa*.[5] Central to this pathogen's success is its ability to form biofilms, which are a community of cells embedded in thick matrix that provides tolerance to antibacterial therapies, macrophages, and neutrophils.[6]

Driven by stress due to oxidation from the innate immune system and by antibiotics from human interventions, *P. aeruginosa* undergoes pathoadaptation to form chronic infections in the lungs of CF patients predominantly in the form of "mucoid type" biofilms.[7] Clinically important, the mucoid type forms a much thicker gel matrix, which reduces antimicrobial effectiveness by creating a greater diffusion barrier.

Despite significant gains in the life expectancy for CF patients in the last several decades, largely due to aggressive antibacterial treatments and better nutrition, CF patients die prematurely due to complications caused by chronic lung infections mainly due to *P. aeruginosa*.[6] Once colonized by *P. aeruginosa*, CF patients are treated in successive on-off treatment cycles lasting 28-days with inhaled nebulized tobramycin for the duration of their lives.[8] However, due to the recalcitrant nature of biofilms, this approach fails to clear the infection completely. To extend the lives of patients with CF, new therapies need to be developed that more effectively target cells within biofilms. Especially therapies that eradicate *P. aeruginosa* before it pathoadapts into a chronic infectious state.

1.2 Biofilms Tolerance Factors

There are several factors that contribute to biofilm tolerance including reduced antimicrobial diffusion, decreased growth rate, and the expression of biofilm specific resistance genes and efflux pumps.[9] Together, these factors make biofilms recalcitrant to antimicrobial therapies.

The diffusion of antimicrobials into biofilms is greatly reduced by the gel matrix, or extra polymeric substance (EPS), that surrounds the cells within a biofilm. The EPS is made up of extracellular DNA, proteins, and polysaccharides, yielding an overall negative charge.[9,10] For this reason, positively-charged aminoglycosides, such as tobramycin, take ~24-hrs to fully penetrate mature *P. aeruginosa* biofilms.[11] Slowed diffusion results in different rates of killings and the opportunity for additional resistance

mechanisms to emerge.[12,13] This diffusion barrier is one of many factors that renders biofilms highly-tolerant to antimicrobials.

Akin to bacteria growing in stationary phase, cells within biofilms are in a less active metabolic state.[14] Because most antimicrobials target metabolically active cellular processes, their activity is reduced against cells growing slowly in a biofilm.[15] Further, biofilms, like stationary cultures, also give rise to persister cells, which are dormant non-growing cells that are tolerant to antimicrobials and can re-populate the biofilm once antimicrobial levels are depleted.[15] Although not completely understood, it is hypothesized that persister cells are produced stochastically within the biofilm or possibly by toxin/antitoxin systems triggered by starvation, which can inhibit translation, reduce adenosine triphosphate levels (ATP), or the proton motive force (PMF).[15-17] However, in *P. aeruginosa* the emergence of persister cells is a incompletely understood mechanism and involves many genes have been implicated including: *rpoS, spoT, relA, dlsA, dinG, spuC, algR, pilH, ycgM,* and *pheA*.[18] Regardless of how persister cells arise, they are a major factor in biofilm tolerance.

Finally, so called “biofilm resistance genes” adds another layer of tolerance against antimicrobial therapies.[19,20] For example, the biofilm-specific global regulator, BrlR, a transcriptional activator belonging to a class of c-di-GMP-responsive regulators, activates efflux pumps systems in biofilms, which have broad substrate specificity.[21] Together, these tolerance factors contribute to the chronic and recalcitrant nature of biofilms.

1.3 Antibacterial Resistance Mechanisms in *P. aeruginosa*

Pseudomonas has several resistance mechanisms spanning three resistance classes: acquired, intrinsic and adaptive (Table 1-1).

Table 1-1. *P. aeruginosa* resistance mechanisms.

Class of Resistance	Mechanisms	Dependent on Environment
Acquired	Horizontal gene transfer Targeted mutations	No
Intrinsic	Outer membrane permeability Efflux pump expression Beta-lactamase production	No
Adaptive	Lipid A modifications Efflux pump overexpression	Yes

Of the two acquired resistance mechanisms (Table 1-1), horizontal gene transfer is thought to play a less significant role in biofilms.[22] Instead, cells within biofilms are known to enter a hyper-mutable state, due to errors in the mismatch repair system induced by reactive oxygen species found within biofilms.[23,24] It is hypothesized, that this hyper-mutable state acts as a kind of "insurance policy," creating as many mutations in the molecular targets of antimicrobials as possible as well as rapidly diversifying the population within the biofilm, yielding a survival advantage.[25]

Pseudomonas has three intrinsic resistance mechanisms, which work in concert to prevent the accumulation of antimicrobials within cells. First, the outer membrane is 100-times less permeable than *Escherichia coli*, due to fewer and less effective porins.[26,27] Second, *Pseudomonas* has a chromosomal encoded β-lactamase, AmpC, which hydrolyzes β-lactam antibiotics.[28,29] Finally, there are at least 12 resistance-nodulation-division (RND) family efflux pump systems encoded for in the genome of *P. aeruginosa,* four of which have been investigated in detail.[30,31] The RND-type efflux pumps have a

broad substrate range including fluoroquinolones, aminoglycosides, and β-lactams.[32] Collectively, these three intrinsic resistance mechanisms make treating *Pseudomonas* infections incredibly difficult.

Pseudomonas also demonstrates adaptive resistance in response to aminoglycoside exposure, which is a phenotype occurring within 1-2-hrs following exposure to an aminoglycoside. During adaptive resistance, the expression of RND-type MexXY-OprM efflux pumps are induced, yielding temporary resistance.[33-35] In addition, lipid A modifications are also responsible for adaptive resistance. Sensor kinases including PhoQ, PmrB, ParS, CprS, and CbrA have been shown to upregulate the expression of *arnBCADTEF-udg* operon, modifying the lipid A structure by adding a 4-aminoarabinose sugar to the lipid A anchor.[36] The modified Lipid A reduces the negative charge of the lipopolysaccharide (LPS), and therefore, reduces the interaction aminoglycosides have with the outer membrane.[36]

1.4 Treatments for *P. aeruginosa* Infections

Due to these resistance mechanisms (Table 1-1), there are only three classes of antimicrobials available for the treatment of *P. aeruginosa* infections: aminoglycosides, third generation β-lactams, and fluoroquinolones.[37] Fluoroquinolones inhibit DNA replication by interfering with the activity of the DNA topoisomerases, DNA gyrase and topoisomerase IV, which are responsible for separating duplex strands of DNA during replication.[38,39] Inhibiting their activity results in breaks in the DNA, halting replication. Because *Pseudomonas* encodes for β-lactamases, only third generation β-lactams (cephalosporins) are effective because their altered β-lactam ring prevents cleavage by

β-lactamases.[40] β-lactams interfere with the synthesis of a cell wall by binding to transpeptidases, blocking peptidoglycan biosynthesis.[41] Thus, cells cannot maintain cell wall integrity or form new cell walls during cell division, resulting in lysis and death. Although these antimicrobials can be used, the mainstay of *Pseudomonal* therapy is aminoglycosides.

Aminoglycosides bind the 16s small ribosomal subunit of the 30s ribosome inducing errors in the synthesis of proteins, which causes misfolding proteins to be inserted in the inner membrane and cellular permeabilization.[42-45] This occurs in three steps, an initial ionic binding phase followed by two energy-dependent transport phases.

In first step, termed self-promoted uptake, aminoglycoside interact with negatively charged phosphates primarily found in the LPS of the outer membrane (OM), displacing cations and creating “cracks” or “fissures” in the membranes of cells.[44] This leads to the diffusion of aminoglycosides into the periplasm in a non-energy dependent manner.[43,44] It is also thought that aminoglycosides can diffuse through porins in the OM.[46]

Subsequent uptake of aminoglycosides from the periplasm into the cytoplasm is energy dependent, termed the slow energy dependent phase I (EDPI). In this phase, aminoglycosides cross the cytoplasmic membrane towards a negatively charged internal membrane potential ($\Delta\psi$).[44,47] It is thought that aminoglycosides enter the cytosol through nonspecific membrane channels, however the exact mechanism remains unclear.[45] Interestingly, this process is dependent on the concentration of aminoglycosides. That is, this effect can be lost by using high concentrations of aminoglycosides, greater than 30 μg/mL.[45] And, EDPI can be blocked by inhibitors of respiration and oxidative phosphorylation.[48,49]

In the fast energy dependent phase II (EDPII), aminoglycosides are rapidly transported across the cytoplasmic membrane using energy from the electron transport chain or ATP hydrolysis.[45] The exact mechanisms by which aminoglycosides are transported into the cytoplasm during EDPII are also unknown. However, it is known that this phase can be inhibited by protein synthesis inhibitors, suggesting translation plays a role in uptake.[50] Once in the cytosol, aminoglycosides bind to the 16s small ribosomal subunit of the 30s ribosome at the P-site, causing translation mismatches resulting in the formation of misfolded proteins and the inhibition of translation.[45] These misfolded proteins are then imbedded in the cytoplasmic membrane causing cellular lysis and the further uptake of aminoglycosides.[42-45]

The current *Pseudomonas* eradication protocol used clinically is 300 mg of aerosolized tobramycin twice a day for 28 days in on-off cycles, reaching mean sputum concentrations of 737 μg/g (~1,576 μM per dose), with little systemic absorption.[51] It has also been found in pediatric CF patients that the mean concentration of bioactive tobramycin within the epithelial lining fluid is 80 μg/mL (~171 μM per dose) ranging from 11 to 265 μg/mL (~23-566 μM) following inhalation.[52] Despite the routine use of *Pseudomonas* eradication therapies, by early adulthood ~80% of CF patients are chronically colonized with *P. aeruginosa*.[53] Numerous retrospective studies have shown that eradication of transient infections by *P. aeruginosa* can extend the lives of CF patients.[54,55] Thus, there is a critical need to identify new agents that target cells within biofilms and avoid selecting for resistance. One possible strategy is to identify anti-resistance compounds or adjuvants, which are effective when combined with antimicrobials but are not effective on their own.[56]

1.5 Alternative Approaches: Antibacterial Adjuvants

It is estimated that at least 2 million people are infected with drug-resistant bacteria in the United States (US) annually.[57] And, up to 70% of hospital-acquired infections in the US are due to pathogens resistant to at least one class of antimicrobials.[58] Making matters worse, the rate of antibiotic development has come to a near stand-still.[59] For example, no new class of broad-spectrum antimicrobials effective against Gram negative bacteria has been deployed in the last 40 years.[60] This is due to costs associated with drug development and the difficulty involved in identifying compounds. Together, these obstacles, have caused pharmaceutical companies to all-but-end antimicrobial development.[61] Because resistance emerges shortly after antibiotics are deployed, the discovery and implementation of new compounds to treat resistant infections becomes a Sisyphean task, doomed to failure.[58] The use of anti-resistance drugs or adjuvants represents a viable alternative to this approach. Antibiotic adjuvants are an attractive option because they do not require activity against essential cellular targets (cell wall synthesis, protein synthesis, DNA replication etc.), but they can inhibit one or more essential or non-essential targets to enhance the activity of an antibiotic.

The first β-lactam, penicillin, was auspiciously discovered in 1928 by Alexander Fleming and was further developed for clinical use in the 1940's by Ernst Chain and Howard Florey, resulting in the creation of one of the most successful classes of antibiotics used today.[62-64] The Achilles heel of all β-lactams, however, is their four-membered β-lactam ring that is easily inactivated by enzymes produce by bacteria (e.g. AmpC). However, β-lactams remain a mainstay therapy for bacterial infections today because of β-lactamase inhibitors. Notable examples include clavulanate, sulbactam

and tazobactam, which have preserved their use clinically and expanded their coverage (reviewed[65]). β-lactamase inhibitors represent the most successful adjuvants used clinically.

Here, we focus on the use of anti-resistance compounds or adjuvants to extend and improve our current antibacterial arsenal, especially as it pertains to combating resistant *P. aeruginosa* biofilms. We will discuss, efflux pump inhibitors[66] and antimicrobial peptides.[67]

1.5.1 Efflux Pump Inhibitors (EPIs)

Antibacterial efflux was first discovered nearly 40 years ago as the mechanism for tetracycline resistance in *enterobacteria*.[68] Since their discovery, it has been found that most reported antibiotics can be effluxed out of the cell, yielding resistance.[69] Efflux pumps are of particular significance in Gram negative bacteria because of their double walled cell envelope consisting of an outer membrane (OM) and inner membrane (IM), interlinked by a thin peptidoglycan layer. The space between the OM and the IM is called the periplasm where antibiotics can be captured and then extruded directly into external medium, termed trans-envelope efflux.[70] For this reason, efflux represents a major resistance target.

There are five families of efflux systems: major facilitator superfamily (MSF), the ATP-binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance family (SMR) and the resistance-nodulation-division (RND) family (reviewed[69]) (Fig. 1). These five families of efflux

pumps obtain energy required for activity from H^+ protons (RND, SMR, MSF), Na^+ (MATE), or by the hydrolysis of ATP (ABC) (Figure 1-1).[32,71]

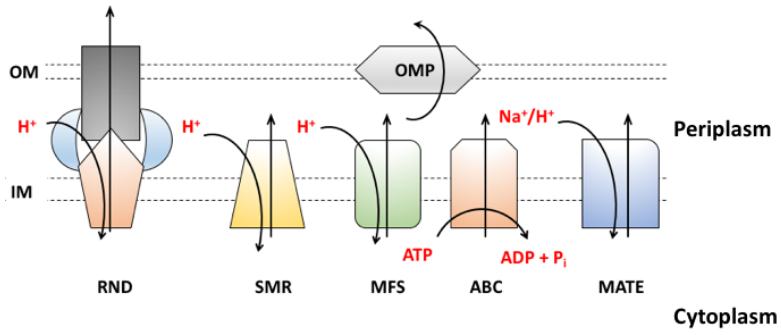

Figure 1-1. Five families of efflux pumps in *P. aeruginosa.*
Abbreviations: Outer membrane protein (OMP), outer membrane (OM), inner membrane (IM), Major facilitator superfamily (MSF), ATP-binding cassette (ABC) family, Multidrug and toxic compound extrusion (MATE) family, Small multidrug resistance family (SMR), Resistance-nodulation-division (RND) family.

RND-type efflux pumps are not specific for one type of antibiotic, and are considered multidrug resistant (MDR) pumps, which can expel a diverse set of compounds, including antimicrobials.[72] There are 12 RND-type efflux pumps encoded in the genome of *P. aeruginosa*.[22] Four are well characterized including, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM.[22] MexAB-OprM is constitutionally expressed and is responsible for intrinsic resistance to fluoroquinolones and β-lactams.[22] And Mex-XY-OprM is also responsible for aminoglycoside efflux, which plays an essential role in resistance in CF. In fact, mutations in the MexZ repressor (or AmrR), which results in the overexpression of MexXY, is the most commonly mutated gene in *P. aeruginosa* CF isolates.[24,32] MexZ mutations can also result in the expression of and possibly MexAB and MexCD.[31] Given their role in resistance, inhibiting their activity is essential for the development of more effective therapies.

The RND-type efflux pumps require a protein gradient (ΔpH) to power a H^+/compound antiport system.[73] The tripartite RND-type efflux pumps are the most clinically significant in terms of antibiotic resistance, and mutations resulting in their overexpression is the most common tobramycin resistance mechanism found in isolates from CF patients.[22,74,75] The RND-type efflux pumps consist of three proteins which span from the inner membrane, through the periplasmic space, and end in the outer membrane (Figure 1-2).[76] The inner membrane protein (IMP, e.g. MexX) provides substrate specificity and catalyzes the H^+ dependent efflux of compounds. The periplasmic membrane fusion proteins (MFP, e.g. MexY) connect the outer membrane protein to the inner membrane protein. Finally, the outer membrane protein (OMP, e.g. OprM) create a channel through which the compound exits.[71]

There are at least six ways to inhibit efflux pumps including, interfering with genetic regulatory pathways responsible for their expression, changing the design of antibiotics so they are no longer a substrate for efflux, inhibiting efflux pump assembly, competing with an antibiotic for efflux, blocking the outer membrane exit channel, and, finally, depleting the energy source required for efflux (Figure 1-2).[66]

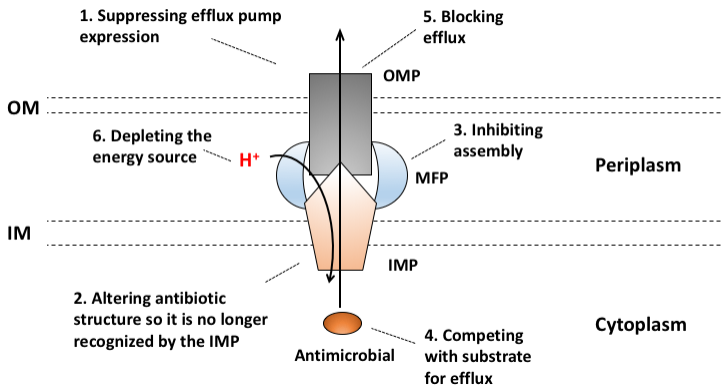

Figure 1-2. Inhibiting RND-type efflux pumps.
Abbreviations: Outer membrane protein (OMP), membrane fusion protein (MFP), inner membrane protein (IMP), outer membrane (OM), inner membrane (IM).

There are few EPIs shown to be effective in Gram negative bacteria. However, countless have been identified for Gram positive bacteria, especially for *Staphylococcus aureus* (reviewed[56]). The first EPI to be identified was the peptidomimetic MC-2077,110, renamed PaβN and it potentiate the fluoroquinolone levofloxacin against *P. aeruginosa* by competing with levofloxacin for efflux.[77]

PaβN is broadly effective, inhibiting 4 RND-type efflux pumps[78], making it a "broad-spectrum EPI." However, not all antibiotic substrates are enhanced by PaβN. For example, it does not improve the import of β-lactams or aminoglycosides, but does potentiate fluoroquinolones, macrolides, oxazolidinones, chloramphenicol, and rifampicin.[77] This is attributed to the fact PaβN competes with antibiotics by acting as a substrate for a specific binding pocket on the IMP, thus, only the efflux of specific antibiotics that can also bind at this site is inhibited, yielding substrate specificity.[78] However, clinical development of PaβN was eventually abandoned due to toxicity concerns.[70] Efforts have been made to develop modifications of PaβN rendering it less toxic such as the derivative MC-207,110.[66]

Other EPIs that have been identified include pyridopyrimidines, which specifically inhibit MexAB-OprM efflux pumps by directly binding to the IMP, competing and occluding antibiotic efflux.[79,80] Another major EPI identified and currently in phase I clinical trials is, MP-601,205.[78] However, few details have been released, including structure, mechanism of action. A list of known EPIs effective against *P. aeruginosa* are summarized in the Table 1-2.

Table 1-2. Efflux pump inhibitors effective against *P. aeruginosa.*

Compound	Antibacterial Synergism	Reference
PaβN, MC-207,110	Levofloxacin, macrolides, oxazolidinones, chloramphenicol, rifampicin	[81]
MP-601,205	Levofloxacin	[78]
4-(3-morpholinopropylamino)- quinazoline	Chloramphenicol, nalidixic acid, sparfloxacin, Norfloxacin	[82]
EA-371α, EA-371δ	Levofloxacin	[83]
Geraniol	Chloramphenicol, β-lactams, fluoroquinolones	[84]
Curcumin	Carbenicillin, gentamicin, ciprofloxacin, meropenem, ceftazidime	[85]
Lanatoside C and daidzein	Levofloxacin, carbenicillin	[86]
Trimethoprim and Epinephrine	Ciprofloxacin	[87]
Chlorpromazine, Amitryptilline, Trans-chlorprothixene	Tobramycin, penicillin, cephalexin	[88]
Pyridopyrimidines e.g. D13-9001	Levofloxacin, Aztreonam	[79,80,89]

To date, no compound specifically classified as an EPI has been approved for clinical use; however, MP-601,205 is currently in Phase I clinical trials. Despite a lack of interest by pharmaceutical companies into the development of new antimicrobials, Mpex pharmaceuticals is invested in developing EPIs for clinical use. Since efflux pumps play a central role in antibacterial resistance, especially in *P. aeruginosa*, the development of new compounds that inhibit these pumps could be a significant clinical benefit.

1.5.2 Antimicrobial Peptides (AMPs)

An essential component of intrinsic resistance in Gram negative bacteria is their impermeable OM.[90] In Gram negative bacteria, the OM is semi-permeable barrier made of an asymmetric lipid bilayer with integrated channel-forming proteins termed porins.[27] *P. aeruginosa* in particular has intrinsic defects in its OM, in which only ~1% of all available porin molecules form functional open channels.[91] Therefore, developing strategies to over-come this resistance mechanism could significantly improve current therapies.

Antimicrobial peptides (AMPs), considered “nature’s antibiotics”, are produced by nearly every class of life as part of an innate immune defense system.[92] AMPs are often short, ranging from 15-30 amino acids, and they have an overall positive charge and a large proportion of hydrophobic residues, making them amphiphilic molecules. This amphiphilic property results in membrane disruption and cellular permeabilization.[93] First, AMPs accumulate on the OM of cells and after reaching a concentration threshold they then fold inward forming peptide-lined pores, though the exact mechanisms is unknown (reviewed[94]). AMPs also have effects beyond OM disruption, including, the downregulation of type IV pili, rhamnolipid, quorum-sensing, and flagellar genes.[95] Finally, AMPs bind to extracellular DNA in the EPS, causing the breakdown of the biofilm.[96]

Importantly, AMPs have a high affinity for bacterial membranes.[93,97] First, the positive charge found in all AMPs is highly selective for the negative anionic phospholipids that make up bacterial membranes. Second, AMPs specificity for bacteria is driven by their highly negatively charged interiors. Together, these electro-

biochemical properties of both bacteria and AMPs results in high levels of binding, efficacy, and selectivity for prokaryotes.[97]

AMPs offer several key advantages over traditional antimicrobials, in that they rapidly kill resistant slow-growing bacteria, such as those classically associated with biofilm growth.[92,96] Further, AMPs can be manipulated to increase antimicrobial activity.[98] For this reason, a great deal of attention has been paid to developing AMPs as antibacterial agents.[99-104]

There are countless examples of AMPs displaying a range of activities. There are 4 AMPs that have made it to phase 3a clinical-efficacy trials: MSI-78 (Pexiganan), IB-367 (Iseganan), rBP23 (Neuprex), and CP-226 (Omigana) for topical applications in the treatment of impetigo, diabetic foot ulcers, mucositis, sepsis, and catheter associated infections, respectively.[92] However, only Pexiganan and Omiganan were shown to be effective in these trials.[92]

Numerous AMPs have been developed for use in combination with antimicrobials against *P. aeruginosa*. Notably, colistin has been combined with ciprofloxacin and tobramycin, as a inhaled treatment for CF patients.[105] Importantly, these combinations improve lung function and possess activity against persister cells.[105] A list of known AMPs effective against *P. aeruginosa* are listed in Table 1-3.

Table 1-3. Antimicrobial peptides effective against *P. aeruginosa.*

AMP	Antibacterial Synergism	Reference
G10KHc (STAMP)	Tobramycin	106
Tachyplesin III	Piperacillin-tazobactam	107
Colistin	Tobramycin, ciprofloxacin	105
GL 13K	Tobramycin	99
LL-37, CAMA, melittin,	Tobramycin, ceftazidime, ciprofloxacin, doripenem, piperacillin, colistin	100-103

Table 1-3 (cont'd)

defensin, magainin-II		
BMAP-27, BMAP-28	Tobramycin	[108]
LL-37	Tobramycin	[109]

Abbreviation: STAMP specifically targeted AMPs[106]

There are several limitations to AMPs including high costs to purify and sequence and possible hemolytic and cytotoxic effects.[92] However, it has been shown that by making modifications to the sequences of AMPs their cytotoxicity can be reduced and their antimicrobial properties improved.[98]

The use of AMPs has a previous history of success. Especially for the treatment of biofilm-associated infections. Notably, colistin or polymyxin E, is routinely used for the treatment of *P. aeruginosa* infections in patients with CF, as well as non-CF patients, alone and in combination with tobramycin.[105] In addition, the AMP polymyxin B has been used for decades in Neosporin®.[110,111] AMPs combined with conventional antibiotics are another strategy to extend the shelf-life of our current antibacterial arsenal.

1.6 Conclusions and Future Directions

P. aeruginosa represents a major cause of nosocomial infections as well as infections associated with patients with compromised immune systems. Currently, there are only three classes of drugs available to treat these infections: fluoroquinolones, third-generation β-lactams, and aminoglycosides. However, each of these antimicrobials are far less effective against *P. aeruginosa* growing as a biofilm, which is the

predominate lifestyle found during infections. Therefore, new approaches to treat these infections are needed.

EPIs and AMPs represent agents that could be used in combination with conventional antimicrobials to extend their shelf-life, inhibit bacterial resistance, and target cells growing as biofilms. Anti-resistance approaches have been successful previously, notably β-lactamase inhibitors, such as clavulanic acid, is routinely used in combination with amoxicillin.[112] Similarly, AMPs have been used successfully clinically. For example, colistin is used with tobramycin to improve threptic outcomes in CF patients.[105,113,114] However, no EPI to-date has been used clinically, but MP-601,205 is currently in Phase I clinical trials.[78]

The Four Core Actions to Prevent Antibiotic Resistance according to the Centers for Disease Control are: (i) preventing the spread of infections, (ii) tracking infections, (iii) antibacterial stewardship, and (iv) the development of new drugs and diagnostics test[57]. We propose an additional core action, the development of adjuvants, or compounds that act to potentiate the activity of conventional antimicrobials against MDR bacteria by targeting resistance mechanisms.

To this end, we performed a high throughput screen (HTS) of 6,080 compounds from four drug repurposing libraries (Prestwick, MS2400, LOPAC, and focus collection libraries) at the University of Michigan Center for Chemical Genomics to identify adjuvants that enhanced tobramycin killing of mature *P. aeruginosa* biofilms.[6]
We identified two novel adjuvants, the fatty acid synthesis inhibitor triclosan and the anthelmintic oxyclozanide both enhanced tobramycin activity.[6] Finally, we evaluated the

synergistic activity of the AMP melittin combined with tobramycin against mature *P. aeruginosa* biofilms.

In Chapter two, we evaluated the Food and Drug Administration (FDA) approved compound, triclosan, combined with tobramycin. We found the combination resulted in a 100-fold reduction of viable cells within biofilms at six hours, but neither compound alone had significant antimicrobial activity against biofilms. This synergistic treatment significantly accelerated killing of biofilms compared to tobramycin treatment alone, and the combination was effective against 6/7 CF clinical isolates compared to tobramycin treatment alone, including a tobramycin resistant strain. Further, triclosan and tobramycin killed persister cells, causing a 100-fold reduction by 8-hrs and eradication by 24-hrs. Finally, using a murine wound model, we show that triclosan and tobramycin are more effective against *P. aeruginosa* biofilms than tobramycin treatment alone *in vivo*.

In Chapter three, we evaluated the veterinary approved anthelmintic, oxyclozanide, combined with tobramycin. We found oxyclozanide combined with tobramycin significantly increased tobramycin killing of mature *P. aeruginosa* biofilms compared with tobramycin treatment alone. This combination also accelerated tobramycin killing of cells within biofilms from 6-hrs to 2-hrs and was effective against 4/6 CF clinical isolates tested, including a tobramycin resistant strain. We also elucidate the mechanism of action of oxyclozanide and tobramycin. We found oxyclozanide acted as a permeabilizer and ionophore, reducing the membrane potential of cells within biofilms, and thus, potentially inhibiting efflux pump activity. In support of this, we found oxyclozanide increased tobramycin accumulation within cells. However, oxyclozanide did

not inhibit RND-type efflux pumps when used alone, suggesting permeabilization may be responsible for increased tobramycin accumulation or another type of efflux pump is inhibited by oxyclozanide.

In Chapter four, we investigated the mechanism of action of triclosan and tobramycin. Similarly, we show that triclosan acts as an ionophore and can permeabilize cells within biofilms. Conversely, we found triclosan inhibited RND-type efflux pump activity and this may result in increased tobramycin accumulated within cells. These data suggest triclosan is an EPI against *P. aeruginosa* growing as a biofilm.

In Chapter five, we demonstrated that the AMP, melittin, is effective alone and in combination with aminoglycosides against mature biofilms formed by *P. aeruginosa*. Melittin and tobramycin resulted in a 100-fold reduction in the number of cells within biofilms after 6-hrs of treatment. Further, the combination demonstrated rapid activity and was effective by 2-hrs, whereas tobramycin was effective by 6-hrs. Melittin and tobramycin showed activity against 7/7 of the CF clinical isolates tested. Finally, we show that melittin permeabilizes cells within biofilms, enhancing the activity of tobramycin. Finally, using a murine wound model, we show that melittin and tobramycin are more effective against *P. aeruginosa* biofilms than tobramycin treatment alone *in vivo*.

Adjuvants represent an underutilized approach to overcome bacterial resistance and improve patient outcomes. Together these data demonstrate that triclosan, oxyclozanide and melittin are potential adjuvants that could be used against MDR bacteria. In this thesis, we describe the development of adjuvants to meet this challenge.

CHAPTER 2

TRICLOSAN IS AN AMINOGLYCOSIDE ADJUVANT FOR THE ERADICATION OF *PSEUDOMONAS AERUGINOSA* BIOFILMS

2.1 Introduction

There is a critical need to identify new agents that target cells within biofilms and avoid selecting for resistance. One possible strategy is to identify anti-resistance compounds or adjuvants, which are effective when combined with antimicrobials but are not effective on their own as described in Chapter 1.[56] To this end, we performed a high-throughput screen (HTS) to identify adjuvants that are effective when combined with tobramycin against *P. aeruginosa* biofilms. From the HTS, we determined that the bisphenol, triclosan, combined with tobramycin was significantly more effective at killing mature *P. aeruginosa* biofilms. Triclosan similarly synergized with the aminoglycosides gentamicin or streptomycin. We also found the combination more effectively killed biofilms of *Burkholderia cenocepacia* and *Staphylococcus aureus,* two bacteria commonly isolated from CF patients. The combination of triclosan with aminoglycosides significantly enhanced the rate of biofilm killing and led to persister cell eradication. Our results suggest that triclosan could provide a potential new aminoglycoside adjuvant for the treatment of *P. aeruginosa* biofilms for multiple indications including CF, diabetic foot ulcers, and burn wounds.[115-117]

Note: Much of this work was previously published.[6] M.M.M and C.M.W., wrote and edited the manuscript. A.A.H & C.M.W., developed and performed the high through put screen. M.M.M. & A.A.H., performed the majority of the experiments. M.P.Z., J.A.G., provided technical assistance. M.H.H. and M.E.H., provided clinical isolates.

2.2 Materials and Methods

2.2.1 Bacterial strains, culture conditions, antibiotics and compounds

Strains used in this study are listed in Table 2-1. Unless stated, bacterial strains were grown in 8 mL glass test tubes (18 X 150mm) at 35 ºC in cation adjusted Müeller-Hinton Broth II (MHB, Sigma-Aldrich) with agitation at 210 revolutions per minute (RPM). Biofilms were grown using the MBEC™ assay (Innovotech) as previously described.[5] For MBEC™ experiments 1 mL of the culture was pelleted and washed three times in 10% MHB in Dulbecco's Phosphate Buffered Saline with magnesium and calcium (DPBS, Sigma-Aldrich) and diluted to an OD_{600} of 0.001. 10% MHB was used to slow bacterial growth and avoid the rapid exhaustion of nutrients found when using more nutrient rich media. 160 µL/well of the diluted culture was seeded into a 96-well MBEC™ plate and incubated for 24-hrs at 35 ºC in a humidified chamber with agitation at 150 RPM. 24-hr old biofilms grown on the peg-lid were then transferred to a 96-well plate filled with DPBS and washed for 5-mins to remove non-adherent cells before being assayed. Bacteria were plated on Dey-Engley neutralizing agar plates (DEA), which neutralizes the activity of disinfectants and antiseptics (Sigma-Aldrich) or tryptic soy agar plates (TSA; Sigma-Aldrich). Antimicrobial activity was neutralized using Dey-Engley (D/E) media before plating as indicated. All antibiotics were obtained from Sigma-Aldrich. Tobramycin sulfate, gentamicin sulfate, and streptomycin sulfate were dissolved in autoclaved deionized water and filter sterilized using 0.22 µM filter membranes (Thomas Scientific). Triclosan (2,4,4'-Trichloro-2'-hydroxydiphenyl ether) and triclocarban (1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl)urea) were dissolved in

100% ethanol. For this study, all susceptibility testing was performed in 1%MHII diluted in DPBS to prevent further growth.

Table 2-1. Bacterial strains used in this study.

Strain	Characteristics	Source or Reference
PAO1	Standard Reference Strain, isolated in 1954 [118]	Martha Mulks
Tn::*fabI*	ISlacZ/hah	[119]
AMT0023_30	Early Isolate 6 MO	[120]
AMT0023_34	Late Isolate 8 YO	[120]
CF_115_J	PA Clinical CF Isolate, Michigan	Martha Mulks
CF_110_N	PA Clinical CF Isolate, Michigan	Martha Mulks
CF_110_O	PA Clinical CF Isolate, Michigan	Martha Mulks
CF_131_M	PA Clinical CF Isolate, Michigan	Martha Mulks
CF_300_A	PA Clinical CF Isolate, Michigan	Martha Mulks
AU1054	BCC Clinical CF Isolate, United States	J. J. LiPuma
PC184	BCC Clinical CF Isolate, Cleveland Ohio	J. J. LiPuma
AU2289	BCC Clinical CF Isolate, Michigan	J. J. LiPuma
H12424	Soil, onion field, New York	J. J. LiPuma
J2315	BCC Clinical CF Isolate, Edinburgh UK	J. J. LiPuma
USA_300_JE2	MRSA, Wound, California	Neal Hammer
COL	MRSA, Colindale Hospital England	Martha Mulks
Newman (25904)	MSSA, Wound, Endocarditis	Neal Hammer ATCC
Wichita (29213)	MSSA, Better Biofilm Former	ATCC

Table 2-1 (cont'd)

Abbreviations: *Pseudomonas aeruginosa* (PA), *Burkholderia cenocepacia complex* (BCC), *Methicillin-resistant Staphylococcus aureus* (MRSA), *Methicillin-sensitive Staphylococcus aureus* (MSSA), American Tissue Type Collection (ATTC). J. J. LiPuma, U.S. *Burkholderia cenocepacia* Research Laboratory and Repository, UM, Ann Arbor MI.

2.2.2 BacTiter-Glo™ Calibration Curve

The BacTiter-Glo™ microbial cell viability assay (Promega) is a bioluminescent assay that determines the number of viable cells present based on quantification of adenosine triphosphate (ATP) concentration as previously described.[121,122] To confirm that BacTiter-Glo™ can be used to reliably determine cellular number a calibration curve was performed. 1 mL of a 16-hr overnight culture of *P. aeruginosa* PAO1 was washed three times and diluted two-fold in a black 96-well ViewPlate (PerkinElmer) with 1% MHB. Aliquots were taken from each dilution series for colony forming unit (CFU) enumeration on TSA. BacTiter-Glo™ was then added according to the manufactures specifications to enumerate cell viability. The plate was incubated in the BacTiter-Glo™ for 5-mins and then luminescence per well was measured using a EnVison Multilabel Plate Reader (PerkenElmer, Waltham, MA). Data was plotted as the average luminescence for each dilution series in triplicate versus the average CFUs/mL for each dilution series in triplicate. We derived a coefficient of determination, r^2=0.9884, for luminescence versus CFU/mL using a linear regression (Figure 2-1), and we found the limit of detection for BacTiter-Glo™ to be 1,000 CFU/mL.

