Antibiotic synergy and mechanisms of action of the antimicrobial peptide glatiramer acetate against *Pseudomonas aeruginosa*

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Cystic Fibrosis and Chronic Lung Infection

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Declaration of originality

I, Ronan A. Murphy, declare that the data presented in this thesis is my own work, unless otherwise stated. All work and data from other sources has been appropriately acknowledged and referenced.

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When I Heard the Learn'd Astronomer

When I heard the learn'd astronomer,

When the proofs, the figures, were ranged in columns before me,

When I was shown the charts and diagrams, to add, divide, and measure them,

When I sitting heard the astronomer where he lectured with much applause in the lecture-room,

How soon unaccountable I became tired and sick,

Till rising and gliding out I wander'd off by myself,

In the mystical moist night-air, and from time to time,

Look'd up in perfect silence at the stars.

- *Walt Whitman*

For Colette

Abstract

Pseudomonas aeruginosa is the most common pathogen infecting the lungs of people with cystic fibrosis (CF), causing both acute and chronic infections. Intrinsic and acquired antibiotic resistance, coupled with the physical barriers resulting from desiccated CF sputum, allow *P. aeruginosa* to colonise and persist in spite of antibiotic treatment. *P. aeruginosa* is also subject to the wider, global issue of antimicrobial resistance. Glatiramer acetate (GA) is a peptide drug, used in the treatment of multiple sclerosis (MS), which has been shown to have moderate anti-pseudomonal activity. Other antimicrobial peptides (AMPs) have been shown to be antibiotic resistance breakers; potentiating activities of antibiotics when given in combination restoring and/or enhancing antibiotic efficacy. Among different antibiotics tested here, the efficacy of tobramycin (TOB) was significantly improved by GA in growth, viability, minimum inhibitory concentration (MIC)-determination and synergy analysis against reference *P. aeruginosa*. This was also the case for clinical strains from people with CF where GA significantly reduced the concentration of TOB required to inhibit *P. aeruginosa* compared with TOB-only. Investigating mechanisms of GA activity showed that GA resulted in significant disruption of outer membranes, depolarisation of cytoplasmic membranes and permeabilisation of *P. aeruginosa* and was the only agent tested (including cationic AMPs) to significantly affect all three; indicating potentiation by permeabilisation. Assays also showed that GA interacts with the lipopolysaccharides (LPS) of *P. aeruginosa* and this interaction may be the mechanism of potentiation of GA against *P. aeruginosa*. GA had moderate impact on expression of genes involved in *P. aeruginosa* LPS modification and on LPS modification, further linking GA activity to LPS interactions. GA is, therefore, a membrane active antibiotic resistance breaker of *P. aeruginosa* and a strong candidate for drug repurposing as an antibiotic adjuvant.

Acknowledgements

On an unspecified date, in a small city in Denmark, in some kind of Group Meeting or Seminar, somebody – a stranger to me – made a passing remark about the resemblance between a drug's activity and that of antimicrobial peptides. Somewhere in the region of 6-7 years later, I am submitting a PhD thesis based on that observation, so firstly I have to acknowledge Chaos.

I have had the extreme good fortune to share a lab and a research group with some brilliant people: great scientists, great colleagues and most of all great people. There are too many to name and thank individually but they have all enriched my work and my life and I hope to be a friend and a peer to them all for many years to come.

I have to thank my parents for all their support and for engendering and encouraging in me a sense of curiosity and wonder. I thank my brother, Eoin, along with his wife, Mairead, for their support – a good brother and a better friend.

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Abbreviations

$^1/_2$max	half maximal growth
95%CI	95% confidence interval
AMK	amikacin
AMP	antimicrobial peptide
ANOVA	analysis of variation
ASL	airway surface liquid
ATM	aztreonam
AUC	Area Under the Curve
Ca	calcium
CARD	Comprehensive Antibiotic Resistance Database
CAZ	ceftazidime
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CFU	colony forming units
CIP	ciprofloxacin
CST	colistin
$DiSC_3(5)$	3,3'-dipropylthiadicarbocyanine Iodide
DMSO	dimethyl sulfoxide
DRAMP	Data Repository of Antimicrobial Peptides
eDNA	extracellular DNA
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	exopolysaccharide
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GA	glatiramer acetate
GEN	gentamicin
HAMP	histidine kinases, adenylyl cyclases, methyl-binding proteins, and phosphatases
HATPase_c	histidine kinase-like ATPase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HisKA	histidine kinase A
HRP	horse radish peroxidase
IL-6	interleukin 6
IL-8	interleukin 8
IV	intra-venous
LAL	Limulus Amebocyte Lysate
L-Ara4N	L-4-aminoarabinose
LB	Luria Bertani
LES	Liverpool Epidemic Strain

ln	natural log
LPS	lipopolysaccharide
m/*z*	mass-to-charge-ratio
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-Time of Flight
MCR	mobile colistin resistance
MDR	multidrug resistant
MEM	minimal essential medium
MEM	meropenem
Mg	magnesium
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
MiST	Microbial Signal Transduction database
MLST	multilocus sequence typing
MRSA	methicillin resistant *Staphylococcus aureus*
MS	multiple sclerosis
NET	neutrophil extracellular trap
NPN	1-*N*-phenylnaphthylamine
NTM	non-tuberculous *Mycobacteria*
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
qRT-PCR	quantitative real time polymerase chain reaction
QS	quorum sensing
RGI	resistance gene identifier
RND	resistance-nodulation-division
rpm	revolutions per minute
RT	reverse transcription
SNP	single nucleotide polymorphism
ST	sequence type
SXT	trimethoprim-sulfamethoxazole
TCS	two component system
TOB	tobramycin
Trans_reg_C	transcriptional regulatory protein C-terminal
UTP	uridine triphosphate
WHO	World Health Organisation

1. Introduction

1.1 *Pseudomonas*

The study of *Pseudomonas* began inauspiciously. The totality of the first description of the genus by Walter Migula[1] in Karlsruhe, Germany in 1894, reads:

"Cells with polar organs of motility. Formation of spores occurs in some species, but it is rare"

as translated by Palleroni in The *Pseudomonas* Story[2]. Much of this very brief description is inaccurate (pseudomonads do not produce spores and are not rare). To compound the vagueness of this initial description, it is unclear what exactly Migula meant by the name *Pseudomonas*, the author having never clarified the etymology[2,3]. Pseudo translated from Greek means 'false' and monas translates as 'unit', also from Greek. However the term 'false unit' is of little descriptive value. The alternative explanation proffered by Palleroni is that 'monas', as used by Migula, was referring to the term coined by Otto Friedrich Müller in the 1770s. Müller was characterising "infusoria" – microscopic aquatic invertebrates – and grouped the nanoflagellate *Monas*[4]. It was a resemblance to these *Monas* to which Migula was actually referring when he described the 'false monas'[2].

The genus *Pseudomonas* falls within the Family Pseudomonadaceae and the Class Gammaproteobacteria and has a Gram-negative cell envelope. The taxonomy of *Pseudomonas* species has been described as "chaotic" and by the middle of the 20th Century there were >800 individually named species in the genus[2]. Today, classification through 16S rRNA gene sequences[5], there are 256 species in within the genus (as of 01/01/2021), with

many previous members having been moved out (including *Burkholderia*, *Stenotrophomonas* and *Ralstonia* species)[5–10].

Members of the *Pseudomonas* species are rod-shaped, motile, aerobic and catalase- and oxidase-positive[11–13]. With a wide range of metabolic diversity, species occupy a diverse range of niches; from opportunistic human pathogen to soil organism to plant pathogen and plant commensal[14]. A recent phylogenomic study showed that *Pseudomonas* contains two well defined species – *P. aeruginosa* and *P. chlororaphis* – as well as four wider groups; *P. fluorescens*, *P. stutzeri*, *P. syringae* and *P. putida*[15].

1.1.1 *Pseudomonas aeruginosa*

The type species of the genus is *P. aeruginosa*. The name 'aeruginosa' pre-dates the genus name and was given by the German botanist Joseph Schröter[16]. Schröter was describing the observed blue-colour of the bacterium – from the Latin aerūgō meaning 'copper rust' or 'verdigris' in colour and -ōsus meaning an 'abundance of'[3]. However, in 1872 Schröter applied his observation and name to the genus *Bacterium* from where Migula later reapplied it to the new genus he was describing[1,16]. The first described isolation of the species occurs between these two events, in 1882, by the pharmacist Carle Gessard who was studying the blue-green bandages of infected wounds of soldiers[17].

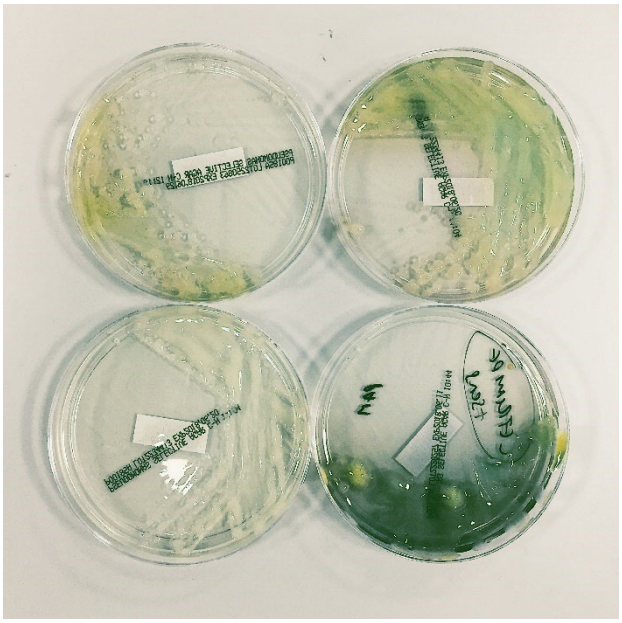


Figure 1-1. P. aeruginosa with examples of its characteristic blue-green colour.

P. aeruginosa is ubiquitous and adaptable bacterium. It can grow across a wide temperature range (4-42°C), can use >100 organic molecules as an energy source, is facultatively aerobic but can grow anaerobically, and can be isolated from wide range of environments (soil, water, associated with plants, and in human infections)[12,13]. Bacterial rods are 0.5–1µm across and 1–5µm long and can be observed in a range of colours (green, red, brown, pink) due the variety of pigments they produce. The blue-green colour which both Schröter[16] and Gessard[12] were describing is as a result of the production of pyocyanin, a unique secondary metabolite believed to be involved in interbacterial warfare[12,13].

P. aeruginosa has a large genome of >6Mbp[18]. This large scale genome allows for its characteristic flexibility and ability to grow in a wide variety of niches, encoding large numbers of genes for transcriptional regulation, transport, catabolism, efflux etc[18–20]. From a study of the complete genome sequences of >1300 *P. aeruginosa* strains, Freschi and colleagues found

665 genes to be core genes, found in all strains, 26420 flexible genes, found in >1 but not all strains, and 27187 unique genes[19].

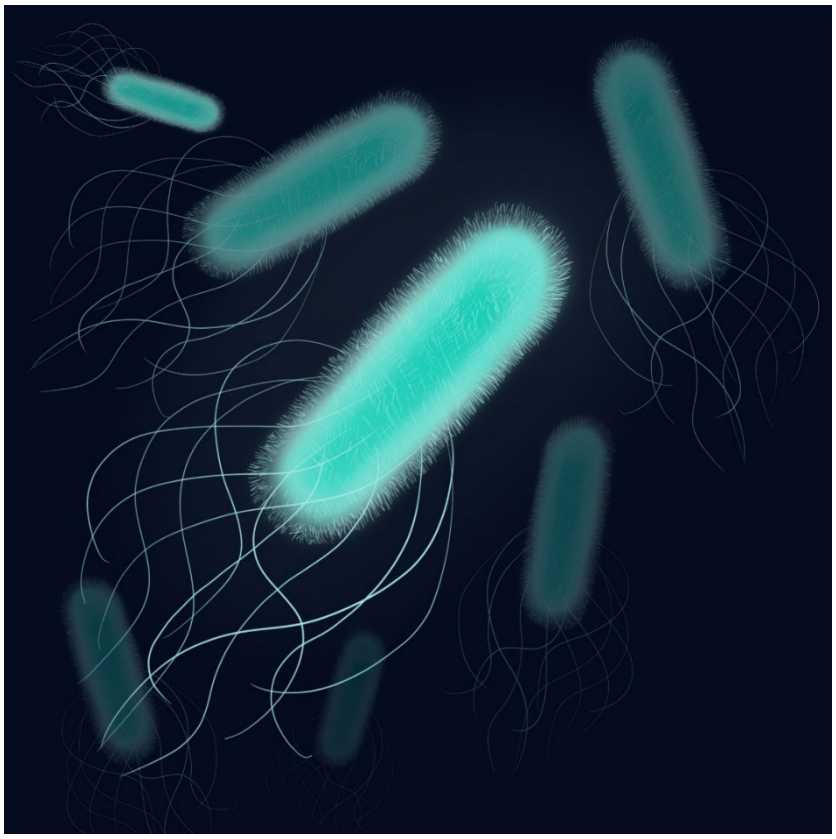


Figure 1-2. Representation of the P. aeruginosa cell. Created by Colette Healy© (@colettehealydesign).

The best characterised and most commonly used strains of *P. aeruginosa* in research are PAO1 and PA14. Strain PAO1 was isolated from a chronic would infection in Australia in the 1950s[21] and has been used a reference strain of *P. aeruginosa* ever since and was the first *P. aeruginosa* strain to be completely sequenced in the genomic era[18]. Strain PA14, a clinical strain from a burn isolated in USA[22], was first identified in the 1990s and was/is of interest due to its high levels of virulence, after initially being reported of being capable of infecting both plants and animals[23]. A further common, but less well studied, reference isolate is strain

PAK[24]. While all 3 reference strains continue to be used frequently in research in labs around the world and are useful benchmarks due to their proliferation and the huge bodies of work with them, genotypic and phenotypic variations have been reported for the strain PAO1 in different locations[13,25].

1.1.1.1 *P. aeruginosa* cell wall

The cell wall of the bacterium is a vital component providing structure, containment and protection for the workings of the cell and preventing entropy. The cell wall of Gram-negative bacteria, including *P. aeruginosa*, consists of three layers; the outer membrane, periplasm and the cytoplasmic membrane (**Figure 1-3**)[26].

