

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

By

Michael M. Maiden

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## ABSTRACT

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

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Michael M. Maiden

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 formidable 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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PREVIEW

## 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 baumannii*, *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 Mueller-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  
 $\Delta$ pH protein gradient  
 $\Delta\psi$  Membrane 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.<sup>1</sup> 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.<sup>2,3</sup> 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*).<sup>4</sup> By the mid-to-late teens, the dominant bacterial pathogen and leading cause of death in CF patients is *P. aeruginosa*.<sup>5</sup> 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.<sup>6</sup>

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.<sup>7</sup> 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*.<sup>6</sup> 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.<sup>8</sup> 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.<sup>9</sup> 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.<sup>9,10</sup> For this reason, positively-charged aminoglycosides, such as tobramycin, take ~24-hrs to fully penetrate mature *P. aeruginosa* biofilms.<sup>11</sup> Slowed diffusion results in different rates of killings and the opportunity for additional resistance

mechanisms to emerge.<sup>12,13</sup> 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.<sup>14</sup> Because most antimicrobials target metabolically active cellular processes, their activity is reduced against cells growing slowly in a biofilm.<sup>15</sup> 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.<sup>15</sup> 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).<sup>15-17</sup> However, in *P. aeruginosa* the emergence of persister cells is an incompletely understood mechanism and involves many genes have been implicated including: *rpoS*, *spoT*, *relA*, *dlsA*, *dinG*, *spuC*, *algR*, *pilH*, *ycgM*, and *pheA*.<sup>18</sup> 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.<sup>19,20</sup> 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.<sup>21</sup> 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.<sup>22</sup> 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.<sup>23,24</sup> 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.<sup>25</sup>

*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.<sup>26,27</sup> Second, *Pseudomonas* has a chromosomal encoded  $\beta$ -lactamase, AmpC, which hydrolyzes  $\beta$ -lactam antibiotics.<sup>28,29</sup> 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.<sup>30,31</sup> The RND-type efflux pumps have a

broad substrate range including fluoroquinolones, aminoglycosides, and  $\beta$ -lactams.<sup>32</sup>

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.<sup>33-35</sup> 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.<sup>36</sup> The modified Lipid A reduces the negative charge of the lipopolysaccharide (LPS), and therefore, reduces the interaction aminoglycosides have with the outer membrane.<sup>36</sup>

#### **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  $\beta$ -lactams, and fluoroquinolones.<sup>37</sup> 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.<sup>38,39</sup> Inhibiting their activity results in breaks in the DNA, halting replication. Because *Pseudomonas* encodes for  $\beta$ -lactamases, only third generation  $\beta$ -lactams (cephalosporins) are effective because their altered  $\beta$ -lactam ring prevents cleavage by

$\beta$ -lactamases.<sup>40</sup>  $\beta$ -lactams interfere with the synthesis of a cell wall by binding to transpeptidases, blocking peptidoglycan biosynthesis.<sup>41</sup> 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.<sup>42-45</sup> 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.<sup>44</sup> This leads to the diffusion of aminoglycosides into the periplasm in a non-energy dependent manner.<sup>43,44</sup> It is also thought that aminoglycosides can diffuse through porins in the OM.<sup>46</sup>

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 ( $\Delta\psi$ ).<sup>44,47</sup> It is thought that aminoglycosides enter the cytosol through nonspecific membrane channels, however the exact mechanism remains unclear.<sup>45</sup> 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  $\mu\text{g/mL}$ .<sup>45</sup> And, EDPI can be blocked by inhibitors of respiration and oxidative phosphorylation.<sup>48,49</sup>

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.<sup>45</sup> 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.<sup>50</sup> 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.<sup>45</sup> These misfolded proteins are then imbedded in the cytoplasmic membrane causing cellular lysis and the further uptake of aminoglycosides.<sup>42-45</sup>

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.<sup>51</sup> 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.<sup>52</sup> Despite the routine use of *Pseudomonas* eradication therapies, by early adulthood ~80% of CF patients are chronically colonized with *P. aeruginosa*.<sup>53</sup> Numerous retrospective studies have shown that eradication of transient infections by *P. aeruginosa* can extend the lives of CF patients.<sup>54,55</sup> 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.<sup>56</sup>