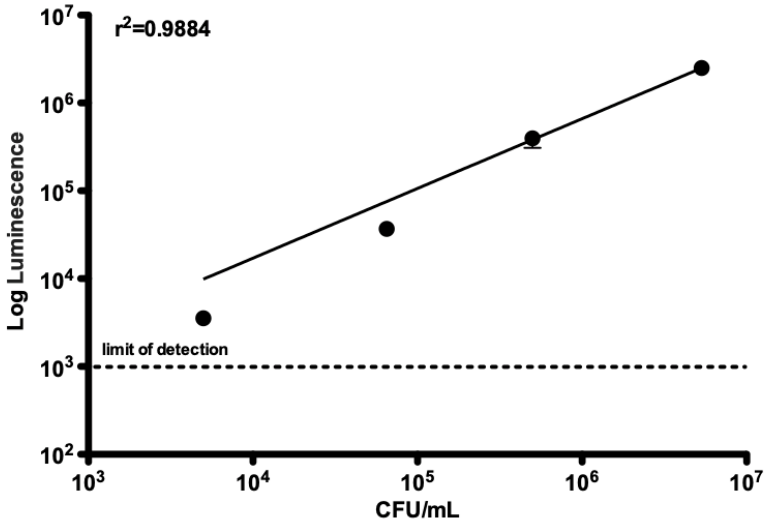

Figure 2-1. Log luminescent units correlate to CFUs. Serial dilutions of cells were plated in triplicate and BacTiter-Glo™ was added to determine cell number. Aliquots were also taken at each dilution to enumerate colony forming units. Log luminescent units versus CFUs/mL were then plotted. The results represent means plus the standard error of the mean (SEM). A linear regression was performed to determine goodness of fit (coefficient of determination r^2=0.9884).

2.2.3 High Throughput Screen

We screened 6,080 compounds from the Prestwick (Prestwick Chemical), MS2400 (Spectrum Collection), LOPAC^{1280} (Sigma Aldrich), and Focused Collections libraries at the University of Michigan (UM) Center for Chemical Genomics (CCG). A overnight culture was prepared as described above and 30 µL/well was seeded into a 384-well plate (Corning) and a 384-pintool (Scinomix) was inserted. The plate-pin combination was incubated for 18-hrs at 37 ºC in a humidified chamber without agitation. The pintool was then transferred to a plate filled with 40 µL/well of DPBS to remove non-adherent cells and debris and then transferred to a 384-well plate filled with 40 µL/well of compounds alone, compounds with tobramycin, or sterile media. Compounds were used at a concentration of 10 µM in 1% MHB. Tobramycin was used

at a concentration of 250 µg/mL (~500 µM, 500X MIC) in 1% MHB. 250 µg/mL of tobramycin was chosen because it leads to ~50% killing of the biofilm (data not shown). Compounds were dissolved in dimethyl sulfoxide (DMSO). Polymyxin B was used as a positive control at 10 µg/mL and 1% DMSO was used as a negative control. After 6-hrs of static treatment at 37ºC, the pintool was washed in a 384-well plate filled with 40 µL/well of DPBS for 5-mins to reduce the carryover of various treatments. Then the pintool was transferred to a 384-white well plate (Greiner), to prevent luminescence crosstalk, filled with 40 µL/well of 25% BacTiter-Glo™ for 5-mins to enumerate cell viability and luminescence per peg were measured using a EnVison Multilabel Plate Reader. Data were plotted as a % reduction ($\%\ \text{reduction} = \left(\frac{\text{Untreated} - \text{Treated}}{\text{Untreated}}\right) \text{X}\ 100$).

2.2.4 Biofilm susceptibility testing using BacTiter-Glo™

24-hr old biofilms formed on the lid of a MBEC™ plate was transferred to the 96-well plate in which the dilutions had been made and incubated for 6-hr at 35 ºC without agitation. The MBEC™ lid was washed for 5-mins in DPBS to reduce carryover and transferred to a black 96-well ViewPlate filled with 160 µL/well of 25% BacTiter-Glo™ to enumerate cell viability as described above. Data was plotted as the average luminescence for each condition tested in triplicate. Dose response curves (DRC), time killing curves, and checkerboard experiments were performed similarly.

2.2.5 Minimum inhibitory concentration (MIC) Planktonically

MICs were determined using the broth microdilution technique.[4] Microdilutions of each aminoglycoside and triclosan were made in a 96-well plate. Cells were added at a concentration of ~1x10^6 CFU/mL. The plates were then incubated for 24-hrs at 35 ºC in a humidified chamber with agitation at 150 RPM. After the 24-hr incubation, the absorbance at 595 nm was measured using a SpectraMax M5 microplate spectrophotometer system (Molecular Devices Sunnyvale, CA). MIC breakpoints were chosen as the minimum concentration in which no turbidity greater than background was measured.

2.2.6 Crystal violet staining

To study biofilm dispersal under static conditions, crystal violet staining was performed as previously described.[7] 24-hr old biofilms formed on MBEC™ plates, as described above, were stained with 0.41% crystal violet solubilized in 12% ethanol in a 96-well plate following a 6-hr treatment.

2.2.7 Flow cell assays

To study biofilm dispersal under flow conditions, biofilms were grown in disposable flow cells (Stovall Life Science, Greensboro, NC) as previously described.[123] Briefly, the inlet side of the flow cell was connected to a reservoir filled with 10% MHB and the outlet side was connected to a waste reservoir. Each flow cell was injected with 0.5 mL of 16-hr overnight culture and the chamber was incubated at 37°C for 1-hr in 100% MHB. The flow was then resumed, with a flow rate of 0.2 mL/min. After 24-hrs,

biofilms were treated for 6-hrs with triclosan and tobramycin, or with each compound alone. Biofilms were then stained with the live/dead cell viability assay using SYTO® 9 and propidium iodide dyes (ThermoFisher Scientific).

2.2.8 Persister enrichment and killing assays

To determine the effects of triclosan combined with tobramycin on persister cells, planktonic stationary-phase cultures were used as previously described.[124] Cultures were grown for 20-hrs and 100 µL/well of cells were dispersed in a 96-well plate. Treatments were added, and the plate was incubated at 37°C without agitation. At hours 2,4,6,8, and 24, 30 µL aliquots were serially diluted, plated on DEA, and CFUs were enumerated. The dilutions that contained 3-30 colonies per 10 µl were counted. Eradication was recorded if there were no colonies found in the drop plating in triplicate of $1x10^{-10}$ dilution and thus the limit of detection for this assay is 10 CFU/mL.

2.2.9 Triclosan toxicity studies

Both studies were performed in collaboration with the Michigan State University *In Vivo* Pharmacology Facility.

1-Day Acute Study: Sprague-Dawley rats were anesthetized with isoflurane and received treatments by trans-oral intratracheal instillation. A control group (N=4) received 1 % DMSO in sterile saline. Dosing groups received triclosan at 10, 30, 100, 300, or 1000 µg/kg, N=3 per each dosing. 24-hrs after dosing, ≥ 2 mL blood was collected under isoflurane anesthesia and animals were subsequently euthanized. Blood samples were transferred to tubes containing dipotassium

ethylenediaminetetraacetic acid, to prevent clotting, or serum separator tubes for complete blood count (CBC), systemic triclosan absorption using liquid chromatography tandem mass spectrometry (LC-MS/MS), and clinical chemistry evaluation. Lungs were collected *en bloc*, weighed, and inflation-fixed in 10% formalin. The left lung was processed using standard hematoxylin and eosin (H&E) staining. Microscopic evaluations of the lungs were blindly examined by the Department of Pathobiology and Diagnostic Investigation at MSU by a board certified veterinary pathologist. Using GraphPad Prism 6.04, statistical analysis of body weight, organ weights, CBC, and clinical chemistry results were compared by a Kruskal-Wallis one-way analysis of variance followed by post-hoc testing using Dunn's multiple comparisons test.

***7-Day Repeated Exposure Study*:** Sprague-Dawley rats were administered vehicle or triclosan at 30, 100, or 300 µg/kg via trans-oral intratracheal instillation daily for 7 consecutive days N=5 per each treatment. Rats were weighed prior to dosing on Day 1 and on Day 7. On day 7, ~1.5-hrs after dosing, ≥ 2 mL blood was collected. Blood samples were processed as described above. Lungs were collected *en bloc*, and processed, evaluated, and analyzed as described above.

2.2.10 Mass spectrometry quantification of triclosan in serum

Serum samples were filtered through a 0.45 µM syringe filter (Titan-PVDF, TheroScientific) using a 1 mL syringe (BD) into a glass insert (Agilent technologies) of a 2-mL clear MS vial (Restek). Samples were analyzed for triclosan via LC-MS/MS on a Quattro Premier XE mass spectrometer (Waters) coupled with a Acquity Ultra

Performance LC system (Waters). Triclosan was detected using electrospray ionization using multiple reaction monitoring in negative-ion mode with at *m/z* 286.94>35.00. The MS parameters were as follows: capillary voltage, 3.5 kV; cone voltage, 35 V; collision energy, 25 V; source temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow (nitrogen), 50 L/h; desolvation gas flow (nitrogen), 800 L/h; collision gas flow (nitrogen), 0.17 mL/min; and multiplier voltage, 650 V. Chromatography separation was reverse phase using an Acquity UPLC BEH C18, 50 x 2.1 mm (Waters), 1.7 µM, column at a temperature of 40℃ with a flow rate of 0.4 mL/min with the following gradient of solvent A (0.1% formic acid) to solvent B (acetonitrile in high-performance liquid chromatography water): t = 0 min; A-95%:B-5%, t = 2-min; A-95%:B-5%, t = 3-min; A-5%:B-95%, t = 4-min; A-5%:B-95%, t = 4.01-min; A-95%:B-5%, t = 5-min; A-95%:B-5%.

2.2.11 Agarose hydrogels

Agarose hydrogels were made by dissolving 1 gm of agarose (Sigma-Aldrich) into 200 mL of Tris-acetate-EDTA (TAE) buffer and heated to form a homogenous solution using a microwave. The 0.5% agarose solution was then allowed to cool and various treatments were added. The solution was then poured into 100 x 15 mm petri dishes (Thermo Fisher Scientific) and stored at 4°C overnight. Prior to treatment, a 4 mm biopsy punch (VWR) was used to create hydrogel wafers.

2.2.12 Murine wound infection model

Wound surgery was performed on 8-9 week-old male and female SKH-1 mice (Charles River), as previously described.[125,126] 24-hr old wounds were infected with

~1x10^9 *P. aeruginosa* cells Xen41 (PerkinElmer), which is a bioluminescent derivative of PAO1 that constitutively expresses *luxCDABE* gene.[125,126] Briefly, 24-hr old biofilms were formed on sterilized polycarbonate membrane filters with a 0.2 μM pore size (Millipore Sigma) by diluting an overnight culture to an OD_{600} of 0.001 and pipetting 100 μl on 4 membranes on a tryptic soy agar (TSA) plate. 24-hr old biofilms were scrapped using L-shaped spreaders (Sigma-Aldrich) from each membrane and re-suspended in 500 μl of DPBS. 20 μl of the biofilm-suspension was inoculated into 24-hr old wounds formed on the dorsal side of the mouse midway between the head and the base of the tail. 24-hrs later the biofilm was imaged using in vivo imaging system (IVIS, Perkin Elmer). The biofilm was then treated by placing a 4 mm 0.5% agarose hydrogel on the wound for 4-hrs. The biofilm was imaged before and after treatment and total flux (photons/sec) was used to quantify bacterial susceptibility.

2.3 Results

2.3.1 Triclosan combined with aminoglycosides results in greater killing of *P. aeruginosa* biofilms

We developed and carried out a HTS of 6,080 compounds from four drug repurposing libraries at the UM CCG to identify compounds that enhanced tobramycin killing of *P. aeruginosa* strain PAO1 biofilms grown on the pegs of a 384-well disposable pin tool. 250 μg/mL tobramycin (~500 μM, 500X planktonic MIC) was used because we found that this concentration had only mild effects on biofilms as seen below. Compounds were added at 10 μM and biofilms were treated for 6-hrs before assaying for cell viability using BacTiter-Glo™. The average plate Z-factor was 0.6. The Z-factor

is a relative score of statistical size effect that utilizes both the divergence and range of the negative and positive controls ranging from 0.0 - 1.0 where 0.5 - 1.0 indicates an excellent assay.[127] 118 compounds exhibiting greater than 3-standard deviations from control and 50% enhancement of biofilm killing were selected as initial hits. The initial hit rate for the screen was 1.9%, but this rate is inflated as these libraries are enriched for biologically active molecules including a number of antibiotics. DRC for each of these 118 hits were generated in duplicate to determine the pAC50 (the inverse log10 of 50% activity) and the maximum killing percentage. This was performed both with and without 250 μg/mL tobramycin. 82 compounds were considered active with pAC50 values ranging from 8.4-3.4 showing a 71.3% confirmation rate. 31 of these compounds were also active on their own and were given lower priority. As we are interested in targeting new pathways that are specific to antibiotic tolerance, we decided to first focus on compounds that are only effective in the presence of tobramycin. After removing compounds that contained problematic toxicity groups and exhibited promiscuous activity in prior HTSs, 26 compounds remained as promising antibiotic adjuvants for further investigation. Here we describe our further analysis of one of these 26 compounds, triclosan.

Triclosan is a broad-spectrum antimicrobial that inhibits the enoyl-acyl carrier protein reductase FabI to prevent fatty acid synthesis in several bacterial species (Fig. 2-2).[128,129]

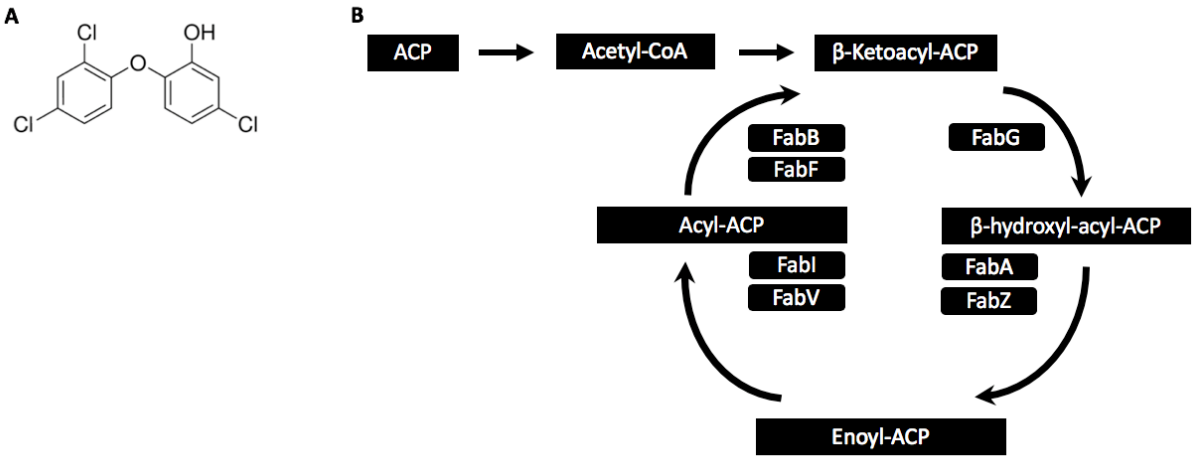

Figure 2-2. Molecular structure of triclosan and type II fatty acid synthesis.

A. Chemical structure of triclosan, 2,4,4'-trichloro-2'-hydroxydiphenylether. **B.** Diagram of the type II fatty acid synthesis (FASII) pathway. Triclosan is known to disrupt FASII by inhibiting FabI. However, FabV is more resistant to triclosan. Abbreviations: ACP=acyl carrier protein.

We chose to further characterize triclosan as it showed significant synergy with tobramycin. Furthermore, it is known that *P. aeruginosa* is inherently resistant to triclosan, making it an intriguing candidate.[130-132] Finally, triclosan has been widely used as a biocide for decades and is still FDA approved to be used in toothpastes at mM concentrations.[133]

To determine the antibiofilm activities of triclosan and tobramycin, 24-hr PAO1 biofilms were treated for 6-hrs with 100 μM of triclosan and 500 μM of tobramycin alone or in combination. Tobramycin alone resulted in a ~2-fold reduction in viable cells compared to untreated biofilms, triclosan alone exhibited no killing, but the combination resulted in a 100-fold reduction in viable cells (Fig. 2-3).

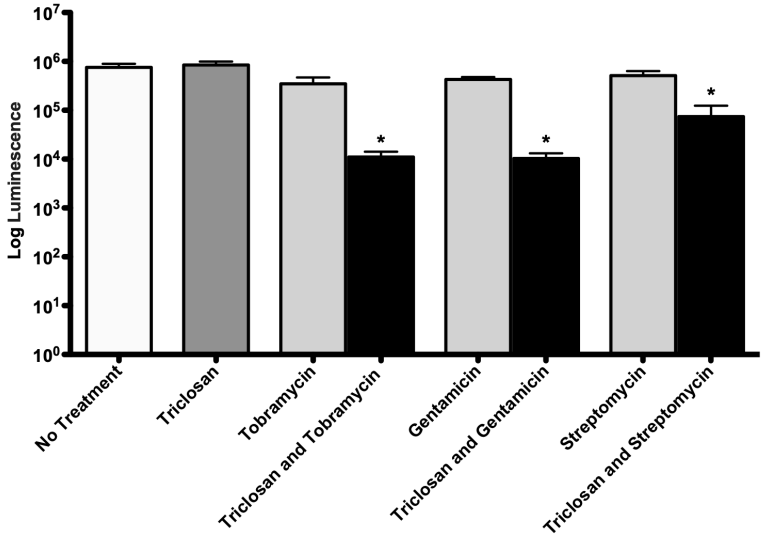

Figure 2-3. Triclosan and aminoglycosides synergize to kill 24-hr old biofilms. Biofilms were treated for 6-hrs with triclosan (100 μM), tobramycin (500 μM), gentamicin (100 μM), or streptomycin (100 μM) alone and in combination, and the number of viable cells within the biofilms were quantified. The assay was performed at least three times in triplicate. The results represent means plus the SEM. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance compared to each aminoglycoside alone (*, $p<0.05$).

These experiments were performed using BacTiter-Glo™ to measure ATP in the biofilm, and in a control experiment we demonstrated that the luminescence generated by this assay shows a direct linear relationship with CFUs (Figure 2-1). We also tested whether triclosan synergized with the antipseudomonal aminoglycosides gentamicin or streptomycin. Biofilms were treated with either 100 μM of gentamicin or streptomycin alone or in combination with 100 μM of triclosan. 100 μM of gentamicin and streptomycin were chosen because these concentrations exhibit 50% reduction in viable cells. After 6-hrs of treatment, gentamicin reduced the number of cells by 2-fold whereas triclosan and gentamicin reduced the number of cells by 100-fold (Figure 2-3).

Likewise, streptomycin alone had little effect on the cells within biofilms whereas triclosan and streptomycin reduced the number of cells by 10-fold.

We also tested if triclosan could enhance several additional antipseudomonal antibiotics, including third and fourth generation carbapenems, which all exhibited poor activity on their own, and found triclosan only enhanced aminoglycosides (Figure 2-4).

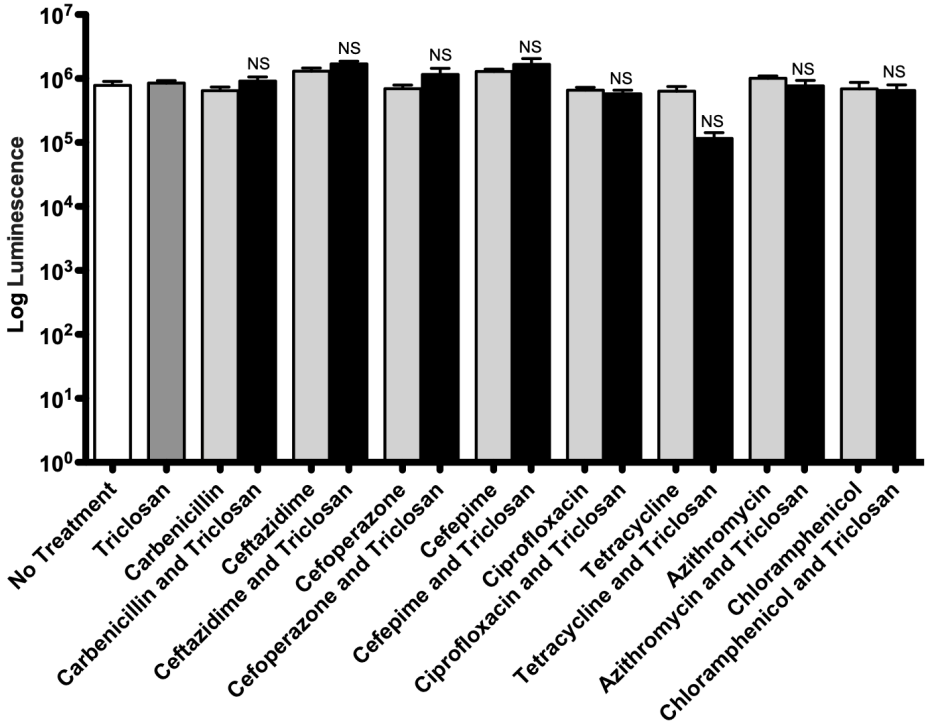

Figure 2-4. Triclosan and non-aminoglycoside antibiotics do not synergize. Biofilms were treated for 6-hrs with triclosan (100 μM) and each antibiotic (100 μM) alone and in combination. The assay was performed two times in triplicate. The results represent means plus the SEM. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance compared to each antipseudomonal alone (NS, not significant).

2.3.2 Triclosan synergizes with aminoglycosides at multiple concentrations

DRC were performed to determine EC_{50} values. Biofilms were treated with dilutions of triclosan and each aminoglycoside ranging from 0.390 to 400 μM in triplicate. Tobramycin alone showed modest activity at concentrations greater than 10 μM, killing approximately 50% of the cells after 6-hrs of treatment (Figure 2-5).

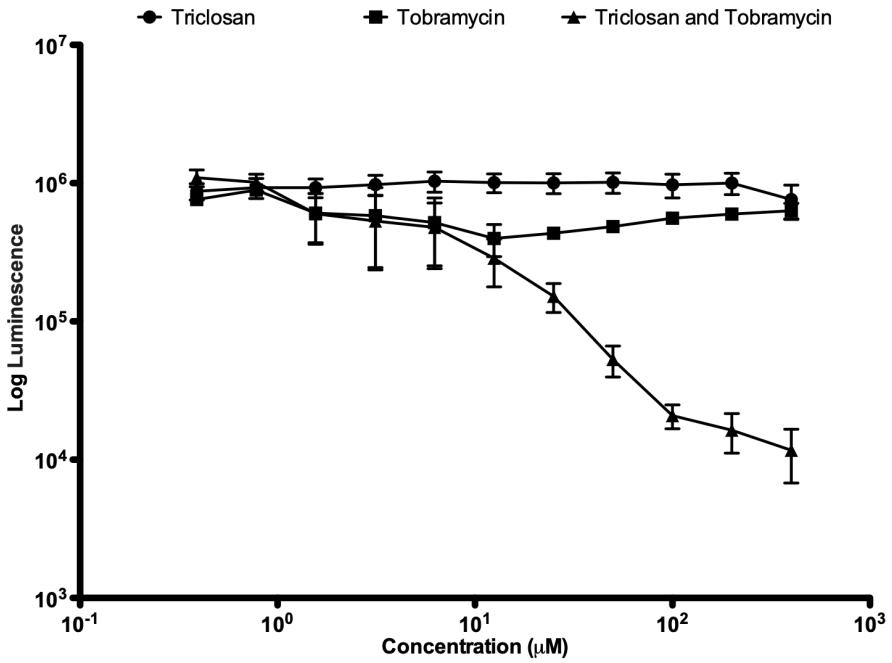

Figure 2-5. Triclosan and tobramycin synergize at multiple concentrations. Biofilms were treated for 6-hrs with 2-fold dilutions of equal concentrations of triclosan combined with tobramycin. The assay was performed at least three times in triplicate. The results represent means ± the SEM.

However, higher concentrations of tobramycin were less effective. This could be due to a phenomenon known as adaptive resistance where aminoglycosides induce antibiotic resistance in *P. aeruginosa* [33,34]. In addition, the paradoxical effects of aminoglycosides in which higher concentrations of aminoglycosides are less effective than lower concentrations has previously been reported [134]. Triclosan did not exhibit activity at any concentration tested. In contrast, the combination of triclosan and tobramycin increased biofilm killing between 25 and 400 μM with maximum activity at

400 μM, demonstrating 100-fold more activity than tobramycin alone. Like tobramycin, gentamicin and streptomycin treatment alone exhibited modest killing activity against biofilms that decreased at increasing concentrations (Figure 2-6A-B).

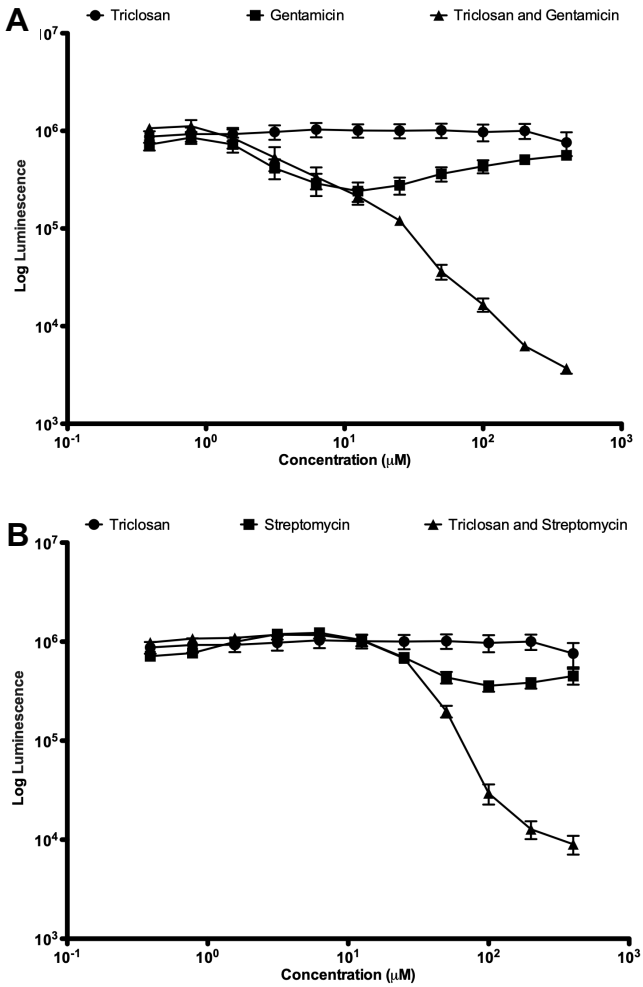

Figure 2-6. Triclosan and gentamicin or streptomycin synergize at multiple concentrations. Biofilms were treated for 6-hrs with 2-fold dilutions of equal concentrations of triclosan combined with gentamicin or streptomycin. The assay was performed at least three times in triplicate. The results represent means ± the SEM.

Triclosan combined with streptomycin or gentamicin at higher concentrations led to a ~100-fold reduction in cells. The EC_{50} values for biofilm killing by these three combinations were between 20 and 30 μM (Table 2-2). The EC_{50} value for tobramycin

and gentamicin with triclosan showed wide variation, with a 95% confidence interval (CI) of 7.65 – 83.78 μM and 12.08-44.02 μM, respectively, whereas streptomycin had a 95% CI of and 24.5 – 34.31.

Table 2-2. Half maximal effective concentration 50 (EC50) values for aminoglycoside combinations.

Antibiotic	Adjuvant	EC50 (μM)	95% Confidence Interval (μM)
Tobramycin	Triclosan	20.50	7.65 – 83.78
Gentamicin	Triclosan	23.06	12.08 – 44.02
Streptomycin	Triclosan	28.96	24.45 – 34.31

EC50 values were calculated using Prism® Version 5. Log10 (inhibitor) vs response – variable slope (four parameters) analyses were performed. EC50 values for aminoglycoside alone were not constructed because no curve was established due to their ineffectiveness against biofilms.

Checkerboard dilution experiments were performed to determine the lowest possible combinations of tobramycin and triclosan that resulted in >1 log10 killing (Figure 2-7). Biofilms were treated with dilutions of triclosan, ranging from 12.5 to 100 μM in triplicate, and tobramycin, ranging from 66 to 534 μM in triplicate. 50 μM of triclosan with 66 to 534 μM of tobramycin resulted in a significant reduction in cells within biofilms. And 25 μM of triclosan combined with 534 or 267 μM of tobramycin resulted in significant killing (Table 2-3). Tobramycin at 66 μM combined with triclosan from 1.5 to 400 μM in showed that triclosan synergized with tobramycin at any concentration greater than 12.5 μM significantly killing cells within biofilms compared to tobramycin alone (Figure 2-8).

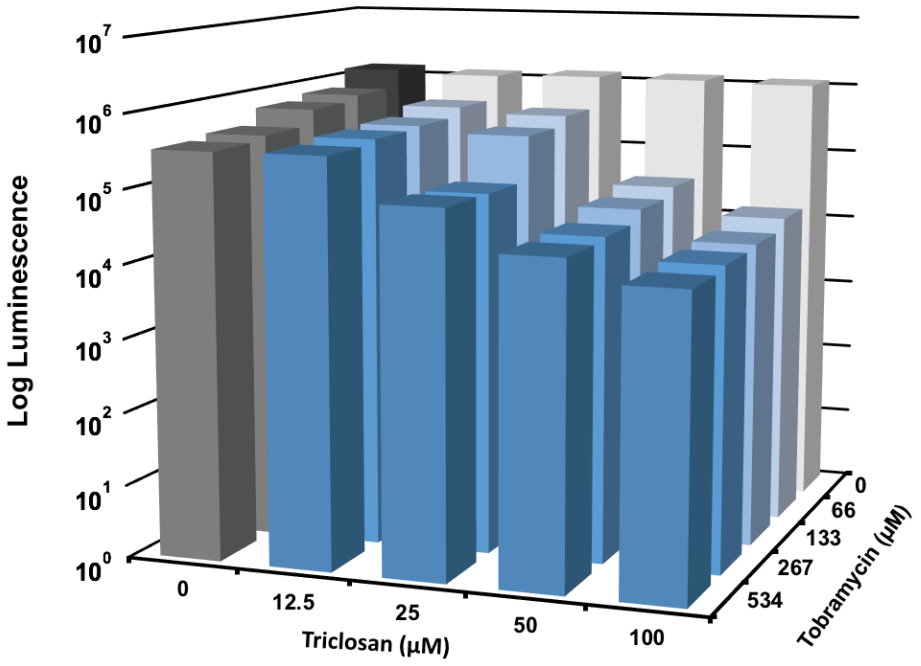

Figure 2-7. Triclosan enhances bacterial killing at lower concentrations of tobramycin. Biofilms were treated for 6-hrs with checkerboard dilutions of triclosan combined with tobramycin. The assay was performed at least three times in triplicate. The results represent means.

Table 2-3. Triclosan enhances low concentrations of tobramycin.

Tobramycin \ Triclosan	100 µM	50 µM	25 µM	12.5 µM	0 µM
534 µM	**$1.2E^4$** ($3.1E^3$)	**$2.4E^4$** ($1.2E^3$)	**$8.8E^5$** ($5.6E^4$)	**$3.5E^5$** ($1.8E^5$)	**$3.5E^5$** ($3.1E^5$)
267 µM	**$1.4E^4$** ($6.4E^3$)	**$2.7E^4$** ($1.6E^4$)	**$8.04E^5$** ($3.3E^4$)	**$3.9E^5$** ($1.6E^5$)	**$3.6E^5$** ($3.3E^5$)
133 µM	**$1.5E^4$** ($6.1E^3$)	**$3.9E^4$** ($9.8E^4$)	**$3.4E^5$** ($4.0E^5$)	**$4.2E^5$** ($1.2E^4$)	**$7.2E^5$** ($3.9E^5$)
66 µM	**$2.1E^4$** ($1.5E^4$)	**$5.1E^4$** ($2.1E^4$)	**$4.7E^5$** ($5.0E^5$)	**$5.5E^5$** ($2.0E^5$)	**$8.8E^5$** ($4.8E^5$)
0 µM	**$1.2E^6$** ($6.7E^5$)	**$1.2E^6$** ($4.8E^5$)	**$1.3E^6$** ($4.1E^5$)	**$1.2E^6$** ($3.8E^5$)	**$1.4E^6$** ($1.8E^5$)

Biofilms were treated for 6-hrs with checkerboard dilutions of triclosan combined with tobramycin. The assay was performed at least three times in triplicate. The results represent means ± the Standard Error Deviation (SD). A two-way ANOVA followed by Bonferroni's test was used to determine statistical significance compared to tobramycin treatment alone. Shaded cells indicate significance ($p<0.05$).

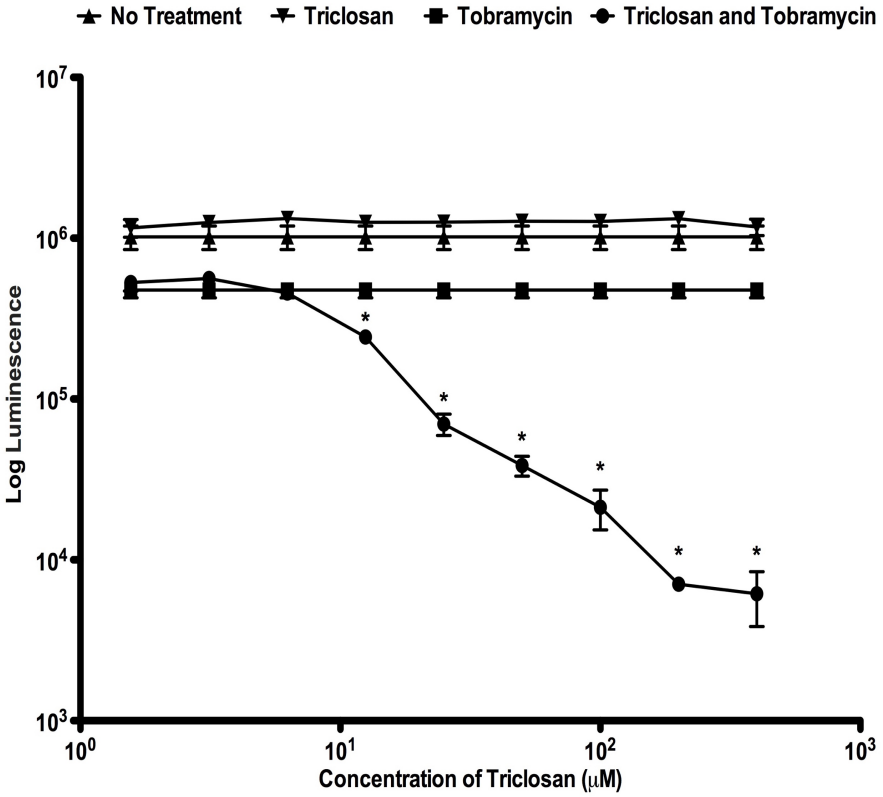

Figure 2-8. Triclosan enhances bacterial killing at lower concentrations of tobramycin. Biofilms were treated for 6-hrs with dilutions of triclosan combined with tobramycin at a fixed concentration of 66 μM. The assay was performed once in triplicate. The results represent means ± the SEM. A two-way ANOVA followed by Bonferroni's test was used to determine statistical significance compared to tobramycin alone. (*, $p<0.05$).