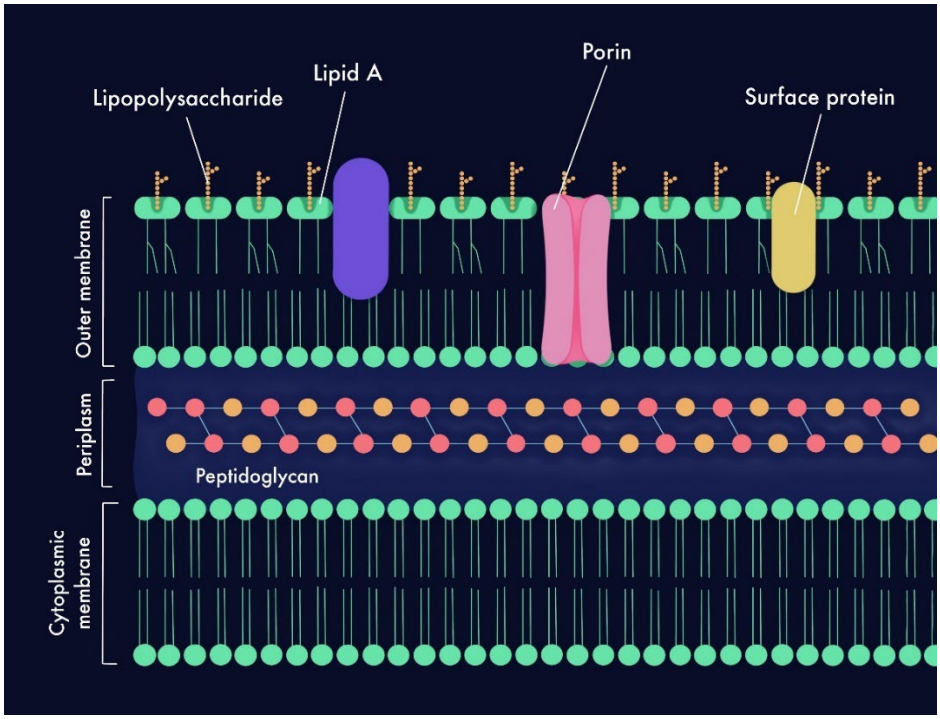


*Figure 1-3. Gram-negative bacterial cell wall. The cell wall consist of three layers; the outer membrane, the Periplasm and the cytoplasmic membrane. The **Outer Membrane** is lipid bilayer with hydrophobic fatty acid tails at centre and hydrophilic head groups on the outer edges. Embedded within the outer membrane are lipopolysaccharides. The **Periplasm** is a gel-like liquid region between the two membranes containing peptidoglycan. The **Cytoplasmic Membrane** is the innermost leaflet of the cells wall and is also a lipid bilayer. Created by Colette Healy© (@colettehealydesign).*

1.1.1.1.1 Outer membrane

The outer membrane is the distinguishing feature of Gram-negative bacteria, absent as it is from Gram-positives[26,27]. It is composed of amphipathic phospholipids which have polar head groups and hydrophobic fatty acid tails. Self-assembly of molecules into a lipid bilayer is driven by hydrophobic interactions: the hydrophobic tails binding together at the centre of the structure and polar head groups at the outermost edges forming a continuous leaflet. The outer membrane functions as a permeability barrier, preventing access to the cellular interior. One of the distinguishing features of the outer membrane (besides its location) is the presence of lipopolysaccharides embedded within it[26].

1.1.1.1.2 Lipopolysaccharides

Lipopolysaccharides (LPS) are chains of mixtures of lipids and polysaccharides protruding from the outer membrane outwards into the cellular environment. LPS chains consist of three regions; (i) Lipid A which embeds the entire LPS structure within the outer membrane, (ii) the core oligosaccharide and, at the outermost, (iii) O-antigen[28–30] (**Figure 1-4**). The LPS plays a number of roles for the bacterium but primarily is a further barrier layer. Although not a contiguous membrane, LPS chains provide a further barrier to the cell surface and can form tightly packed structures due to the ability to cross-link chains (particularly in the presence of divalent cations Mg^{++}) forming a non-fluid continuum as a barrier to hydrophobic molecules.

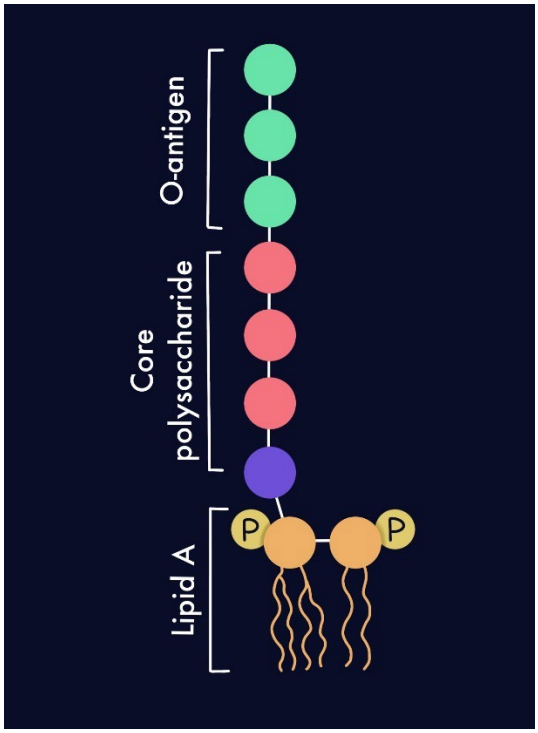


Figure 1-4. Lipopolysaccharide chains of Gram-negative bacteria consist of three elements; the highly heterogenous O-antigen, the Core polysaccharide and Lipid A which anchors the structure in the outer membrane. Created Colette Healy© (@colettehealydesign).

1.1.1.1.2.1 LPS O-antigen

The outermost element of the LPS is a heterogenous oligosaccharide called the O-antigen. The composition of O-antigens show both intra- and inter-strain variability due the sugar assembly process within the cell and the genetics of LPS production systems[28,29,31]. The variability in O-antigen structures among bacterial strains is responsible for classification of many Gram-negative species into serotypes, including *P. aeruginosa*, with the expressed O-antigens used to group strains. Although now largely defunct as a typing scheme, due to being superseded by genomic techniques. O-antigens are non-essential to the bacterium and *P. aeruginosa* lacking O-antigens are phenotypically known as "rough"[31].

1.1.1.1.2.2 Core polysaccharide

The central region of the LPS is the core oligosaccharide which can be further subdivided into outer- and inner-core. Made up of hexose and heptose sugars the core of *P. aeruginosa*'s negative charge allows divalent cation binding and bridging of neighbouring LPS chains[32].

1.1.1.1.2.3 Lipid A

LPS is anchored in the outer membrane by Lipid A through hydrophobic interactions. Lipid A molecules are tightly packed together facilitating the extra-barrier function to the membrane provided by LPS and the bridging together of LPS chains. Lipid A is also subject to modification by *P. aeruginosa* in response to environmental stimuli such as divalent cation deficiency[33] and attack by cationic AMPs[34].

1.1.1.1.3 Periplasm

The periplasm is a gel-like liquid region containing many proteins between the two lipid bilayers of Gram-negatives. Also within this space is the peptidoglycan layer. Although much less abundant than in Gram-positive bacteria, peptidoglycan still performs important cell structure roles, providing a rigid exoskeleton[26].

1.1.1.1.4 Cytoplasmic membrane

The innermost membrane of the cell wall of Gram-negative bacteria is the cytoplasmic membrane. Though there are similarities, it differs from the outer membrane in its protein composition and the cytoplasmic membrane is more porous than the outer membrane due to a lesser need to exclude molecules and a greater need to embed and export molecules[26].

1.1.2 *P. aeruginosa* pathogenesis

The term 'opportunistic' is usually attached to descriptions of the human pathogenesis by *P. aeruginosa*. This is because infections by *P. aeruginosa* are usually precipitated by some other factor(s), the most common being burns, wounds and the impaired host defence with the respiratory systems of people with cystic fibrosis (CF). *P. aeruginosa* also causes nosocomial infections, eye infection associated with contact lens use, urinary tract infections and can colonise medical devices. As with the ability to grow in a diverse range of ecological niches, *P. aeruginosa* pathogenesis is rooted in its large, complex and versatile genome which encodes different factors which allow colonisation of the human host[13].

Also known as endotoxin, *P. aeruginosa* LPS (and other Gram-negative LPS) is strongly immunogenic, especially in the blood stream where low levels can result in Septic Shock[35]. LPS also stimulates immune response is the already inflammatory environment of the cystic fibrosis (CF) lung[36–38]. Further LPS is significance to *P. aeruginosa* is discussed in **Introduction 1.3.6**.

P. aeruginosa produce exopolysaccharides alginate, PSL and PEL, major factors in the extracellular matrix of biofilms; a communal mode of bacterial life (**Introduction 1.2.4.1**). These polysaccharides provide structure to the biofilm as well as a physical barrier to the bacterial cells. *P. aeruginosa* strains which produce high levels of alginate are known as 'mucoid' and the mucoid phenotype is associated with the switch to chronic infections in the lungs of people with CF[39]. The community behavior exemplified by *P. aeruginosa* biofilms is mediated by quorum sensing (QS) molecules – signaling molecules which allow for cell-to-cell communication and coordinated behavior, such as biofilm formation[39].

P. aeruginosa cells are motile through both pili and flagella and can swim, swarm and twitch. As well as movement, pili and flagella have been implicated in adherence and aggregation to surfaces and to epithelial cells[40,41] and strains lacking both elements have been shown to be less virulent.

The pathogenesis of *P. aeruginosa* is also related to its ability to outcompete other microbial species in the environments in which it finds itself. This is of particular importance in the polymicrobial communities of the CF lung[42,43] where *P. aeruginosa* employs a number of tactics to become the dominant species including secreted antimicrobials (pyocyanin, hydrogen cyanide), cell puncturing and toxin delivery (secretion systems)[44] and iron chelating siderophores (pyoverdine and pyochelin)[13].

Interestingly, while many of the factors described here are important in the establishment of infection and the ability of *P. aeruginosa* to colonise a host, *P. aeruginosa* tends to downregulate or even lose many of these factors as infections progress[45]. This is particularly the case in CF as infections progress over many years or decades. *P. aeruginosa* have been shown to lose parts of LPS chains, pili and flagella as they become persistent colonisers[46,47].

1.2 Cystic Fibrosis

Cystic fibrosis is a juxtaposition of the very old and the very new – reaching back to "hexes" and "bewitching" through to gene therapy and the modulation of a genetic defect through drug treatment. The defect causing CF has been lurking for many thousands of years[48] and descriptions of CF-like conditions can be found centuries ago both in medical reports and in folklore[49,50]. Yet, in the 20th century, CF has frequently been to the forefront of medical and

scientific advances; the early adoption of antibiotics[51], the development of a rapid diagnostic test[52], the discovery of the discrete genetic cause of the disease[53–55], newborn genetic screening[56], successful correction of the disease causing defect[57,58]. The rapid advances have meant that in ~85 years since Dorothy Andersen first described the new condition "Cystic fibrosis of the pancreas"[59] the progression, manifestations and demographics of the disease have changed utterly, to point where the name Cystic Fibrosis has become something of a misnomer[49,50,60–62].

Dorothy H. Andersen was describing fibrotic cysts in the pancreases of babies she had autopsied when describing the pathology of the condition she had observed[59]. Today, CF is mainly associated with respiratory manifestations but up until the early part of the 20th century children with CF died of pancreatic insufficiency and malnutrition within the first months of life[49], while now predicted survival of a person with CF is >50 years in the UK[63]. This switch is a marker of the changes which have occurred in the intervening years; correction of the nutritional deficit leading to people with CF living for longer and respiratory symptoms coming to the fore.

The underlying cause of CF is mutations in the gene for the cystic fibrosis conductance regulator (CFTR), a channel which facilitates the movement of chloride and bicarbonate across the cell surface[60,62]. To date, over 2000 mutations have been discovered many of which have disease causing effects, to differing degrees[64]. These mutations are broken into 6 classes which range in outcomes for CFTR; from no protein being expressed on the cell surface through to scarce or unstable protein in the membrane (**Figure 1-5**)[58]. CF is a multisystem disorder as CFTR is expressed across the body including the digestive system (hence pancreatic insufficiency[59]), sweat glands ("salty" children are "hexed"[49]), the reproductive

systems and most significantly, currently, epithelial surfaces[60–62]. The result of CFTR defects is the reduction or absence of chloride movement which causes “low volume” mucus on cell surfaces; dehydrated secretions which are excessively thick and become resistant to normal movement by cilia. In the case of exocrine systems like the pancreas, this leads to blocking of ducts but in the respiratory system the results are most deleterious.

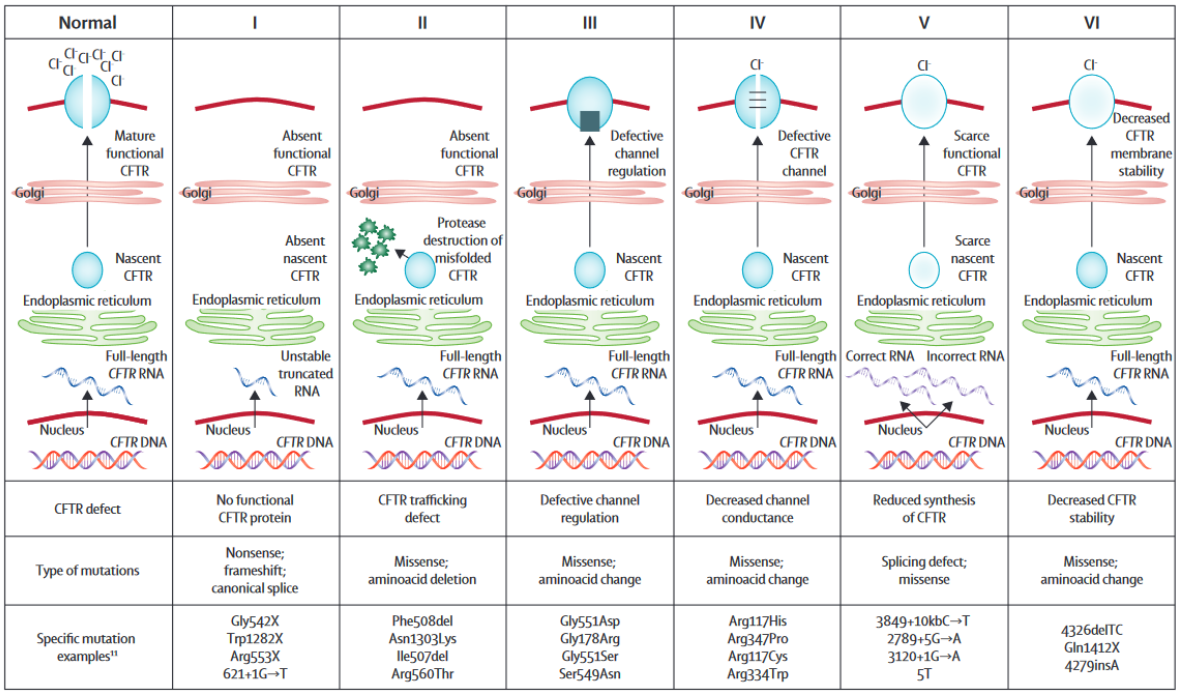


Figure 1-5. Different classes of CFTR mutation resulting cystic fibrosis. Reproduced from Boyle and De Boeck[58].

1.2.1 Diagnosis of CF

In folklore, CF-like conditions were often associated with the sufferer being “salty” – “if it tastes salty when someone is kissed on the brow, then this person is hexed” – and this property was confirmed in the heat-wave summer of 1948 when Paul di Sant’Agnese, a paediatrician in New York, noticed that many of children suffering heat stroke also had CF. di

Sant'Agnese demonstrated abnormally high levels of sodium and chloride in CF children's sweat (and simultaneously demonstrated the systemic nature of the underlying defect) which remained high beyond that summer[52]. This observation was used to develop the first rapid diagnostic test for CF and sweat chloride is still used today as a diagnostic and as a biomarker of CFTR function[60,62,65].

Due to the high prevalence in many Western countries and the importance of early interventions to limit the severity of CF and its burdens and costs of care[66], newborn screening is used to diagnose CF. A heel prick blood sample is taken soon after birth and subjected to immunoreactive trypsinogen concentration and/or DNA mutation analysis[60].