2.3.3 The planktonic MICs of tobramycin, gentamicin, or streptomycin are not changed when used in combination with triclosan

We wondered if the synergy of triclosan with aminoglycosides was specific to biofilm growing cells. We therefore determined the MIC of the combinations against planktonically grown cells. Surprisingly, we found that triclosan combined with tobramycin, gentamicin, or streptomycin did not impact the planktonic MIC of the aminoglycosides to kill *P. aeruginosa*. This suggests that triclosan and these aminoglycosides functionally synergistically specifically against biofilm-growing bacteria.

Table 2-4. Triclosan does not alter MIC values of cells growing planktonically.

Antimicrobial	Alone (μM)	+ Triclosan (μM)
Tobramycin	1.5 - 3.125	1.5 - 3.125
Gentamicin	6.25 - 12.5	6.25 - 12.5
Streptomycin	50.0	50.0

MIC were determined as the minimum concentration that no turbidity greater than background was measured.

2.3.4 Triclosan combined with tobramycin resulted in accelerated killing of biofilm cells.

Time killing assays were performed to determine the rate of killing of 100 μM triclosan, 500 μM tobramycin, or the combination of the two. Triclosan was ineffective alone, whereas tobramycin increased ATP concentrations and or cell number in the biofilm at 2 and 4-hrs (Figure 2-9). This may be due to a stress response initiated by bacteria experiencing antimicrobial toxicity.[135] Triclosan combined with tobramycin resulted in a shorter onset of action with killing observed at 2-hrs compared to 6-hrs for tobramycin alone (Figure 2-9).

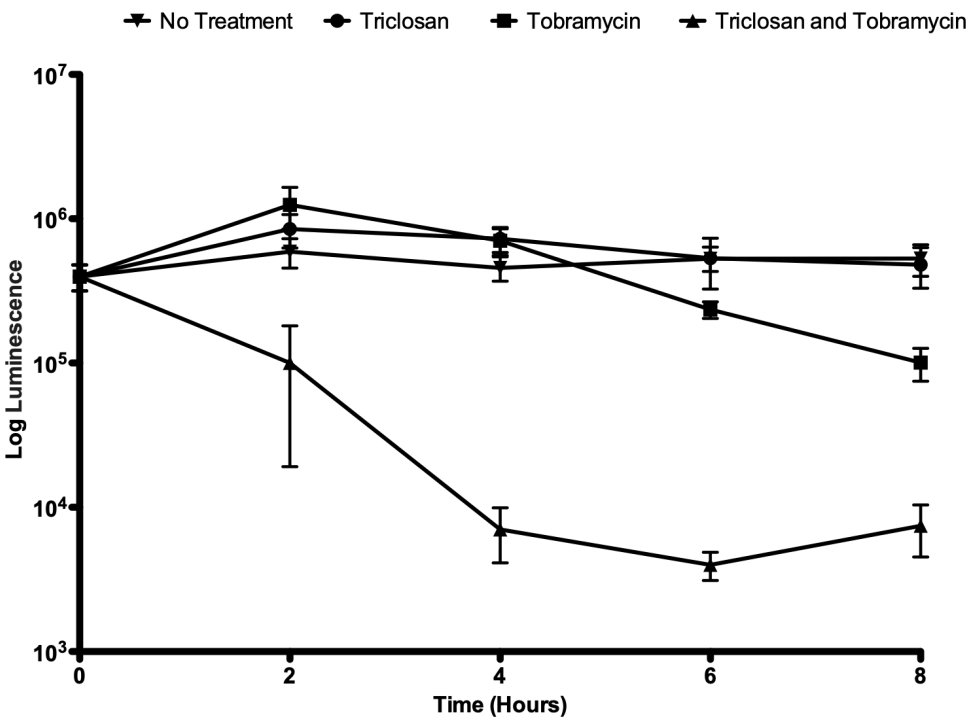

Figure 2-9. Triclosan enhances the onset and maximum efficacy of tobramycin. Biofilms were treated with triclosan (100 μM) or tobramycin (500 μM) alone and in combination. At 0, 2, 4, 6, and 8-hrs the number of viable cells within the biofilms were quantified. The assay was performed at least three times in triplicate. The results represent means ± SEM.

In addition, the combination exhibited killing at 2-hrs that was similar to that observed for tobramycin alone at 8-hrs and reduced the number of cells within the biofilm by over 100-fold at 4-hrs compared to tobramycin alone (Figure 2-9). Analogous experiments were repeated with streptomycin and gentamicin with similar outcomes (Figure 2-10A-B).

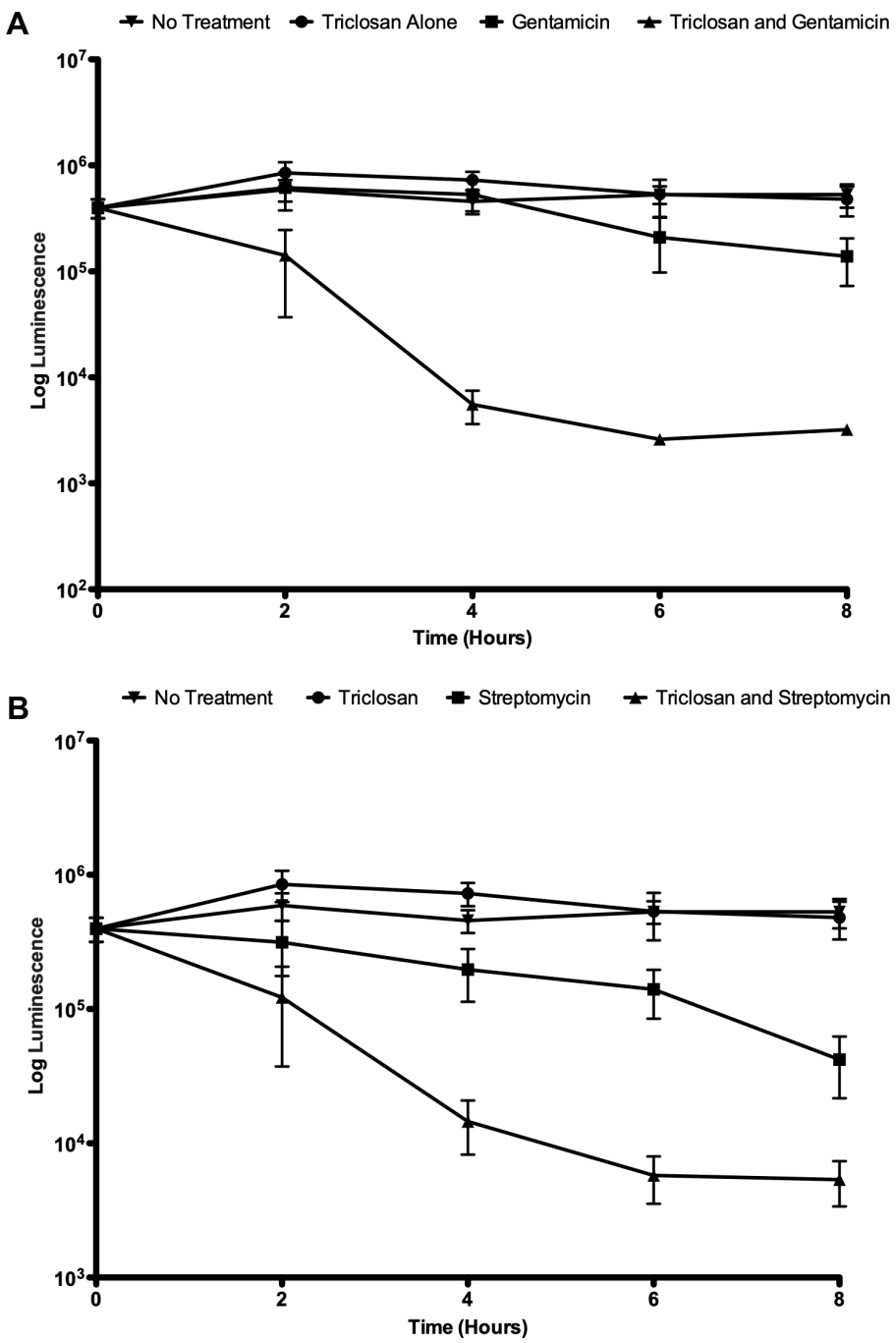

Figure 2-10. Gentamicin and streptomycin have a shorter onset of action and enhanced killing when combined with triclosan. Biofilms were treated with triclosan (100 μM), gentamicin (100 μM), or streptomycin (100 μM) alone and in combination for 8-hrs. At 0, 2, 4, 6, and 8-hrs the number of cells within the biofilms were quantified. The assay was performed at least three times in triplicate. The results represent means ± the SEM.

2.3.5 Triclosan and tobramycin effectively kill biofilms of CF clinical isolates

We examined triclosan and tobramycin activity against seven clinical isolates that were collected from patients at the MSU or the University of Washington CF Clinic. Importantly, two clinical isolates, AMT0023_30 and 34, were isolated longitudinally from the same patient at either 6 months or 8 years of age, respectively. In addition, clinical isolates CF110_N and CF110_O were isolated longitudinally from the same patient 3 months apart (Table 2-1). Using isolates collected from the same patient at different times allowed us to test the activity of triclosan and tobramycin against *P. aeruginosa* isolates unsuccessfully exposed to eradication therapies using tobramycin.

100 μM of triclosan combined with 500 μM tobramycin resulted in significantly greater killing of 6/7 *P. aeruginosa* clinical isolates compared to tobramycin treatment alone (Figure 2-11). Strain 300_A without any treatment formed minimal biofilms that were barely above the limit of detection of our assay (10^3 luminescence). However, treatment with tobramycin led to a 50-fold increase in the biofilm. The combination of tobramycin and triclosan reduced biofilm formation of this strain to background levels, indicating that the combination therapy is much more effective than tobramycin alone. Importantly, triclosan and tobramycin were active against the tobramycin resistant strain AMT023_34. This clinical isolate has mutations in the *mexZ* repressor causing increased expression of the MexXY-OprM multidrug efflux pump that transports tobramycin and in the *mutS* gene resulting in a hypermutator state. Moreover, this strain produces persister cells at an increased frequency.[23]

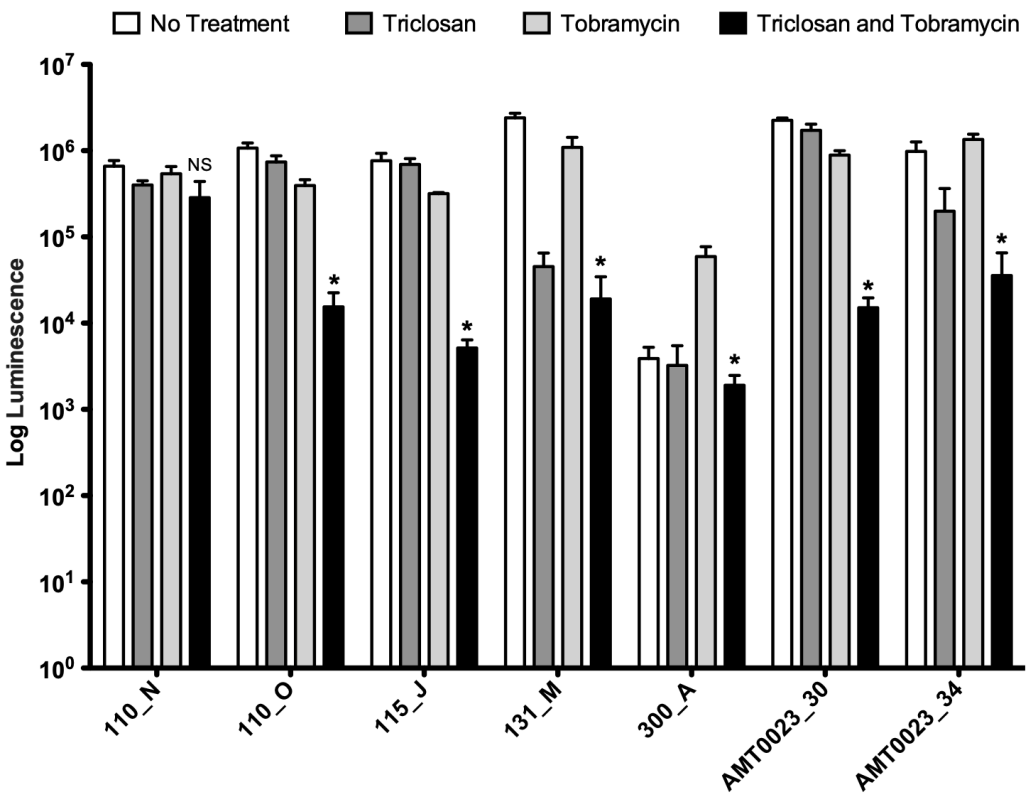

Figure 2-11. Tobramycin and triclosan are effective against clinical isolates. Biofilms were treated with triclosan (100 μM) or tobramycin (500 μM) alone and in combination for 6-hrs. The number of viable cells within the biofilms were quantified. The assay was performed at least three times in triplicate. The results represent means plus the SEM. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance compared to tobramycin alone (*, $p<0.05$, NS, not significant).

As CF infections are polymicrobial, we assessed the activity of tobramycin and triclosan against other bacterial pathogens associated with CF. We first assessed the activity against five *Burkholderia cenocepacia complex (Bcc)* isolates, four CF clinical isolates and one environmental isolate (Figure 2-12). Two of the *Bcc* strains formed robust biofilms in our conditions and the combination reduced viable cells ~1000-fold compared to no treatment. In the other three isolates, which all poorly formed biofilms, the combination reduced viable cells in two of them, although this difference was not

statistically significant is due to the luminescence values being near the limit of detection.

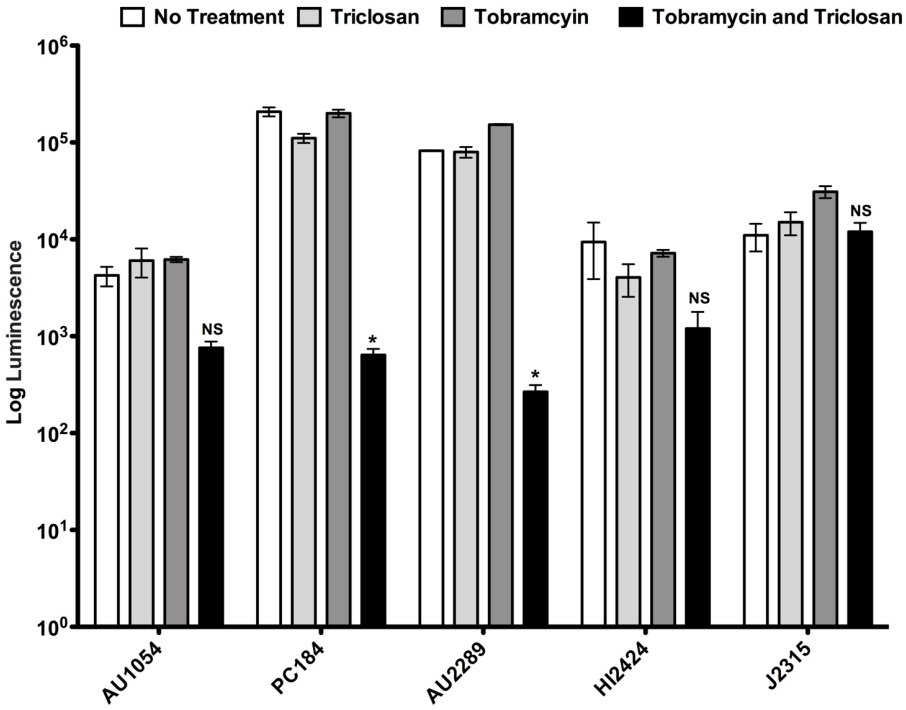

Figure 2-12. Tobramycin and triclosan are effective against isolates of *Burkholderia cenocepacia.* Biofilms grown were treated with triclosan (100 μM) or tobramycin (500 μM) alone and in combination for 6-hrs. The number of viable cells within the biofilms were quantified. The assay was performed once in triplicate. The results represent means plus the SEM. A two-way ANOVA followed by Bonferroni's test was used to determine statistical significance compared to tobramycin alone (*, $p<0.05$, NS, not significant).

We also assessed activity against 4 strains of *Staphylococcus aureus*. Each strain increased in cellular number and/or ATP in response to tobramycin, but all were sensitive to triclosan alone and the activity of the combination was driven primarily by triclosan (Figure 2-13). This is expected because *S. aureus* encodes only *fabI* which is known to be sensitive to triclosan.[136]

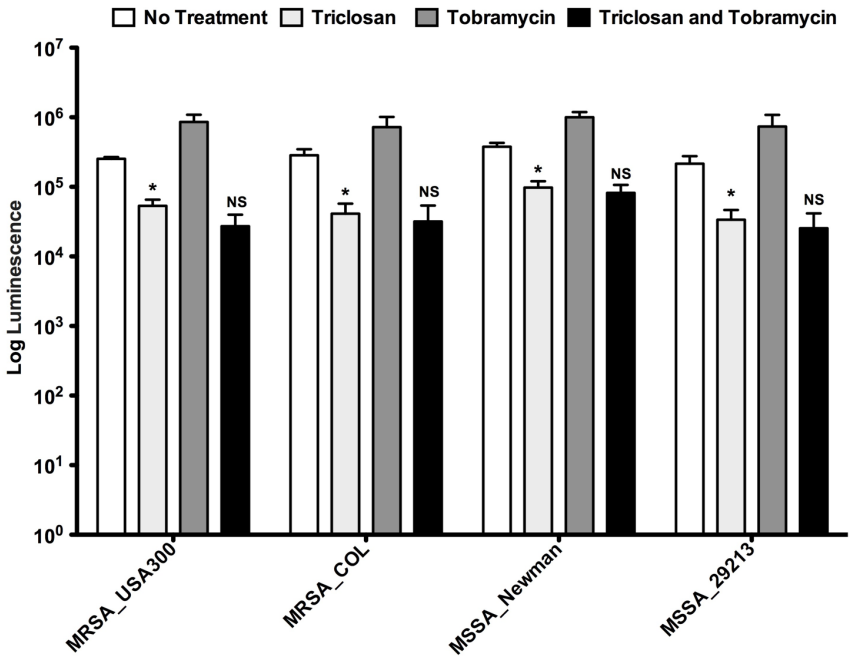

Figure 2-13. Triclosan alone is effective against *S. aureus.* Biofilms were treated with triclosan (100 μM) or tobramycin (500 μM) alone and in combination for 6-hrs. The number of viable cells within the biofilms were quantified. The assay was performed at least three times in triplicate. The results represent means plus the SEM. A two-way ANOVA followed by Bonferroni's test was used to determine statistical significance compared to no treatment and triclosan alone (*, $p<0.05$, NS, not significant).

2.3.6 Triclosan combined with aminoglycosides does not increase dispersal of *P. aeruginosa* biofilms

We examined whether triclosan combined with tobramycin, gentamicin, or streptomycin dispersed *P. aeruginosa* PAO1 biofilms grown in static conditions using crystal violet staining. Biofilms were treated for 6-hrs with 100 μM of triclosan, gentamicin, or streptomycin or 500 μM tobramycin alone or in combination. Triclosan treatment alone had no significant effect while all three aminoglycosides caused biofilm dispersal. However, triclosan combined with the aminoglycosides did not significantly increase biofilm dispersal versus the aminoglycoside alone (Figure 2-14A). To further evaluate the effect of tobramycin and triclosan on biofilm dispersal, we evaluated

biofilms formed under flow conditions for 24-hrs treated for 6-hrs with 100 μM triclosan, 500 μM tobramycin, or the combination of the two. Following treatment, cells were stained with SYTO®9 and propidium iodide to measure live (green) and dead (red) cells, respectively. We found that the combination did not significantly disperse the biofilm but did increase cell death (Figure 2-14 and Figure 2-15).

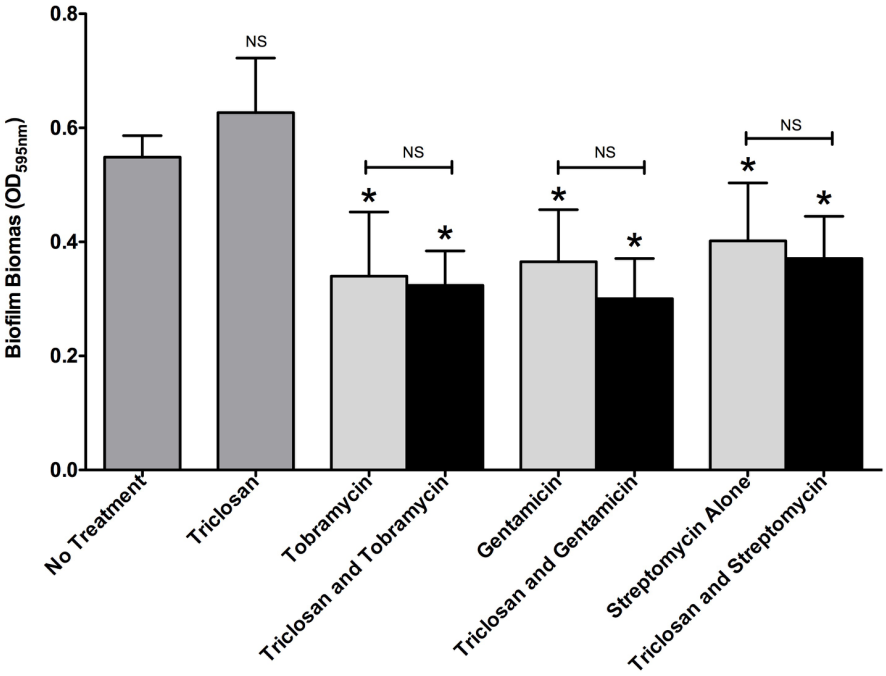

Figure 2-14. Aminoglycosides combined with triclosan do not increase biofilm dispersal using a crystal violet assay. Biofilms were treated with triclosan (100 μM), tobramycin (500 μM), gentamicin (100 μM), or streptomycin (100 μM) alone and in combination. The experiment was performed five times in triplicate. The results represent the means plus SEM. A one-way ANOVA followed by Dunnett's posttest was used to determine statistical significance compared to no treatment (*, $p<0.05$, NS, not significant).

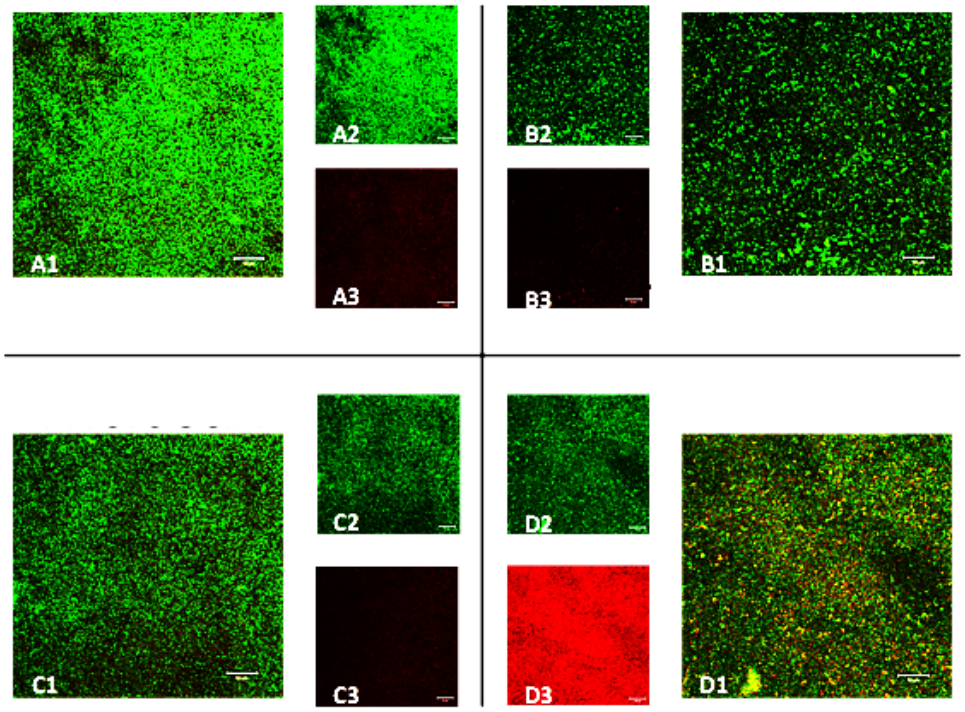

Figure 2-15. Aminoglycosides combined with triclosan do not increase biofilm dispersal using a flow cell. 24-hr old biofilms grown in flow cells were treated with triclosan (100 μM), tobramycin (524 μM), or the combination for 6-hrs. Live cells are stained green and dead cells are stained red. Representative images are shown: no treatment (top left panel), tobramycin alone (top right panel), triclosan alone (lower left panel), combination (lower right panel). Inserts are shown for the live channel (A2, B2, C2, D2) and for the dead channel (A3, B3, C3, D3) for each condition.

2.3.7 FabI is not the target of the combination therapy.

To determine if triclosan inhibition of FabI accounts for the observed synergy, we measured the activity of triclosan and tobramycin against biofilms of a FabI deficient strain (Tn::*fabI*), which has a ISlacZ/hah transposon inserted in the FabI gene.[119] If the synergistic activity of triclosan is solely due to FabI inhibition, we would expect the *fabI* transposon mutant to be sensitive to tobramycin alone in the absence of triclosan. Biofilms were treated with 100 μM of triclosan, gentamicin, or streptomycin or 500 μM of tobramycin alone or in combination for 6-hrs. Contrary to our expectation, biofilms of the *fabI* transposon mutant did not exhibit increased sensitivity to aminoglycosides and triclosan continued to significantly enhance tobramycin, gentamicin, and streptomycin

killing of biofilms (Figure 2-15A). In addition, the activity of triclocarban, which is a triclosan analog and is thought to also inhibit FabI [137,138], did not enhance tobramycin activity against biofilms (Figure 2-15B).

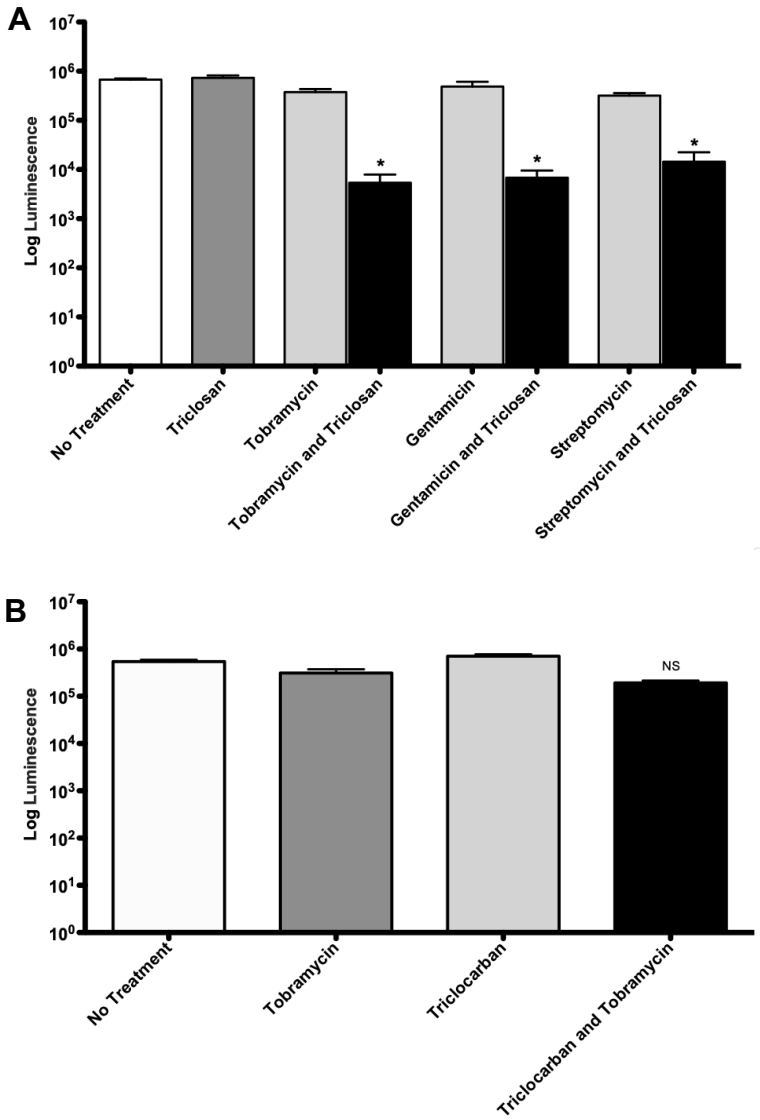

Figure 2-16. FabI is not the target of the combination therapy. A. Biofilms formed by a FabI *P. aeruginosa* deficient strain (*Tn::fabI*) were treated with triclosan (100 μM), tobramycin (500 μM), gentamicin (100 μM), or streptomycin (100 μM) alone and in combination. **B.** Biofilms formed by PAO1 were treated with triclocarban (100 μM) or tobramycin (500 μM) alone or in combination. The assays was performed at least three times in triplicate. The results represent means plus the SEM. A one-way ANOVA followed by Bonferroni's posttest was used to determine statistical significance compared to tobramycin alone (*, $p<0.05$, NS, not significant).

2.3.8 Triclosan and tobramycin are more effective at killing persister cells

We hypothesized that the combination therapy may enhance killing by targeting persister cell populations within biofilms. Persister cells are dormant, non-growing cells that are recalcitrant to antimicrobial therapy.[139] We examined the ability of triclosan and tobramycin to kill *P. aeruginosa* PAO1 persister cells by performing a time killing assay on 20-hr old stationary cells, which are enriched for persister cells.[15] We found that the combination of triclosan and tobramycin significantly enhanced persister cell killing compared to either antimicrobial alone (Figure 2-17). By 8-hrs, the combination resulted in a ~2-log10 reduction in persister cells compared with tobramycin alone and the classic persister biphasic killing pattern was not observed. At 24-hrs, the combination exhibited a 6-log10 increase in killing versus tobramycin alone and viable cells could not be recovered (<10 CFU/mL).

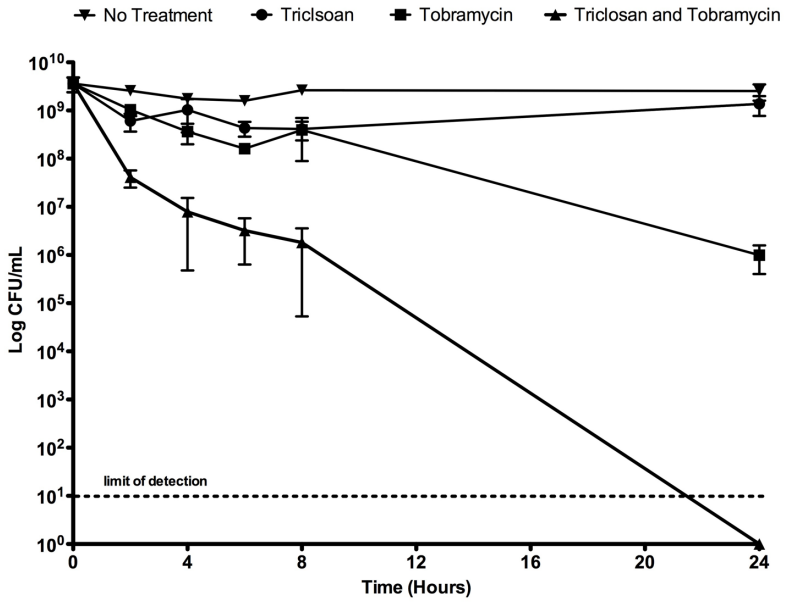

Figure 2-17. Tobramycin combined with triclosan kills persister cells. 20-hr old stationary-phase cells were treated with triclosan (100 μM) or tobramycin (50 μM) alone and in combination. At 0, 2, 4, 6, 8, and 24-hrs aliquots were taken for CFUs/mL enumeration. The experiment was performed three times in triplicate. The results represent means ± the SEM.

2.3.9 Intratracheal administration of triclosan to the lungs of rats exhibits mild clinical symptoms

As there is limited data regarding the toxicity of triclosan delivered directly to the lungs, we performed a 1-day acute and 7-day repeated exposure rat toxicity study. In the 1-day acute exposure study, triclosan concentrations of 10, 30, 100, 300, and 1000 μg/kg were delivered by trans-oral intratracheal installation into the lungs of Sprague-Dawley rats, and 24-hrs following treatment animals were sacrificed for analysis. For reference, ~10 μg/mL corresponds to the effective dose that we determined *in vitro.* Overall, both CBC and serum chemistry analyses for triclosan treated animals were within reference ranges with the exception of neutropenia observed at the highest triclosan dose of 1000 μg/kg. The concentration of triclosan in plasma was assessed by LC-MS/MS, and triclosan was not detected in any animal, suggesting limited to no systemic absorption. Histopathology assessment revealed mild perivascular edema within adventitia around pulmonary veins and mild type II pneumocyte hyperplasia. In general, pathological changes were mild and may not be sufficiently severe to present clinical signs.

In the repeated exposure toxicity study, doses of 30, 100, and 300 μg/kg triclosan were administered by intratracheal instillation consecutively for 7 days. We found no significant difference in body weight of the rats treated compared to controls. CBC and serum chemistry analyses for triclosan treated animals were within reference ranges. At the conclusion of the study, blood was analyzed for triclosan by LC-MS/MS. Triclosan was below the limit of detection of our assay in each sample tested, again indicating limited to no systemic absorption despite repeated exposures. The histopathology

assessment revealed perivascular edema, with severity correlating with increasing doses of triclosan. The overall histopathology assessment concluded that the observed triclosan-dependent changes were mild and of insufficient severity to present clinical signs.

2.3.10 Triclosan and tobramycin show enhanced efficacy in an *in vivo* wound model

Because both tobramycin and triclosan are widely used in human applications, there is significant clinical potential for this combination to treat biofilm-based infections. To determine if triclosan and tobramycin are more effective against biofilms *in vivo,* we tested their activity using a murine wound model.[125,140] In this model, a wound is generated on the back of an SKH-1 hairless mice and infected with ~1x10^9 *P. aeruginosa* cells growing as a biofilm. We utilized the bioluminescent derivative XEN 41 to allow real-time imaging of the infection using the In Vivo Imaging System (IVIS).[126] After establishing in the wounds for 24 hours, biofilms were treated for 4-hrs using an agarose hydrogel imbedded with either triclosan or tobramycin alone and in combination. We found that triclosan treatment alone had no effect compared to untreated controls while tobramycin treatment alone resulted in 2.5-fold-reduction in bioluminescence after 4-hrs of treatment; however, this difference was not statistically significant compared to an untreated control (Figure 2-18). Triclosan combined with tobramycin resulted in 4.5-fold-reduction in bioluminescence and was both statistically significant compared with tobramycin treatment alone and untreated controls.

Interestingly, the combination resulted in an 8 to 9-fold reduction three separate times, whereas tobramycin treatment only resulted in an ~6-fold reduction once.

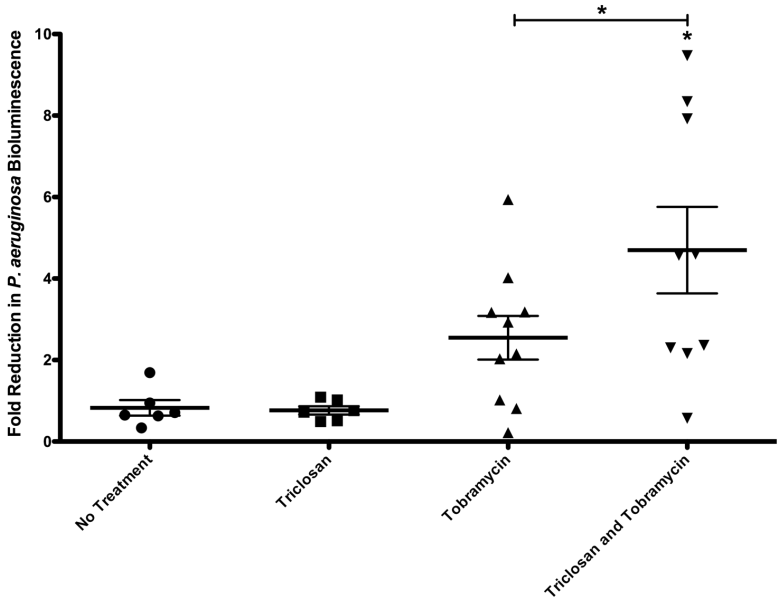

Figure 2-18. Triclosan and tobramycin are more effective in vivo. 24-hr old bioluminescent biofilms formed within wounds were treated with triclosan (100 μM), or tobramycin (500 μM), alone and in combination for 4-hrs. Reduction in the number of cells within biofilms was quantified using IVIS. The results are fold reduction of three separate experiments plus the SEM, no treatment n=6, triclosan n=6, tobramycin n=10, triclosan and tobramycin n=9. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between each treatment and the untreated control and tobramycin treatment alone was compared with tobramycin and triclosan (*, $p<0.05$).

2.4 Discussion

We report, triclosan, combined with three antipseudomonal aminoglycosides, killed highly tolerant and tobramycin resistant *P. aeruginosa* biofilms. Our results show that the combination increases both the rate and degree of killing of cells within *P. aeruginosa* biofilms which has important clinical implications for the treatment of biofilm infections in CF patients. Moreover, killing activity against a variety of CF *P. aeruginosa* clinical isolates, including tobramycin resistance isolates, further suggests clinical potential for the treatment of lung infections in CF patients. Importantly, we found that

significantly lower concentrations of tobramycin can be used when combined with triclosan for maximum efficacy. This reduction in aminoglycosides would have significant benefits as these antimicrobials are known to be nephro- and ototoxic.[141,142]

Interestingly, we found the combination was effective against persister cells, which are a major biofilm tolerance factor. And high persister cells mutants are often isolated from CF patients.[23] There are currently few treatments that eradicate persister cells, and thus the combination of triclosan with aminoglycosides could potentially be used to eradicate these dormant cells.

Triclosan has been used for the past three decades as a general antimicrobial and antifungal in toothpaste and plastics.[133] Because of decades of overuse, the FDA has recently restricted the use of triclosan due to concerns over bioaccumulation and the potential for induction of resistance to other antibiotics in bacteria. Importantly, in these rulings the FDA declared that there was not enough evidence to consider triclosan a Generally Recognized as Safe (GRAS) compound, but it did not otherwise address potential toxicity. However, numerous safety studies have concluded that triclosan has acceptable safety parameters when administered to humans. For example, a 4-year study on humans found that routine use of toothpaste containing 0.3% triclosan, as is found in Colgate Total®, had no adverse effects on the endocrine system [143]. Importantly, this is 333x the concentration of triclosan that is effective at enhancing aminoglycoside activity.

A second study evaluated the accumulation of triclosan in humans via exposure to consumer products and also found no adverse health outcomes.[144] Furthermore, a human safety study, totaling 1,246 participants that used toothpaste and mouthwash

containing up to 0.6% of triclosan for up to 12 weeks found no adverse effects.[145] Finally, the Scientific Committee of Consumer Products of the European Union recently released a comprehensive report that summarized hundreds of triclosan toxicity studies, including human oral dose toxicity studies[146]. The toxicity level for the majority of the studies are >50 mg/kg body weight, again orders of magnitude higher than the effective dose we report in our work. Together, these studies show that triclosan is safe when used appropriately.

2.5 Conclusions

We envision the use of triclosan at low concentrations, ~ 30 μM, in combination with tobramycin or other aminoglycosides, as an inhaled aerosolized solution into the lungs of CF patients. This route of administration provides many benefits, including fewer side effects, due to reduced systemic absorption, and enhanced activity, due to direct delivery to the lungs.[147,148] To further assess triclosan toxicity when administered to the lungs, we performed both single and repeated intratracheal instillation toxicity studies on Sprague-Dawley rats using concentration up to 1000 μg/kg of triclosan and found only mild clinical symptoms with little significant change to lung histology or blood chemistry. Furthermore, we developed a sensitive LC-MS/MS assay for triclosan and no triclosan was detected in the blood when administered to the lung, indicating little systemic absorption (limit of detection 300 ng/mL). Finally, we found triclosan and tobramycin in combination in a novel hydrogel were more effective in a murine wound model. Although more studies are needed, the safety profile of triclosan suggests that it is a worthy candidate for further exploration.

CHAPTER 3

OXYCLOZANIDE ENHANCES TOBRAMYCIN KILLING OF *PSEUDOMONAS AERUGINOSA* BIOFILMS BY DEPOLARIZING THE MEMBRANE POTENTIAL AND PERMEABILIZING CELLS

3.1 Introduction

Here, we report the characterization of another compound identified in the adjuvant HTS described in Chapter 2, oxyclozanide (Figure 3-1). This molecule is a proton ionophore that disrupts proton motive force (PMF), and it is approved for the treatment of parasitic worm infections in cattle.[149] We found that oxyclozanide had weak antibacterial activity on its own while oxyclozanide combined with tobramycin significantly enhanced the rate and degree of aminoglycoside killing of *P. aeruginosa* biofilms. The combination was effective against CF clinical isolates, including a tobramycin resistant isolate, and multiple *S. aureus* isolates growing as biofilms. We further show that oxyclozanide both permeabilized *P. aeruginosa* and functioned as a proton ionophore, reducing the membrane potential of *P. aeruginosa*. Our findings suggest that tobramycin combined with oxyclozanide represents a potential new antimicrobial therapy for the treatment of *P. aeruginosa* and *S. aureus* biofilms in CF patients, as well as other biofilm-based infections such as diabetic foot ulcers and burn wounds.[115-117]

Note: Much of this work was previously published.[213] M.M.M and C.M.W., wrote and edited the manuscript. M.M.M. & M.P.Z., performed the experiments.

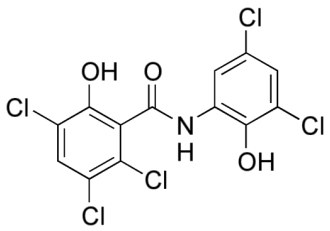

Figure 3-1. Oxyclozanide structure.

3.2 Materials and Methods

3.2.1 Bacterial strains, culture conditions, and compounds

All strains used in this study are listed in Table 3-1. Bacterial strains were grown in glass test tubes as described previously in Chapter 2.

Table 3-1. Bacterial strains used in this study

Strain	Characteristics	Reference
PAO1	PA Standard Reference Strain, isolated in 1954[118]	[6]
CF_110_N	PA Clinical CF Isolate, Michigan	[6]
CF_110_O	PA Clinical CF Isolate, Michigan	[6]
CF_115_J	PA Clinical CF Isolate, Michigan	[6]
CF_131_M	PA Clinical CF Isolate, Michigan	[6]
AMT0023_30	PA Early Isolate 6 MO	[120]
AMT0023_34	PA Late Isolate 8 YO	[120]
USA_300_JE2	MRSA, Wound, California	[150]
COL	MRSA, Colindale Hospital, England	[151]
Newman (25904)	MSSA, Endocarditis, ATCC	[152]
Wichita (29213)	MSSA, Better Biofilm Former, ATCC	[153]

Abbreviations*: Pseudomonas aeruginosa* (PA), *Methicillin-resistant Staphylococcus aureus* (MRSA), *Methicillin-sensitive Staphylococcus aureus* (MSSA), American Tissue Type Collection (ATTC).

3.2.2 Minimum inhibitory concentration (MIC) of cells growing planktonically

MICs were determined as described previously in Chapter 2.

3.2.3 Biofilm susceptibility testing using BacTiter-Glo™

We used the Minimum Biofilm Eliminating Concentration (MBEC™, Innovotech) assay to measure antimicrobial susceptibility as described previously in Chapter 2. However, for this chapter percent reduction was used and calculated as:

$$\%\, reduction = 1 - \left(\frac{Luminesces\ of\ Treated\ Biofilm - Background}{Luminesces\ of\ Untreated\ Biofilm - Background}\right) \mathrm{X}\ 100.$$

3.2.4 Crystal violet staining

To study biofilm dispersal under static conditions, crystal violet staining was performed as described previously in Chapter 2.

3.2.5 BacLight™ membrane potential assay and live/dead TO-PRO-3 Staining

24-hr old biofilms were formed in glass test tubes (18 x 150 mm) in 1 mL of 10% (v/v) MHB at 35°C and agitated at 150 RPM. Cells were then washed in DPBS to remove non-adherent cells and treated with oxyclozanide and tobramycin for 2-hrs or 6-hrs. Following treatment cells were washed in PBS without magnesium and calcium and the biofilm was disrupted from the air-liquid interface using an autoclaved wooden stick. The cells were stained in 1 mL of PBS for 20-mins using the BacLight™ bacterial membrane potential kit in combination with flow cytometry (Thermo Fisher Scientific). This assay uses the dye $DiOC_2(3)$, which fluoresces in the FITC channel within all cells. However, greater membrane potentials drives accumulation and self-association of the

dye in the cell cytoplasm, shifting its fluoresces to the phycoerythrin (PE) channel. To this kit we added the TO-PRO-3 iodide live/dead stain that is impermeable to live cells but can accumulate within cells that have permeabilized membranes characteristic of dead cells (Thermo Fisher Scientific). Once inside cells with compromised membranes, this dye fluoresces in the allophycocyanin (APC) channel upon intercalating DNA. Single cell flow cytometry was performed on an LSR II (BD Biosciences), with excitation from 488 mm and 640 mm lasers, and analyzed in FITC/PE and APC channels, respectively.

3.2.6 Stationary-phase killing assay

Cultures were grown for 20-hrs and 100 µL/well of stationary-phase cells were added to individual wells of a 96-well plate.[124] Treatments were added and the plate was incubated at 37°C without agitation. At hours 2,4,6,8, and 24, aliquots were serially diluted, plated on Dey-Engley neutralizing agar plates, which neutralizes the activity of disinfectants and antiseptics (Sigma-Aldrich), and cfus were enumerated. The dilutions that contained 3-30 colonies per 10 µl were used to quantify cfus/mL.

3.2.7 Tobramycin accumulation assay

To measure the accumulation of tobramycin within cells in biofilms, tobramycin was conjugated to Texas Red (Sigma-Aldrich) using an amine conjugation reaction, as previously described [154,155]. Briefly, conjugated tobramycin was used at a concentration of 250 µg/mL (~500 µM) alone and in combination with 100 µM of triclosan against 24-hr old biofilms formed in glass test tubes at the air-liquid interface, as described in

Chapter 2. Following treatments, biofilms were washed in DPBS for 3-mins, and then disrupted with autoclaved wooden sticks into 1 mL of 0.2% Trition X-100 to lyse cells (Sigma-Aldrich). Lysed cells were then transferred to spectrophotometer cuvettes (Thermo Fisher Scientific) and read using a SpectraMax M5 microplate spectrophotometer system ($\lambda_{excite,}$ 595 nm and $\lambda_{emit,}$ 615 nm).

3.3 Results

3.3.1 Oxyclozanide potentiates tobramycin activity against *P. aeruginosa* and *S. aureus* biofilms

In Chapter 2, we performed a HTS to discover compounds that increased tobramycin activity against *P. aeruginosa* biofilms.[6] From this screen, the proton ionophore oxyclozanide used for the treatment of parasitic worm infections in cattle was identified as a compound that significantly increased tobramycin killing of biofilms.[149,156] To confirm the results of the screen, *P. aeruginosa* PAO1 biofilms were exposed to 100 μM oxyclozanide or 500 μM tobramycin (~500x planktonic MIC) alone and in combination for 6-hrs, and the efficacy was determined using BacTiter-Glo™.[6] Oxyclozanide and tobramycin alone resulted in ~2-fold fewer viable cells in the biofilm compared to the untreated control. However, the combination of oxyclozanide and tobramycin was more effective eradicating 87% (7.7-fold reduction) of the cells within a biofilm (Figure 3-2). Oxyclozanide combined with the aminoglycosides gentamicin or streptomycin killed 96% (25-fold reduction) or 91% (11.1-fold reduction) of the cells in a biofilm, respectively. We also tested for synergy with third and fourth generation cephalosporins, β-lactams, fluoroquinolones, and tetracycline, and determined that in

addition to aminoglycosides oxyclozanide only enhanced tetracycline, increasing killing of the biofilm to 86% (Figure 3-2).

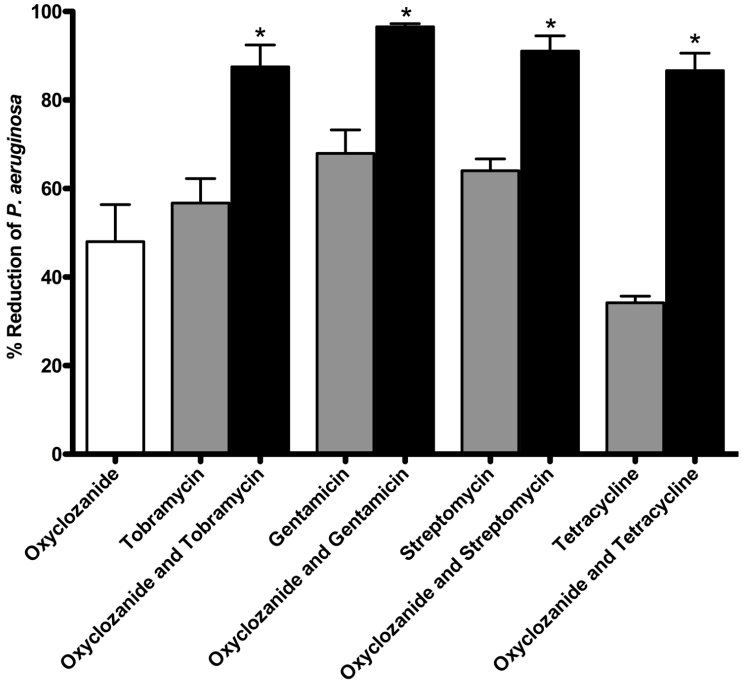

Figure 3-2. Oxyclozanide enhances aminoglycosides and tetracycline. Biofilms were treated for 6-hrs with oxyclozanide (100 μM), tobramycin (500 μM), gentamicin (100 μM), streptomycin (100 μM), or tetracycline (100 μM) alone and in combination, and the number of viable cells within the biofilms were quantified. The assay was performed at least three times in triplicate. The results represent means plus the standard error of the mean (SEM). A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between the combination and each antibiotic alone (*, $p<0.05$).

We also tested the combination against four strains of *S. aureus.* and found oxyclozanide was effective alone, killing ~90% of each strain grown as a biofilm (Figure 3-3). Interestingly, *S. aureus* biofilms were resistant to tobramycin alone and the biofilm increased in cellular number and/or ATP after 6-hrs of treatment. Supporting our observation, it has been observed clinically that high doses of inhaled tobramycin has no effect on methicillin resistant *S. aureus*.[157] This resistance was lost when tobramycin

was used in combination with oxyclozanide, resulting in 90% killing on average for each strain of *S. aureus* tested and a maximum ~36-fold reduction (MSSA_29213) compared to tobramycin treatment alone.

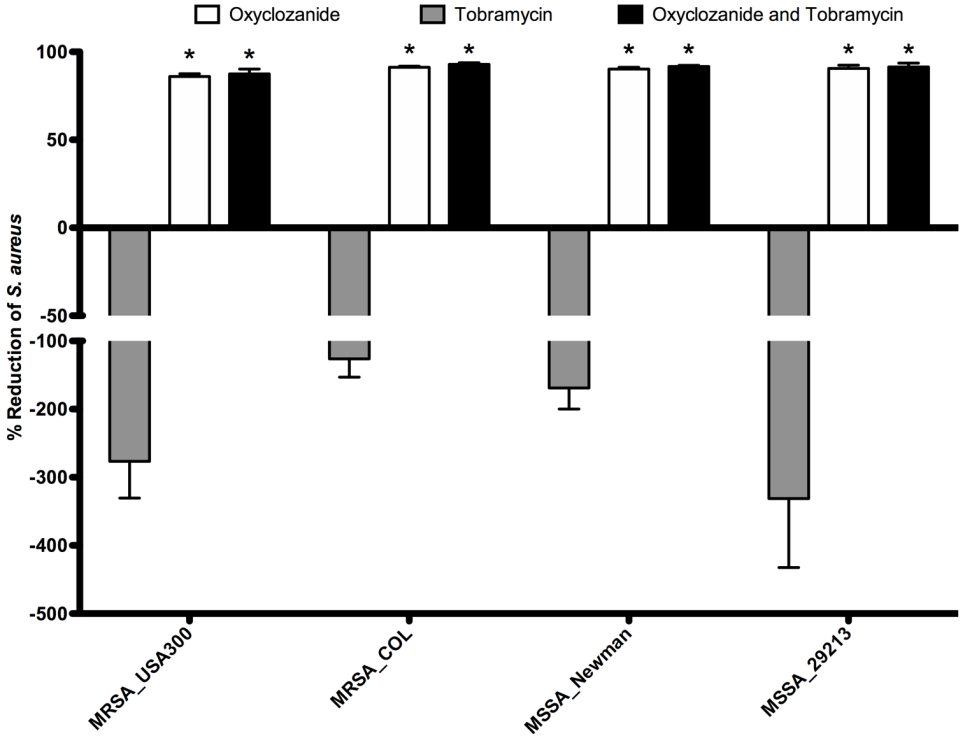

Figure 3-3. Oxyclozanide kills *S. aureus* biofilms. Biofilms were treated with oxyclozanide (100 μM) or tobramycin (100 μM) alone and in combination for 6-hrs. The number of viable cells within the biofilms were quantified. The assay was performed two times in triplicate. The results represent means plus the SEM. A two-way ANOVA followed by Bonferroni's posttest was used to determine statistical significance between either oxyclozanide alone and tobramycin alone or the combination and tobramycin alone (*, $p<0.05$).

We also performed MIC assays to determine if oxyclozanide and these antibiotics were more effective against planktonic cells. The MIC for tobramycin, gentamicin, streptomycin, and tetracycline, did not change when used in combination with oxyclozanide against PAO1 planktonic cells, suggesting oxyclozanide specifically

enhanced the activity of these antibiotics against cells when growing in a biofilm (Table 3-3).

Table 3-2. The MIC values were not changed by oxyclozanide in planktonic cells.

Antimicrobial	Alone (μM)	+ Oxyclozanide (μM)
Tobramycin	1.5 - 3.125	1.5 - 3.125
Gentamicin	6.25 - 12.5	6.25 - 12.5
Streptomycin	50.0	50.0
Tetracycline	100.0	100.0

MIC were determined as the minimum concentration that no turbidity greater than background was measured.

3.3.2 Dose response curves of oxyclozanide and aminoglycosides

To determine the effective ranges of the combinations, we performed dose response curves (DRCs). Tobramycin treatment showed modest activity at concentrations between 25 and 400 μM, reducing the number of the cells within biofilms between 50 and 60% after 6-hrs of treatment (Figure 3-4). Similarly, oxyclozanide treatment exhibited modest activity between 25 and 200 μM, killing ~20-40% of the cells within biofilms, but at 400 μM, oxyclozanide alone killed ~80% of the cells within biofilms. The combination of oxyclozanide and tobramycin significantly increased killing of biofilms compared with tobramycin treatment alone between 100 and 400 μM of each compound, with maximum efficacy of 99% killing (100-fold reduction) seen at 200 μM and 400 μM of oxyclozanide and tobramycin. DRCs were also performed with gentamicin, streptomycin, and tetracycline either alone or in combination with oxyclozanide ranging from 25 to 400 μM (Figure 3-5 and Figure 3-6). The results were

similar with significant killing observed when biofilms were treated at 100, 200, or 400 μM of the combinations versus tobramycin alone.

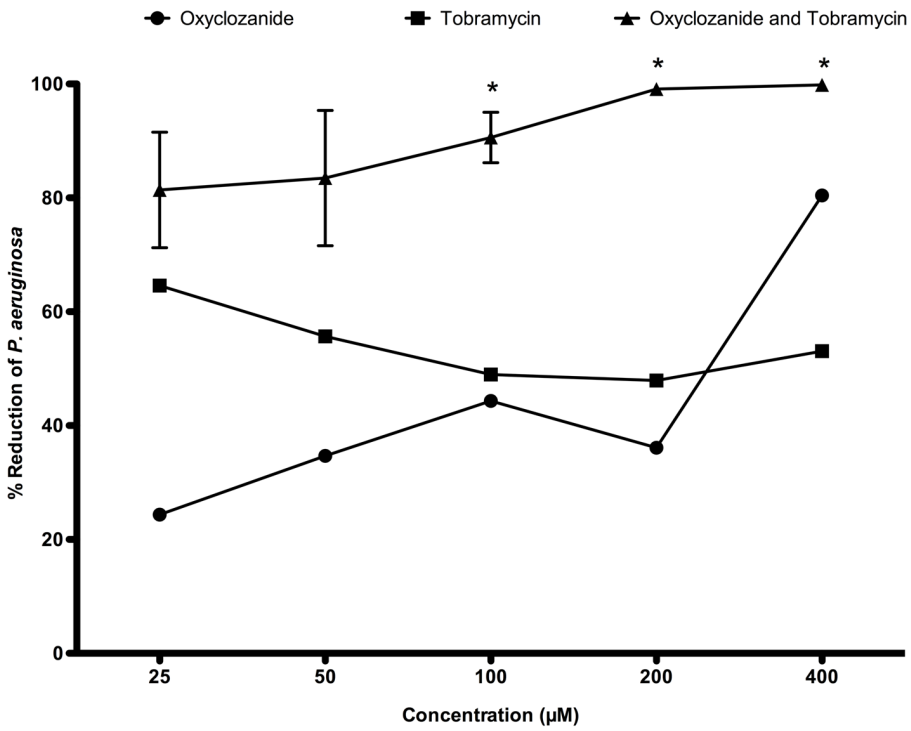

Figure 3-4. Dose response curves of oxyclozanide or tobramycin alone and in combination. Biofilms were treated for 6-hrs with oxyclozanide or tobramycin alone and in combination in equal molar two-fold dilutions, and the number of viable cells within the biofilms were quantified. The assay was performed at least three times in triplicate. The results represent means ± the SEM. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between the combination and tobramycin alone (*, $p<0.05$).

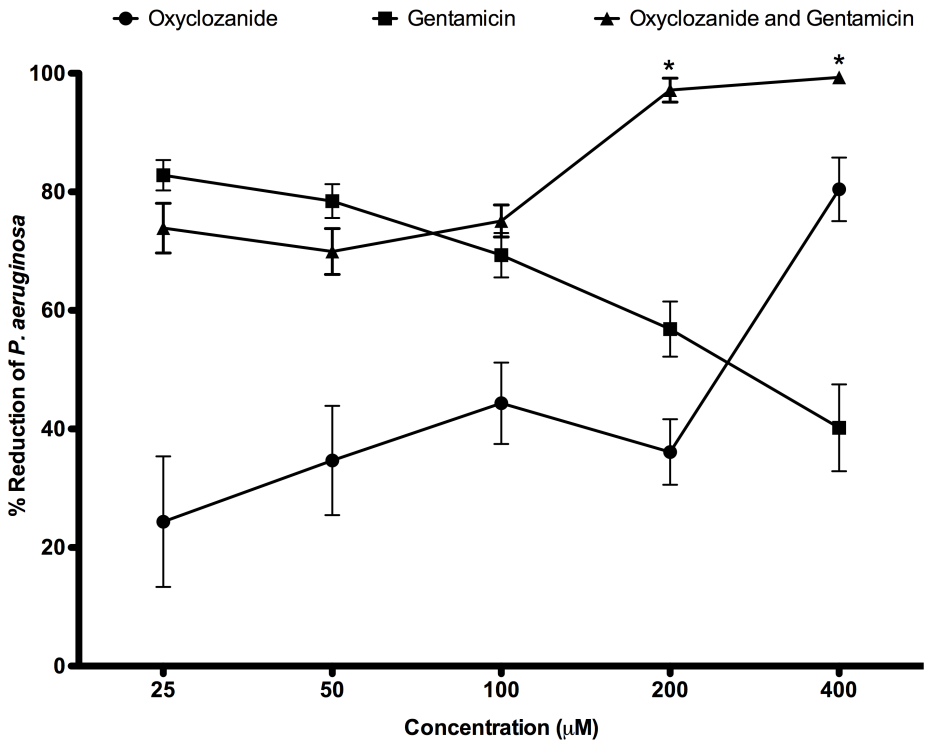

Figure 3-5. Dose response curves of oxyclozanide or gentamicin alone and in combination. Biofilms were treated for 6-hrs with two-fold dilutions of equal concentrations of oxyclozanide combined with gentamicin. The number of viable cells within the biofilms were quantified. The assay was performed at least three times in triplicate. The results represent means ± the SEM. The same oxyclozanide data set was re-plotted in each graph for comparison. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between the combination and gentamicin alone (*, $p<0.05$).

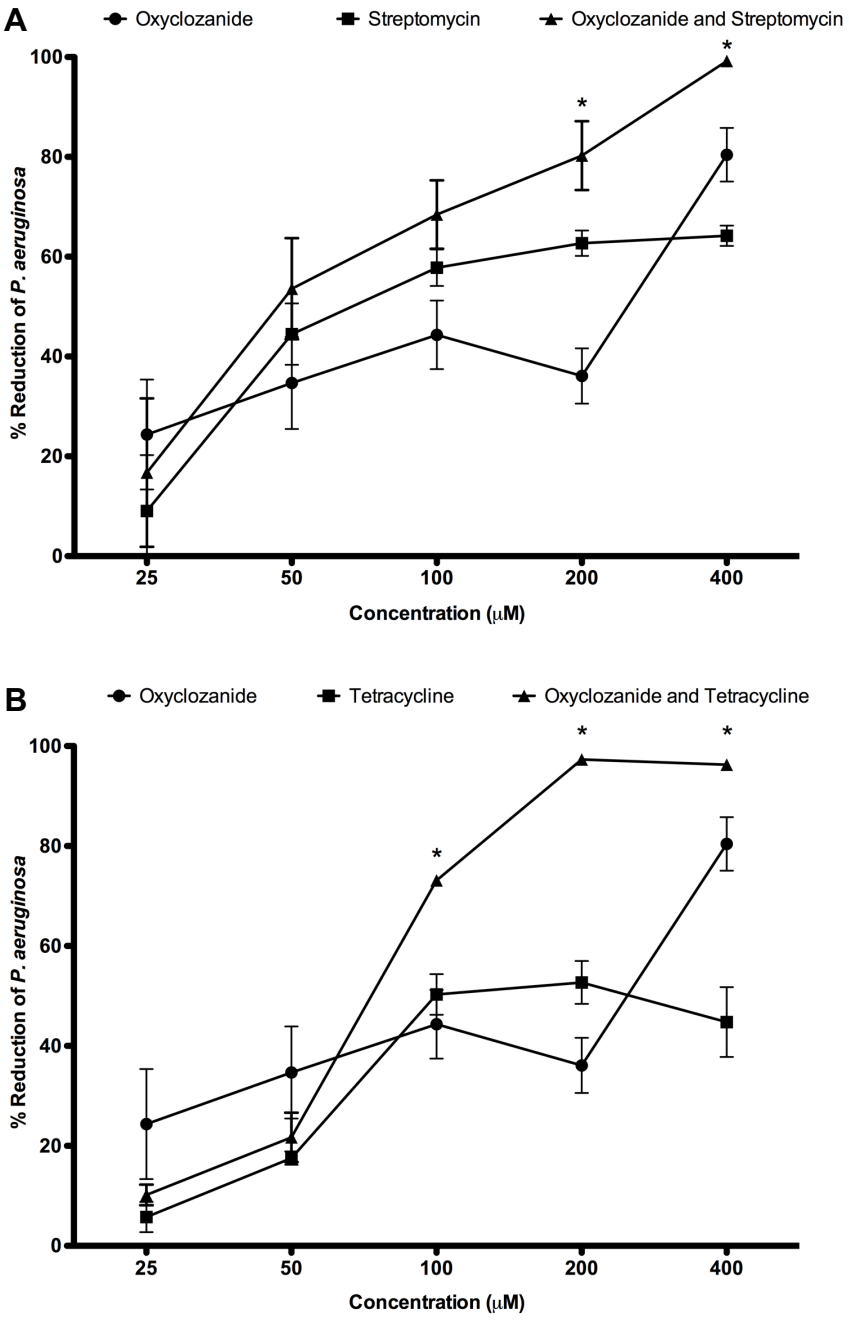

Figure 3-6. Dose response curves of oxyclozanide in combination with streptomycin and gentamicin. Biofilms were treated for 6-hrs with two-fold dilutions of equal concentrations of oxyclozanide combined with (**a**) streptomycin or (**b**) tetracycline. The assay was performed three times in triplicate. The results represent means ± the SEM. The same oxyclozanide data set was re-plotted in each graph for comparison. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between the combination and streptomycin alone and tetracycline alone (*, $p<0.05$).

To determine the lowest possible combinations of oxyclozanide and tobramycin that resulted in significant killing, we performed chequerboard assays (Table 3-4). Biofilms were treated with dilutions of oxyclozanide, ranging from 12.5 to 100 μM, and tobramycin, ranging from 50 to 400 μM. 12.5 μM of oxyclozanide and 50 μM of tobramycin resulted in 86% reduction in cells within the biofilms. The greatest reduction was seen at 100 μM of oxyclozanide and 400 μM of tobramycin reducing 92% of the cells within the biofilms. Shaded cells indicate statistically significant killing compared to tobramycin alone.

Table 3-3. A checkerboard dilution series of oxyclozanide and tobramycin.

Tobramycin \ Oxyclozanide	100 μM	50 μM	25 μM	12.5 μM	0 μM
400 μM	92% (+/- 4.8)	85% (+/- 5.1)	71% (+/- 3.7)	61% (+/- 3.7)	60% (+/- 1.4)
200 μM	90% (+/- 5.1)	80% (+/- 9.1)	67% (+/- 4.2)	64% (+/- 2.4)	67% (+/- 1.5)
100 μM	88% (+/- 6.9)	84% (+/- 4.2)	80% (+/- 3.9)	77% (+/- 3.8)	76% (+/- 0.46)
50 μM	88% (+/- 7.8)	88% (+/- 5.7)	86% (+/- 5.4)	86% (+/- 2.3)	75% (+/- 1.9)
0 μM	31% (+/- 1.9)	32% (+/- 6.3)	26% (+/- 1.9)	29% (+/- 4.7)	

Biofilms were treated with varying combinations of oxyclozanide and tobramycin for 6-hrs. The number of viable cells within the biofilms were quantified by BacTiter-Glo™. The assay was performed twice in triplicate. The results represent means ± the Standard Deviation (SD). A two-way ANOVA followed by Bonferroni's test was used to determine statistical significance between the combination and tobramycin alone. Shaded cells indicate significance compared to tobramycin alone ($p<0.05$).

3.3.3 Oxyclozanide accelerates tobramycin killing of cells within biofilms

Another important property of these antimicrobials, which is highly clinically relevant, is the rate at which biofilms are killed. To determine the rate of killing of cells within biofilms by tobramycin, oxyclozanide, or the combination, we measured percent reduction over the course of 8-hrs. Oxyclozanide alone did not exhibit significant killing until 6-hrs (Figure 3-7). Rather than cell death, tobramycin treatment alone led to an increase in cell number and/or ATP at 2-hrs, which was lost by ~4-hrs. We hypothesize that this increase is due to the mechanism of adaptive resistance that occurs in *P. aeruginosa* in response to aminoglycosides or protein synthesis inhibitors/corruptors. Adaptive resistance, which has been demonstrated in *P. aeruginosa* growing planktonically, in biofilms, and in human lungs, is a transient phenotype that occurs within the first 1-2-hrs of exposure.[158,35,159] This phenomenon results in reduced intracellular accumulation of aminoglycosides due to the activation of RND-type efflux pumps such as the MexXY-OprM tripartite efflux pump among other mechanisims.[35,159] Importantly, this temporary resistance was abolished in the combination treatment as oxyclozanide significantly shortened the onset of action of tobramycin from 6-hrs to 2-hrs, resulting in 50%, 78%, and 85% killing of the cells within the biofilm at 2, 4, and 6-hrs, respectively (3, 4, and 6-fold reductions). Thus, at 2-hrs the combination of oxyclozanide and tobramycin was 100-fold more effective at killing biofilms than tobramycin treatment alone, indicating this combination exhibits both enhanced activity and accelerated action.

Oxyclozanide significantly accelerated the onset of action of gentamicin and tetracycline (Figure 3-8AB). However, unlike the other aminoglycosides examined,

streptomycin did not exhibit a delay in biofilm killing, and minimal enhancement when combined with oxyclozanide was only observed at 6-hrs (Figure 3-8C).