1.2.2 CF lung infection

The main cause of morbidity and mortality of CF is lung damage. Impaired mucociliary clearance of the thick, dehydrated sputum results in bacterial colonisation and chronic infections as the environment becomes more favourable to bacterial growth and the underlying defect compromises the immune response to infection[67]. This is not to say that immune response is dampened – in fact the conditions on the CF lung are thought to be pro-inflammatory – and cycles of infection and inflammation result in progressive bronchiectasis and eventual respiratory failure[39,60–62].

As mentioned, the CFTR channel is involved in bicarbonate transport, as well as chloride. This has two particular implications for infection in the CF lung: (i) reductions in the anionic content of the mucus results in structural changes of the mucus, an increase in viscosity and impaired mucociliary clearance[68] (ii) the pH of airway surface liquid (ASL) is reduced. In both pig[69] and ferret[70] models of CF lung disease, the bicarbonate defect and

acidification of the ASL resulted in defects in innate immunity and increase bacterial survival. In CF pigs, aerosolised bicarbonate restored some of the bacterial killing defect[69].

The CF lung is a relatively hospitable environment for bacteria to grow providing moisture, humidity, both aerobic and anaerobic pockets and nutrition sources. A wide variety of bacterial species are capable of causing infections including Gram-positives and Gram-negatives and both aerobes and anaerobes[63]. Viral and fungal infections are also common; viruses in children[71,72] and fungi[73] increasing in prominence as bacterial treatments become more effective.

The progress of bacterial infection in CF is, commonly, colonisation with *S. aureus* early in life which is replaced by *P. aeruginosa* during the teenage years and the dominant mode of *P. aeruginosa* becoming chronic infection (**Figure 1-6**). By the age of 36, >40% of people with CF in the UK are chronically infected with *P. aeruginosa*[63]. *P. aeruginosa* infection is associated with worse outcomes in people with CF: frequent exacerbations, a rapid decline in lung function and increased mortality[74,75]. Other pathogenic bacteria found in CF infections include species of *Burkholderia*, *Stenotrophomonas* (both close relatives of *Pseudomonas*), *Achromobacter, Haemophilus* and methicillin-resistant *S. aureus* (MRSA) is an increasing pathogen in USA CF populations[76]. Non-tuberculous *Mycobacteria* (NTM) (particularly *M. abscessus* complex) are an increasing concern in CF[77].

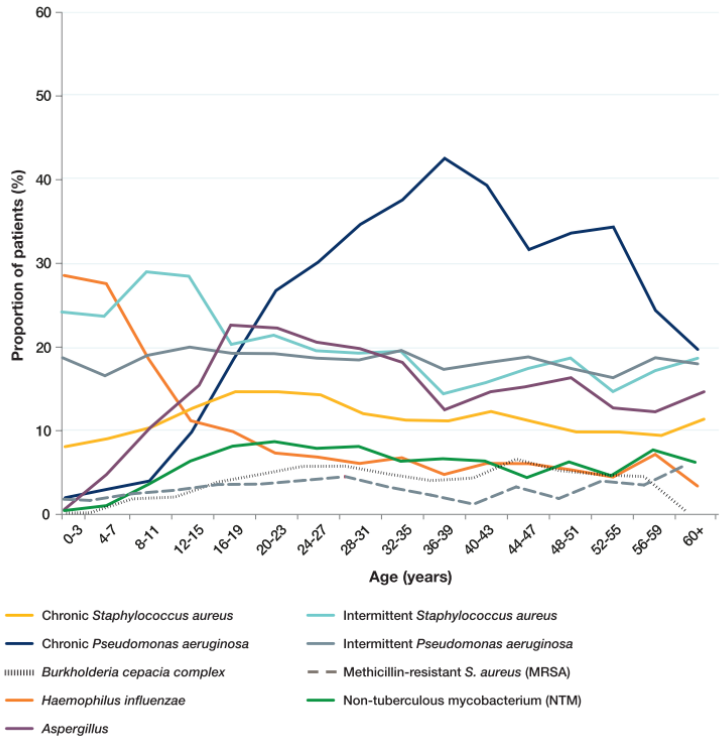


Figure 1-6. Infection rates of different bacterial pathogens in people with CF with different bacterial pathogens by age in 2020[63].

The genomics era has revealed that the CF lung is a polymicrobial environment containing multiple bacterial species. As well as the mentioned bacterial pathogens, there are also abundant anaerobic species present in CF sputum including members of *Prevotella*, *Porphyromonas* and *Veillonella*[78,79]. Recent studies have shown that composition of the microbiome of the CF lung is associated with the outcomes of people with CF[42,43] – higher levels of bacterial diversity (and the abundance of anaerobes) results in better outcomes, while dominance by a single species (usually *P. aeruginosa*) results in poorer outcome. Therefore, *P. aeruginosa* infection is not detrimental solely through host-pathogen interaction but also the ability to dominate the polymicrobial community and drive down diversity.

1.2.3 Treatment of CF

For much of the time since CF was recognised as a distinct condition, treatment was focused on managing and treating the disease manifestations: better nutrition and dietary supplementation to counteract malnutrition, hypertonic saline to rehydrate mucus and physiotherapy to remove it, antibiotics to treat the infections, among others. Once bronchiectasis became too advanced, lung transplantation also became an option[60]. While all of these have played a role in the gradual increase in CF life expectancy seen across the second half of the 20th century, none of them directly address the underlying CFTR mutation.

Modulators of CFTR activity are small molecules which target the defects associated with different classes of mutation seeking to restore the function of the protein by allowing proper processing and transport of the protein to the membrane, gating of the channel or stabilisation of the protein in the membrane[57]. These modulators can be administered alone or in combinations and improve outcomes for people with CF[57].

In infection in CF, the effect of modulators has been studied by tracking the microbiomes of the lungs of people with CF during modulator treatments with mixed results noted to date[80,81]. Encouragingly, studies have shown decreases in the abundance of pathogens, including *P. aeruginosa*, and bacterial load after commencement of modulator therapy as well as increases in the diversity of the bacterial population present[82–84]. However, other studies have reported no changes in microbiome composition or abundance after modulator therapies and studies have shown that *P. aeruginosa* can return to pre-modulator levels in patients who initially saw reductions[85,86]. It has been observed that the least improvements were made in older patients, indicating that those with already advanced lung

disease at the time of beginning modulators were less likely to see changes in their infections and to still require antibiotic treatments[87].

Modulator therapies may also directly impact the infecting bacteria with decreases in both *P. aeruginosa* and *S. aureus* noted after exposure to Ivacaftor[88]. Intriguingly, there have also been reports of synergistic interactions between CFTR modulators and antibiotics against *P. aeruginosa*, resulting in greater levels of bacterial killing than by the antibiotic alone[89,90].

1.2.3.1 Anti-pseudomonal antibiotics in CF

Antibiotics from a range of different classes are used in the treatments of *P. aeruginosa* infections on CF and can be administered orally, intravenously (IV) or as inhaled treatments nebulised directly to the lung[60–62,91,92].

1.2.3.1.1 Aminoglycosides

Aminoglycoside antibiotics are derived from species of *Streptomyces*[93]. They function by binding to the bacterial ribosome, disrupting or preventing bacterial protein synthesis and stopping successful cell division. The first-in-class drug from the family was streptomycin which was discovered in 1943. Subsequent members of the family include amikacin, gentamycin, kanamycin and tobramycin[93]. Predominantly used in the treatment of Gram-negatives, aminoglycosides have some activity against Gram-positives.

Tobramycin is an aminoglycoside and was derived from the species *Streptomyces tenebrarius* and was approved for medical use in 1974. As with other aminoglycosides, tobramycin binds to bacterial ribosomes, preventing successful translation of proteins and leading to cells death[93]. Tobramycin has also been shown to be membrane active and to mediate its own uptake in *P. aeruginosa*; The low positive charge of the molecules allow binding to the negatively charged bacterial cell and at high enough level can cause enough

disruption of the bacterial membrane to allow itself to cross into the cytoplasm and access its ribosomal targets[94,95].

Resistance to tobramycin in *P. aeruginosa* is mediated by several mechanisms, both intrinsic to bacterial strains and acquired by them[96,97]. Impermeability, antibiotic efflux, target alteration and enzymatic degradation have all been implicated in tobramycin resistance by *P. aeruginosa*[96].

Tobramycin is an effective anti-pseudomonal agent and is commonly used in the treatment of *P. aeruginosa* infections in people with CF either as eradication of early infection, or suppression of infection once it is chronic. It is also widely used in the treatment of pulmonary exacerbations – poorly understood episodes of increased symptoms and decreased lung function. Administration in CF is via either IV or inhalation[63]. Long-term exposure to tobramycin has been associated with nephrotoxicity, particularly via the systemic route, and hearing loss in people with CF[98–100].

1.2.3.1.2 Cephalosporins

Cephalosporin antibiotics are derived from species of the fungus *Acremonium*. Discovered in 1945, they have been in clinical use since 1964. They are β-lactam antibiotics and function in a similar manner to other β-lactams by preventing peptidoglycan synthesis in the bacterium and denying it the cell wall integrity provided by the peptidoglycan layer[101]. Ceftazidime is a third generation cephalosporin.

1.2.3.1.3 Polymyxins

Polymyxins are peptide antibiotics produced by *Paenibacillus polymyxa*. Formed of 10 amino acids residues, polymyxins are cyclic with a hydrophobic tail. The cyclic portion is cationic which facilitates binding of the peptide to bacterial LPS of Gram-negatives and the

hydrophobic tail causes membrane damage and bacterial cell death. Polymyxins are used in the treatment of Gram-negative infections.

Polymyxin E, also known as colistin, is often described as an antibiotic of 'last resort' in the treatment of Gram-negative infections, although it is in fact a commonly used inhaled therapy in CF[63]. Rarely used for many decades due to its nephrotoxicity, the crisis of antimicrobial resistance has led to IV colistin re-emergence as an important anti-Gram-negative agent. Colistin is active against *P. aeruginosa*.

Resistance to colistin is relatively rare in *P. aeruginosa* strains but is increasing. Resistance is mediated by bacterial target alteration; the LPS chains which colistin usually targets are modified by the bacterium to become less susceptible to colistin binding[102]. These modifications of LPS result in a reduction in the normal negative charge of the bacterial cell, negating the cationic nature of colistin in binding and attack the cell (for a more comprehensive review of *P. aeruginosa* LPS modification, see **Introduction 1.2.6**). While most *P. aeruginosa* have some ability to sense colistin (or the actions of colistin on the cell) and modify LPS in response, the level of response and subsequent resistance varies. These modifications are mediated in strains by intrinsic factors (two component systems [TCS]) or acquired resistance (mobile colistin resistance [MCR]) [103].

1.2.3.1.4 Fluoroquinolones

Fluoroquinolones are a family of quinolone derived antibiotics[104]. They prevent bacterial replication by binding to and disabling enzymes involved in DNA unwinding during cell division. Without the ability to unwind coiled DNA, bacteria cannot make a new copy and divide successfully. Fluoroquinolones have broad spectrum activity and are used in the treatment of both Gram-positives and Gram-negatives[104].

Ciprofloxacin is a second generation fluoroquinolone and the most commonly used member of the fluoroquinolones and one of the most commonly used antibiotics in the world due to its broad spectrum activity. Ciprofloxacin resistance in *P. aeruginosa* is as result of target alteration and antibiotic efflux[105].

Antibiotics administration to people with CF is via 3 routes; intravenous, inhaled and by mouth. Intravenous antibiotic administration was categorised in the top 5 of most burdensome treatments by both Lay and Professional groups in a study on the treatment burden in CF care[106]. This was despite neither group considering it in the top 5 most important treatments. Ten percent of people with CF were on IV antibiotics at the time of that study. Of the top 5 most burdensome categorised by the Lay group, 3 featured antibiotic administration of some kind; IV, nebulised or by mouth[106]. To this end, shortening the duration of IV antibiotic administration has been studied and it was found that shortening antibiotic courses from 14days to 10days was not inferior in lung function tests while 21days was found not to be superior to 14days[107]. However, interestingly, both clinicians and patients/caregivers surveyed expressed concern about incomplete treatments resulting from too short a duration of antibiotic, despite the know consequences of antibiotic over-use; burden, toxicity, resistance development[108].

1.2.4 *P. aeruginosa* antibiotic resistance

P. aeruginosa is of particular concern due to the variety of ways it can avoid killing by antibiotics. Some of these methods are specific (e.g. aminoglycoside deactivating enzymes) and some are general (e.g. biofilm production) and *P. aeruginosa* has both innate resistances (e.g. antibiotic efflux pumps) and the ability to acquire antibiotic resistance via horizontal gene transfer (e.g. MCR). For these reasons, *P. aeruginosa* is a member of the ESKAPE

pathogen group, a group of bacteria which are of particular concern for their ability to 'escape' antibiotic treatments: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp[109,110]. Members of this group are frequently multi-drug resistant (MDR), exhibiting resistance to antibiotics from three or more antibiotics from different classes[111,112]. *P. aeruginosa* is also considered to be of the highest priority (CRITICAL) for the development of new antimicrobials by the World Health Organisation (WHO), specifically due to carbapenem resistance[113].

P. aeruginosa's armory of antibiotic resistance mechanisms means it evades eradication by heritable and non-heritable methods[114–116]. This is of particular importance in CF; once chronic infection becomes established bacteria-evolved resistance (mutations in resistance genes) and lifestyle resistance (biofilm, persister cells) become entrenched thus making antibiotic treatments even less effective. Heterogeneity of distribution of inhaled antibiotics[117] also means *P. aeruginosa* potentially being exposed to sub-inhibitory antibiotic concentrations, further facilitating resistance development[118]. For these reasons, not just new treatments but new treatment strategies are warranted[87,119] (see **Introduction 1.4** for more). Some of the mechanism by which *P. aeruginosa* survive antibiotic treatments are summarized here.

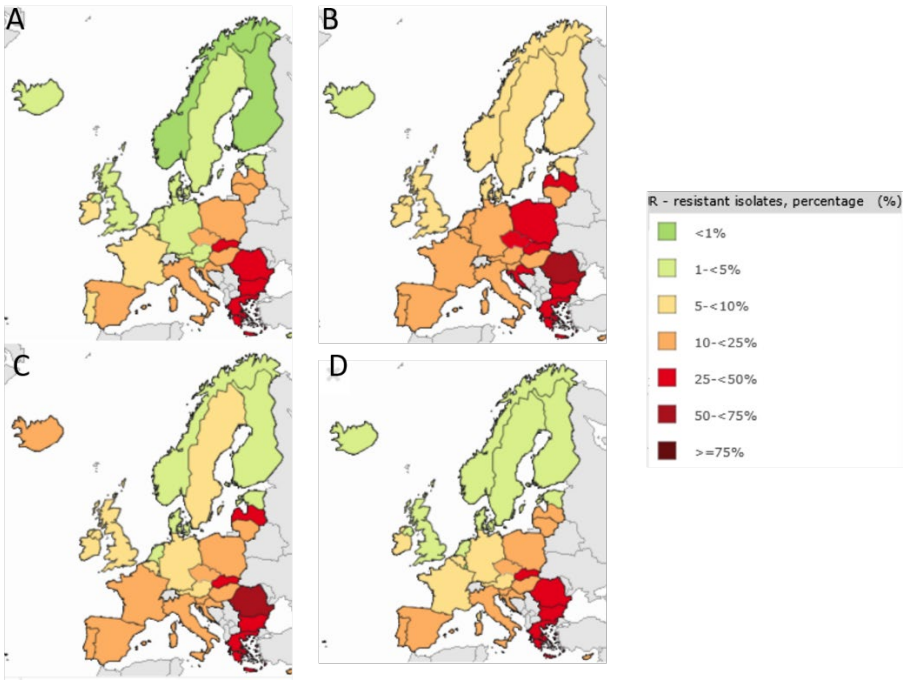


*Figure 1-7. Rates of P. aeruginosa resistance to **A.** aminoglycosides **B**. fluoroquinolones **C**. ceftazidime and **D**. multidrug resistance (MDR, ≥3 different antibiotic classes) in Europe in 2019 according to European Centre for Disease Prevention and Control (https://www.ecdc.europa.eu/en. Accessed 08/01/2022).*

1.2.4.1 Biofilm

A biofilm is "an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance (EPS) matrix"[120]. Bacterial cells switch from living as individual cells (planktonic) to living and behaving as a group within an EPS matrix and in communication with each other. The EPS is a complex substrate made up of self-produced polysaccharides and environmental components such as extracellular DNA (eDNA). The switch to a biofilm lifestyle is complex and multifactorial and the advantages to the bacterium are not limited or specific to antibiotic resistance. Resistance to antibiotics by biofilms is related to physical properties of the biofilm such as the EPS providing a barrier to the bacterial cells, the net charge of the biofilm being different to planktonic bacteria and preventing binding by charged antibiotics. Cells within bacterial biofilms also frequently exhibit lower

growth rates than planktonic bacteria of the same species, reducing susceptibility to antibiotics which require cell division for their activity.