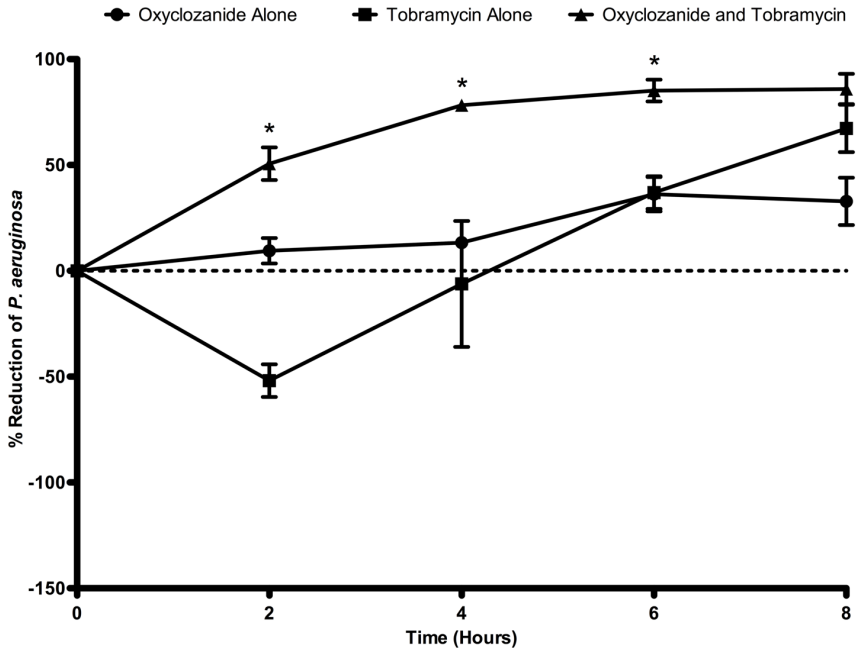

Figure 3-7. Oxyclozanide accelerates tobramycin killing of *P. aeruginosa* biofilms. Biofilms were treated with oxyclozanide (100 μM), or tobramycin (500 μM), alone and in combination. At 0, 2, 4, 6, and 8-hrs the number of viable cells within the biofilms were quantified. The assay was performed at least three times in triplicate. The results represent means ± the SEM. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between the combination and tobramycin alone (*, $p<0.05$).

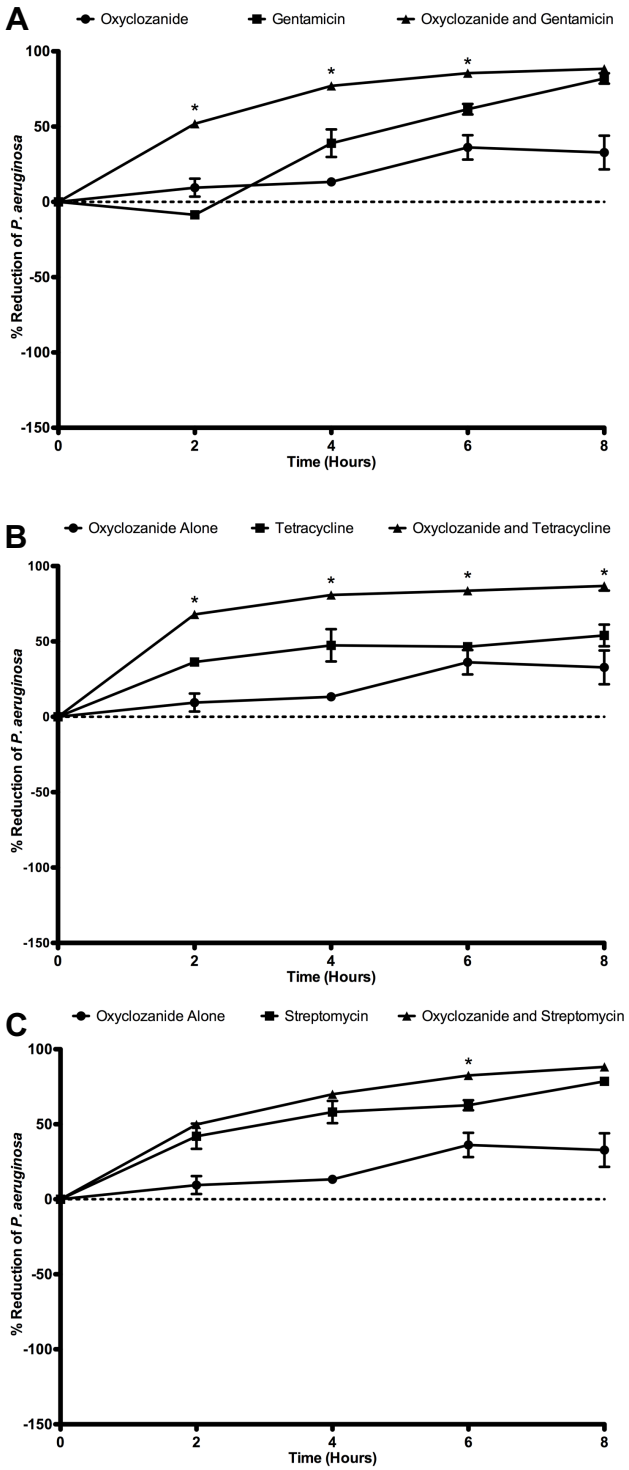

Figure 3-8. Oxyclozanide accelerates the onset of action of several antibiotics. Biofilms were treated with oxyclozanide (100 μM), (**A**) gentamicin (100 μM), (**B**) tetracycline (100 μM), or (**C**) (streptomycin 100 μM) alone and in combination for 8-hrs. The assay was performed three times in triplicate. The results represent means ± the SEM. The same oxyclozanide data set was re-plotted in each graph for comparison. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between the combination each antimicrobial (*, $p<0.05$).

3.3.4 Oxyclozanide combined with tobramycin does not increase biofilm dispersal

We tested the ability of oxyclozanide to disperse *P. aeruginosa* biofilms using a crystal violet staining assay. Biofilms were treated with oxyclozanide or tobramycin alone and in combination from 50 μM – 400 μM (Figure 3-9). Tobramycin significantly reduced biofilm biomass at all concentrations examined. Conversely, oxyclozanide did not cause biofilm dispersal and the combination of tobramycin and oxyclozanide did not cause an increase in biofilm dispersal compared to tobramycin alone. This experiment suggests that oxyclozanide is not acting by inducing biofilm dispersal.

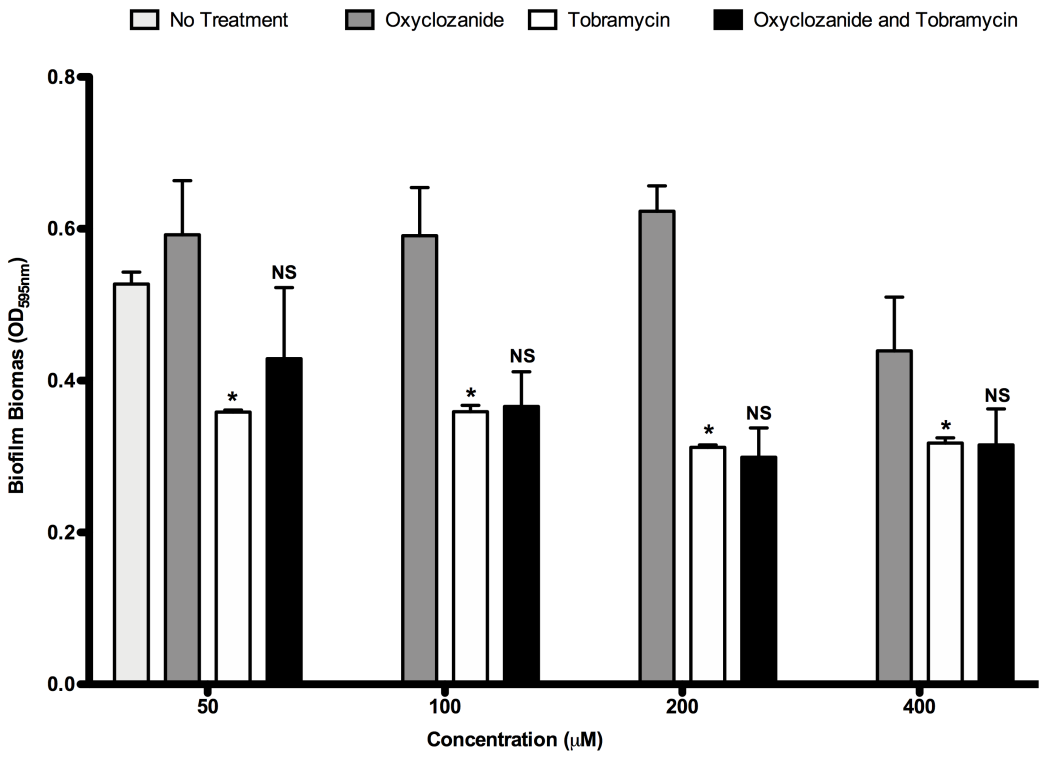

Figure 3-9. Oxyclozanide does not induce biofilm dispersal. Biofilms were treated with oxyclozanide, tobramycin, or the combination in equal molar two-fold dilutions. The experiment was performed twice in triplicate. The results represent the means plus SEM. A one-way ANOVA followed by Bonferroni's posttest was used to determine statistical significance between tobramycin alone and the untreated control (*, $p<0.05$). And between the combination and tobramycin alone (NS, not significant).

3.3.5 Oxyclozanide and tobramycin are effective against CF isolates of *P. aeruginosa*

To determine if the combination of oxyclozanide and tobramycin exhibits activity against clinical isolates, we tested its activity against six *P. aeruginosa* CF clinical isolates. Two clinical isolates were isolated longitudinally from the same patient at 6 months of age and 8 years of age: AMT0023_30 and 34, respectively. In addition, clinical isolates CF_110_N and CF_110_O were isolated longitudinally from the same patient 3 months apart (the strains are described in Table 1). These six clinical isolates were treated with 100 μM oxyclozanide or 500 μM of tobramycin alone or in combination. All isolates displayed no to modest susceptibility to oxyclozanide or tobramycin treatment alone and exhibited the greatest susceptibility to the combination (Figure 3-10). This increase was statistically significant in 4/6 isolates. Importantly, the combination significantly enhanced killing of strain AMT0023_34, which over expresses the RND-type MexXY-OpRM tripartite multidrug efflux pump, rendering it resistant to tobramycin[23], resulting in a ~16-fold reduction in viable AMT0023_34 cells compared with tobramycin treatment alone (Figure 3-10).

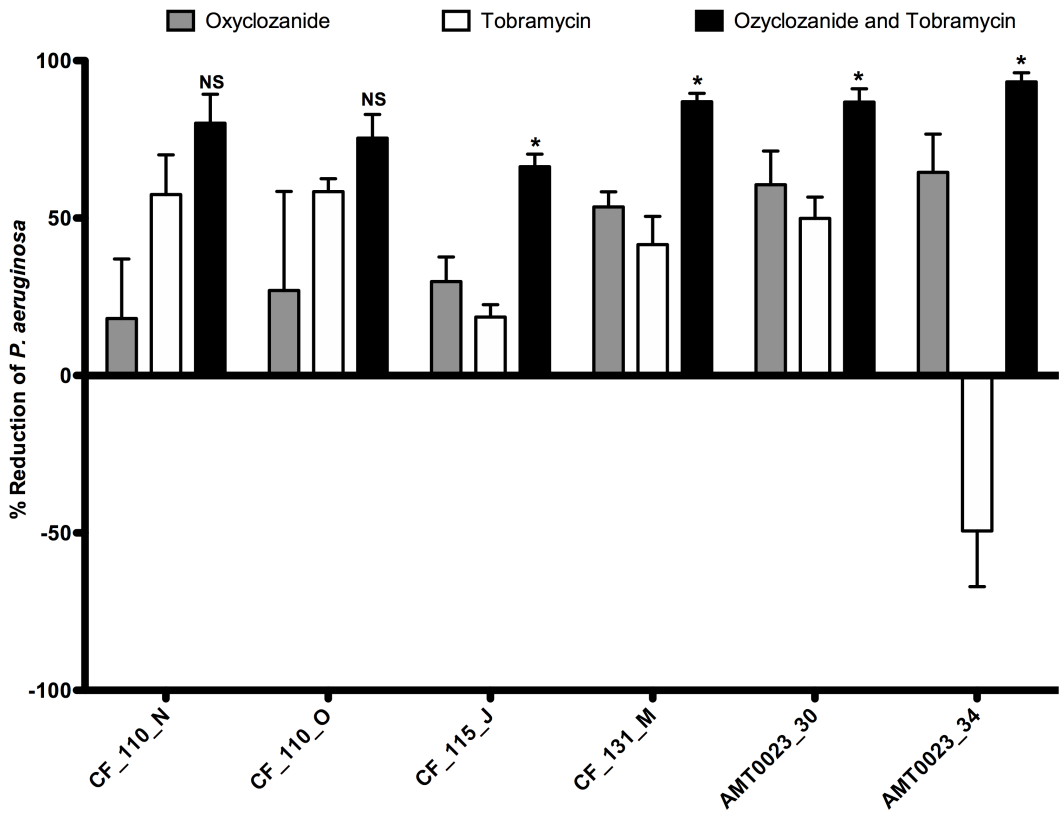

Figure 3-10. Oxyclozanide is broadly active against *P. aeruginosa*. Biofilms were treated with oxyclozanide (100 μM), or tobramycin (500 μM), alone and in combination for 6-hrs and the number of viable cells within the biofilms were quantified. The assay was performed three times in triplicate. The results represent means plus the SEM. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between the combination and tobramycin alone (*, $p<0.05$, NS, not significant).

3.3.6 *Oxyclozanide and tobramycin are more effective against stationary-phase cells*

We hypothesized that the combination therapy may also be effective against stationary-phase cells that mimic cells growing in biofilms as they are in a lower metabolic state and enriched for persister cells. Persister cells are metabolically dormant and thus highly tolerant to antimicrobials that target actively growing cells.[14,15] To test this, we analyzed the activity of oxyclozanide (100 μM), tobramycin (50 μM), or the combination on cultures of *P. aeruginosa* grown for 20 hours. Similar to our results

with biofilms, we found that the combination of oxyclozanide and tobramycin significantly reduced viable cells as measured by quantifying cfus, exhibiting a 10-fold reduction in viable cells compared with tobramycin alone at 8 and 24-hrs (Figure 3-11).

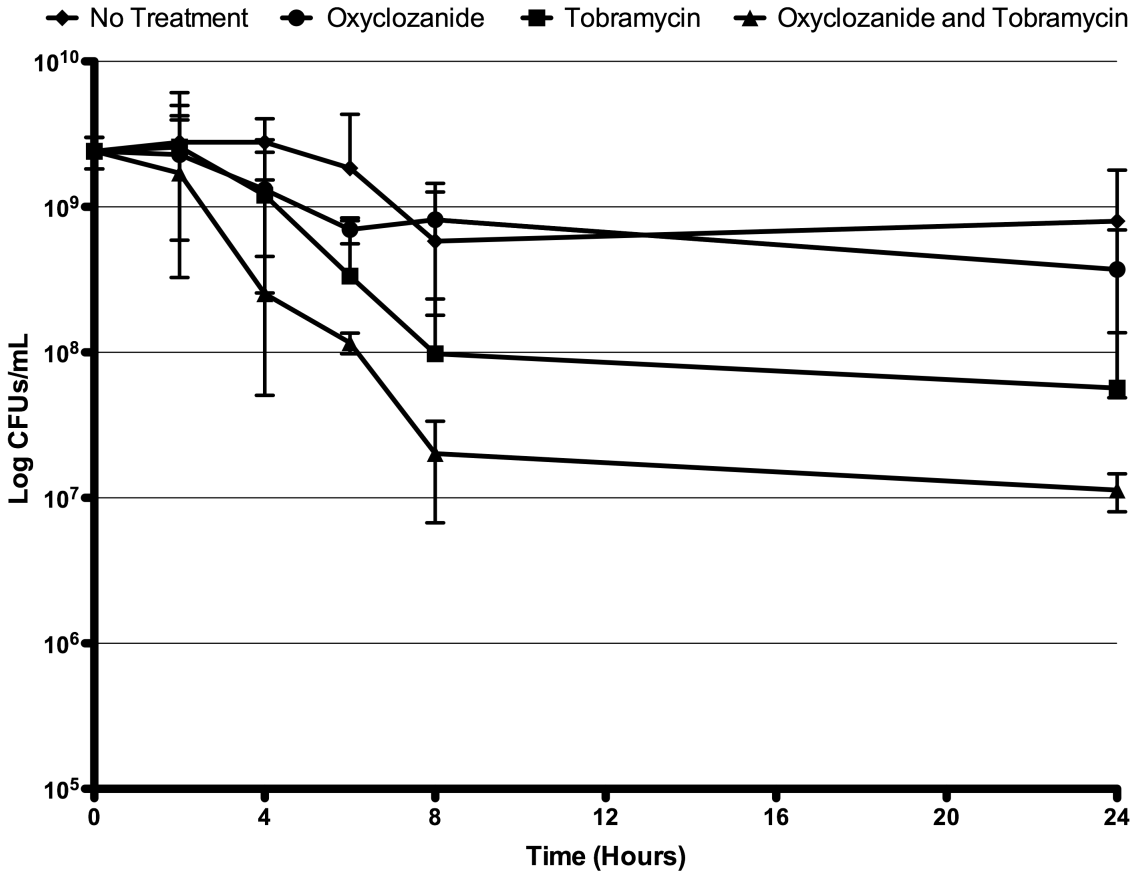

Figure 3-11. **Oxyclozanide and tobramycin are more effective against stationary-phase cells.** 20-hr old stationary-phase cells were treated with oxyclozanide (100 μM) or tobramycin (50 μM) alone and in combination. At 0, 2, 4, 6, 8, and 24-hrs aliquots were taken for CFUs/mL enumeration. The experiment was performed twice times in triplicate. The results represent means ± the SD.

3.3.7 Oxyclozanide causes cellular permeabilization and the loss of a membrane potential in P. aeruginosa biofilms

The mode of action of oxyclozanide in parasitic flatworms is the uncoupling of oxidative phosphorylation by the translocation of protons through the inner mitochondrial membrane, disrupting the PMF and eliminating the membrane potential ($\Delta\psi$).[149] Additionally, oxyclozanide has been shown to induce cellular permeabilization in the Gram-positive pathogen, *S. aureus*.[160] However, ionophore activity nor membrane disruption of oxyclozanide has not been demonstrated in Gram-negative bacteria or bacteria growing in a biofilm.

To test if oxyclozanide exhibits ionophore activity against *P. aeruginosa*, biofilms exposed to tobramycin, oxyclozanide, or the combination were assayed with BacLight™, which consists of the dye DiOC2(3). This dye is a marker for cellular membrane potential ($\Delta\psi$), and cells were analyzed by flow cytometry. Because oxyclozanide has been shown to cause cell permeabilization, we also added to this assay TO-PRO-3 iodide, which stains the DNA of bacteria with disrupted outer membranes, characteristic of dead cells. The combination of these two dyes allowed us to determine both cellular permeabilization and membrane potential in the same assay. For those cells that have an intact outer membrane and do not stain with TO-PRO-3, import of DiOC2(3) into the cell cytoplasm, where self-association of this dye shifts its fluorescent spectrum to the phycoerythrin (PE) channel, indicates an intact $\Delta\psi$.

TO-PRO-3 staining indicated that oxyclozanide caused significant cellular permeabilization and death, increasing the population of permeabilized cells from 10% in untreated biofilms to 27% when treated for 2 hours (Figure 3-12A). Tobramycin

treatment alone similarly increased permeabilized cells, but this increase was not statistically significant compared to oxyclozanide alone. This is not unexpected, given both oxyclozanide and tobramycin treatment alone caused a ~50% reduction of viable cells within biofilms (Figure 3-2). However, oxyclozanide combined with tobramycin significantly increased the population of permeabilized and dead cells compared to tobramycin treatment alone, 59% versus 19%, respectively.

Subsequent analysis of the cells that were living (i.e. TO-PRO-3 negative) by DiOC2(3) staining demonstrated that oxyclozanide showed significant ionophore activity against cells within biofilms after 2-hrs of treatment (Figure 3-12B). The population of cells maintaining a $\Delta\psi$ was reduced from 39% in untreated biofilms to 19% in oxyclozanide treated biofilms. Alternatively, tobramycin treatment alone significantly increased the population of cells maintaining a $\Delta\psi$ to 69%. This dramatic increase in the population of cells maintaining a $\Delta\psi$ is, again, indicative of adaptive resistance.[35,159] Oxyclozanide combined with tobramycin abolished this effect, reducing the population of cells maintaining a $\Delta\psi$ to 19%.

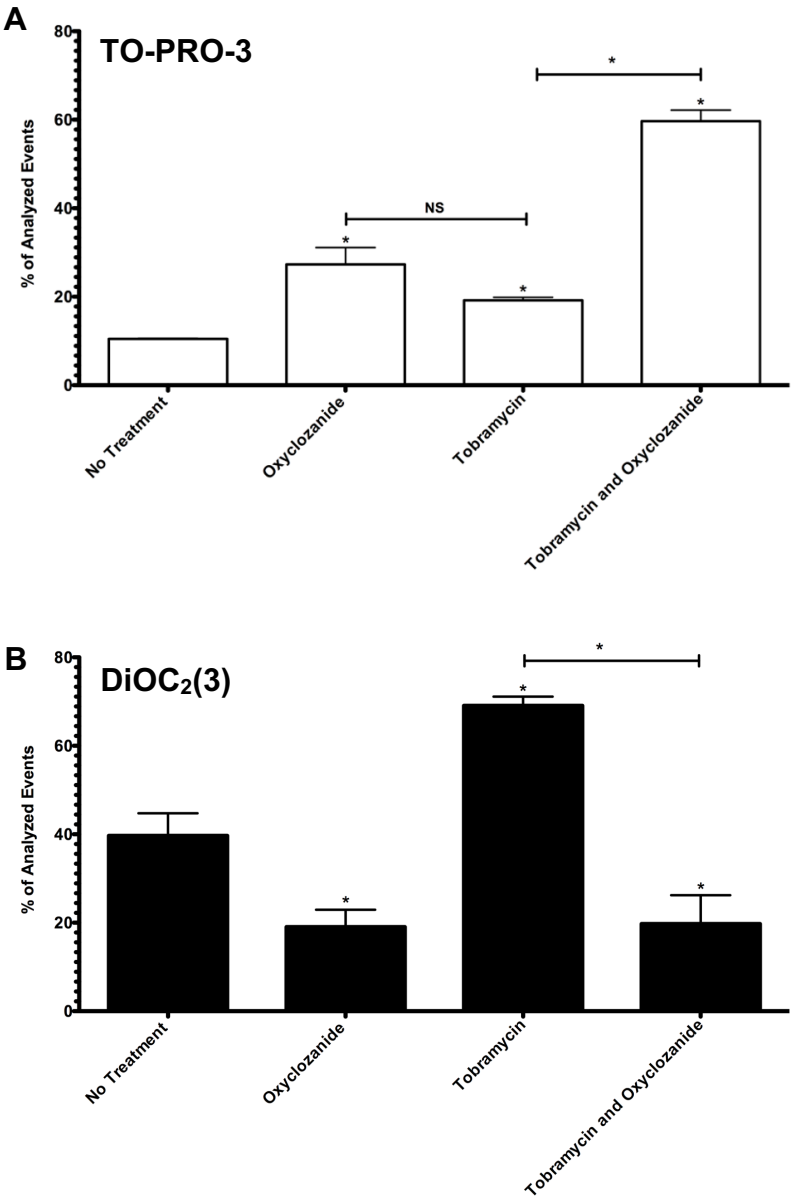

Figure 3-12. Oxyclozanide induces permeabilization and depolarizes the membrane potential of *P. aeruginosa*. Biofilms were treated with oxyclozanide (100 μM), or tobramycin (500 μM), alone and in combination for 2-hrs. **A**. Cells were stained with TO-PRO-3 to determine the number of cells that were permeabilized. **B**. Cells were also stained with DiOC2(3) to determine the number of cells maintaining a membrane potential. Permeabilized cells were excluded from membrane potential analysis. The experiment was performed two separate times in duplicate. The results are percent averages plus the SEM. Percent values indicate the average relative abundance of events within each gate normalized to the total number of events analyzed, excluding artifacts, aggregates and debris. A one-way ANOVA followed by Dunnett's multiple comparison test was used to determine statistical significance between each treatment and the untreated control (*, $p<0.05$). Oxyclozanide treatment alone was compared with tobramycin treatment alone in panel A (NS, not significant). A one-way ANOVA followed by Bonferroni's multiple comparison test was used to compare tobramycin to the combination in panel A and B (*, $p<0.05$).

Similar results were obtained after 6-hrs of treatment. TO-PRO-3 staining indicated that oxyclozanide increased the population of permeabilized and dead cells from 2.6% in untreated biofilms to 13% when treated for 6-hrs (Figure 3-13A). Tobramycin did not significantly cause permeabilization and death. However, the combination of oxyclozanide and tobramycin significantly increased the population of permeabilized and dead cells to 38% compared to tobramycin treatment alone. Again, similar results were obtained after 6-hrs of treatment with the exception that tobramycin did not result in a significant increase in the population of cells maintaining a $\Delta\psi$ (Figure 3-13B). This is expected if the increased $\Delta\psi$ is due to adaptive resistance as this is known to be temporal in nature and a short-lived response.[33] Strikingly, by 6 hours virtually no cells exhibited a $\Delta\psi$ when treated with the combination of oxyclozanide and tobramycin.

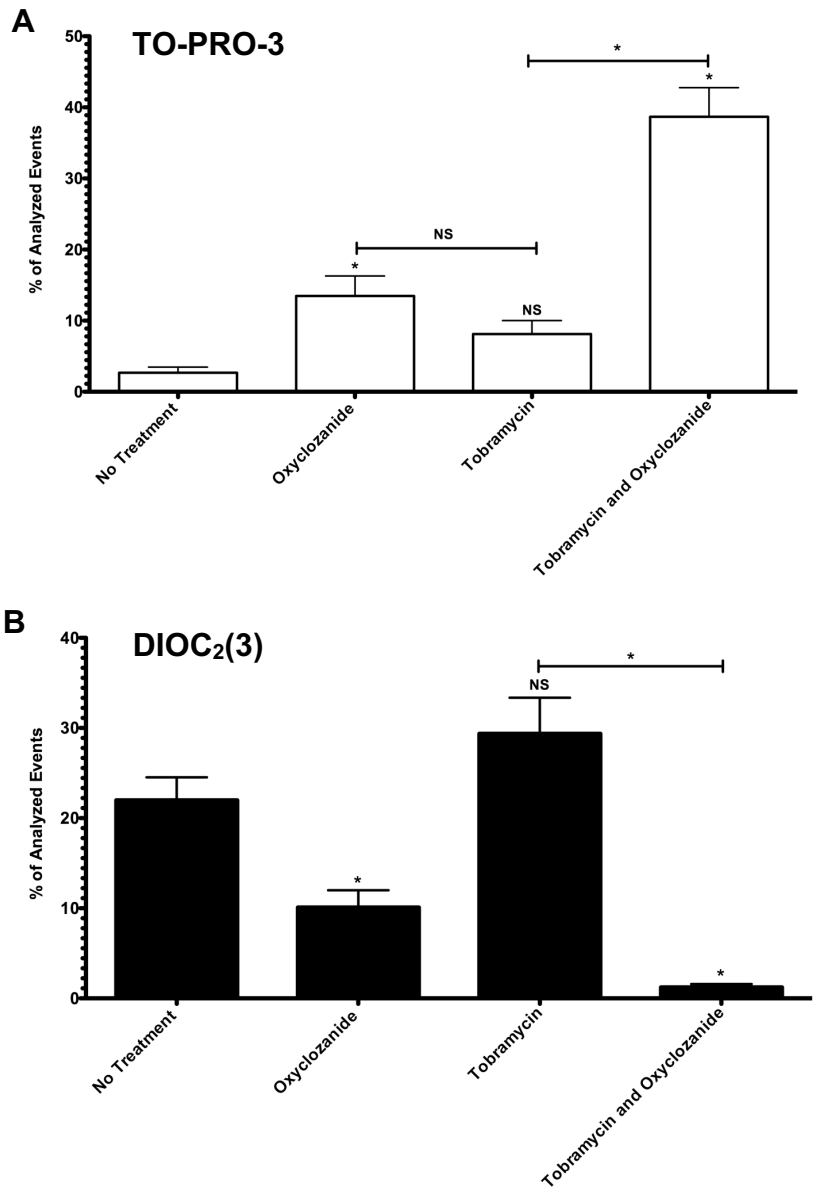

Figure 3-13. Oxyclozanide induces permeabilization and depolarizes the membrane potential of *P. aeruginosa*. Biofilms were treated with oxyclozanide (100 μM), or tobramycin (500 μM), alone and in combination for 6-hrs. **A**. Cells were stained with TO-PRO-3 to determine the number of cells that were permeabilized. **B**. Cells were also stained with DiOC2(3) to determine the number of cells maintaining a membrane potential. Permeabilized cells were excluded from membrane potential analysis. The experiment was performed two separate times in duplicate. The results are percent averages plus the SEM. Percent values indicate the average relative abundance of events within each gate normalized to the total number of events analyzed, excluding artifacts, aggregates and debris. A one-way ANOVA followed by Dunnett's multiple comparison test was used to determine statistical significance between each treatment and the untreated control (*, $p<0.05$). Oxyclozanide treatment alone was compared with tobramycin treatment alone in panel A (NS, not significant). A one-way ANOVA followed by Bonferroni's multiple comparison test was used to compare tobramycin to the combination in panel A and B (*, $p<0.05$).

Finally, DRCs were performed with oxyclozanide to further characterize its effects on the Δψ and its ability to permeabilize and kill cells within biofilms. Untreated controls were re-plotted for comparison. After 2-hrs of treatment, oxyclozanide caused permeabilization and cell death at 200 μM (Figure 3-14), resulting in very few cells with a Δψ. However, this effect was lost from 100 to 25 μM while a reduction of the Δψ compared to no treatment could be observed.

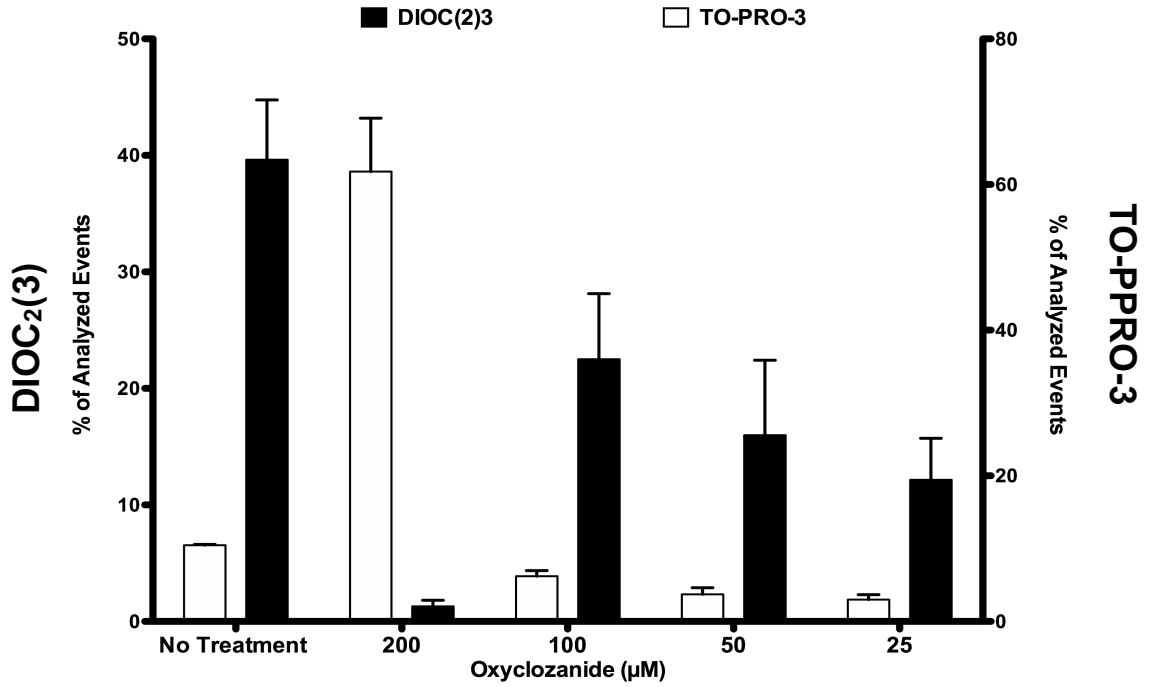

Figure 3-14. Dose response curves of oxyclozanide measuring the membrane potential and permeabilization of *P. aeruginosa*. 24-hr old biofilms were treated with oxyclozanide (100 μM), or tobramycin (500 μM), alone and in combination for 2-hrs. No treatment controls were re-plotted for comparison. Cells were stained with TO-PRO-3 to determine the number of cells that were permeabilized. Cells were also stained with DiOC2(3) to determine the number of cells maintaining a membrane potential. Permeabilized cells were excluded from membrane potential analysis. The experiment was performed two separate times in duplicate. The results are percent averages plus the SEM. Percent values indicate the average relative abundance of events within each gate normalized to the total number of events analyzed, excluding artifacts, aggregates and debris.

3.3.8 Oxyclozanide causes increased tobramycin accumulation

Since oxyclozanide causes cellular permeabilization, reduces membrane potential, and causes accelerated activity of tobramycin, we sought to determine if oxyclozanide increased the accumulation of tobramycin associated with cells. To do this, we fluorescently labeled tobramycin with Texas Red and measured its accumulation within cells of a mature biofilm following 30-mins of treatment. We found triclosan in combination with tobramycin resulted in significantly more tobramycin accumulation within cells (Figure 3-15).

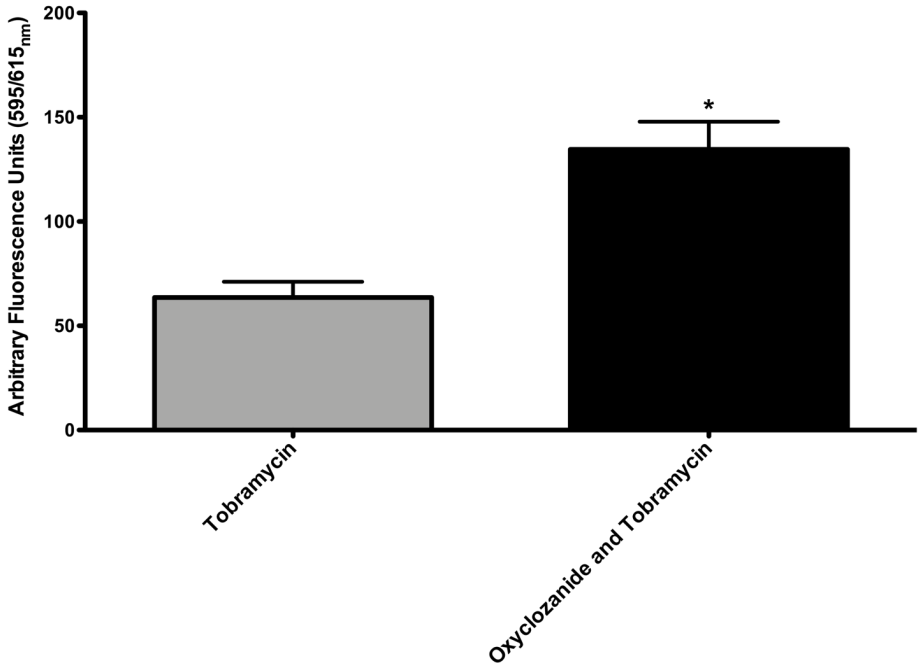

Figure 3-15. Oxyclozanide results in increased cellular accumulation of tobramycin. 24-hr old biofilms grown in glass test tubes were treated for 30-mins with oxyclozanide (100 μM) and Texas Red™ labeled tobramycin (250 μg/mL). Then cells were lysed with 0.2% Trition-X 100® to measure intercellular accumulation of labeled tobramycin. Uptake of labeled tobramycin was measured by relative fluoresce units using excitation 595_{nm} and emission 615_{nm}. The assay was performed twice in duplicate. The results represent means plus the SEM. An unpaired t-test was performed comparing tobramycin versus triclosan and tobramycin (*, $p<0.05$).

3.4 Discussion

Drug repurposing affords many benefits for developing novel antimicrobials, including reduced costs and accelerated deployment.[161] We recently demonstrated that triclosan combined with tobramycin can be repurposed to eradicate biofilms formed by both Gram-negative and Gram-positive bacteria.[14] Here, we show that the anthelmintic drug, oxyclozanide, enhances the activity of aminoglycosides and tetracycline to eradicate antibiotic tolerant biofilms. Importantly, we found that the combination has activity against cells within biofilms formed by both Gram-negative and Gram-positive pathogens. Young CF patients are often colonized with *Staphylococcus* organisms, making tobramycin combined with oxyclozanide an applicable therapy for both early and late CF lung pathogens.[1]

Oxyclozanide is given orally at a dose of 10-15 mg/kg (~25-37 μM/kg).[149] We envision the use of oxyclozanide in conjunction with tobramycin at a concentration between 50-100 μM as an inhaled aerosolized solution for the treatment of CF. This route of administration provides many benefits including reduced side effects and enhanced activity, which could deliver higher concentrations locally while limiting systemic exposure.[162,163] We also imagine the use of tobramycin combined with oxyclozanide as a topical therapy for the treatment of diabetic and burn wounds at concentrations similar to what is currently used clinically.

Oxyclozanide has been reported to have two modes of action. It has been shown to function as an ionophore in parasitic worms[149], and it was found to permeabilize the Gram+ bacterium *S. aureus* growing planktonically.[29] Our results indicate that oxyclozanide exhibits both activities against *P. aeruginosa* biofilms, which could

contribute to the observed increase of tobramycin in cells in the presence of oxyclozanide that we observed, and we suggest that each is important for oxyclozanide and tobramycin synergy.

The PMF is one component of the Δψ that has been shown to contribute to efflux of antibiotics and adaptive resistance in bacteria. A gradient of protons across the inner membrane drives the production of ATP as they are imported via the phosphorylation of ADP to ATP by ATP synthase.[164] Importantly, the PMF also functions as the energy source for efflux pumps, including those in the RND superfamily, to export antimicrobials. The RND efflux superfamily is responsible for MDR and adaptive resistance in *P. aeruginosa*.[33,35,74] Our results are consistent with previous results as we observed *P. aeruginosa* growing in a biofilm increases the Δψ in response to tobramycin, which may be responsible for the increased ATP that we observed during the first few hours of tobramycin treatment. Whether uncoupling of the Δψ by oxyclozanide inhibits efflux pump activity remains to be examined.

Based on our data, we propose a model where oxyclozanide enhances tobramycin accumulation in *P. aeruginosa* by abolishing adaptive resistance through permeabilizing and disrupting the Δψ of cells. This causes increased tobramycin accumulation in the cells, yielding accelerated activity and increased effectiveness. In support of this model, oxyclozanide inhibits the large increase in the population of cells maintaining a Δψ, which was seen in response to tobramycin and significantly increases the rate of killing by tobramycin. Oxyclozanide also resulted in increased tobramycin accumulation within cells in mature biofilms. Uncoupling the individual role of

permeabilization and inhibition of membrane potential in the synergy of oxyclozanide and tobramycin requires further investigation.

Although an intact PMF is considered one of the main mechanisms of aminoglycoside influx into cells, it is well known that, independently of respiration, aminoglycosides can accumulate within cells through a process termed self-promoted uptake.[44] In this process, aminoglycoside interact with the membrane surface by displacing cations, creating “cracks” or “fissures” in the outer membrane of cells. This leads to the diffusion of aminoglycosides into the cytosol of cells, which in turn contributes to additional membrane damage by the insertion of misfolded proteins in the outer membrane.[43,44] Thus, at the concentrations we are examining, it is likely that the increased killing in the absences of a Δψ can largely be attributed to the effects of self-promoted uptake combined with the permeabilizing effects of oxyclozanide.
We found that oxyclozanide only enhanced antimicrobials that targeted the ribosome, namely, aminoglycosides and tetracycline. We hypothesize that this is due to the fact that only ribosomal inhibitors and not antimicrobials that target cellular pathways triggers the induction of MexXY efflux pumps and adaptive resistance.[165,166] Oxyclozanide over-comes this mechanism by both permeabilizing and disrupting the Δψ of cells within biofilms.

3.5 Conclusion

Many bacterial species including *S. aureus* and *P. aeruginosa* can be found growing as biofilms in non-healing chronic wounds such as diabetic foot ulcers and burns [115-117]. Previously, it has been shown that oxyclozanide has activity against

planktonic *S. aureus* and cancerous cells, indicating broad applicability (21). The repurposing of veterinary drugs has a proven history of success, notably the ionophore anthelmintic ivermectin, which has been repurposed for the treatment of several diseases in humans [167]. Oxyclozanide combined with tobramycin could be a potential new treatment for the eradication of *P. aeruginosa* and *S. aureus* infections in CF patients as well as diabetic foot ulcers and burn wounds. This is yet another proof of principle demonstrating the role adjuvants could have in improving current therapies, as described in Chapter 1.

CHAPTER 4

TRICLOSAN ABOLISHES ADAPTIVE RESISTANCE IN *PSEUDOMONAS AERUGINOSA* BIOFILMS

4.1 Introduction

In this chapter, we investigated the mechanism of action of tobramycin and triclosan. We found triclosan inhibits RND-type efflux pumps by acting a protonophore, thus abolishing adaptive resistance, permitting tobramycin to accumulate rapidly within cells and cause permeabilization. Collectively, these results suggest that triclosan could be developed further for use as an efflux pump inhibitor (EPI) that abolishes adaptive resistance, thus, rendering cells susceptible to aminoglycosides.

4.2 Materials and Methods

4.2.1 Bacterial strains, culture conditions, and compounds

All strains used in this study are listed in Table 2-1. Bacterial strains were grown in glass test tubes as described previously in Chapter 2.

Table 4-1 Bacterial strains used in this study.

Strain	Characteristics	Reference
PAO1	*PA* Standard Reference Strain, isolated in 1954[118]	[6]
Xen41	Bioluminescent PAO1 derivative: constitutively expresses *luxCDABE* gene	PerkinElmer [126]
F305	**fusA1*, L40Q (C**T**G→C**A**G)	This Study
F306	**fusA1*, L40Q (C**T**G→C**A**G)	This Study
30B28	**fusA1*, T64A (**A**CC→**G**CC)	This Study
30B230	**fusA1*, T64A (**A**CC→**G**CC)	This Study

Table 4-1 (cont'd)

30B257	**fusA1*, T64A (**A**CC→**G**CC)	This Study
30B282	**fusA1*, T64A (**A**CC→**G**CC)	This Study

*All additional SNPs in evolution mutants are listed in supplemental file 1.

4.2.2 Measurement of bacterial growth

Cells were diluted to an optical density at 600 nm (OD_{600}) of 0.001 in 10% v/v MHB diluted in Dulbecco's Phosphate Buffered Saline with magnesium and calcium (DPBS, Sigma-Aldrich), and 200 µl of cells were loaded into a 96-well flat bottom Costar® plates (Corning). OD_{600} was read every hour for 24-hrs, using a SpectraMax M5 microplate spectrophotometer system at 35°C (Molecular Devices).

4.2.3 Biofilm susceptibility testing using BacTiter-Glo™

As prescribed described as described previously in Chapter 2.

4.2.4 Selection for *P. aeruginosa* evolution mutants

To select mutants resistant to tobramycin and triclosan, we modified a protocol by Lindsey and colleages and serially passaged biofilms under gradual, moderate and sudden selection pressure.[168] 24-hr biofilms were formed as described above and treated with triclosan and tobramycin for 24-hrs. After each treatment, biofilms were sonicated for 15-mins (Branson Ultrasonics) to disperse surviving cells from the pegs into 10% v/v MHB recovery media. Biofilms were then allowed to re-form on new pegs overnight. The next day, passaged biofilms were treated at a slightly higher concentration of triclosan and tobramycin. Following the treatment, the recovery

process was repeated, and biofilms were re-formed. In each subsequent cycle, an increasing concentration of triclosan and tobramycin was used. By serially passaging biofilms and gradually increasing the concentration of triclosan and tobramycin two mutant pools were created. From these mutant pools, 191 single colony resistant mutants were isolated.

4.2.5 Sequencing

Genomic deoxyribonucleic acid (gDNA) was isolated from each mutant using the Wizard® genomic DNA purification kit (Promega). Illumina NextSeq was then performed by the Genomic Services Facility at Indiana University Center for Genomics and Bioinformatics, using pair end reads. To identify single nucleotide polymorphisms (SNPs), sequencing results were first verified for quality using FASTQC and aligned to the PAO1 reference genome[169], which can be downloaded from the *Pseudomonas* Genome Database (http://www.pseudomonas.com) using the *Breseq* pipeline, which can be downloaded from (http://barricklab.org).[170] All identified SNPs are listed in Table 4-2.

4.2.6 Ethidium bromide efflux pump assay

Intercellular accumulation of the DNA-intercalating agent ethidium bromide, which is a substrate for RND-type efflux pumps, was measured fluorometrically as previously described.[171] The premise of this assay is that lower levels of RND-type efflux pump activity will result in increased concentrations of ethidium bromide within cells, which only fluoresces when bound to DNA.[172,173] Briefly, 24-hr old biofilms were

formed in 96-well black ViewPlate (PerkinElmer) as described above. 10 μg/mL of ethidium bromide was added to each well along with various treatments. Fluoresces was recorded every 2-hrs for 6-hrs using a SpectraMax M5 microplate spectrophotometer system ($\lambda_{excite,}$ 530 nm and $\lambda_{emit,}$ 600 nm).

4.2.7 BacLight™ membrane potential assay and cell permeabilization assay

As described previously in Chapter 3.

4.2.8 Tobramycin accumulation assay

As previously as described previously in Chapter 3.

4.2.9 Agarose hydrogels

As previously as described previously in Chapter 2.

4.2.10 Murine wound infection model

As previously as described previously in Chapter 2.

4.3 Results

4.3.1 Tobramycin and triclosan do not synergize by targeting FabI

Triclosan is known to inhibit FabI, the enzyme responsible for the final elongation step in fatty acid synthesis (Figure 2-1)[128,129], however our prior results show that the inhibition of FabI is not the primary mechanism of synergy because a *fabI* mutant is not sensitive to tobramycin treatment alone.[6] *P. aeruginosa* also encodes *fabV,* which

encodes a functional analog of FabI that is more resistant to triclosan.[130] Therefore, to further explore if inhibition of fatty acid biosynthesis is responsible for synergy with tobramycin, we examined killing of *P. aeruginosa* biofilms by the fatty acid synthesis inhibitor AFN-1252, which forms a binary complex with the active site of FabI and is thought to also inhibit FabV.[174-176] We found that AFN-1252 alone and in combination with tobramycin was not significantly effective against mature biofilms (Figure 4-1). However, at the maximal concentrations of tobramycin and AFN-1252 used, we observed slightly more than 2-fold killing that was not statistically significant, which is far weaker than the ~100-fold killing observed when triclosan is combined with tobramycin.[6] Thus, our results using both genetic mutation and chemical inhibition failed to support the hypothesis that triclosan synergizes with tobramycin by disruption of membrane biogenesis.

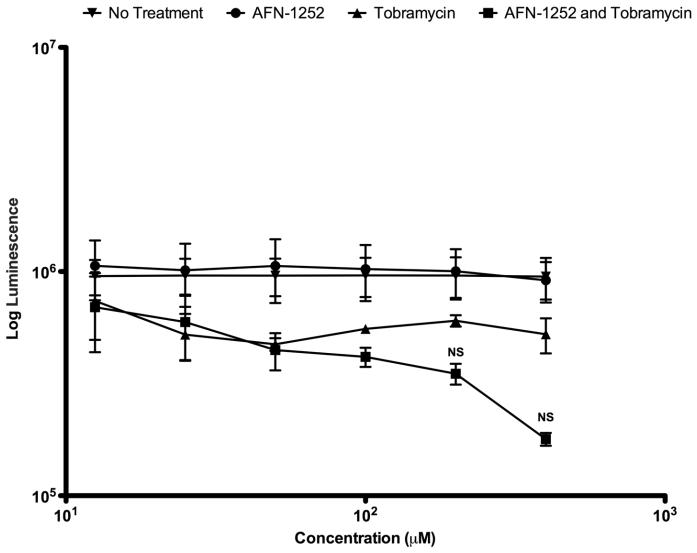

Figure 4-1. AFN-1252 alone or in combination with tobramycin is not effective against mature *P. aeruginosa* biofilms. Biofilms were treated for 6-hrs with AFN-1252 (400 μM), tobramycin (400 μM), alone and in combination in two-fold dilutions, and the number of viable cells within the biofilms were quantified. The assay was performed twice in in duplicate. The results represent means ± the standard error mean *(*SEM). A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between tobramycin versus the combination (NS, not significant).

4.3.2 Selection of resistant mutants

We hypothesized that triclosan synergized with tobramycin via an undescribed mechanism. To further elucidate this mechanism we selected for resistance mutants using experimental evolution combined with whole genome sequencing.[170,177] To select for mutants resistant to the combination we modified an evolution protocol developed by Lindsey and colleagues that selected for antibiotic resistance in *Escherichia coli.*[168] 27 *P. aeruginosa* biofilms were serially passaged in sudden, moderate, and gradual treatment regimens that were exposed to varying degrees of increasing concentrations of tobramycin and triclosan in parallel. All of the populations in the sudden and moderate treatment groups were eradicated, consistent with previous results demonstrating sudden treatment with antibiotics can restrict beneficial mutations from occurring.[168] However, after 30 passages, two mutant populations from the gradual treatment regimen were isolated, from which 191 clonal isolates were obtained that were found to be resistant to the combination.

To determine the mutation(s) responsible for the resistance to the combination we performed whole genome sequencing on two mutants from one mutant population and four mutants from the other mutant population. These 6 mutants all showed resistance to tobramycin and to the combination compared to the ancestral strain (Figure 4-2).

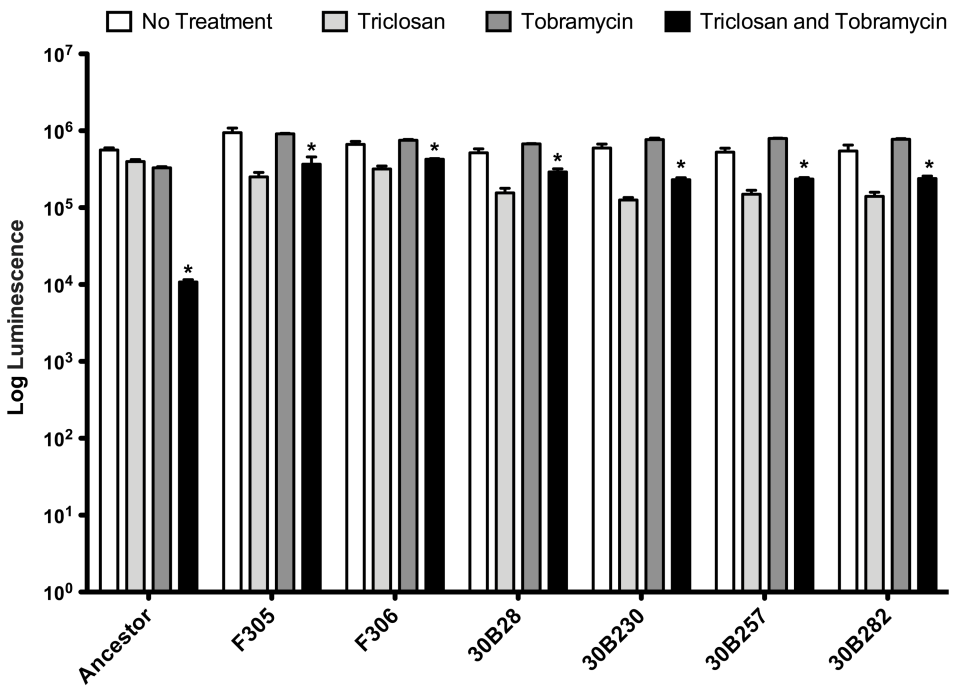

Figure 4-2. Six tobramycin and triclosan resistant mutants. Biofilms were treated with triclosan (100 μM) and tobramycin (500 μM) alone and in combination and the number of viable cells within biofilms were quantified. The assay was performed twice in triplicate. The results represent the means plus SEM. For the resistant mutants, a two-way ANOVA followed by Bonferroni's posttest was used to determine statistical significance between tobramycin alone and the combination (*, $p<0.05$).

However, these mutants were not completely resistant as there was still a significant decrease in the number of cells within a biofilm after treatment with the combination for 6-hrs compared to tobramycin treatment alone. Part of this activity may be attributed to triclosan as all six of these mutants exhibited increased sensitivity to triclosan treatment alone versus the ancestral strain. This phenomenon is termed collateral sensitivity, where resistance to one antimicrobial simultaneously results in sensitivity to unrelated antimicrobials.[178] We also found that the resistant mutants showed a fitness cost, both growing more slowly and lacking diauxic growth, compared to the ancestral strain (Figure 4-3).

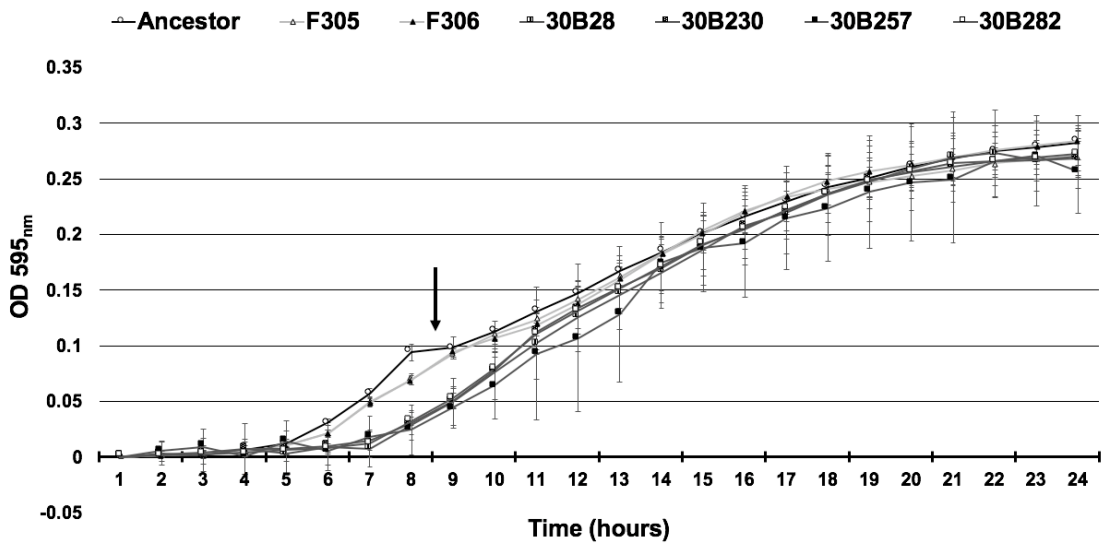

Figure 4-3. Resistant mutants show a growth defect and lack diauxic growth. The ancestor strain and evolution mutants were back-diluted to an OD of 0.001 in MHII broth and plated into a 96-well plate. Growth was measured over time using optical density (OD) 595_{nm}. The results represent the means ± standard deviation (SD). Black arrow indicates lag phase seen during diauxic growth in the ancestor strain. OD 595_{nm} at time zero was subtracted from all readings. The assay was performed once in triplicate.

All six mutants had a single nucleotide polymorphisms (SNPs) in the *fusA1* gene which encodes for elongation factor G (EFG1A), a protein responsible for ribosomal translocation and recycling.[179-181] Two unique mutations, a SNP at residues L40Q or T64A located in domain I responsible for GTPase hydrolysis, were identified (Figure 4-4). Because all mutants having a L40Q SNP came from mutant pool 1 and all mutants having a T64A SNP came from mutant Pool 2, we speculated that these SNPs occurred early on, allowing for clonal expansion.[182] However, no two mutants have the exact same set of SNPs, indicating these are not clonal and each is a distinct mutant. Interestingly, mutations in *fusA1* have previously been shown to confer resistance to tobramycin.[183] Thus, the results of our experimental evolution study show that tobramycin must inhibit translation in order for the combination to be effective.

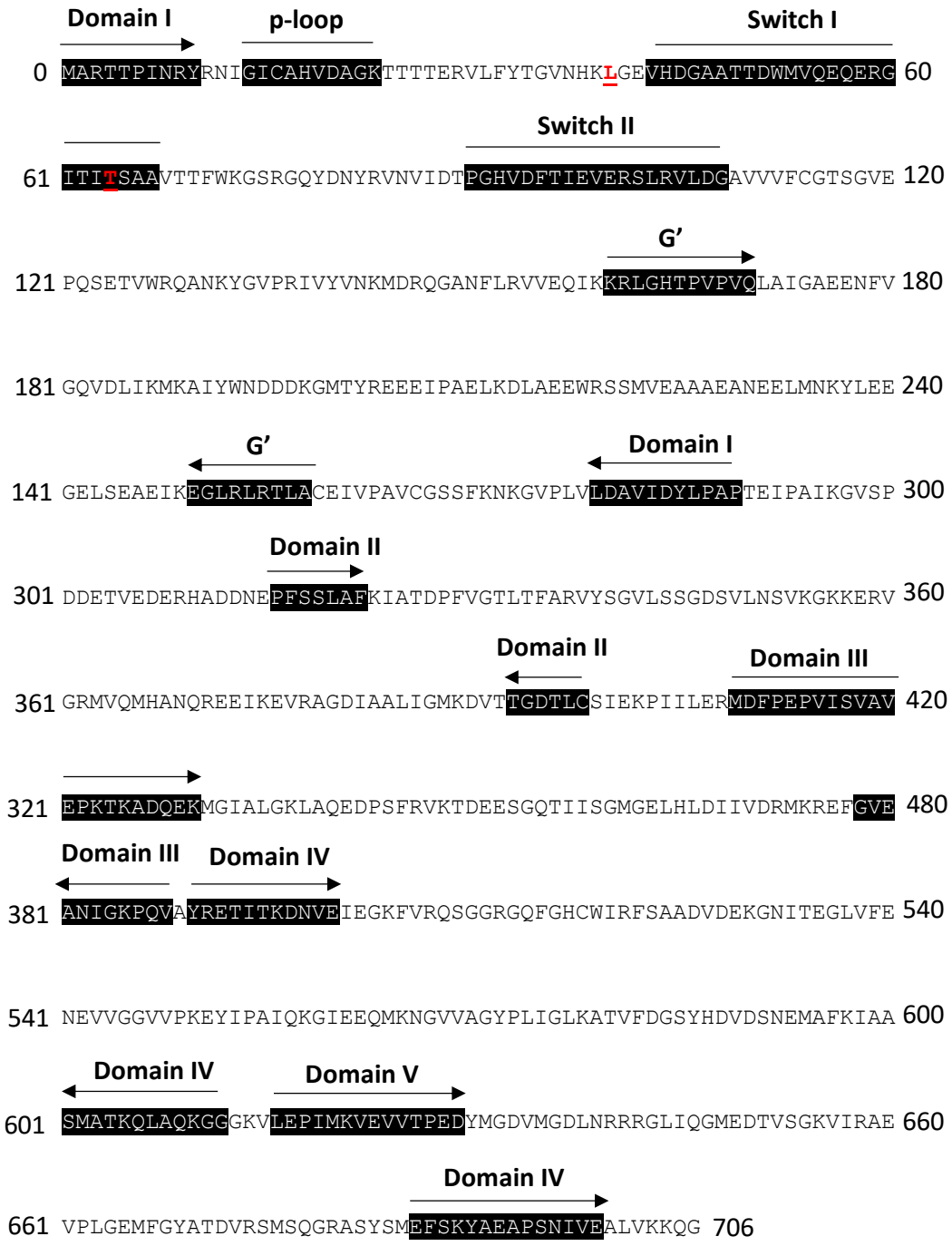

Figure 4-4. The protein sequence of elongation factor g (EFGIA) and locations of single nucleotide polymorphisms (SNPs). SNPs occur at residue L40Q and T64A, which is located within the switch I domain required for hydrolysis of guanosine triphosphate (GTP) and the "power-stroke" of EFGIA.

4.3.3 Triclosan inhibits RND-type efflux pumps

We therefore hypothesized that triclosan enhanced the activity of tobramycin via an unidentified mechanism. As efflux pumps are a major mechanism of antibiotic resistance in *P. aeruginosa,* we measured the activity of RND-type efflux pumps to export ethidium bromide in mature biofilms after treatment with tobramycin alone and in combination with triclosan. Ethidium bromide is a substrate of RND-type efflux pumps, and its accumulation within cells can be used as a proxy for efflux pump activity.[172,173] Our results show that triclosan resulted in reduced efflux pump activity as indicated by increased ethidium bromide accumulation within cells, while tobramycin treatment alone had no effect (Figure 4-5). Because triclosan does not kill cells these results cannot be explained by the binding of DNA. Together, these data suggest triclosan decreases efflux pump activity.

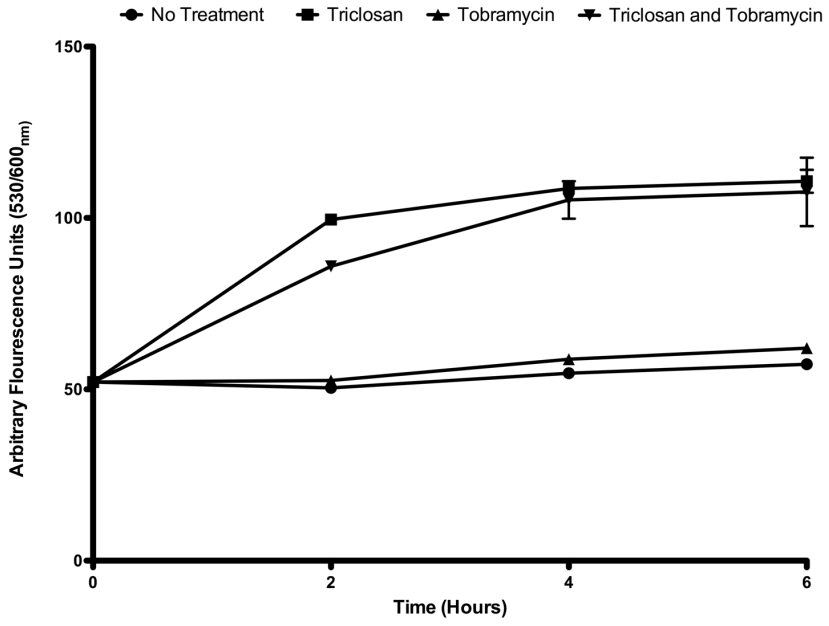

Figure 4-5. Triclosan inhibits RND-type Efflux Pumps. Biofilms were stained with ethidium bromide (10 µg/mL) to measure accumulation. Biofilms were treated with triclosan (100 µM), tobramycin (100 µM) alone and in combination. Fluorescence was read every 2-hrs for 6-hrs. The assay was performed twice in triplicate. Results represent the average arbitrary fluorescence units ± SEM.

4.3.4 Triclosan acts as a proton ionophore

The next question we addressed was the mechanism by which triclosan inhibited efflux pumps. Triclosan possess a hydroxyl group with a dissociable proton that has been shown to shuttle protons across the inner membrane of mitochondria, dissipating the proton motive force [184,185]. Because RND-type efflux pumps import a H^+ from the periplasm to power the extrusion of antimicrobials across the outer membrane, we speculated that protonophore activity by triclosan could reduce the proton motive force, thus, inhibiting efflux pump activity.[30,73]

The activity of RND-type efflux pumps is constrained by the proton motive force (PMF), which consists of two components, the membrane potential (Δψ) and proton gradient (ΔpH) [164]. To assess dissipation of the PMF we measured the Δψ component because it is more readily quantified, whereas changes to the proton gradient can be difficult to measure due the buffering capacity of the cytoplasm.[186,187] We measured changes in the Δψ of cells within biofilms using BacLight™. This assay consists of the dye DiOC2(3), which is driven into the cell cytoplasm by a Δψ where self-association of this dye shifts its fluorescent spectrum to the phycoerythrin channel.

In support of our hypothesis, we found triclosan treatment alone for 2-hrs reduced the population of cells maintaining a Δψ from 18% in untreated biofilms to 3% (Figure 4-6). Although protonophore activity by triclosan has recently been described against mammalian mitochondria[184,185,188], this activity has not been described against Gram negative cells within biofilms. Tobramycin treatment resulted in an increase in the population of cells with a Δψ, increasing the population to 32%, which is indicative of adaptive resistance (Figure 4-6).[33,34,159] That is, the induction of RND-type efflux pumps

is reflected by a surge in the number of cells maintaining a Δψ to meet the energy demand. The combination of triclosan and tobramycin reduced the population of cells maintaining a Δψ to 5%, thus abolishing adaptive resistance.

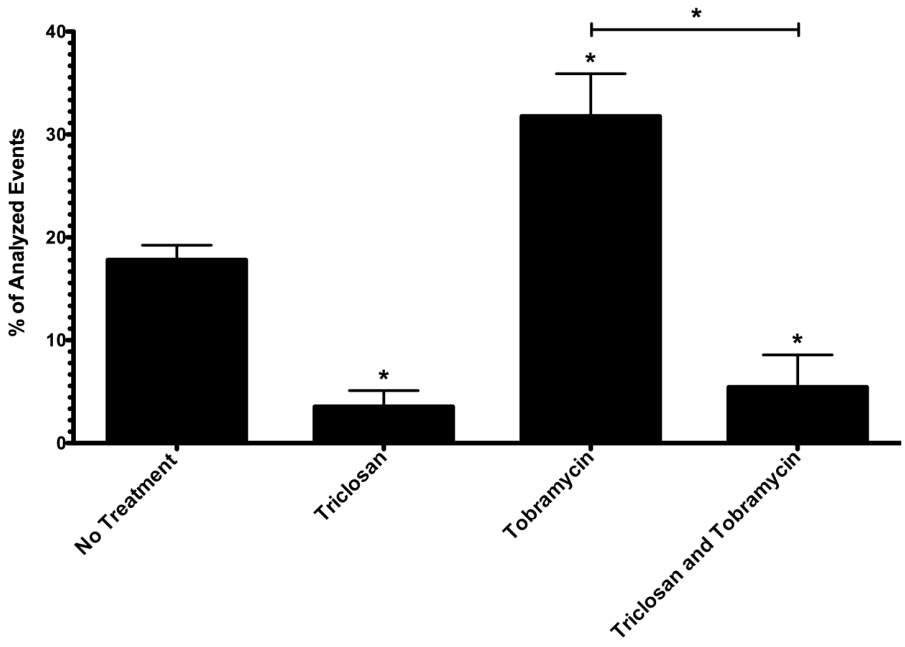

Figure 4-6. Triclosan acts a proton ionophore and abolishes adaptive resistance. Biofilms were treated with triclosan (100 μM), or tobramycin (500 μM), alone and in combination for 2-hrs. Cells were stained with DiOC2(3) to determine the number of cells that maintained a membrane potential. Dead or permeabilized cells were excluded from membrane potential analysis. The experiment was performed two separate times in duplicate. The results are percent averages plus the SEM. Percent values indicate the average relative abundance of events within each gate normalized to the total number of events analyzed, excluding artifacts, aggregates and debris. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between each treatment and an untreated control, and tobramycin treatment alone was compared with the combination (*, $p<0.05$).

4.3.5 Protonophore activity is required to abolish adaptive resistance

To further examine the function of triclosan as a protonophore, we treated cells with the chemical derivative methyl triclosan that lacks a dissociable proton and therefore cannot act as a protonophore.[185,189,190] Methyl triclosan had little to no effect on the population of cells growing in a biofilm that maintained a Δψ compared to the

untreated or tobramycin treated biofilms (Figure 4-7). These results further support the conclusion that triclosan functions as a protonophore against *P. aeruginosa* growing in a biofilm to abolish the adaptive resistance response.

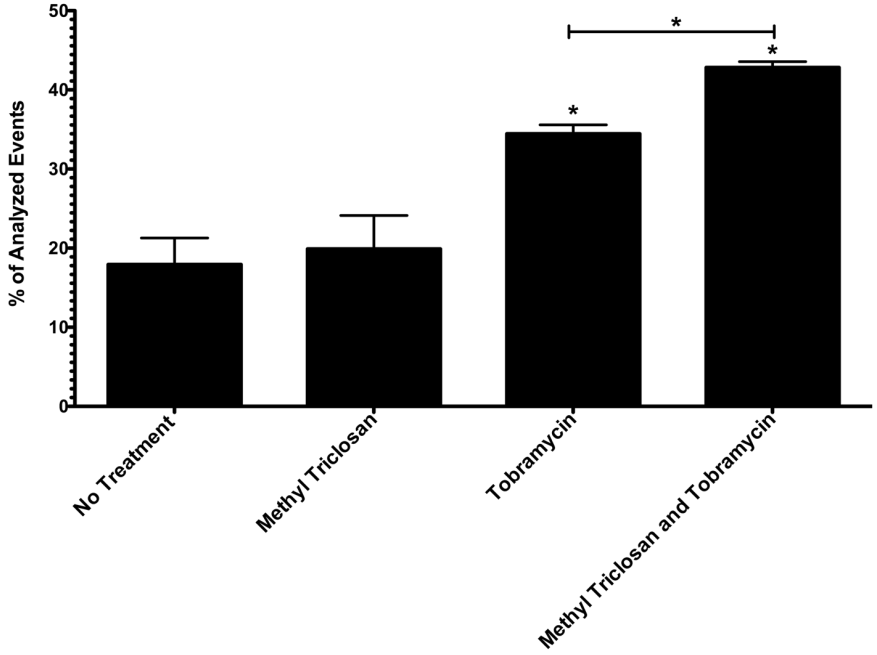

Figure 4-7. Methyl triclosan alone and in combination with tobramycin does not abolishes adaptive resistance. Biofilms were treated with methyl triclosan (100 μM), or tobramycin (500 μM), alone and in combination for 2-hrs. Cells were stained with DiOC2(3) to determine the number of cells that were maintain a membrane potential. Dead or permeabilized cells were excluded from membrane potential analysis. The experiment was performed two separate times in duplicate. The results are percent averages plus the SEM. Percent values indicate the average relative abundance of events within each gate normalized to the total number of events analyzed, excluding artifacts, aggregates and debris. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between each treatment and an untreated control, and tobramycin treatment alone was compared with the combination (*, $p<0.05$, NS, not significant).

4.3.6 Triclosan results in increased cellular accumulation of tobramycin

Since triclosan inhibits RND-type efflux pumps, we sought to determine if triclosan increased the accumulation of tobramycin within cells. To do this, we fluorescently labeled tobramycin with the Texas Red® dye and measured its accumulation within cells by lysing the cells of a mature biofilm following 30-mins of treatment. We found triclosan in combination with tobramycin resulted in significantly more tobramycin within cells (Figure 4-8).