P. aeruginosa is particularly adept at biofilm formation and forms biofilms in both environmental and infection settings[121,122]. In *P. aeruginosa*, biofilms are associated with increased tolerance to metabolic process targeting, including by aminoglycosides and fluoroquinolones[123]. This is not the case for cell surface targeting treatments such as AMPs with slow metabolic rates of biofilm cells serving to prevent reactive resistance and repair membrane damage inflicted[124]. However, this strategy does not eliminate all bacteria with small subpopulations surviving, as do subpopulations after combination treatments with antibiotics with different mechanisms of action[122,124]. This highlights the community approach to bacterial survival of biofilms, allowing at least some cells to survive.

P. aeruginosa from people with CF form biofilms in the lung. The dehydrated, poorly-cleared sputum of the CF airway, presence of polysaccharides, eDNA and other factors promote biofilm formation by *P. aeruginosa* and the presence of biofilm forming *P. aeruginosa* in CF lungs is long established[125,126]. eDNA has been shown to be of particular importance to biofilm formation by *P. aeruginosa* and may further be important in resistance to antibiotic treatment in biofilms, the charged DNA molecules able to bind an sequester positively charged antimicrobials such as aminoglycosides and polymyxins[127–130].

1.2.4.2 Impermeability

P. aeruginosa is good at preventing antibiotics from crossing its cell wall and accumulating in its cytoplasm[27,97,131–133]. *P. aeruginosa* cells are >100-times less permeable than *E. coli*[133], for example, despite both have a Gram-negative cell wall. This is achieved by a number of mechanisms. *P. aeruginosa* produces and employs tightly packed LPS molecules

on its cell surface providing a barrier to antibiotic binding to the outer membrane itself. Further to the physical barrier, the bacteria can reduce the cell surface charge by cross-linking individual LPS, such as by environmental divalent cations, to preventing binding of cationic antibiotics like aminoglycosides and polymyxins[134,135]. *P. aeruginosa* can go a step further than this, reacting to antibiotic attack by modifying LPS and reducing the overall charge of the cell[95,136–138].

P. aeruginosa also keeps tighter control of molecules crossing it's membrane than other Gram-negatives. This is achieved by employing fewer non-specific porins in its outer membrane which allow large molecular weight molecules to pass through and thus excluding many antibiotics[131,139].

In *P. aeruginosa* isolated from people with CF, impermeability has been shown to be a vital mechanism of surviving eradication from the CF lung during treatment with tobramycin[97].

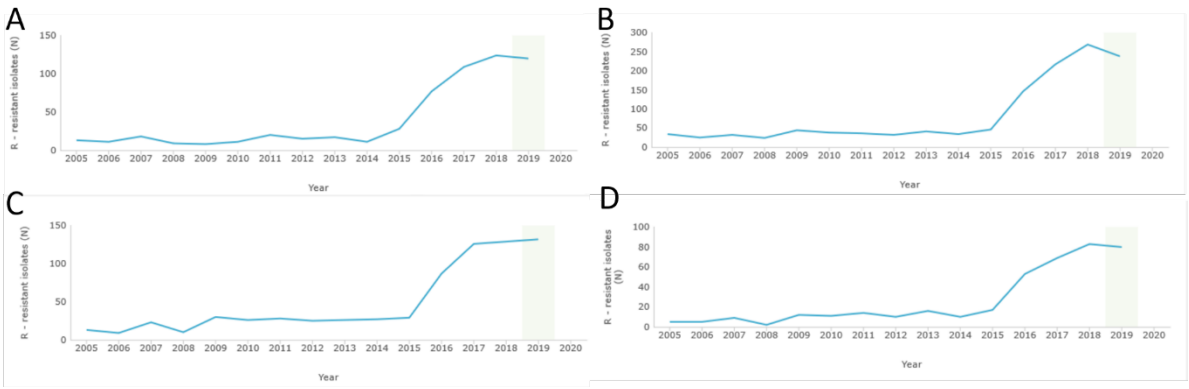


Figure 1-8. Numbers of P. aeruginosa resistant to ***A.*** *aminoglycosides* ***B.*** *fluoroquinolones* ***C.*** *ceftazidime and* ***D.*** *multidrug resistance (MDR, ≥3 different antibiotic classes) in the UK from 2005-2019 according to European Centre for Disease Prevention and Control (https://www.ecdc.europa.eu/en. Accessed 08/01/2022).*

1.2.4.3 Antibiotic efflux

Should an antibiotic make it across the *P. aeruginosa* membrane and into the cytoplasm, the bacterium employs a series of efflux pumps to prevent antibiotic accumulation and cell death. *P. aeruginosa* employs resistance-nodulation-division (RND) family of efflux proteins – three component efflux systems formed of a cytoplasmic membrane bound antiporter and a membrane protein channel linked by a periplasmic protein. In *P. aeruginosa* the systems are Mex-Opr systems with paired cytoplasmic and periplasmic proteins (both Mex) and the outer membrane factor (Opr)[96,140–142].

P. aeruginosa strains harbour a number of these systems and they have varying functions with some cross-over: MexAB-OprM[124,143] (fluoroquinolones, cephalosporins, peptides), MexCD-OprJ (aminoglycosides, fluoroquinolones,)[144], MexEF-OprN (fluoroquinolones)[141], MexGHI-OmpD (fluoroquinolones)[145], MexXY-OprM (aminoglycosides, cephalosporins)[141,146]. The regular functions of these pumps is the prevention of antibiotics accumulating to toxic levels in the cytoplasm and mutations within these systems has also been shown to increase resistance[147]. Furthermore, regulators of these systems and mutations within them have been implicated in antibiotic resistance in *P. aeruginosa*[143,148–152].

1.2.4.4 Antibiotic inactivation

P. aeruginosa employs a number of enzymes capable of modifying antibiotics and inactivating them[96]. *P. aeruginosa* can modify and inactivate aminoglycoside antibiotics via acetylation[153] (AAC enzymes), phosphorylation[154] (APH enzymes) and adenylation[155] (ANT enzymes)[96]. *P. aeruginosa* can also encode for beta-lactamase enzymes such as oxacillinases[156] (OXA) and PDC[157] which act against cephalosporins.

1.2.4.5 Target alteration

Regulated and mutational changes to the targets of antibiotic treatment can also be methods of antibiotic resistance and this includes the previously mentioned bridging and medication of LPS (more of which in **Introduction 1.2.6**) conferring aminoglycoside and polymyxin resistance[134,135,158,159]. Other target alteration leading to resistance to antibiotic treatment by *P. aeruginosa* include mutations in DNA gyrase and topoisomerase IV genes, resulting to reduced susceptibility to fluoroquinolones[160].

1.2.5 LPS modification

The LPS embedded in the outer membrane of *P. aeruginosa* is the outermost part of the bacterial cell and the first barrier to antibiotic treatments but also a point of binding and attack by many antimicrobials, such as AMPs. The importance of LPS was noted in the >60 years ago, when it was shown that the permeability of bacteria was increased by treatment with ethylenediaminetetraacetic acid (EDTA)[161] and that this was as a result of EDTA removing LPS from the outer membrane[162–164]. As a chelator of metal ions, the effect of EDTA was further investigated and it was shown that divalent cations of magnesium (Mg^{++}) and calcium (Ca^{++}) were the most important in the stability of LPS and cell premeability[165–168].

1.2.5.1 Divalent cations

With the observations that chelation of Mg^{++} and Ca^{++} increased permeability of bacteria, including *P. aeruginosa*, it was further reported that presence of these ions was both protective and restorative against the activities of both aminoglycosides and polymyxins[32,134,135,168–173]. This is due the ability of *P. aeruginosa*, and other Gram-negatives, to modify their LPS using environmental divalent cations – binding to the Lipid A portion of LPS chains competitively with cationic agents[138,174,175].

The binding of cations to LPS provides a physical barrier to the cell wall as individual LPS chains are cross-linked together by the cations forming a continuous barrier, protecting the outer membrane from direct interactions with antibiotics. Attachment of positively charged ions to the negatively charged Lipid A also serves to neutralise the bacterial cell and protecting from mildly cationic agents such as some aminoglycosides.

1.2.5.2 Aminoarabinose

It was noted in early studies on the effects of EDTA on permeability of *P. aeruginosa* that growing the bacteria in Mg-limited conditions before EDTA treatment meant the chelator did not have as detrimental an effect and indicating other mechanisms of LPS stabilisation and modification[167]. The best characterised of these is the reactive addition of L-4-aminoarabinose (L-Ara4N) to Lipid A of LPS[103]. Addition of L-Ara4N serves the same purpose as divalent cations in reducing the charge of the LPS in order the protect against the self-mediated uptake of cationic agents such as AMPs.

In *P. aeruginosa* LPS modification by L-Ara4N is carried out by the Arn (*arnBCADTEF*) operon and regulated by a selection of Two Component Systems[34,176–178]. The operon encodes the genes to synthesise, transport and attach L-Ara4N to Lipid A[176] (**Figure 1-9**).

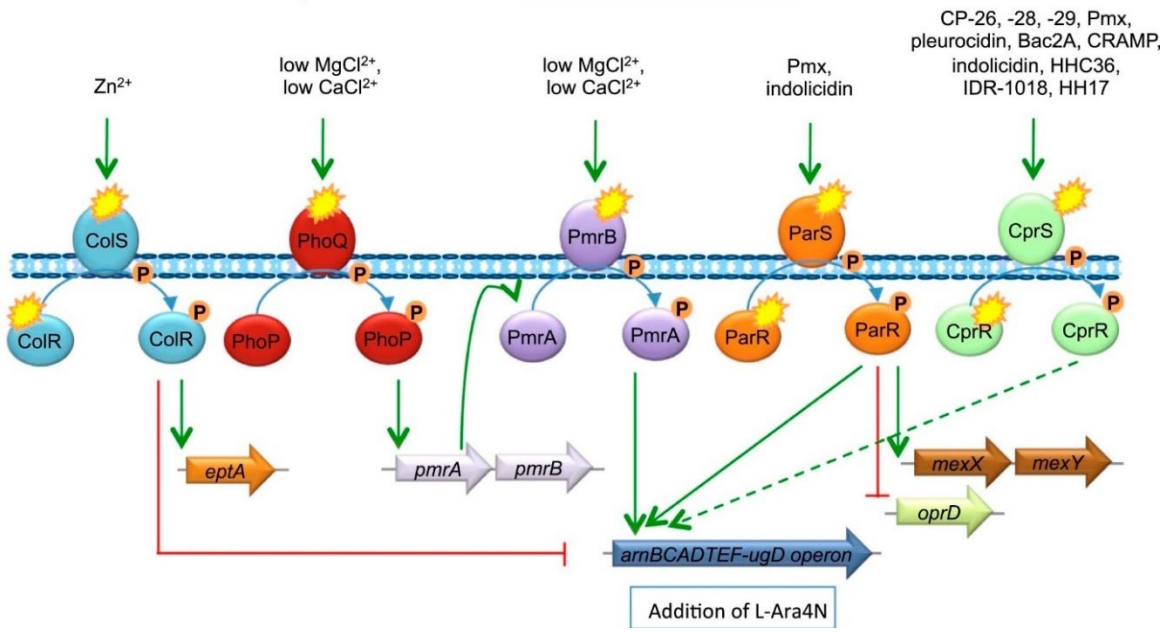


Figure 1-9. Two Component Systems of P. aeruginosa which feed environmental signals to and regulate LPS modification by addition of L-Ara4N via arnBCADTEF. Reproduced from Jeannot et al,2017[103].

1.2.6 Two Component Systems

Target alteration is a major method of AMP resistance in *P. aeruginosa*. Positively charged AMP attaches to the negatively charged bacterial cell wall where it has its effect or gains access to the bacterial cell. To prevent this, *P. aeruginosa* can make modifications to its outer membrane, particularly the LPS component, to neutralise it's charge and prevent AMPs from binding[177,179–182]. This involves the addition of positively charged moieties such as L-4-aminoarabinose (L-Ara4N) to LPS which serve to reduce the negative charge of the cell and reduce binding opportunities for antimicrobials.

These modifications are regulated in *P. aeruginosa* by a selection of Two Component Systems (TCS); paired proteins one of which is a Sensor, taking in information, and the second of which is a Response Regulator, passing the information from the Sensor onto the process which the TCS regulates[183] (**Figure 1-10**). TCSs are found widely across bacterial species[184,185].

According to the Microbial Signal Transduction (MiST) database *P. aeruginosa* has 68 TCS encoded on its genome and they sense a range of stimuli and regulate a wide variety of cellular processes[184].

The sensor component of TCSs sit across the bacterial membrane and is composed of a periplasmic domain, protruding outwards to sense the environmental, sitting between 2 transmembrane domains[183,185,186]. Signals are transferred from the periplasmic domain via a HAMP domain (histidine kinases, adenylyl cyclases, methyl-binding proteins, and phosphatases) by transfer of a phosphate to a histidine kinase A (phosphoacceptor) domain (HisKA) using a histidine kinase-like ATPase (HATPase_c). The phosphate is transmitted to the receiver (REC) domain of the Response Regulator activating the transcriptional regulatory protein C-terminal (Trans_reg_C) domain (**Figure 1-10**).

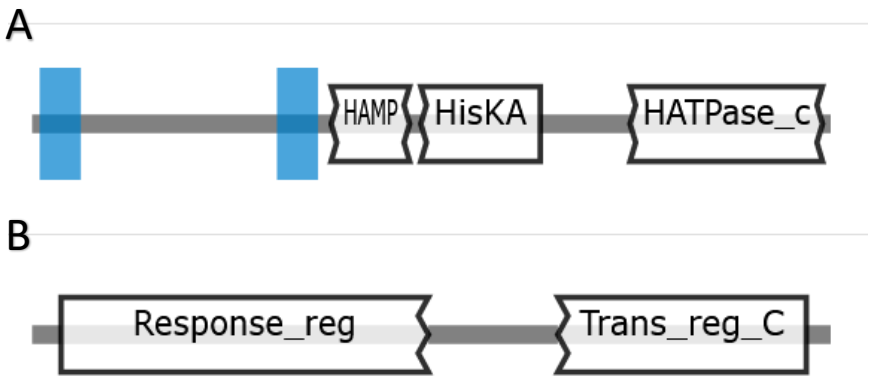


Figure 1-10. Structures of genes involved in Two Component Systems for signal transduction in bacteria. ***A.*** *The Sensor protein straddles the bacterial membrane with two Transmembrane domains (blue) between which is the Periplasmic domain which takes in signals from the bacterial environment. Phosphorylation of the Histidine kinase (HisKA) via the HAMP domain (histidine kinases, adenylyl cyclases, methyl-binding proteins, and phosphatases) from ATP by Histidine kinase-like ATPase (HATPase_c) results.* ***B****. The Response Regulator protein receives the phosphate from the Sensor in its receiver domain (Response_reg) and activates its transcriptional regulator C-terminal domain (Trans_reg_C). Diagram from the Microbial Signal Transduction (MiST) database*[184]*.*

Five *P. aeruginosa* TCSs have been shown to play a role in LPS modification, some better studied than others: PhoPQ[136,137,159,187–189], PmrAB[33,159,180,188,190], CprSR[188,191], ParSR[188,192] and ColSR[193,194]. The usual function of these systems is to modify LPS in response to AMPs. However, these TCS have also been shown to have an important role in AMP resistance (usually through study of colistin resistance) when SNPs in their gene sequences lead to specific non-synonymous amino acid changes in the TCS proteins[158,159,188,190,192,195,196]. While some SNPs in the Response Regulators have been proposed to result in AMP-resistance, the majority of changes leading to resistance are found in the TCS Sensors. Unusually, many of the resistance associated amino acid changes in Sensor genes are loss-of-function mutations, with the absence in environmental feedback from the Sensor causing constitutive expression downstream and permanent LPS modification[102,197]. Summaries of discrete amino acid changes which have been associated with AMP resistance in *P. aeruginosa* have been compiled by Olaitan et al[102] and Poirel et al[197] but new mutations are being discovered.

Most of the TCS mutations involved in AMP resistance have been discovered through the study of colistin resistance in *P. aeruginosa*, either by screening colistin resistant clones or by resistance generation experiments. However, colistin resistance seen is not limited to the polymyxin and is applicable against other cationic AMPs[191,192,198] and is also associated with changes in susceptibility to other antibiotics[199]. Cross-talk between different TCS has also been reported[159,177,181,188,198]. Colistin/AMP resistance mediated by TCS mutation have been identified in *P. aeruginosa* isolates from people with CF[158,159,193,195].

Upregulation of TCS gene expression and modification to LPS has also been reported in divalent cation depleted media illustrating the importance of divalent cations in protecting *P. aeruginosa* from attack by cationic agents[33,137].

1.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are found abundantly in nature across all kingdoms of life as part of immune defences against infection and different AMPs provide defence against bacteria, viruses, fungi and parasites[200–206]. The first AMP was discovered in 1939 by Dubos, isolated from a *Bacillus* found in soil and investigated as an antibacterial[207,208]. In a somewhat common theme for AMPs, it was found to be useful topically but not systemically, due to cytotoxicity[209]. Today, the best known clinical AMP is the polymyxin, colistin[210,211]. Since then, thousands of AMPs have been discovered and created and the current number in the Data Repository of Antimicrobial Peptides (DRAMP) database (http://dramp.cpu-bioinfor.org/), including synthetic AMPs, is >20000 (accessed 01/01/2022)[212].

Short in length, most AMPs are <50 amino acids long, although the DRAMP database includes AMPs up to 100 amino acids in length[212]. For many years it was thought that all AMPs were cationic and were predominantly antibacterial via this property. However, it is now known that AMPs can also function by inhibition of DNA synthesis, inhibition of protein synthesis and inhibition of cell division[202,206]. The antibiotic resistance crisis in bacteria has led to an increased interest in AMPs, both natural and synthetic, as source of new antimicrobials or as enhancers of the activity of existing antibiotics[213–217].

AMPs are broadly broken into four structural categories; α-helical, β-sheet, linear extension and complex peptides which contain both helical and sheet elements. Peptides have been identified which do not conform to these categories and some AMPs adopt different structures under different conditions[218]. Many antibacterial AMPs are also amphipathic in nature, containing both hydrophobic and hydrophilic regions[202,206].

1.3.1 Cationic AMPs

Cationic antibacterial AMPs are the most common and best studied AMPs. Their antibacterial activities are intrinsically linked to their positively charged nature which facilitates binding to negatively charges cell surface structures of bacteria, resulting in disrupted bacterial membranes, membrane thinning, pore-formation and membrane disintegration[202,203,206]. Initial attachment is electrostatic followed by hydrophobic interactions between the amphipathic AMP and the bacterial membrane with binding, insertion and disruption of the lipid bilayer resulting. Even though the target is common (cell membranes) and outcomes similar (disruption and cell death), there is diversity in the mechanism of membrane disruption caused by AMPs (**Figure 1-11**).

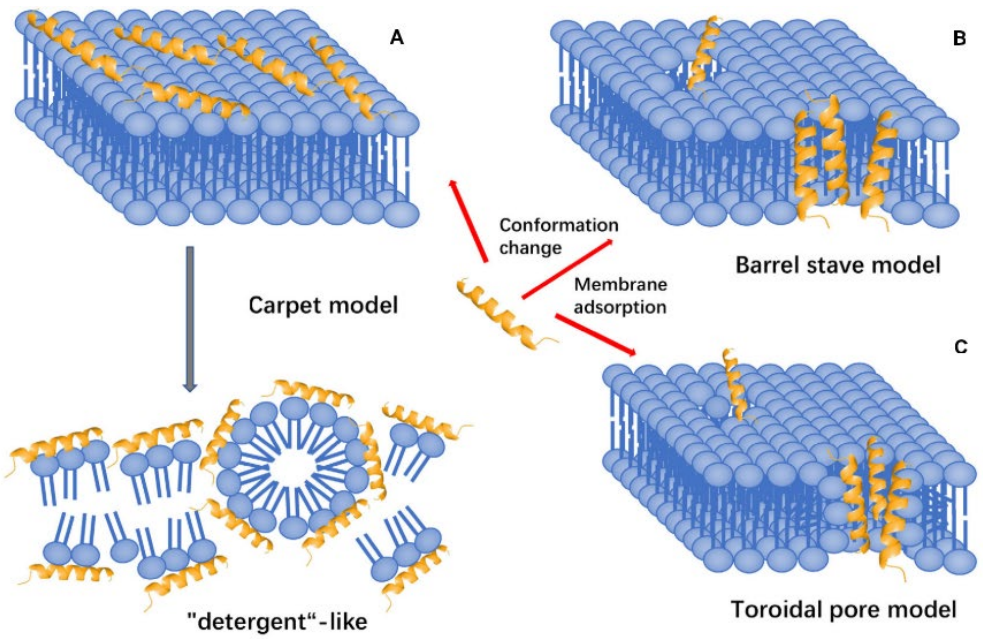


Figure 1-11. Antimicrobial peptides cause membrane disruption via A. Carpet model of detergent-like membrane sloughing B. Barrel-Stave pore formation C. Toroidal pore formation. Reproduced from Huan et al, 2020[206].

1.3.1.1 Carpet model

The Carpet model is the detergent-like activity of AMPs resulting from parallel interactions on the cell surface. The AMPs associate with small areas of the bacterial cell surface coating or 'carpeting' it. Lipid bilayer and AMP interactions lead to gaps being formed between lipid molecules and pores being formed. Membrane sloughing and thinning results in cell death. AMP effects via the carpet model have been shown to be related to charge accumulation rather than AMP concentration, with a specific charge threshold required to be reached before effectiveness. Human derived cathelicidin AMP LL-37 has been shown to function via the Carpet model AMP[219].

1.3.1.2 Barrel-Stave model

In the Barrel-Stave model, AMPs associate with each other forming a stave before insertion into the bacterial membrane. The resulting pore (barrel) is formed by the AMP excluding the cell membrane lipids and resulting in membrane collapse or loss of bacterial cytoplasmic contents. Fungal α-helical AMP Alamethicin is Barrel-Stave former[220].

1.3.1.3 Toroidal Pore model

Similar to the Barrel-Stave model, in that AMPs insert perpendicular to the membrane, forming pores/channels. The difference with the Toroidal pore model is that the AMPs in the pore are associated with the lipids of the membrane turning lipid hydrophilic headgroups inwards, rather than excluding the bacterial lipids. Magainin and Melittin, AMPs from frog skin and bee venom, respectively, form Toroidal pores[221,222].

In Gram-negative bacteria, all of these membrane disruption processes may be facilitated by or as a direct result of the ability of AMPs to target LPS structures including for *P. aerugninosa*[32,138,223]. AMPs can attach to LPS electrostatically, again exploiting their positivity. Bound AMPs force LPS chains apart to cause membrane ruptures or facilitate their own uptake by exposing more of the bacterial membrane. They can also displace divalent cations which Gram-negative's use to cross-link and stabilise LPS chains, again facilitating their own uptake and attack or the attack of further agents. Indeed, the AMP colistin continues its targeting of LPS into the cytoplasmic membrane[224].

Membrane disruption is not the only method by which AMPs kill bacteria. Some AMPs have been shown to have dual targets in the bacterial cells while some work exclusively on intracellular process. The AMP Indolicidin, from bovine neutrophils, is capable of membrane permeabilisation and also prevents DNA biosynthesis[225]. The pig intestine AMP Cecropin PR-39 can prevent bacterial protein synthesis[226]. Indeed, membrane disruption is not a pre-requisite for AMP function with examples like Apidaecin, from insects, which is bactericidal without the ability to form pores in bacterial membranes[227].

As mentioned the human cathelicidin AMP, LL-37, is an innate defence peptide of humans[228,229]. While difficult to fully distinguish, its mechanism of action against bacteria is thought to be via the Carpet model of membrane disruption[228,230]. It has also been shown to diffuse into the lipid bilayer. However, given LL-37's reported activity against both Gram-negative and Gram-positive bacteria, with their different membrane structures, the activity of LL-37 is non-specific but features electrostatic binding to negatively charged bacterial cells – lipid A in Gram-negatives and teichoic acid in Gram-positives[228]. LL-37 has been shown to

synergise with several antibiotics against *P. aeruginosa* including tobramycin, ciprofloxacin and colistin (among others)[231–234].

The activity of LL-37 has been shown to be ablated in CF, leading to a reduction on its antimicrobial effect. This is due to the commonly known physical barriers of CF sputum (reduced viscosity and increased ionic strength)[235,236]. On top of these factors, the acidic pH of the CF lung environment may reduce LL-37 activity and high levels of free DNA may aggregate the peptide and lower its bioavailability[69,229,237].

The activity of the AMP colistin against *P. aeruginosa* is well studied and colistin is in-use today in treatments of *P. aeruginosa* infections in people with CF as an inhaled treatment[238].

Table 1-1. Examples of AMPs.

	Structure	**Size**	**Source**	***P. aeruginosa* MIC (Strain)**	**Citation**
LL-37	α-helical	37aa	human	16mg/L (PAO1)	Strempe et al, 2013[239]
Melittin	α-helical	26aa	honeybee venom	20mg/L (ATCC 27853)	Akbari et al, 2019[240]
Pexiganan	α-helical	22aa	synthetic	1mg/L (clinical strains)	Ge et al, 1999[241]
Melimine		30aa	synthetic	125mg/L (ATCC 27853)	Yasir et al, 2020[242]
Alamethicin	α-helical	21aa	fungus		
Magainin	α-helical		frog	6.25mg/L (clinical strains)	Levison et al, 1993[243]
Apidaecin	mixed	18aa	insect	8mg/L (clinical strains)	Berthold et al, 2013[244]
Indolicidin		13aa	bovine	8mg/L (ATCC 27853)	Giacometti et al, 1999[245]
Cecropin P1	two helices	31aa	nematode	4mg/L (ATCC 27853)	Giacometti et al, 1999[245]

1.4 Antibiotic resistance crisis

As early as 1945, the discoverer of penicillin, Sir Alexander Fleming was sounding the alarm about antibiotic overuse and resistance[246]:

"The microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out which can be passed to other individuals and perhaps from there to others until they reach someone who gets a septicemia or a pneumonia which penicillin cannot save"

This warning came before what is sometimes referred to as the 'glorious era of antibiotic discovery'[247] from the 1940s-1960s when many of the major classes of antibiotics were discovered and brought to market. Antibiotics played a vital role in the increase in life expectancies across the 20th century; from 47 to 80 years for females between 1900 and 2000[248]. However, even as antibiotics were being discovered and implemented, and as per Fleming's warning, resistance to antibiotics was increasing. Shortly after penicillin went into use in 1941 a resistant *S. aureus* was discovered clinically[209] and resistance *Streptococcus pneumoniae* was found in 1967[249]. Methicillin resistant *S. aureus* (MRSA) were discovered contemporaneously with the release of the drug in the early 1960s[250] (**Figure 1-12**).

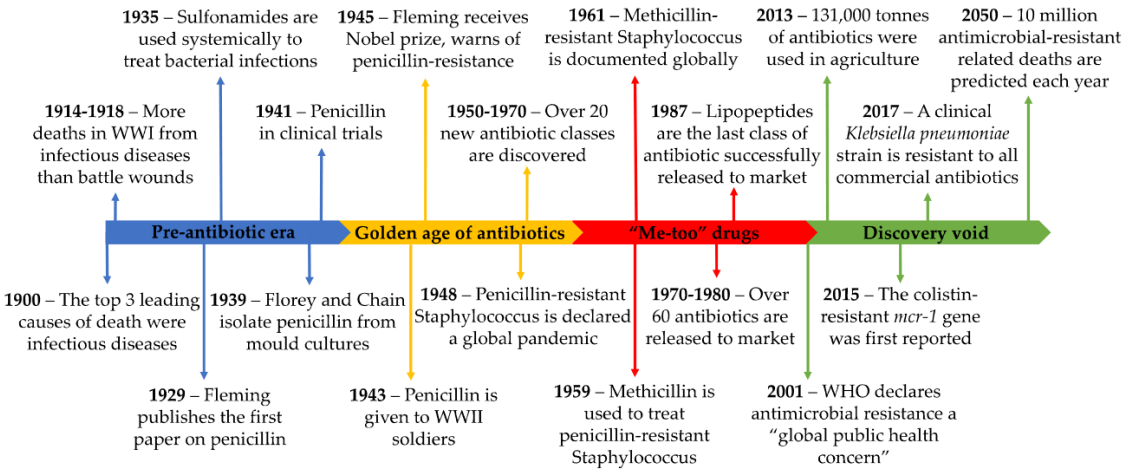


Figure 1-12. Timeline of antibiotic resistance development. Reproduced from Browne et al, 2020[251].

While resistance development by bacteria was to be expected, the confluence of resistance development, antibiotic overuse and the lack of development of new antimicrobials has led to, what is now, a crisis of antibiotic resistance[247,252–256]. At the current trajectories, it has been predicted that global deaths due antimicrobial resistance will reach 10 million people per year by 2050, surpassing cancer by that time[253].