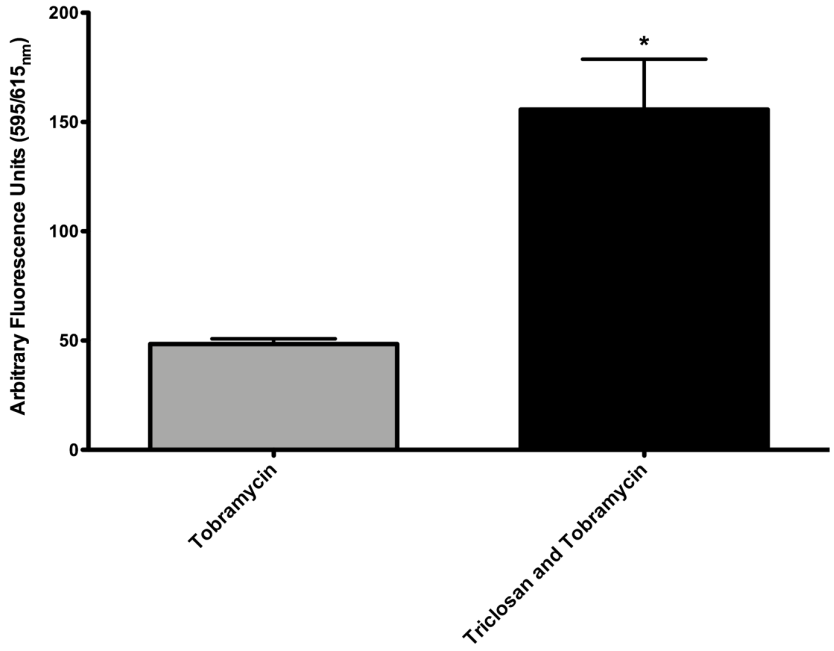

Figure 4-8. Triclosan results in increased cellular accumulation of tobramycin. Biofilms grown in glass test tubes were treated for 30-mins with triclosan (100 μM) and Texas Red labeled tobramycin (250 μg/mL). Then cells were lysed with 0.2% Trition-X 100® to measure intercellular accumulation of labeled tobramycin. Uptake of labeled tobramycin was measured by relative fluoresce units using excitation 595_{nm} and emission 615_{nm}. The assay was performed twice in duplicate. The results represent means plus the SEM. A unpaired t-test was performed comparing tobramycin versus triclosan and tobramycin (*, $p<0.05$).

4.3.7 Triclosan in combination with tobramycin causes increased permeabilization

Tobramycin corrupts protein synthesis resulting in the misfolding of proteins which are inserted in the inner membrane, causing permeabilization.[42-45] To determine if greater tobramycin accumulation in the cell in the presence of triclosan resulted in increased activity, cellular permeabilization was measured using the cell impermeable dye TO-PRO-3, which binds DNA and emits a fluorescent signal only when cells have become permeabilized.

We found that triclosan treatment alone resulted in ~20% of the cells within a mature biofilm to become permeabilized after 2-hrs (Figure 4-9). As expected, tobramycin treatment alone also significantly permeabilized cells within a biofilm, resulting in ~15% of cells compared to less than 5% of cells in an untreated control. Both of these treatments were statistically significantly greater than cells that were not treated. However, the combination of triclosan and tobramycin resulted in nearly 60% of cells becoming permeabilized after only 2-hrs of treatment. Previous kinetic experiments support these conclusions showing that triclosan and tobramycin were effective in as little as 2-hrs, whereas tobramycin alone required at least 6-hrs before having an appreciable effect.[6] Collectively, our current and past results suggest that triclosan inhibits RND-type efflux, allowing tobramycin to accumulate rapidly within the cell, increasing and accelerating its effectiveness.

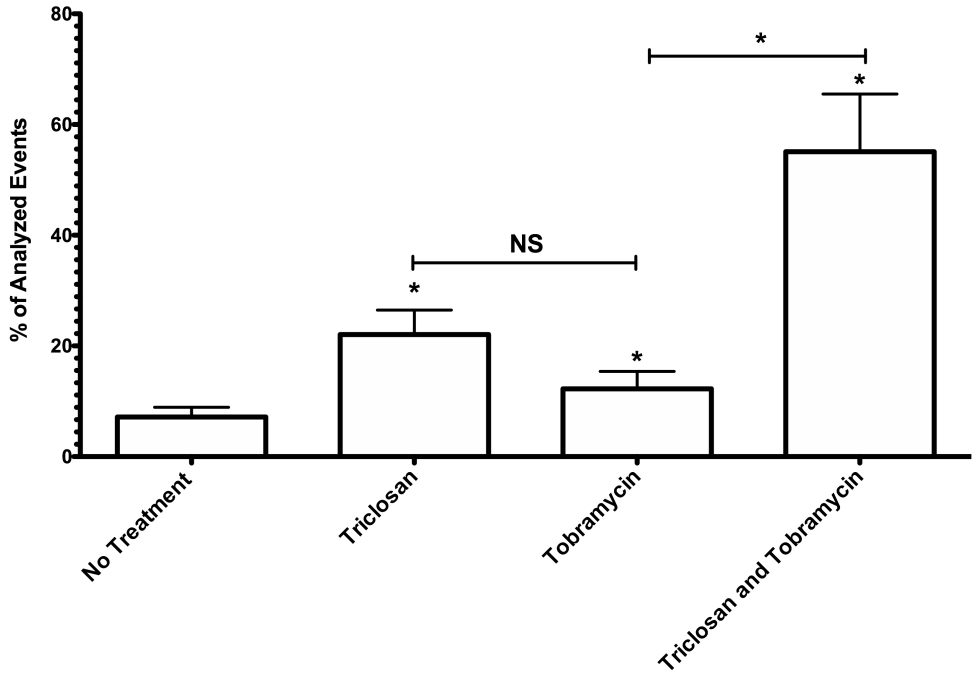

Figure 4-9. Triclosan in combination with tobramycin results in increased permeabilization. Biofilms were treated with triclosan (100 μM), or tobramycin (500 μM), alone and in combination for 2-hrs. Cells were stained with TO-PRO-3 to determine the number of cells that were permeabilized. The experiment was performed two separate times in duplicate. The results are percent averages plus the SEM. Percent values indicate the average relative abundance of events within each gate normalized to the total number of events analyzed, excluding artifacts, aggregates and debris. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between each treatment and the untreated control (*, $p<0.05$). And triclosan treatment alone was compared with tobramycin treatment (NS, not significant).

4.4 Discussion

Adaptive resistance is inseparably linked to the activity of efflux pumps, which are powered by the movement of protons from the periplasm into the cytoplasm, driving the extrusion of compounds across the outer membrane.[35,70] Our data demonstrate that triclosan acts as a protonophore, shuttling protons across the inner membrane, depleting the energy required for RND-type efflux pump activity to abolish adaptive resistance (Figure 4-10).[75] We show that in the presence of triclosan, tobramycin

accumulated within bacteria at an accelerated rate and its activity was enhanced (Figure 4-8 and 4-9). Importantly, all of these results were obtained with *P. aeruginosa* growing in a mature biofilm, which is reflective of the state of these bacteria in chronic infections.

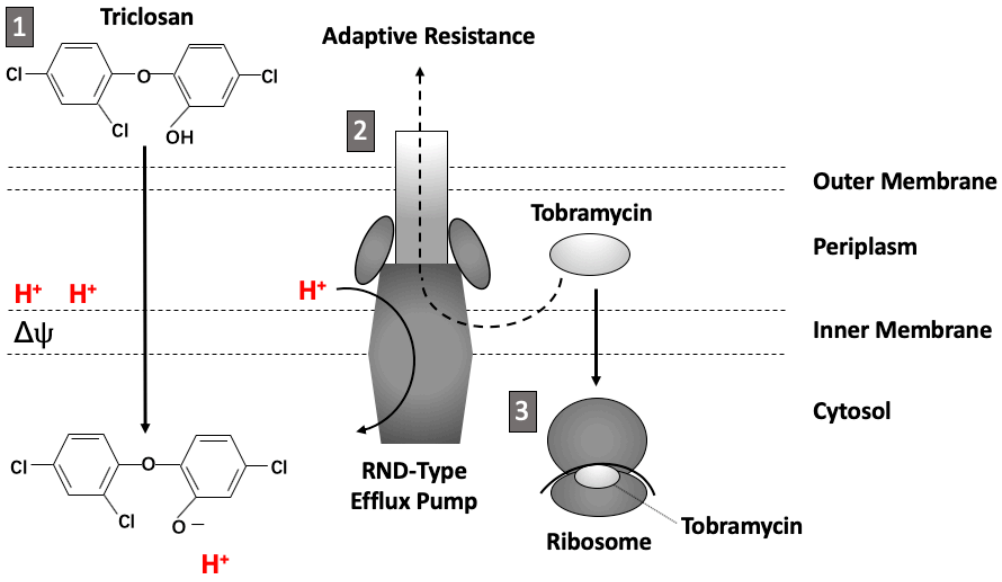

Figure 4-10. Triclosan sensitizes *P. aeruginosa* to tobramycin by abolishing adaptive resistance. Within 2-hrs of exposure to tobramycin, adaptive resistance occurs, which is due to the induction of RND-type efflux pumps, resulting in reduced accumulation of tobramycin within the cytosol. (1) Triclosan permeabilizes cells and shuttles protons across the inner membrane, collapsing the proton motive force and depolarizing the membrane potential (Δψ). (2) As a consequence, efflux pump activity is inhibited and there is enhanced accumulation of tobramycin within the cytosol. (3) Tobramycin binds to the P-site of the ribosome, corrupting protein synthesis and causing membrane permeabilization. Overall, triclosan accelerates and increase the effectiveness of tobramycin by reducing its efflux from the cell.

We cannot rule out the possibility that in addition to its protonophore activity, triclosan also inhibits fatty acid synthesis, which could destabilize the membrane of *P. aeruginosa* rendering it susceptible to tobramycin. However, our data do not support this model, as tobramycin itself is not effective against a *fabI* mutant (Tn::*fabI),* which is the molecular target of triclosan, as would be predicted if the synergy was due to FabI

inhibition by triclosan.[6] Further, the fatty acid synthesis, AFN-1252, which also targets FabI, did not synergize with tobramycin (Figure 4-1).[174-176] *P. aeruginosa* possess a second enzyme, FabV, that is functionally equivalent to FabI but is highly resistant to triclosan treatment. Therefore, in all of the experiments described FabV is expected to be functional and maintain membrane biogenesis.

It is curious that triclosan both inhibits efflux pumps and, yet, efflux pumps appear to be one of the resistance mechanism against triclosan in *P. aeruginosa*.[131] We speculate that although triclosan inhibits RND-type efflux pumps by disrupting the proton gradient across the inner membrane, this is not sufficient to render cells sensitive to triclosan alone. We hypothesize that the degree of efflux pump inhibition by triclosan does not allow enough triclosan to accumulate to inhibit fatty acid synthesis. Again, this residual resistance may be mediated by FabV, where high concentrations of triclosan are required, and these concentrations are only achievable in complete eff lux pump mutants of *P. aeruginosa*.[130] This hypothesis is also supported by the trajectory of the evolution experiments, which resulted in mutations rendering the cells resistant to tobramycin and not triclosan, suggesting tobramycin is responsible for cell death (Fig. 4-2).

Interestingly, *P. aeruginosa* possesses two genes that encode for elongation factor G (EF-G), *fusA1* and *fusA2.*[191] *In vitro* translation studies have suggested that *fusA2* is the primary EF-G responsible for ribosome translocation, while the function of *fusA1* remains unclear. *fusA1* and *fusA2* are 98% identical and 84% similar, but an interesting observation is that *fusA1* expression is induced in cells growing as biofilms compared to cells growing planktonically.[192] Approximately 30% of bacterial species

have two copies of EF-G, and the role of these alternate EF-Gs is an interesting question for further investigation.[191]

Our work and others have now showed that mutations in *fusA1* confers aminoglycoside resistance in *P. aeruginosa*. Recently, Bolard and colleagues have identified a new resistance mechanism caused by mutations in *fusA1* by sequencing *P. aeruginosa* clinical isolates taken from CF patients.[183,193] These researchers identified L40Q in clinical isolates, one of the mutations that we report here, and showed that generation of this mutation in PAO1 rendered it resistant to tobramycin.[183] These results support our findings, that mutations in EFG1A reduce susceptibility to aminoglycosides. However, the mechanism by which such mutations in *fusA1* render *P. aeruginosa* resistant to tobramycin is unknown. Similar mechanisms exist involving mutant forms of EF-Gs. For example, TetO is an EF-G-like protein that can confer resistance to tetracycline by preventing binding of tetracycline at the A-site of the 30S subunit.[194-196] We hypothesize that the mutations in *fusA1* may function through a similar mechanism, clearing the P-site of tobramycin and preventing protein synthesis corruption.

As efflux pumps are major mechanisms of resistance in bacteria, they are attractive targets for anti-resistance strategies, but there are few reported efflux pump inhibitors (EPIs) in development. The first reported EPI, MC-207,110 inhibits several efflux pumps and has been shown to potentiate the effects of the fluoroquinolone levofloxacin against lab strains and clinical strains of *P. aeruginosa.*[197] MC-207,110 does not affect the proton gradient required for drug extrusion by RND-type efflux pumps, but instead it is a substrate of efflux pumps itself and competes with the efflux of other substrates.[197] Another notable EPI is produced by Mpex™ Pharmaceuticals, MP-

601,205, is currently in phase I clinical trials.[78,198] Its mechanism of action and structure have not been disclosed.

4.5 Conclusion

Importantly, triclosan is not a new chemical entity (NCE). Owing to decades of overuse, the FDA has restricted the use of triclosan due to concerns over bioaccumulation and the potential for induction of resistance to other antibiotics in bacteria. However, triclosan maintains FDA approval for use in Colgate® total toothpaste at 100-times the concentrations used in this study. In support of this decision, numerous toxicity studies and the Scientific Committee on Consumer Safety published by the European Union has found that triclosan is safe when used appropriately.[143-146] Furthermore, envisioning triclosan as an adjuvant for tobramycin therapy for cystic fibrosis, we previously performed an acute and long-term triclosan toxicity study in rats and showed that direct delivery of triclosan to lungs did not elicit significant toxicity, described in Chapter 2.[6] Likewise, niclosamide is a protonophore that has been widely used in humans and is being considered for the treatment of diabetes and cancer.[149,160] Thus, EPIs that function as protonophores is a new strategy to limit bacterial resistance to antibiotics.

CHAPTER 5

THE ANTIMICROBIAL PEPTIDE, MELITTIN, IS AN ANTIBIOFILM AGENT AGAINST *PSEUDOMONAS AERUGINOSA* IN VITRO AND IN VIVO

5.1 Introduction

Besides the use of efflux pump inhibitors (EPIs), antimicrobial peptides (AMPs) combined with conventional antibiotics represents another strategy that could be used to extend the shelf-life of our current antibacterial arsenal, as described in Chapter 1. In this chapter, we described the AMP, melittin, enhances the aminoglycoside, tobramycin, activity against mature biofilms.

We evaluated the AMP, melittin, for activity alone or in combination with aminoglycosides against mature biofilms formed by *P. aeruginosa* and *S. aureus*. Melittin is derived from the venom of European honey bee *Apis mellifera* and is a cationic amphiphilic linear peptide (NH2-GIGAVLKVLTTGLPALISWIKRKRQQ-CONH2).[97] Upon contact with cells, melittin causes membrane permeabilization.[97] At high concentrations, melittin can induce pain and inflammation, however, concentrations up to ~35 mM have been shown to have anti-nociceptive, anti-inflammatory and antimicrobial properties.[97,199,200]

We found that melittin in combination with tobramycin or gentamicin showed enhanced activity reducing the number of cells within mature *P. aeruginosa* biofilms by ~2-$logs_{10}$ after 6-hrs of treatment compared to untreated controls. The combination was also effective in as-little-as 2-hrs. And we found melittin alone, and in combination with tobramycin, resulted in significant cellular permeabilization and dispersal of *P.*

aeruginosa biofilms. Melittin in combination with tobramycin was also effective against 7/7 of the CF isolates of *P. aeruginosa* tested. And melittin was effective alone against 4/4 of the *S. aureus* isolates tested. Finally, melittin combined with tobramycin in a novel hydrogel significantly reduced biofilm bioluminescence after 4-hrs using a murine wound model. Our findings suggest that melittin alone or in combination with aminoglycosides could represent a potential new therapy for the treatment biofilm-associated infections in diabetic foot and burn wounds using a novel hydrogel or in CF using an aerosolized formulation.

5.2 Materials and Methods

5.2.1 Bacterial strains, culture conditions, and compounds

All strains used in this study are listed in Table 5-1. Bacterial strains were grown in glass test tubes as described previously in Chapter 2.

Table 5-1. Bacterial strains used in this study.

Strain	Characteristics	Reference
PAO1	PA Standard Reference Strain, isolated in 1954[118]	[6]
CF_110_N	PA Clinical CF Isolate, Michigan	[6]
CF_110_O	PA Clinical CF Isolate, Michigan	[6]
CF_115_J	PA Clinical CF Isolate, Michigan	[6]
CF_131_M	PA Clinical CF Isolate, Michigan	[6]
USA_300_JE2	MRSA, Wound, California	[150]
AMT0023_30	PA Early Isolate 6 MO	[120]
AMT0023_34	PA Late Isolate 8 YO	[120]
COL	MRSA, Colindale Hospital, England	[151]

Table 5-1 (cont'd)

Newman (25904)	MSSA, Endocarditis, ATCC	[152]
Wichita (29213)	MSSA, Better Biofilm Former, ATCC	[153]

Abbreviations*: Pseudomonas aeruginosa* (PA), *Methicillin-resistant Staphylococcus aureus* (MRSA), *Methicillin-sensitive Staphylococcus aureus* (MSSA), American Tissue Type Collection (ATTC).

5.2.2 Minimum inhibitory concentration (MIC) of planktonically growing cells

As described previously in Chapter 2.

5.2.3 Biofilm susceptibility testing using BacTiter-Glo™

As described previously in Chapter 2.

5.2.4 Crystal violet staining

As described previously in Chapter 2.

5.2.5 Membrane permeabilization assay

24-hr old biofilms were formed in glass test tubes (18 x 150 mm) in 1 mL of 10% (v/v) MHB at 35°C and agitated at 150 RPM, as previously described (REF Oxy Paper). Cells were then washed in DPBS to remove non-adherent cells and treated with melittin and tobramycin for 2-hrs. Following treatment cells were washed in PBS (phosphate buffered solution without magnesium and calcium) and the biofilm was disrupted from the air-liquid interface using an autoclaved wooden stick. The cells were stained with TO-PRO-3 iodide, which fluoresces in cells that have compromised membranes by intercalating deoxyribonucleic acid (DNA). Single cell flow cytometry was performed on an LSR II (BD Biosciences), with excitation from the 640 mm lasers.

5.2.6 Agarose hydrogels and murine wound infection model

As described previously in Chapter 2.

5.3 Results

5.3.1 Melittin is effective on its own and enhances aminoglycosides against *P. aeruginosa* biofilms

DRCs were performed alone and in combination with tobramycin, gentamicin or streptomycin against mature biofilms. Melittin was most effective at 100 μM, resulting in a ~1.5-$\log_{10}$ reduction in the number of cells within a biofilm compared to untreated controls after 6-hrs of treatment (Figure 5-1). Tobramycin showed activity from 1.5 – 25 μM, killing ~half-a-$\log_{10}$ of cells within biofilms. However, when 100 μM melittin was used in combination with 100 μM tobramycin, a ~2-$\log_{10}$ cellular reduction compared to untreated controls was observed. And synergy was seen when 50 μM melittin was combined with 50 μM tobramycin, resulting in ~1.5-$\log_{10}$ cellular reduction compared to untreated controls.

DRCs were also performed with gentamicin or streptomycin. Melittin and no treatment were re-plotted for comparison in this figure. Gentamicin showed activity from 6.25 – 100 μM, resulting in a maximal ~1-$\log_{10}$ cellular reduction compared to untreated controls (Figure 5-2A). Maximal enhancement was seen when melittin and gentamicin were used in equal molar combinations of 100 μM, resulting in a ~1.5-$\log_{10}$ cellular reduction compared to gentamicin treated biofilms. Whereas, the streptomycin and melittin combination showed less activity. This is not surprising, as it is known that streptomycin has reduced activity against *P. aeruginosa* and is not used clinically [37]. Streptomycin showed modest activity between 50 and 100 μM, resulting in ~half-a-$\log_{10}$

cellular reduction (Figure 5-2B). Maximal enhancement was seen when melittin and streptomycin were used in equal molar combinations of 100 μM, resulting in a ~1-$\log_{10}$ cellular reduction compared to streptomycin treated biofilms. These data indicate that melittin is effective alone and has greater efficacy (defined as maximum cellular reduction in μM) in combination with tobramycin or gentamicin.

Effective concentration 50 (EC50) values were calculated to determine the potency of the combinations (defined as EC50 values in μM). The EC50 value for melittin was decreased 2.8 and 4.6-fold when used in combination with tobramycin or gentamicin, from 46.26 to 16.92 (14.45-19.93 μM) and 10.68 μM (9.79-11.65 μM), respectively (95% confidence intervals) (Table 5-2). An EC50 value could not be determined for the streptomycin and melittin combination. This is not surprising, as the combination only showed weak enhancement in a DRC. EC50 values for tobramycin, gentamicin or streptomycin when used alone could not be determined using the concentrations tested against cells growing as biofilms.

MICs were performed to determine if melittin in combination with aminoglycosides were more effective against planktonic cells. The MIC for tobramycin, gentamicin or streptomycin did not change when used in combination with melittin, indicating enhancement occurs against cells growing as biofilms only (Table 5-3).

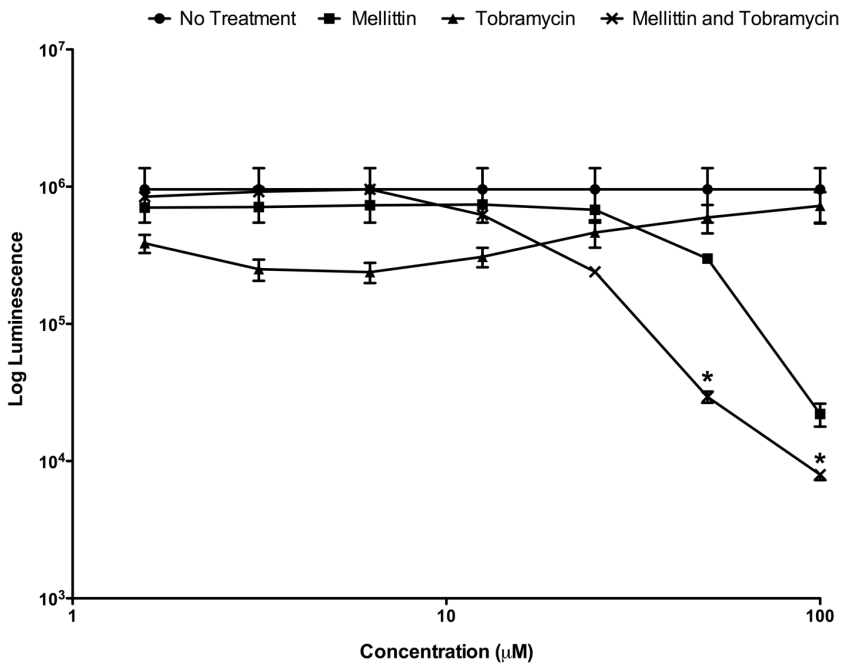

Figure 5-1. Melittin is effective alone and enhances tobramycin against *P. aeruginosa* biofilms. Biofilms were treated with melittin or tobramycin alone and in combination in two-fold dilutions and the number of viable cells was quantified. The assay was performed two times in triplicate. The results represent means plus SEM. A two-way ANOVA was performed followed by a Tukey's multiple comparisons test to determine statistical significance between tobramycin and the combination (*, $p<0.05$).

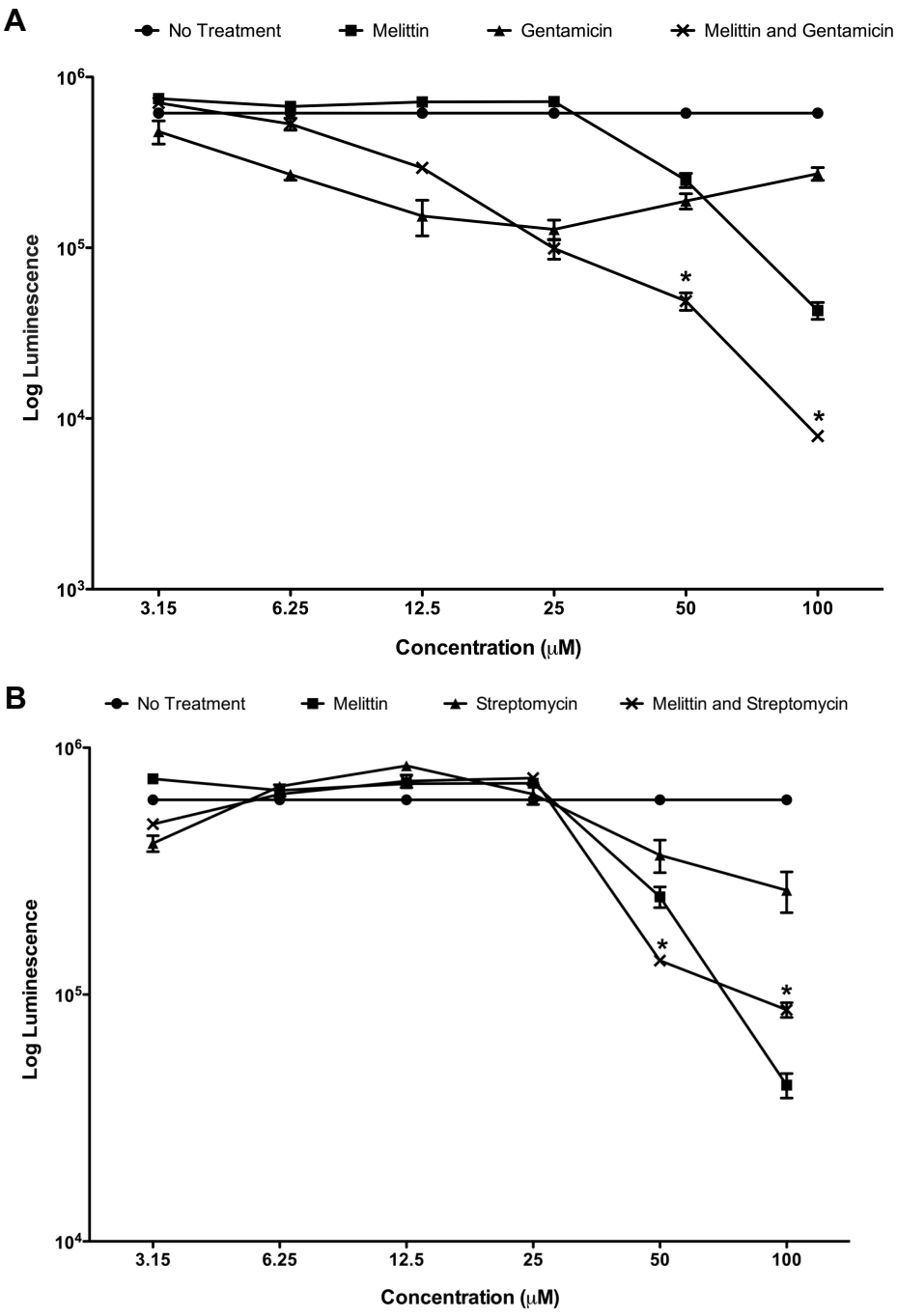

Figure 5-2. Melittin in combination with gentamicin or streptomycin is effective against *P. aeruginosa* biofilms. Biofilms were treated with melittin alone or in combination with (**a**) gentamicin or (**b**) streptomycin in two-fold dilutions and the number of viable cells were quantified. Melittin and no treatment were re-plotted in each figure for comparison. The assay was performed once in triplicate. The results represent means ± SEM. A two-way ANOVA was performed followed by a Tukey's multiple comparisons test to determine statistical significance between gentamicin and the combination and between streptomycin and the combination (*, $p<0.05$. NS, not significant).

Table 5-2. Half maximal effective concentration 50 (EC50) values for aminoglycoside combinations.

Antibiotic	Antimicrobial Peptide	EC50 (μM)	95% Confidence Interval (μM)
Melittin	-	46.26	39.1 – 60.6
Tobramycin	Melittin	16.92	14.45 – 19.93
Gentamicin	Melittin	10.68	9.79 – 11.65
Streptomycin	Melittin	N/A	N/A

Table 5-3. MIC values for planktonic cells are not changed by melittin.

Antimicrobial	Alone (μM)	+ Melittin (μM)
Melittin	62.5 – 200	–
Tobramycin	3.125 – 6.25	3.125 – 6.25
Gentamicin	6.25 – 12.5	12.5
Streptomycin	25.0 – 50.0	25.0 – 50.0

MIC were determined as the minimum concentration that no turbidity greater than background was measured.

5.3.2 Melittin has a shorter onset of action alone and in combination with aminoglycosides

Time-killing curves were performed to study the pharmacokinetic properties of melittin alone and in combination with tobramycin, gentamicin or streptomycin. 50 μM of melittin and 100 μM of tobramycin showed activity by 2-hrs, whereas tobramycin required 6-hrs (Figure 5-3). By 6-hrs, the combination resulted in ~1-$\log_{10}$ cellular reduction, whereas melittin resulted in ~half-a-$\log_{10}$ cellular reduction and tobramycin showed little activity. It is not surprising that tobramycin was ineffective, it is known to penetrate the biofilm poorly and can require up to 24-hrs to diffuse into biofilms.[11]

Similarly, melittin in combination with gentamicin or streptomycin showed activity by 2-hrs (Figure 5-4A and B). Melittin and no treatment were re-plotted for comparison in this figure.

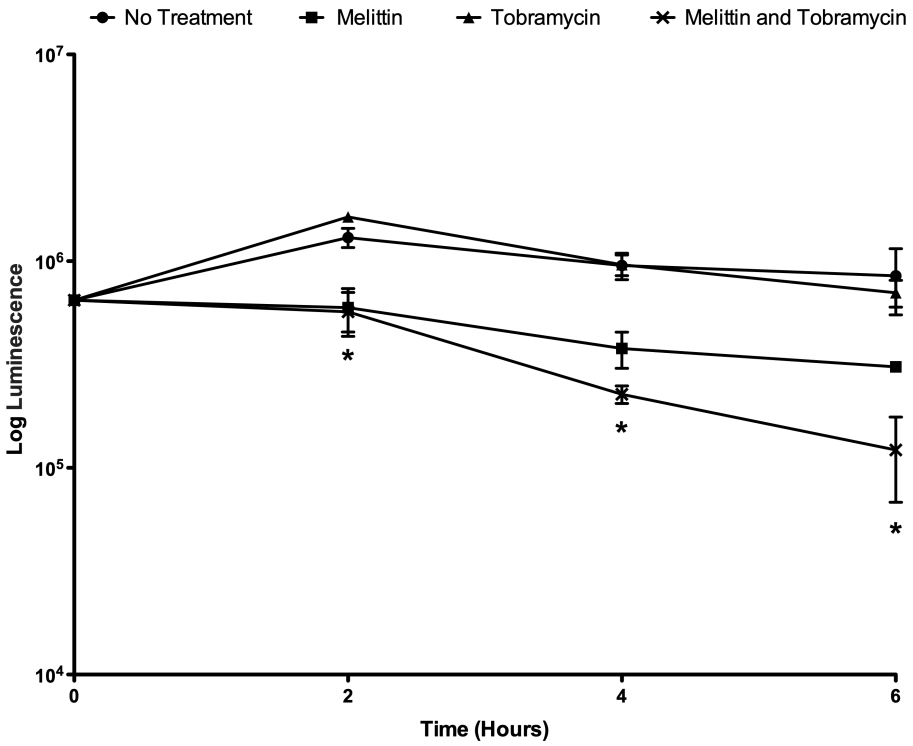

Figure 5-3. Melittin has a shorter onset of action than tobramycin. Biofilms were treated with melittin (100 μM) or tobramycin (400 μM) alone and in combination. At 0, 2, 4, and 6-hrs the number of viable cells within the biofilms were determined by BacTiter-Glo™. The assay was performed two times in triplicate. The results represent means ± SEM. A two-way ANOVA was performed followed by a Tukey's multiple comparisons test to determine statistical significance between tobramycin and the combination (*, $p<0.05$. NS, not significant).

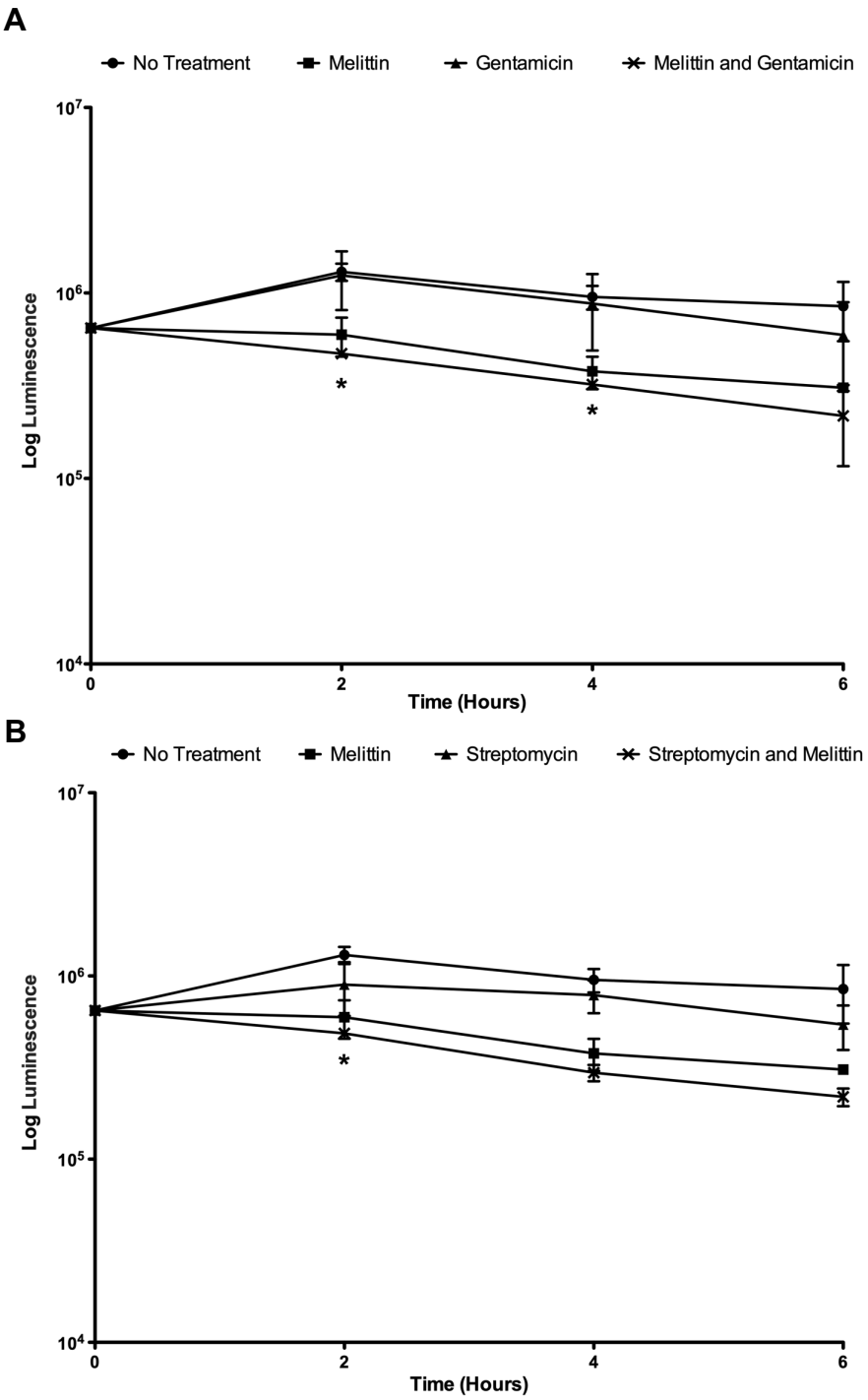

Figure 5-4. Melittin has a shorter onset of action than gentamicin or streptomycin. Biofilms were treated with melittin (100 μM) and (**A**) gentamicin (100 μM) or (**B**) streptomycin (100 μM) in combination and the number of viable cells within the biofilms were determined. The assay was performed two times in triplicate. The results represent means ± SEM. A two-way ANOVA was performed followed by a Tukey's multiple comparisons test to determine statistical significance between aminoglycosides and the combination (*, $p<0.05$. NS, not significant).

5.3.3 Melittin and tobramycin are effective at micromolar concentrations

Checkerboard experiments were performed to determine the lowest concentration of melittin and tobramycin that was effective against *P. aeruginosa* biofilms. Melittin was effective alone at 100 μM, resulting in a ~1.5-$\log_{10}$ cellular reduction compared to untreated controls (Figure 5-5). Maximal effect was observed when 100 μM of melittin was combined with 400 μM of tobramycin, resulting in ~2-$\log_{10}$ cellular reduction compared to untreated controls. Melittin and tobramycin showed enhancement when used in combination between 12.5 – 50 μM of melittin and between 50 – 400 μM of tobramycin, resulting in a ~1-$\log_{10}$ cellular reduction within biofilms compared to either tobramycin or melittin alone (Table 5-4).

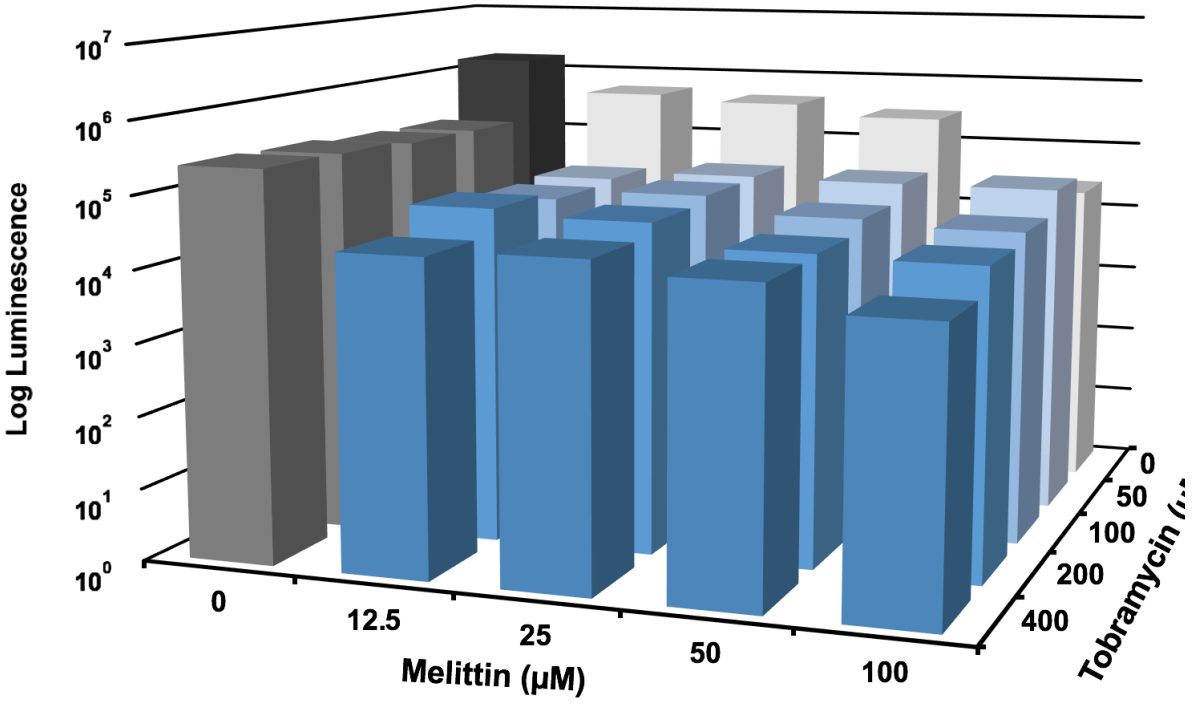

Figure 5-5. Melittin and tobramycin are effective at micromolar concentrations. Biofilms were treated for 6-hrs with checkerboard dilutions of melittin combined with tobramycin and the number of viable cells within the biofilms were quantified. The assay was performed two times in triplicate. The results represent means.

Table 5-4. Melittin enhances micromolar concentrations of tobramycin.

Tobramycin \ Melittin	100 µM	50 µM	25 µM	12.5 µM	0 µM
400 µM	7.96E3 (±7.14E3)	1.79E4 (±2.28E4)	2.64E4 (±3.55E4)	2.16E4 (±2.79E4)	2.44EE5 (±1.45E5)
200 µM	1.77E4 (±1.45E4)	1.94E4 (±1.26E4)	3.95E4 (±4.13E4)	4.83E4 (±4.74E4)	2.27E5 (±9.90E4)
100 µM	2.40E4 (±1.24E4)	2.88E4 (±1.99E4)	4.94E4 (±4.96E4)	3.50E4 (±3.65E4)	1.91E5 (±7.15E4)
50 µM	5.03E4 (±5.87E3)	5.03E4 (±2.52E4)	5.23E4 (±3.56E4)	3.90E4 (±2.49E4)	1.82E5 (±8.91E4)
0 µM	2.51E4 (±9.58E3)	2.87E5 (±3.11E5)	4.22E5 (±4.82E5)	5.18E5 (±5.90E5)	1.61E6 (±1.96E5)

Biofilms were treated for 6-hrs with checkerboard dilutions of melittin combined with tobramycin. Number of viable cells within the biofilms were quantified by BacTiter-Glo™. The assay was performed two times in triplicate. The results represent means plus/minus the Standard Deviation (SD). A two-way ANOVA followed by Sidak's multiple comparison was used to determine statistical significance compared to tobramycin treatment alone. Shaded cells indicate significance ($p<0.05$) and dotted cells are not significant because melittin is effective alone at these concentrations.

5.3.4 Melittin is effective against clinical isolates of *P. aeruginosa* and *S. aureus* alone and in combination with tobramycin

We tested 4 *S. aureus* and 7 CF *P. aeruginosa* clinical isolates (Table 1). CF_110_N and CF_110_O were isolated longitudinally from the same patient 3 months apart.[6] And AMT0023_30 and 34 were isolated longitudinally from the same patient at 6 months and 8 years of age, respectively.[120]

Melittin in combination with tobramycin was effective against 7/7 CF isolates tested, resulting in a maximal ~1.5-$\log_{10}$ cellular reduction compared to untreated controls (CF_115_J) (Figure 5-6). Isolate CF_300_A formed biofilms with fewer cells and was sensitive to melittin or the combination. Importantly, the combination significantly enhanced killing of strain AMT0023_34 compared to tobramycin alone,

which over expresses the RND-type MexXY-OpRM efflux pump, rendering it resistant to tobramycin.[23]

Melittin alone was effective against 4/4 *S. aureus* isolates tested, resulting in a maximal ~3-$\log_{10}$ cellular reduction compared to controls (MSSA_Newman) (Figure 5-7). Interestingly, tobramycin was ineffective against each strain of *S. aureus* tested and resistance was observed. Resistance was lost, however, when used in combination with melittin.

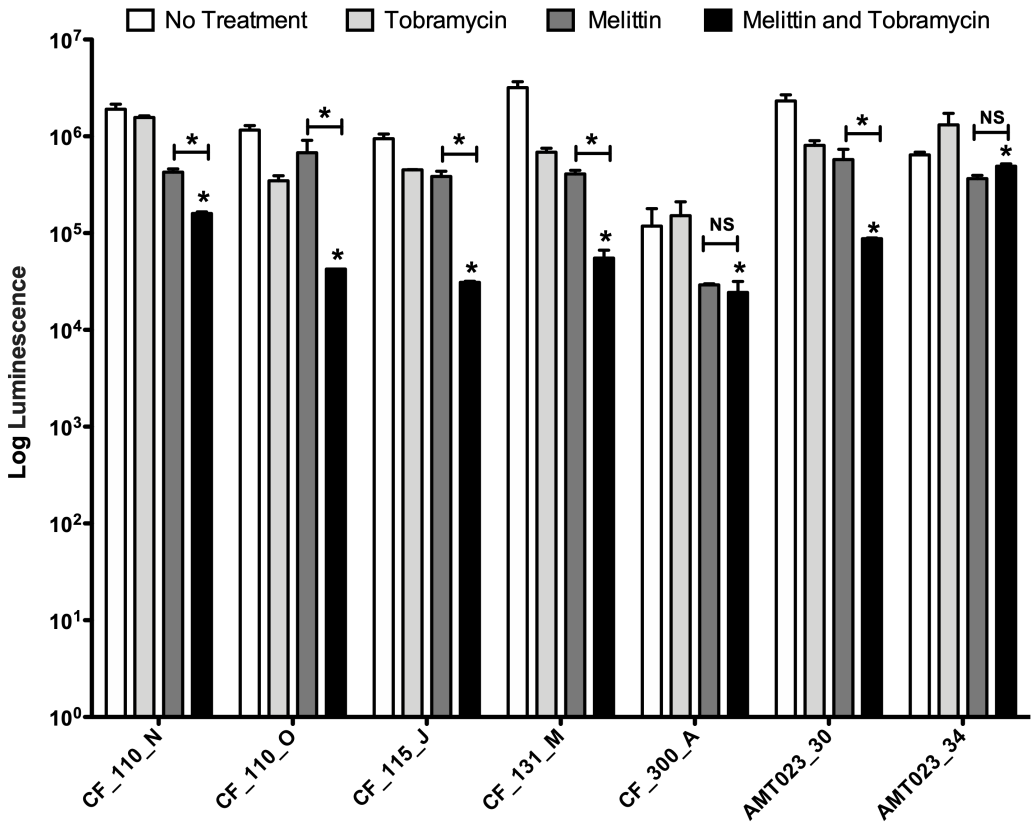

Figure 5-6. Melittin is effective in combination with tobramycin against *P. aeruginosa* isolates. Biofilms were treated with melittin (50 μM) or tobramycin (400 μM) alone and in combination and the number of viable cells were quantified. The assay was performed twice in triplicate. The results represent means plus SEM. A two-way ANOVA was performed followed by a Tukey's multiple comparisons test to determine statistical significance between tobramycin and the combination and between melittin and the combination (*, $p<0.05$. NS, not significant).

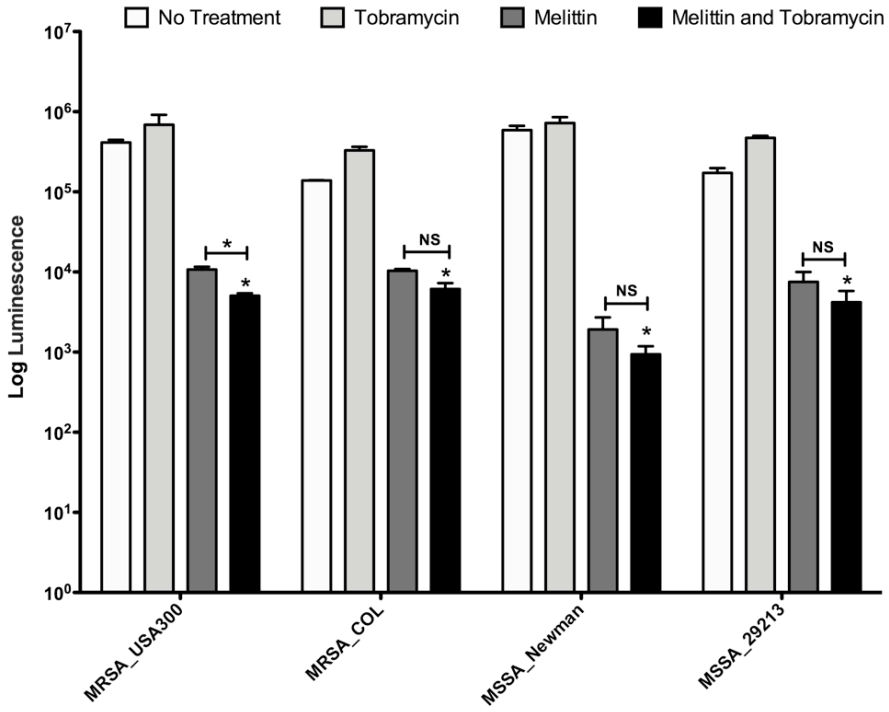

Figure 5-7. Melittin is effective in combination with tobramycin against *S. aureus* isolates. *S. aureus* biofilms were treated with melittin (100 μM) or tobramycin (100 μM) alone and in combination and the number of viable cells were quantified. The assay was performed twice in triplicate. The results represent means plus SEM. A two-way ANOVA was performed followed by a Tukey's multiple comparisons test to determine statistical significance between tobramycin and the combination and between melittin and the combination (*, $p<0.05$. NS, not significant).

5.3.5 Melittin causes biofilm dispersal alone and in combination with tobramycin

We hypothesized that melittin may cause biofilm dispersal. To test this hypothesis, we measured biofilm dispersal using crystal violet assays. At all concentrations tested the combination resulted in significant biofilm dispersal compared to either compound alone (Figure 5-8). And either 50 or 100 μM of melittin or tobramycin alone resulted in significant biofilm dispersal compared to untreated controls. These data suggest that the mechanism of action of melittin alone and in combination with tobramycin is both biofilm dispersal and cellular permeabilization.

The mechanism of action of AMPs against cells within biofilms could also be through cellular permeabilization.[201] To test this hypothesis, we used the TO-PRO-3 dye that stains cells with compromised membranes and performed single cell flow cytometry. After 2-hrs of treatment, 100 μM of melittin significantly increased the population of permeabilized cells within biofilms compared to untreated controls, 78% versus 6%, respectively (Figure 5-9). And 100 μM melittin combined with 400 μM tobramycin significantly increased the population of permeabilized cells compared to tobramycin treatment alone, 82% versus 6%, respectively. This finding is in agreement with Figures 5-5 and 5-3, where melittin accounted for the majority of the effect observed and tobramycin had no effect after 2-hrs of treatment, respecitvely.

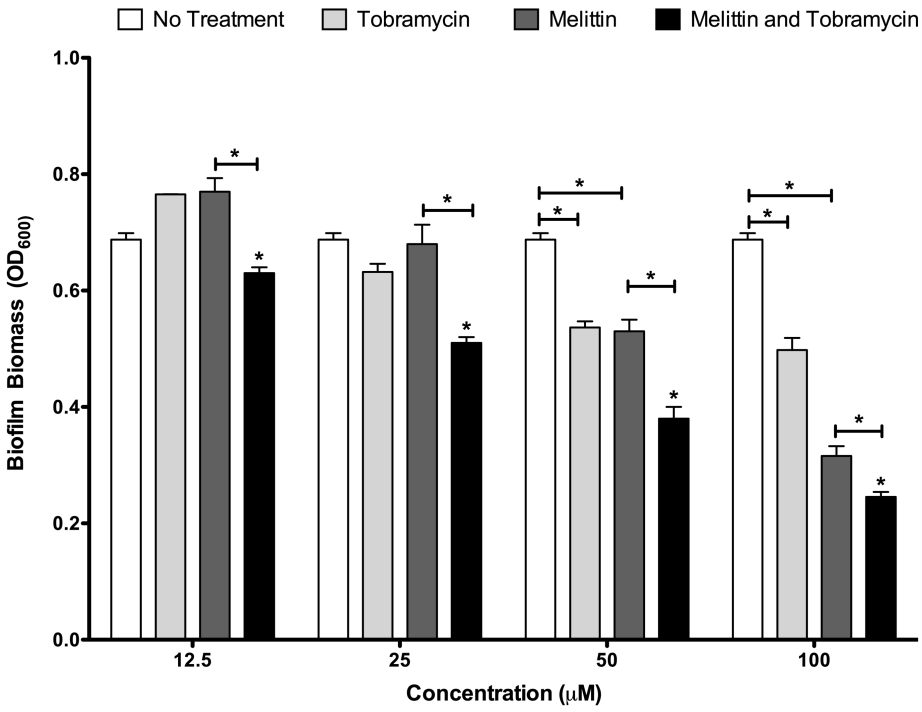

Figure 5-8. Melittin causes biofilm dispersal alone and in combination with tobramycin. Biofilms were treated for 6-hrs and biofilm dispersal was quantified using crystal violet staining. The assay was performed at least four times in triplicate. The results represent means plus SEM. A two-way ANOVA was performed followed by a Tukey's multiple comparisons test to determine statistical significance between tobramycin and the combination and between melittin and the combination (*, $p<0.05$).

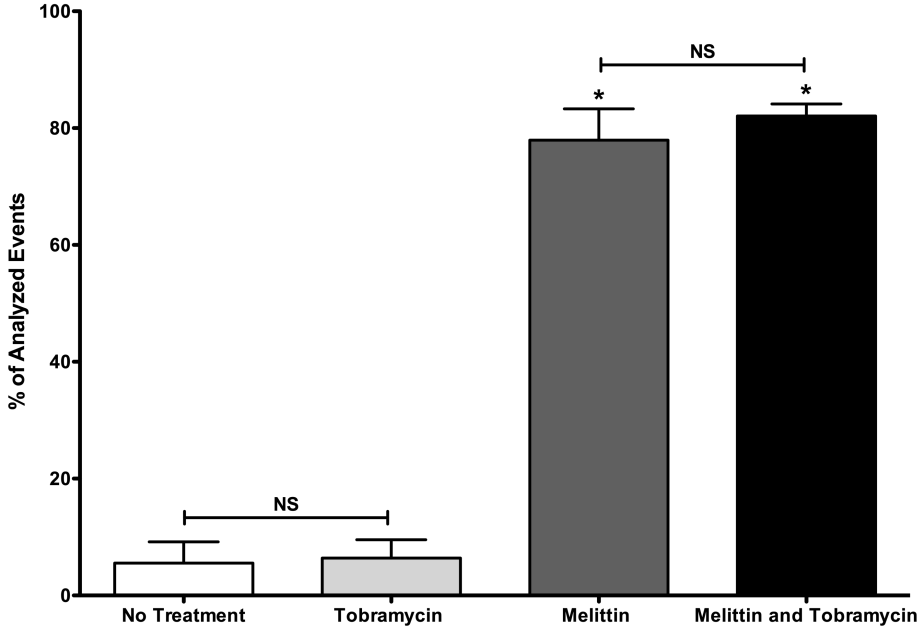

Figure 5-9. Melittin causes permeabilization of *P. aeruginosa* cells within biofilms. Biofilms were treated with melittin (100 μM), or tobramycin (400 μM), alone and in combination for 2-hrs. **A.** Cells were stained with TO-PRO-3 to determine the number of cells that were permeabilized. Percent values indicate the average relative abundance of events within each gate normalized to the total number of events analyzed, excluding artifacts, aggregates and debris. The experiment was performed two separate times in duplicate. The results are percent averages plus the SEM. A one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine statistical significance between tobramycin versus melittin, tobramycin versus the combination, and melittin versus the combination (*, $p<0.05$).

5.3.6 Melittin in combination with tobramycin in a novel hydrogel is effective in vivo

To determine if melittin and tobramycin were effective against biofilms in vivo, we tested their activity using a IVIS murine wound model.[125,140] Mature biofilms were treated for 4-hrs using a novel hydrogel imbedded with either 100 μM of melittin or 400 μM of tobramycin alone and in combination. This combination was chosen because it caused the maximum amount of killing in vitro (Figure 5-5). Surprisingly, melittin hydrogels had no effect compared to biofilms treated with control hydrogels which did not contain tobramycin or melittin (Figure 5-10). This is likely due to protease activity in

the wound released by neutrophils.[202] Tobramycin hydrogels resulted in 1.8-fold-reduction in biofilm bioluminescence after 4-hrs, however, this was not significant compared to controls. Hydrogels containing melittin and tobramycin resulted in a significant 4.2-fold-reduction in biofilm bioluminescence compared to tobramycin or controls. Interestingly, aminoglycosides are known to be heparin mimics that can inhibit proteases released by neutrophils and *Bacillus anthracis.*[203,204] This may explain why the combination is effective, whereas melittin alone is not. The combination resulted in a maximal reduction in biofilm bioluminescence of 7.8 and 9.8-fold. The size of wounds between mice were similar and we hypothesize differences in protease levels within wounds may account for this variability.[202]

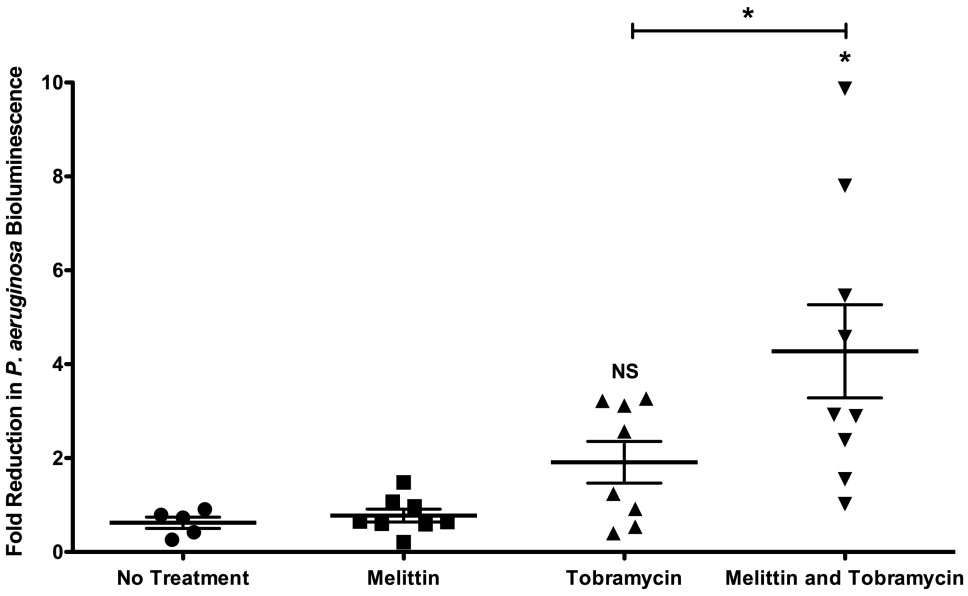

Figure 5-10. Melittin in combination with tobramycin in a novel hydrogel is effective *in vivo*. 24-hr old bioluminescent biofilms formed within wounds were treated with melittin (100 μM), or tobramycin (400 μM), alone and in combination for 4-hrs. Reduction in the number of cells within biofilms was quantified using IVIS. The results are fold reduction of two separate experiments ± the SEM, control hydrogels n=5, melittin hydrogels n=8, tobramycin hydrogels n=8, melittin and tobramycin hydrogels n=9. A one-way ANOVA followed by Bonferroni's posttests was used to determine statistical significance between each treatment and controls and as indicated by black bars (*, $p<0.05$). Abbreviations: NS, not significant.

5.4 Discussion

Melittin has been shown to be effective against *S. aureus* and *P. aeruginosa* growing planktonically and can inhibit cell surface attachment, which is the first step in biofilm formation.[100,200,205] Here, we demonstrate that melittin alone and in combination with tobramycin or gentamicin has potent and rapid activity against mature biofilms. We also demonstrate that melittin and tobramycin are effective at micromolar concentrations, potentially lowering the concentration of tobramycin needed for treatment, reducing its nephro-ototoxic side effects.[206] Because the biofilm biomass causes frustrated phagocytosis and neutrophilic collateral tissue damage[3], compounds that disrupt biofilms, such as inhaled DNase, and those that reduce the immune response, such as anti-inflammatory drugs, are the cornerstone of CF therapies.[207] Importantly, we showed that the mechanism of action of melittin is both biofilm dispersal and permeabilization, suggesting that melittin may provide potential anti-inflammatory benefits by dispersing the biofilm and enabling a more effective immune response. It has been reported that AMPs can disrupt metabolism, cell wall, nucleic acid and protein synthesis.[93] These activities may also contribute to the effectiveness of melittin alone and in combination with tobramycin. Finally, we show melittin combined with tobramycin in a novel hydrogel is effective in vivo using a murine wound model.

Melittin used in this study was ≥85% pure and was derived from honey bee venom. Variations in melittin purity between batches likely account for the variability observed between experiments. Melittin can induce an IgE response in 1/3rd of patients sensitive to honeybee venom, however it has been speculated this is due to additional

compounds found in bee venom.[200] Synthetically produced melittin is potentially more efficacious, less allergenic and will be evaluated in future studies.

There are numerous studies demonstrating melittin's anti-bacterial properties against several pathogens, including, *Borrelia burgdorferi*, *S. aureus*, *Escherichia coli*, *K. pneumoniae*, and *P. aeruginosa* growing planktonically.[101,103,205] Melittin has also been shown to have anti-inflammatory properties in acne vulgaris, atherosclerosis and arthritis.[200] This study adds to the growing literature demonstrating melittin's anti-bacterial properties, warranting further development.[92,208]

AMPs are effective and routinely used clinically. For example, colistin or polymyxin E is used for the treatment of Gram-negative bacteria infections in patients with CF [105,113,114,141,209] and polymyxin B is used in Neosporin®.[110,111] Taken together, these data demonstrate that alone or in combination with aminoglycosides, melittin could be a new AMP therapy for the treatment of biofilm-associated infections in chronic wounds using a novel hydrogel or in CF using an aerosolized solution.

5.5 Conclusion

This study adds to the growing literature demonstrating melittin's anti-bacterial, warranting further development.[92,208] Together, these data demonstrate that alone or in combination with aminoglycosides, melittin could represent a vital new therapy for the treatment of chronic wounds such as diabetic foot ulcers and burn wounds.[115-117] Finally, this body of work, spanning Chapters 2-5, demonstrates how adjuvants can extend the life of antimicrobials and enhance their effectiveness.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS

Gram negative multi-drug-resistant bacteria, such as *Pseudomonas aeruginosa*, represents a substantial threat to human health because it encodes for numerous resistance mechanisms and primarily exists in a biofilm state during infection, being up to 1000-times more tolerance to conventional therapeutic approaches.[22,26,210] Currently, only three classes of antimicrobials are effective against *P. aeruginosa,* fluoroquinolones, β-lactams, and aminoglycosides.[37] However, none of these antibiotics are able to eradicate *P. aeruginosa* once it pathoadapts into a hyper-biofilm state, leading to a chronic infection in up to 80% of patients with cystic fibrosis (CF).[53]

As our antibacterial arsenal continues to dwindle, the development and deployment of new antibiotics becomes a Sisyphean task, doomed to failure, in the face of never-ceasing resistance.[60] Thus, new antimicrobial strategies are needed, especially those that are effective against cells in a biofilm state. Here, we describe one such approach, the use of adjuvants, that synergize with conventional antimicrobials by inhibiting resistance mechanisms or by targeting additional cellular processes.[56]

In this work, we describe three adjuvants, triclosan, oxyclozanide and melittin, that synergized with the aminoglycosides against mature *P. aeruginosa* biofilms *in vitro*. Finally, we found that tobramycin and triclosan in a novel hydrogel were more effective than either treatment alone at killing *P. aeruginosa* growing as a biofilm in a murine wound model *in vivo*.

We also identified the mechanism of synergy for triclosan, finding it act as protonophore, collapsing the proton gradient required to power efflux pumps. As a consequence, because adaptive resistance is inseparable from efflux pump activity, triclosan abolished adaptive resistance, accelerating the maximum activity of tobramycin from 24-hrs to 2-hrs. Together, these results defined triclosan as an efflux pump inhibitor (EPI) against Gram negative bacteria, a new mechanism of action for this antimicrobial that has been widely used for decades.

Oxyclozanide also acted as a protonophore, collapsing the proton gradient; however, this activity did not result in inhibition of efflux pumps when used alone. Instead, oxyclozanide and tobramycin synergized by permeabilization and protonophore activity through an incompletely understood mechanism. And we found the AMP, melittin, which has been shown to permeabilize cells growing planktonically[100,200,205], could also permeabilize cells within mature biofilms, leading to synergy with tobramycin.

Neither triclosan nor oxyclozanide are new chemical entities (NCE). Triclosan has been used for clinical applications in humans for at least 3 decades and is approved by the Food and Drug Administration for use in Colgate® Total Toothpaste.[143-146] Further, oxyclozanide is routinely used in cattle for the treatment of worm infections.[149,156,160] The repurposing of veterinary drugs for use in humans has a proven history of success, notably ivermectin. Finally, AMPs have been used clinically as adjuvants. For example, colistin, is routinely used in combination with tobramycin to improve *P. aeruginosa* eradication therapies in CF patients.[105,113,114,141,209] Future work is warranted to develop these compounds for their use clinically. Collectively, this body of work is a proof-of-principle, demonstrating the use of anti-resistance compounds or

adjuvants to extend and improve our current antibacterial arsenal, giving Sisyphus a helping hand in the never-ending bacterial arms race.

6.2 Future Directions

This work leaves many unanswered questions and several avenues for future research. First, the mechanism of *fusA1* resistance remains undetermined. Second, additional *in vivo* efficacy studies are needed to develop these adjuvants as clinical therapies. Third, a high throughput screen (HTS) to identify protonophores that target adaptive resistance could be developed based on the findings presented in this thesis. Finally, the identification of compounds that are structurally similar to triclosan and the modification of triclosan to discover and create additional EPIs is a final avenue of exploration.

P. aeruginosa expresses two elongation factor G (EF-G) proteins, EF-G1A and EG-G1B, encoded by the *fusA1* and *fusA2* genes, respectively.[191] EF-G1A and EF-G1B are both GTPase translocases that catalyze the translocation step during protein synthesis by hydrolyzing GTP.[179] Interestingly, EF-G1A has been shown to be predominately expressed in biofilms, whereas EF-G1B is expressed in cells growing planktonically [192].

We and others have found that mutations in *fusA1* render *P. aeruginosa* resistant to aminoglycosides.[183] How these mutations lead to resistance remains unknown. One hypothesis is that mutations in *fusA1* allows for increased expression of the RND-Type efflux pump MexXY-OprM, which is frequently overexpressed in tobramycin resistant CF isolates[22,74]. However, *Bolard et al.,* investigated the expression of MexXY-OprM

and found no induction of the gene. Further, deletion of the MexXY-OprM gene did not eliminate resistance but did reduce the benefit of the *fusA1* mutation.[183]

Another hypothesis is that *fusA1* mutations may result in reduced protein synthesis, inhibiting the activity of tobramycin. To assess the impact of *fusA1* mutations on translation, Bolard and colleagues performed growth curves. They observed reduced growth rates in two *fusA1* mutants, which either had a single nucleotide polymorphism (SNP) at residue T456A or A371C.[183] Similarly, we observed that the evolved resistant mutants grew more slowly than the ancestral strain, which either had a SNP at residue L40Q or T64A. These data suggest protein synthesis may be reduced in *fusA1* mutants, contributing to tobramycin resistance.

A final hypothesis is that mutations in *fusA1* may result in the expression of an EF-G1A that is able to clear the P-site of the ribosome of tobramycin. Such a resistance mechanism exist for tetracycline through the activity of two ribosomal protection proteins (RPPs) TetM and TetO, which clear the A-site of the ribosome of tetracycline, allowing for translation to continue uninterrupted.[194] RPPs are nearly structurally identical to EF-G and only differ in domain I, which is responsible for the GTPase activity of the protein.[179,194] Interestingly, the evolution mutants contained SNPs in domain I only.

Further work is needed to elucidate the mechanism of action of *fusA1* resistance. Radiolabeled tetracycline techniques to measure ribosomal clearance by RPP TetM and TetO have already been developed.[195] Such methods could be adapted for use with tobramycin, either radiolabeled or fluorescently labeled, to measure clearance from the ribosome in a *fusA1* mutant. Further, rates of translation can be measured using a bioorthogonal noncanonical amino acid tagging (BONCAT) assay.[211] And to directly

assess the roles of L40Q and T64A *fusA1* mutations, site-directed mutagenesis experiments need to be carried out and these mutants tested growing as a biofilm against aminoglycosides. Alternatively, the L40Q mutant strain has already been created and could be requested for further testing.[183] Moreover, the evolution mutants contain additional SNPs in genes with unknown and known roles, including Lipid A modification, biofilm formation and metabolism.[22] Further investigation of these SNPs could yield insight into ancillary mechanisms of resistance.

Additional *in vivo* studies are required to develop triclosan oxyclozanide and melittin as clinical options. Using our murine would model, different concentrations of triclosan and tobramycin should be tested to determine if greater efficacy can be achieved *in vivo.* Similar experiments should be performed with oxyclozanide and melittin. In terms of developing these adjuvants for CF therapies, testing the combinations in artificial CF media and a CF mouse model or pig model would also be beneficial.[212]

Finally, additional EPIs could be discovered by searching for compounds with similar structures and/or chemical properties to triclosan. In addition, triclosan could be modified to design a broad-spectrum EPI, which would be advantageous for the eradication of *P. aeruginosa* and additional pathogens. It is curious that triclosan acted as EPI only for aminoglycosides. One would hypothesize that the general disruption of the $\Delta\psi$ would yield broad-spectrum EPI effects. This may simply be an artifact of the pathogen evaluated in this thesis. That is, *P. aeruginosa* is sensitive to few antimicrobials and most sensitive to the aminoglycoside tobramycin.[37] The specificity of triclosan as an EPI for aminoglycosides is yet another avenue for future research.

The HTS used in this study used BacTiter-Glo™ to quantify killing within mature biofilms, which uses adenosine triphosphate (ATP) to determine cell death. This had the unexpected result of identifying compounds that disrupt ATP synthesis, and thus, adaptive resistance, yielding the protonophores triclosan and oxyclozanide. A more targeted approach using BacLight™, which measures membrane potential, and a shorter treatment, lasting 1-2-hrs, could be used as to identify additional protonophores that disrupt adaptive resistance in *P. aeruginosa*. Importantly, BacLight™ can easily be adapted for use in a HTS.[76]

This thesis offers a foundation for future scientific exploration and drug discovery, especially as it pertains to targeting adaptive resistance. Adaptive resistance is a untapped target compared to β-lactam resistance, which has been extensively thwarted by the development of numerous β-lactamases [65]. This thesis illuminates a path to identify adjuvants that abolish adaptive resistance and enhance antimicrobial activity against *P. aeruginosa*. Such a path will likely come in the form of HTS coupled to BacLight™ utilizing short treatments and a large drug repurposing library.

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