The dearth in development of new treatments has been due to lack of investment in new antimicrobials is as a result the reduction in the numbers of pharmaceutical companies ('merger mania') and a linked reduction in Research and Development[247]. The movement in focus to developing 'blockbuster' drugs and drugs to be taken chronically has reduced industrial interest in less profitable drugs with the increased time and cost of bringing new antimicrobials to market[247,257–259].

Antibiotic misuse and poor stewardship is also a major factor in the resistance crisis. Prescription of broad-spectrum, non-targeted antibiotic treatments of infections or in the treatment of viral infections has been a major driver of resistance development. There is also a prophylactic overuse of antibiotics in agriculture as growth promoters[252,253].

There are a variety of proposed strategies for dealing with resistance crisis[113,252,253]. These range from increased investment in drug discovery and new antimicrobials to combining drugs to restore/protect antibiotic efficacy to better stewardship programs facilitated by accurate rapid diagnostic tests. Among these different strategies AMPs feature prominently[213,216,260].

1.4.1 AMPs as new antibiotics

According to Theuretzbacher et al, 2020[213], there were 135 projects using AMPs as direct new antimicrobials under investigation at the time of that publication, including both synthetic and natural AMPs.

1.4.2 AMPs as antibiotic potentiators

Potentiators are drugs which have little or no antimicrobial activity but augment or restore the activity of another antibiotic. Also referred to as 'antibiotic resistance breakers', for their ability to 'break' the resistance of bacteria to antibiotic(s), AMPs have been proposed as antibiotic adjuvants to be given in combination with existing antibiotics by exploiting the abilities of many AMPs to breach bacterial cell walls and thereby permeabilise the bacterial cell[251,260–263]. Permeabilisers are highly desirable and many AMPs and their derivatives have been investigated as permeabilising resistance breakers[261,264,265].

Other kinds of resistance breakers/potentiators have also been investigated: efflux pump inhibitors, β-lactamase inhibitors, quorum sensing modulators, LPS modification prevention, virulence inhibitors, among others[261,264,265]. However, while synergies have been described for combinations of existing antibiotics (and antibiotics may be administered clinically with this in mind) the only clinically in-use antibiotic resistance breakers currently are not AMPs but β-lactamase inhibitors, such as avibactam[266,267].

1.4.3 Repurposed drugs

Drugs which have been approved for use for another condition or antimicrobials which are being reexamined for new activity and targets have the potential to be repurposed as antibiotics. The repurposing of existing drugs can cut down on time and cost of development due to the body of research and knowledge already in place[213,268]. Fifteen repurposing projects were identified by Theuretzbacher et al, 2020[213].

1.4.4 Glatiramer acetate

Glatiramer acetate (GA) is a drug currently used in the treatment of multiple sclerosis (MS). Originally designed to resemble myelin basic protein (MBP) in the study of MS, GA was found to suppress an animal model of MS[269]. Further studies and clinical trials led to introduction of GA as an MS treatment in the 1990s[270–275]. GA is sold under the name Copaxone™.

GA is formed by the random co-polymerisation of 4 amino acids; L-glutamate, L-lysine, L-alanine, and L-tyrosine (**Figure 1-13**). GA is extremely heterogenous, not only due to the random addition of the amino acids, but the polymerisation technique yields chains of variable length. GA peptides range from 45-80 amino acid residues and between 5-9kDa and, taking into account these properties, there are $>10^{30}$ GA peptide configurations possible[276].

GA is cationic (+3, approximately) and amphipathic and form α-helices in membrane-like environments[277]. In addition to the heterogeneity produced as a result of the manufacturing process, oligomers of GA[277,278] have also been seen and in solution GA has been observed in at least two distinct populations – string-like fibers and spherical particles[278].

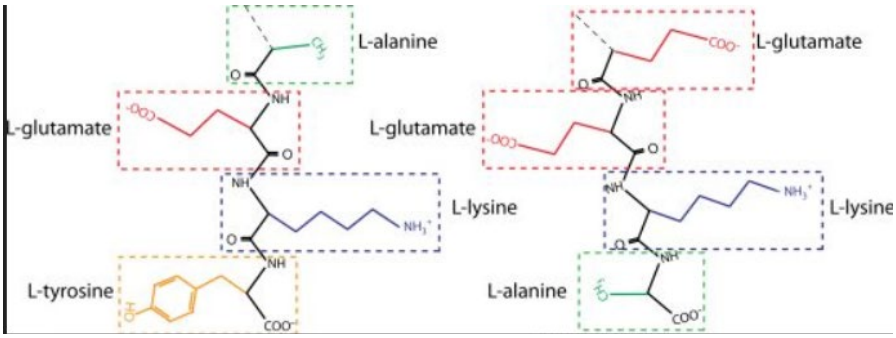


Figure 1-13. Glatiramer acetate is formed by the random co-polymerisation of four amino acids L-glutamate, L-lysine, L-alanine, and L-tyrosine. Reproduced from Jalilian et al, 2012[276]*.*

The pharmacokinetics of GA are not well studied – it's heterogeneity being a barrier to quantification – with the main human findings coming from a study performed to quantify GA in human sera after subcutaneous injection with a single, supratherapeutic dose of 60mg administered to healthy volunteers[279]. Nine of 17 subjects (healthy male volunteers) had levels of GA in plasma which was quantifiable. The time to reach the maximum concentration (T_{max}) of GA in those 9 subjects was 15-30mins and the maximum plasma concentration (C_{max}) of GA was 69-605ng/ml. However, the variability in concentration seen was wide (AUCs in the range 1644-67532). Concentrations of GA returned to baseline 30-60mins post-injection but 4 of the test subjects also had quantifiable GA at the later time points of 240-360mins. After 24hrs GA levels of the in the serum of 15 of the 17 subjects had dropped below the limit of detection (25-50ng/ml)[279].

In MS, it is accepted that the mechanisms of action of GA are complex, multifactorial and incompletely understood. What is also well accepted is that GA is an immunomodulator

in that disease[274,280–282]. Due to the resemblance of GA to MBP, the purpose for which it was created, it has been proposed to act as an antagonist to the autoimmune effects of the disease and studies have shown a reduction in MBP-specific T cells after GA treatment[275,283]. GA has been shown to be anti-inflammatory; GA treated macrophages and monocytes produced increased amounts of the anti-inflammatory cytokine IL-10 and GA treated dendritic cells promoted the switch from pro-inflammatory T-helper 1 (Th1) cells towards anti-inflammatory Th2 cells[284,285]. GA has also been shown to restore the functioning of immune cells which are known to be deficient in autoimmune conditions such as MS, including Natural Killer (NK) cells and Regulatory T (Treg) cells[274,280,286].

In addition to this knowledge of GA-immunomodulation, and a crucial juncture in the subsequent investigations of GA as an antimicrobial, in 2017 Christiansen et al showed that GA rapidly kills human leukocytes via membrane damage[277]. Interestingly, the activity of GA against leukocytes was limited to cells with anionic elements (sialic acid) on their cell surface, indicating that the cationic nature of GA was vital in this activity, allowing electrostatic binding to the cell surface and facilitating insertion and disruption (**Figure 1-14**)[277].

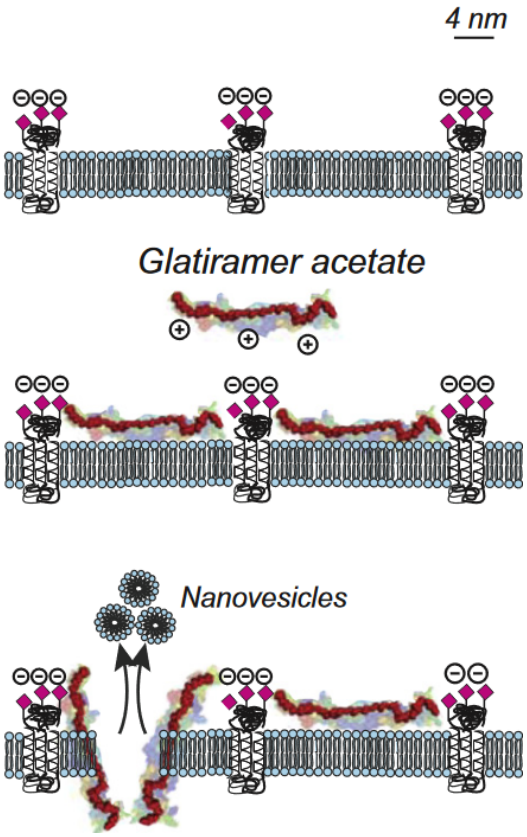


Figure 1-14. Proposed mechanism of GA action against human leukocytes via membrane disruption with the anionic moieties being present on the cell surface being vital for GA activity. Electrostatic interactions allow GA bind to the cell surface followed by membrane insertion. Adapted from Christiansen et al, 2017[277].

The peptide composition of GA, along with its cationic and amphipathic properties and its ability to rapid kill leukocytes via membrane damage, led to the investigation of GA as an AMP against bacteria[277,287]. In an earlier collaborative project[287], we demonstrated rapid cell killing of Gram-negatives (*P. aeruginosa*, *E. coli* and *Acinetobacter baumannii*) with bacterial cell death occurring after 30min exposures, but not for a Gram-positive bacterium (*S. aureus*). *P. aeruginosa* was the most effected by GA treatment with a greater proportion of cell death than *E. coli*, for reference strains of each. Furthermore, GA was found to be effective against CF isolates of *P. aeruginosa* after the same short exposure time (30min) and at the same GA

dose (50mg/L)[287]. However, half of the clinical *P. aeruginosa* tested (22/44) were resistant to the rapid killing effect of GA in that model.

Cell envelope stress was predicted to be the mechanism of GA activity against the Gram-negative bacteria, with uptake of the membrane impermeable dye propidium iodide seen and evidence of 'cellular ghosts'[287] – bacteria which had been emptied of their cytoplasmic contents – an observation which has been made in AMP activity previously[288]. There was an increase in cellular debris after GA treatment, although interestingly this was not limited to GA-susceptible species, as *S. aureus* also showed increased debris. This was speculated to be a defence mechanism of the Gram-positive bacterium, selectively blebbing off parts of its membrane affected by GA. Dense aggregates were also observed in some bacterial cells, suggesting intracellular activity of GA and the possibility of DNA-aggregation by GA[287].

In MS, GA is administered by subcutaneous injection and has a long, remarkably good safety profile and few side-effects[281,289]. An investigation into the effects of an nebulised formulation of GA on the lungs of pigs revealed no adverse effects on the animals and the authors of that study propose GA as a candidate for repurposing as an antimicrobial[290].

1.5 Summary

P. aeruginosa is an important and serious pathogen with its ubiquity, versatility, ability to cause infection opportunistically and complex and multifaceted antibiotic resistance mechanisms making it of particular concern[109,110,113]. *P. aeruginosa* is of particular significance in infections of people with CF where it is the most common and most significant infecting bacterium and can cause chronic infections lasting decades[39,60,61]. The versatility and

adaptability of *P. aeruginosa* mean that the CF lung environment allows the bacteria to use and evolve its intrinsic resistance to evade antibiotic treatments successfully – switching to a mucoid and biofilm lifestyle , accumulating mutations which make their efflux systems and LPS modification more active.

The ongoing crisis in antimicrobial resistance is a global threat[252,253] and is of particular concern in *P. aeruginosa* which is of 'CRITICAL' priority for new treatments[113]. Strategies to combat this crisis include deployment of AMPs (both natural and synthetic), use of antibiotic resistance breakers which potentiate the efficacy of existing antibiotics and the repurposing of existing drugs with strong safety profiles for use as antimicrobials[213,216,268,291].

Glatiramer acetate is an existing peptide drug used in multiple sclerosis. Though its mechanism of action is incompletely understood in that condition, it is known to be an immunomodulator with an excellent safety record and few side-effects[274,276,281,282]. Of particular interest is recent research which showed that GA attaches to and disrupts the membrane of human leukocytes in a manner that very much resembles the way in which AMPs function against bacterial cells[277]. Further to this observation, GA has been shown to kill *P. aeruginosa* cells, albeit only over short exposures and in non-dividing cultures[287]. Therefore, GA has a combination of properties of interest in combatting the antimicrobial resistance crisis against *P. aeruginosa*.

1.5.1 Hypotheses

I hypothesise that:

- Co-administration of GA with commonly used antibiotics in CF makes the antibiotics more effective against *P. aeruginosa*

- Co-administration of GA with TOB makes TOB more effective against *P. aeruginosa* from people with cystic fibrosis
- Glatiramer acetate will rapidly kill *P. aeruginosa* as a result of disruption of its bacterial membranes
- GA targets *P. aeruginosa* LPS and strains of *P. aeruginosa* react to this targeting by modifying their LPS structures to protect against GA activity
- Elements of the CF airway will interact with GA either interfering with its antimicrobial activity or leading to a host inflammatory response

1.5.1.1 Aims

To investigate these hypotheses, I will:

1

- Measure the effect of GA on growth and viability of *P. aeruginosa* strains
- Compare the growth and viability of reference *P. aeruginosa* exposed to antibiotics with and without GA co-treatment
- Calculate whether the activities of GA in combination with each antibiotic result in synergy, additivity or antagonism of the agents in their activities against *P. aeruginosa*

2

- Expand the knowledge and methods of Chapter 3 to a larger cohort of clinical *P. aeruginosa* from people with cystic fibrosis: growth and viability tests, minimum inhibitory concentrations, synergy analyses

3

- Assess the impact of GA on *P. aeruginosa* strains during short exposures
- Test the effect of GA on *P. aeruginosa* membranes including GA dosing and comparison with other cationic agents
- Contrast and compare the effects of GA on *P. aeruginosa* cell membranes with that on other species of bacteria from CF samples

4

- Test the effects of divalent cations and *P. aeruginosa* LPS on GA activity
- Asses the *P. aeruginosa* response to GA treatment for known mechanisms of AMP resistance in *P. aeruginosa*

5

- Assess the impact of GA on *P. aeruginosa* biofilms
- Test the effect of eDNA on the activity of GA
- Assess the impact of GA on paired epithelial cell lines

2. Materials and Methods

2.1 Materials

Table 2-1. Materials used in this study.

Product	Source
1.5mL tubes	ThermoFisher Scientific
15mL Centrifuge tubes	VWR
1-*N*-Phenylnaphthylamine	Merck/Sigma Aldrich
3,3'-Dipropylthiadicarbocyanine Iodide	ThermoFisher Scientific
50mL Centrifuge tubes	VWR
96-well plate (flat bottom, untreated, sterile)	ThermoFisher Scientific
96-well plate (flat bottom, high bind, sterile)	Corning
96-well plate (flat bottom, black, sterile)	PerkinElmer
96-well plate seal (breathable, sterile)	VWR
96-well plate seal (optically clear, breathable, sterile)	4titude
AnaeroGen anaerobic gas pack (3.5L)	Oxoid
Casein hydrosolate	Merck/Sigma Aldrich
Ceftazidime hydrate	Merck/Sigma Aldrich
Cetrimide Agar	Merck/Sigma Aldrich
Ciprofloxacin	Merck/Sigma Aldrich
Colistin sulphate salt	Merck/Sigma Aldrich
CXCL8 DuoSet ELISA kit (DY208)	R&D Systems
Direct-zol RNA Miniprep kit (R2053)	Zymo Research
Fetal bovine serum	Merck/Sigma Aldrich
Filters (0.44µM)	VWR
Glatiramer acetate	Biocon
Glucose	Merck/Sigma Aldrich
HEPES Buffer (1M)	Merck/Sigma Aldrich
IL-6 DuoSet ELISA kit (DY206)	R&D Systems
Inoculating Loops (10µL)	VWR
KAPA SYBR FAST One-Step qRT-PCR (KK4651)	Merck/Sigma Aldrich
Lennox LB agar	Merck/Sigma Aldrich
Lennox LB both	Merck/Sigma Aldrich
LL-37 trifluoroacetate	Merck/Sigma Aldrich
M9 broth	Merck/Sigma Aldrich
$MgSO_4$	Merck/Sigma Aldrich
MicroAmp Fast Optical 96-Well Reaction Plate	ThermoFisher Scientific

MicroAmp Optical Adhesive Film	ThermoFisher Scientific
Microbank Vials	Pro Lab Diagnostics
Minimal Essential Medium (phenol red and L-glutamine)	Merck/Sigma Aldrich
Mueller-Hinton Broth	Merck/Sigma Aldrich
NaCl	Merck/Sigma Aldrich
Syringe Needles	VWR
Syringes (10mL)	VWR
NucleoSpin Microbial DNA Mini kit (740235)	Macherey-Nagel
Phosphate buffered saline (10 X)	Fisher Scientific
Propidium iodide	Merck/Sigma Aldrich
Pseudomonas aeruginosa lipopolysaccharide	Merck/Sigma Aldrich
Reagent Diluent Concentrate (DY995)	R&D Systems
Round Petri Dishes (sterile, 90mm, vented)	VWR
Square Petri Dishes (sterile, 120mm, vented, with grid)	
Stop solution for ELISA (DY994)	R&D Systems
Streptavidin-HRP for ELISA (DY998)	R&D Systems
Substrate Reagents for ELISA (DY999)	R&D Systems
Tobramycin	Merck/Sigma Aldrich

Table 2-2. Equipment used in this study.

Equipment	**Model**	**Manufacturer**
Centrifuge	5810R	Eppendorf
Imager	ChemiDoc MP	BioRad
Incubator	SM-30/TH-30	Edmund Buhler
Incubator (CO2)	FisherBrand	ThermoFisher Scientific
Microbiological Safety Cabinet		Nuaire
Microbiological Safety Cabinet		Kojair
Microcentrifuge	5424R	Eppendorf
Platereader	FLUOstar Omega	BMG Labtech
Platereader Software	Omega (ver 5.11)	BMG Labtech
Real-Time PCR	QuantStudio 7 Flex	ThermoFisher Scientific
Vortexer/Mixer	MixMate	Eppendorf

2.2 Methods

2.2.1 Bacterial storage and growth conditions

Reference strains of *P. aeruginosa*, PAO1, PA14 and PAK, were sourced from collaborator labs. Clinical bacterial strains were sourced from the Clinical Microbiology Laboratory at the Royal Brompton Hospital and were processed, purified isolates from respiratory samples from people with cystic fibrosis. Clinical isolates were collected weekly on agar plates, once Clinical Microbiology had completed processing and testing, and stored by inoculating a single colony into Microbank vials (ProLab Diagnostics) containing plastic beads with a sterile 10µL inoculation loop; (i) bacteria added to the vial, (ii) briefly shaken by hand to emulsify, (iii) bacteria allowed to bind to beads for ≥2mins, (iv) media removed and (v) vial transferred to -80°C.

From these Master Stocks, bacteria were revived by retrieving a single bead from the vials (without allowing vial to fully thaw) using a sterile needle and the bead placed onto an agar plate, allowed to rest on the agar for ≥5mins followed by spreading the bead across the agar surface with a 10µL inoculating loop and streaking for single colonies. *P. aeruginosa* were revived onto Cetrimide Agar and non-*Pseudomonas* isolates onto LB agar. Plates were inverted and placed in an incubator at 37°C overnight.

Agar plates were inspected for purity and single colony growth. Once these criteria were met, new -80°C stocks were made as above to avoid repeated freeze-thaw cycles, contamination and depletion of Master Stock of each strain. The new stocks were stored at -80°C, discreetly from the Bacterial Repository, and used exclusively for this project. This process of creating sub-stocks was repeated on one further occasion during the project, on

depletion of the first sub-stock, where 3 new sub-stocks were created simultaneously from a single pure agar culture of each isolate.

For subsequent experimental use, purified bacteria were revived as above onto LB agar and incubated for at least one overnight growth cycle. Bacteria which were slow to revive were incubated for a further night or subcultured onto a further LB agar plate and re-incubated. All strains used grew consistently from -80°C using this approach. Bacterial agar plates were stored at 4°C for up to a week for further usage. After this, if strains were still required, a further subculture onto LB agar was made if necessary but serial passages over extended periods of time was avoided and revival from -80°C stocks was instead undertaken.

Eleven strains of *P. aeruginosa* isolated from people with CF in the Microbiology Laboratory of the Royal Brompton Hospital and stored in the CF&CLI CF Bacterial Strain Repository at Imperial College London, were select for experimentation to represent a variety of antibiotic susceptibility profiles and mucoidy.

Table 2-3. Clinical P. aeruginosa strains from people with CF used in this study. All data and results from the Clinical Microbiology Laboratory at the Royal Brompton Hospital prior to this study.

	Sample Type	***Sample Date***	***Mucoid***	***VNTR***	***AMK***	***ATM***	***CAZ***	***CIP***	***CST***	***SXT***	***GEN***	***MEM***	***TOB***	***Profile***
RBH750	SS	Feb 2016	✓	12,5,5,3,4,4,8,3,7	S	I	S	S	S	R	S	S	S	
RBH899	SS	Jan 2016			S	I	S	S	S	S	S	S	S	
RBH550	SS	June 2014		11,6,-,2,1,3,-,3,14	S	S	S	S	S	S	S	S	S	
RBH294	SS	Jan 2016	✓		S	R	S	S	S	R	S	S	S	
RBH982	SS	May 2016			S	I	S	S	S	S	S	S	S	
RBH422	SS	Apr 2016		11,-,4,-,3,3,7,2,10	S	I	S	R	R	S	S	S	S	
RBH519	SS	Apr 2015	✓	11,4,5,2,3,2,8,4,15	R	S	S	R	S	S	R	S	R	MDR
RBH461	CS	July 2014		11,7,-,4,4,3,5,2,7	R	R	R	R	S	R	R	R	R	MDR
RBH490	SS	Sep 2014		8,2,3,-,3,2,5,3,9	S	R	R	R	S	R	R	R	R	MDR
RBH065	SS	Feb 2016			R	I	S	R	S	S	R	S	R	MDR
RBH072	SS	Nov 2015		11,6,2,2,1,3,7,2,10	S	I	S	R	S	R	R	S	R	MDR

SS -spontaneous sputum, CS – Cough Swab

AMK – amikacin, ATM – aztreonam, CAZ – ceftazidime, CIP – ciprofloxacin, CST – colistin, SXT – trimethoprim-sulfamethoxazole, GEN – gentamicin, MEM – meropenem, TOB – tobramycin

S – susceptible, I – intermediate, R – resistant

MDR – multidrug resistant according to criteria of Magiorakos et al, 2012[112]

The following non-pseudomonal strains from people with CF were used:

Table 2-4. Non-pseudomonal bacterial species from people with CF used in this study.

	Species
RBH699	*Staphylococcus aureus*
RBH743	*Burkholderia cepacia*
RBH834	*Achromobacter xylosoxidans*

For proliferation of bacterial numbers for experimental use broth cultures were used; single colonies were inoculated into the required (details in individual experimental methods), experiment-specific, sterile liquid media using 10µL inoculation loop, vortexed briefly where applicable and grown aerobically (unless stated otherwise) at 37°C with agitation at 200rpm overnight (16-18hrs) to late-exponential phase. Growth was carried out in 10mL volumes of liquid media in 50mL centrifuge tubes to allow for sufficient oxygenation. After overnight growth, cultures were centrifuged at 3500g for 15mins, depleted media removed and pellet resuspended in an experimentally appropriate medium.

After resuspension, if required, bacterial concentrations were estimated using optical density (OD); measurements were carried out at 600nm (OD_{600}) in FLUOstar Omega platereader (BMG Labtech) in 200µL volumes in clear, sterile flat-bottomed 96-well plates. The OD_{600} of cultures and suspensions were adjusted according to the bacterial concentration and volume requirements of the individual experiments, using the formula:

$$C_1\ (concentration\ of\ solution\ 1)\ \times\ V_1\ (volume\ of\ solution\ 1) =$$

$$C_2\ (concentration\ of\ solution\ 2)\ \times\ V_2\ (volume\ of\ solution\ 2)$$

where the target concentration (C_1) in OD_{600} and volume (V_1) in mL of the adjusted bacterial culture and the concentration of the overnight culture (C_2) were used the calculate the required volume of overnight culture (V_2) to produce the new, adjusted bacterial suspension. (Colony counts of strain PAO1, carried out per **Methods 2.2.5**, showed that an OD_{600} of 1.0 equates to ~8 X 10^8 CFU/mL).

2.2.2 Master stock solutions of antimicrobials

2.2.2.1 Glatiramer acetate

Glatiramer acetate was produced by Biocon (India) as a powder and stored at -20°C. According to the manufacturers Certificate of Analysis, the purity of the provided GA powder was 99.7% (by HPLC). The average molecular weight of the GA provided was 6584 (SD 4202-13222) Da, which is in line with what is expected of GA given its known and expected heterogeneity[274,276]. Solutions were prepared at 50000mg/L as a Master Stock by dilution of 250mg of GA in 5mL of sterile de-ionised water and allowed to dissolve at room temperature. The solution was filter sterilised using a 0.44μM filter and aliquoted into 1.5mL eppendorf tube in 1mL volumes. Aliquots were stored at -20°C and diluted according experimental requirements in experiment-specific media.

2.2.2.2 Antibiotic solutions

Solutions of the antibiotics used were prepared as Master Stocks at a volume of 10000mg/L using the formula:

$$W = \frac{1000}{P} \times V \times C$$

where W is the weight (mg) required of antibiotic powder, P is the potency (µg/mg) of the antibiotic powder, V is the desired volume (L) of the antibiotic solution being made and C is the desired concentration (mg/L). Potency of each Lot of each antibiotic was sourced from the manufacturer's website. Antibiotic solutions were prepared in sterile de-ionised water and stored at -20°C and further diluted according experimental requirements and in experiment-specific media.

Table 2-5. Antibiotics used in this study.

Antibiotic	Class
Tobramycin	Aminoglycoside
Ceftazidime hydrate	Cephalosporin
Colistin sulphate salt	Polymyxin
Ciprofloxacin	Fluoroquinolone

2.2.2.3 LL-37

Solutions of LL-37 (Merck/Sigma Aldrich) were prepared to a concentration of 16000mg/L by dissolving 1mg (the pack size) in 62.5µL sterile deionised water and stored at -20°C and further diluted according to experimental requirements in experiment-specific media.

2.2.3 Media, buffers and solutions

Table 2-6. Media and Buffers used in this study.

	Name	Composition	Preparation	Sterilisation
Media	Cetrimide Agar (powder)	Agar (15.0g/L) Cetiltrimethyl-ammonium bromide (0.3g/L) Gelatin peptone (20g/L) Magnesium chloride (1.4g/L) Potassium sulfate (10g/L)	46.7g/L in deionised water	Autoclave
	Lennox LB Broth with Agar (powder)	Agar (15g/L) Sodium chloride (5g/L) Tryptone (10g/L) Yeast Extract (5g/L)	35g/L in deionised water	Autoclave
	Lennox LB Broth (powder)	Sodium chloride (5g/L) Tryptone (10g/L) Yeast Extract (5g/L)	20g/L in deionised water	Autoclave
	Mueller-Hinton Broth (powder)	Beef infusion solids (2.0g/L) Casein hydrolysate (17.5g/L) Starch (1.5g/L)	21g/L in deionised water	Autoclave
	M9 Broth (powder)	Ammonium chloride (1g/L) Disodium hydrogen phosphate (6g/L) Potassium dihydrogen phosphate (3g/L) Sodium chloride (0.5 g/L)	10.5g/L in deionised water	Autoclave
Buffers	Phosphate buffered saline (10x liquid)	1.37M NaCl 0.027M KCl 0.119M phosphates	Diluted 1:10 in deionised water	Autoclave
	HEPES (liquid)	1M N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)	Diluted to 5mM in deionised water	Filter
Others	Glucose (crystals)		Dissolved in deionised water	Filter
	NaCl (crystals)		Dissolved in deionised water	Filter
	$MgSO_4$ (crystals)		Dissolved in deionised water	Filter
	Casein hydrosolate (powder)		Dissolved in deionised water	Filter
	P. aeruginosa lipopolysaccharide (lyophilised powder)		Dissolved in Sterile PBS	

2.2.4 Broth microdilution assays

Overnight *P. aeruginosa* broth culture pellets grown in Mueller-Hinton broth (MHB), were resuspended in fresh sterile MHB in the same volume as the overnight culture. Optical density at 600nm (OD_{600}) was measured and, in a new sterile centrifuge tube, OD_{600} was adjusted to a concentration of 0.1 at a volume specific to the experiment (**Methods 2.2.1**).

Antibiotic Working Solutions were prepared from Master Stocks (**Methods 2.2.2**) in the desired, antibiotic-specific range of concentrations in log2 increments. Initially, solutions were prepared at 2 X the final concentrations desired. Solutions were prepared in sterile MHB using $C_1V_1 = C_2V_2$ with volumes sufficient to carry out all required tests.

Adjusted bacterial cultures, at OD_{600} 0.1, were combined separately with each 2 X antibiotic solution in a 1:1 ratio, in a sterile 1.5mL eppendorf tube, such that the bacterial concentration was now OD_{600} 0.05 and antibiotic is at 1 X concentration. The same was done for bacterial cultures combined with MHB without antibiotic, as untreated culture. Volumes used were experiment-specific but final volume was always ≥700µL to allow for 3 X technical replicates of each experiment, plus excess. Sealed eppendorfs were vortexed for 5secs and 200µL volumes aliquoted to the wells of a sterile, clear, flat-bottomed 96-well plate. Uninoculated MHB was included, also in 3 X 200µL volumes, as sterility control and blank.

2.2.4.1 Minimum inhibitory concentration determination by broth microdilution

Once plates were prepared, they were sealed with a breathable seal to allow gas exchange and placed in an incubator at 37°C with agitation at 200rpm for 18-20hrs. After incubation, wells in the 96-well plate were visually inspected for growth; turbidity of the wells of a given strain at a given concentration of agent indicating resistance, clarity indicating susceptibility.

The MIC of GA against *P. aeruginosa* was tested for against reference strains PAO1, PA14 and PAK. GA was tested in log2 increments in the range 50-1600mg/L. Prepared broth microdilution plates were incubated at 37°C with agitation at 200rpm for 18-20hrs. Following incubation wells of the plates were visually inspected for turbidity.

To further confirm viability of bacteria in turbid wells, 20µL volumes from each well was spotted onto LB agar and LB plates incubated overnight at 37°C and agar plates examined for growth.

2.2.4.1.1 Anaerobic growth

Prepared broth microdilution plates were plate-sealed as before and placed in a sealable container with an AnaeroGen (Oxoid) anaerobic atmosphere generating sachet per manufacturer's instructions; sachet was unsealed and placed in an airtight 2.5L container with culture plate. The container was sealed and placed in an incubator at 37°C with agitation at 200rpm for 18-20hrs. Visual inspections were carried out as before.

2.2.4.2 Growth Curves

Growth curves were prepared using **Methods 2.2.3** for each antibiotic in the desired concentration range. Bacteria-antibiotic suspensions were prepared in volumes allowing for at least 6 wells (containing 200µL each) of 96-well plate. The first set of triplicates (3 X 200µL) were distributed to a 96 well plate. To the remainder of the suspension, GA was added from 50000mg/L stock solution in 1:1000 dilution, allowing for a final concentration of 50mg/L GA without diluting the prepared bacteria-antibiotic suspension. Bacteria-antibiotic-GA suspensions were distributed to triplicate (200µL) wells of the same 96-well plate.

Bacteria in MHB with and without 50mg/L GA were also included on each plate as growth controls, as were uninoculated broths as blanks and sterility controls. After

preparation broth microdilution plates were sealed with breathable, optically clear seals (4titude). Sealed plates were placed in a FLUOstar Omega platereader with temperature set at 37°C. Measurements of OD_{600} were taken at time 0hrs and then every 30mins for 18hrs. Between readings plates were agitated at 200rpm.

2.2.4.3 Growth curve analyses

2.2.4.3.1 Optical density plots

For each plate, the OD_{600} of each well was 'blanked' by subtracting the mean OD_{600} of uninoculated broth wells, at the same timepoint. Blanked results were used to draw XY plots in GraphPad Prism with time on the x-axis and blanked OD_{600} on the y-axis. For each biological experiment, the mean OD_{600} of the 3 technical replicates at each timepoint was plotted with the standard deviation.

2.2.4.3.2 Semilog plots

Prism was used to transform OD_{600} results via natural log ($\ln(OD_{600})$) of each well and mean $\ln(OD_{600})$ of the 3 technical replicates at each timepoint was plotted with their standard deviation for each biological experiment.

2.2.4.3.3 Non-linear fit of optical density plots

Optical density results were fitted via nonlinear regression using the Logistic Growth model for each strain under each treatment. This model is defined by early exponential growth with growth rates slowing as the maximum population is reached and has the equation:

$$Y = \frac{YM \times Y0}{(YM \times Y0)^{(-k \times X)} + Y0}$$

where Y is the population (in OD_{600}), X is time (mins), YM is the maximum population, Y0 is the starting population and k is the rate constant (i.e. the growth rate). Time values X and their corresponding Y values (as technical replicates) were used with Y0 constrained to 0.05, the starting OD_{600} of the growth curves. The Best Fit values of YM, k and xint (OD_{600} doubling time) were derived by Prism. These values were calculated for each biological replicate using the technical replicates.

2.2.4.3.4 $^1/_2$max calculation

For each biological replicate of each strain, the YM derived from Logistic Growth curves (rather than the measured maximal OD_{600}) of untreated growth was used to calculate half the maximal growth level OD_{600}, of that strain under those conditions on that day, by dividing YM by 2.

The resulting OD_{600} of untreated growth was used to interpolate the time taken to reach this OD_{600} by the same strain under treatments, on the same plate, from their previously calculated, fitted growth curves.

2.2.4.3.5 Area Under the Curve

The Area Under the Curve (AUC) was calculated for 18hr growth by Prism for OD_{600} over time using the trapezoid method.

2.2.5 Bacterial viability

Viable colony counts were performed using the Miles-Misra Method[292]. After experimentally relevant incubation and/or treatments, cultures were transferred to 1.5mL eppendorfs in volumes dependent on the experiment. Centrifugation at 15000g for 10mins was carried out and supernatant removed. Pellets were resuspended in the equal volumes of

sterile PBS. Serial dilutions were performed in 1.5mL eppendorfs; 100µL of culture was added to 900µL of sterile PBS, vortexed briefly, 100µL removed and added to further eppendorf with 900µL. This process was repeated to dilutions of 10^{-8}.

Following serial dilution, 20µL volumes were removed from dilution eppendorfs and spotted onto LB agar, ≥5 spots per dilution as technical replicates. Spots were allowed to dry at room temperature followed by incubation at 37°C overnight. Colonies were counted with colony numbers between 2-20 per spot being included for analysis. Means of technical replicates were analysed. Colony forming units per millilitre (CFU/mL) were calculated as

$$= mean\ colony\ count\ \times dilution\ factor\ \times dilution$$

Triplicate technical replicates from growth curve plates were pooled in 1.5mL tubes and centrifuged for 10mins at 15000g. The supernatant was removed and pellet resuspended in an equal volume of sterile PBS. Suspensions were vortexed briefly and serial dilutions and plating performed.

2.2.6 Inhibition Curves

The percentage inhibition resulting from treatments was calculated for biological replicates of CFU/mL results as a percentage of untreated control CFU/mL. Inhibition curves were calculated using Nonlinear Regression analysis with the model [Inhibition] vs response (three parameters) which has the equation:

$$Y\ =\ Bottom + \frac{(Top - Bottom)}{1 + (\frac{X}{IC50})}$$

where X is the concentration of agent being tested and Y is the response in Inhibition. Agent concentrations which resulted in 50% and 90% inhibition were interpolated from these curves as the minimum inhibitory concentration for 50% (MIC_{50}) and the minimum inhibitory concentration for 90% (MIC_{90}), respectively.

2.2.7 Synergy calculations

Synergy between the activities of agents was tested using results of colony counts, as previously described[293]. The Inhibition Effects in CFU/mL caused by agent A (E_A) and agent B (E_B) were used to calculate Expected Effect (E_{Exp}) of combining them at each concentration of A and B tested, where:

$$E_{EXP} = E_A + E_B - (E_A \times E_B)$$

The Observed Effect (E_{OBS}) of the combined treatments from experimentation was compared to the calculated E_{EXP} whereby:

$$E_{OBS} > E_{EXP} \rightarrow Synergy$$

$$E_{OBS} = E_{EXP} \rightarrow Additivity$$

$$E_{OBS} < E_{EXP} \rightarrow Antagonism$$

Synergy was reported when the above criteria were met and the resulting values had non-overlapping 95% confidence intervals.

2.2.8 Genomics

DNA extraction and genomic sequencing were kindly performed by Dr. Laura Nolan at the Quadram Institute, Norwich, using Promega Maxwell™ RSC Cell DNA Purification Kit and the Promega Maxwell RSC 48 Instrument (automated nucleic acid purification). DNA concentration was evaluated using a Qubit dsDNA BR assay kit and Qubit Fluorometer. Sequencing was on a standard Illumina HiSeq platform.

Genomes were assembled with Shovill[294], annotated using Prokka[295,296] with quality control by Quast[297–300] on the Galaxy platform[301].

Multilocus Sequence Types (MLST) gene sequences (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE*) and Sequence Types (ST) of each strain were derived from the assembled genomes using PubMLST (pubmlst.org) database[302]. Minimum spanning tree of ST data for each strain (including known reference strain STs) using allele numbers was generated in PHYLOViZ[303,304].

Resistance genes were identified in the genomes of each clinical strain using the Resistance Gene Identifier (RGI) tool on The Comprehensive Antibiotic Resistance Database (CARD) using Strict and Perfect matches[305].

The sequences for sensor genes *phoQ*, *pmrB*, *cprS* and *parS* from PAO1 were taken from https://pseudomonas.com and extracted from genomic data 11 clinical *P. aeruginosa* using the MyDbFinder tool (https://cge.cbs.dtu.dk/services/MyDbFinder/).

Gene sequences of clinical strains were aligned using MEGA-X[306] and translated to aligned protein sequences from which amino acid changes were identified.

2.2.9 1-*N*-phenylnaphthylamine assay for outer bacterial membrane integrity

Disruption of the outer bacterial membrane was measured using the fluorescent probe 1-*N*-phenylnaphthylamine (NPN)[224] (Merck/Sigma-Aldrich). Solutions of NPN were prepared by dissolving 11mg NPN crystals in 10mL ethanol for a solution of 5mM and further diluted to 40µM in HEPES buffer as a working solution. Late-exponential phase cultures were washed twice and adjusted to an OD_{600} of 0.5 in 5mM HEPES buffer. Agents to be tested were prepared to 4 X the required concentration in 5mM HEPES buffer.

In the wells of a black 96-well microtitre plate (Perkin-Elmer) buffer, NPN, bacteria and treatments were combined in proportions according to the **Table 2-7**. Final volume of all wells was 200µL.

Table 2-7. Required wells and their combinations of reagents-bacteria-agent included on every plate to measure and calculate NPN Uptake Factors. All suspensions were prepared in 5mM HEPES buffer.

Blank Well	200µL 5mM HEPES			
Blank Well	150µL 5mM HEPES	50µL 40mM NPN		
Blank Well	150µL 5mM HEPES			50µL Test Agent
Blank Well	100µL 5mM HEPES	50µL 40mM NPN		50µL Test Agent
Control Well	100µL 5mM HEPES		100µL Adjusted bacteria	
Control Well	50µL 5mM HEPES	50µL 40mM NPN	100µL Adjusted bacteria	
Test Well	50µL 5mM HEPES		100µL Adjusted bacteria	50µL Test Agent
Test Well		50µL 40mM NPN	100µL Adjusted bacteria	50µL Test Agent

Fluorescence was measured in a platereader at excitation 355nm, emission 460nm every 30secs for 15mins. Blank wells were meaned and used to calculate NPN Uptake Factor for control and test wells using:

$$\frac{Fluorescence\ of\ Sample\ with\ NPN - Fluorescence\ of\ Sample\ without\ NPN}{Fluorescence\ of\ Buffer\ with\ NPN - Fluorescence\ of\ Buffer\ without\ NPN}$$

For all experimental iterations, triplicate wells of each condition and blank were measured.

For comparisons of cationic agents treatments were prepared in HEPES buffer at the following concentrations for addition to the relevant wells of 96-well black microtitre plate, 50µL added for 1:4 dilution:

Table 2-8. Concentrations of working solutions of treatments prepared and their final concentrations in the wells of 96-well black microtitre plate after combination with other reagents and/or bacteria.

Working Solution	Final Concentration
25mg/L GA	6.25mg/L GA
50mg/L GA	12.5mg/L GA
100mg/L GA	25mg/L GA
200mg/L GA	50mg/L GA
400mg/L GA	100mg/L GA
8mg/L CST	2mg/L CST
64mg/L LL-37	16mg/L LL-37
16mg/L TOB	4mg/L TOB
200mg/L GA + 16mg/L TOB	50mg/L GA + 4mg/L TOB

Working solutions were prepared from previously prepared Master Stocks of each agent (**Methods 2.2.2**).

To test the effect of Mg^{++} divalent cations on GA activity, the assay was carried out as previously but with all solutions (**Table 2.7**) prepared in 5mM HEPES buffer with $MgSO_4$ at either 0.5mM or 1mM. Buffer with 1mM NaCl was used a monovalent salt control/comparator.

P. aeruginosa LPS (Merck/Sigma Aldrich) was used at physiologically relevant concentrations (0.01-0.02mg/mL)[36–38] and a supra-physiological concentration (0.1mg/mL).

To test GA-LPS pre-incubations*, P. aeruginosa* LPS and GA were incubated together in 1.5mL eppendorfs at 4 X final concentrations in HEPES buffer in experiment-specific volumes according to:

Table 2-9. Working solutions prepared for pre-incubation of GA with P. aeruginosa LPS for administration in NPN assays.

Working Solution	Final Concentration
0.04mg/mL LPS	0.01mg/mL LPS
0.04mg/mL LPS + 200mg/L GA	0.01mg/mL LPS + 50mg/L GA
0.08mg/mL LPS	0.02mg/mL LPS
0.08mg/mL LPS + 200mg/L GA	0.02mg/mL LPS + 50mg/L GA
0.4mg/mL LPS	0.1mg/mL LPS
0.4mg/mL LPS + 200mg/L GA	0.1mg/mL LPS + 50mg/L GA

Working solutions were incubated at 37°C for 30mins before 50µL was added to appropriate wells with the remaining assay components, as per **Table 2.7** Measurements and data analysis were performed as before.

To test background LPS, bacterial suspensions and NPN solutions were prepared in 5mM HEPES buffer containing:

Table 2-10. Working solutions prepared for background of P. aeruginosa LPS for administration in NPN assays.

Working Solution	Final Concentration
0.013mg/mL LPS	0.01mg/mL LPS
0.026mg/mL LPS	0.02mg/mL LPS
0.13mg/mL LPS	0.1mg/mL LPS

Suspensions were distributed to wells as per **Table 2.7**. GA solutions were prepared to a concentration of 200mg/L in 5mM HEPES without LPS in order to prevent any pre-binding of LPS-GA and added in 50µL to appropriate wells.

eDNA was tested at the physiologically appropriate concentration of 1mg/mL[307]. eDNA and GA were incubated together in 1.5mL eppendorfs at 4 X final concentrations in HEPES buffer in experiment-specific volumes according to:

Table 2-11. Working solutions prepared for pre-incubation of GA with eDNA for administration in NPN assays.

Working Solution	Final Concentration
4mg/mL DNA	1mg/mL DNA
4mg/mL LPS + 200mg/L GA	1mg/mL LPS + 50mg/L GA

Working solutions were incubated at 37°C for 30mins before 50µL was added to appropriate wells with the remaining assay components.

2.2.10 3,3'-dipropylthiadicarbocyanine iodide assay for cytoplasmic membrane polarisation

Polarisation of the cytoplasmic membranes was measured using the fluorescent probe 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$)[308] (ThermoScientific). A 0.1M solution was prepared by dissolving 100mg in 1.83mL DMSO. Late-exponential phase cultures were washed twice and adjusted to an OD_{600} of 0.05 in 5mM HEPES-20mM glucose. Agents to be tested were prepared to 20 X target concentrations in buffer.

Dye was added to adjusted bacterial cultures in a 1:1000 dilution for a final $DiSC_3(5)$ concentration of 1µM. Similarly, dye was added to buffer as control. Suspensions were

aliquoted to wells of a black 96-well microtitre plate in 200µL volumes. Fluorescent signal of $DiSC_3(5)$ was allowed to quench in the dark for 30mins, by which point the fluorescent signal became stable.

After quenching, agents were added in 10µL volumes with 10µL of buffer added to control wells. Fluorescence was measured in a platereader at excitation 544nm, emission 620nm every 30secs for 1hr.

For all experimental iterations, triplicate wells of each condition and blank were measured.

For comparisons of cationic agents, treatments were prepared in HEPES-glucose buffer at the following concentrations for addition to the relevant wells of 96-well plate:

Table 2-12. Concentrations of working solutions of treatments prepared and their final concentrations in the wells of 96-well black microtitre plate after combination with other reagents and/or bacteria.

Working Solution	Final Concentration
125mg/L GA	6.25mg/L GA
250mg/L GA	12.5mg/L GA
500mg/L GA	25mg/L GA
1,000mg/L GA	50mg/L GA
2,000mg/L GA	100mg/L GA
160mg/L CST	2mg/L CST
320mg/L LL-37	16mg/L LL-37
80mg/L TOB	4mg/L TOB
1,000mg/L GA + 80mg/L TOB	50mg/L GA + 4mg/L TOB

Working solutions were prepared from previously prepared Master Stocks of each agent (**Methods 2.2.2**).

To test the effect of Mg^{++} divalent cations on GA activity, the assay was carried out as previously but with all solutions prepared in 5mM HEPES buffer with $MgSO_4$ at either 0.5mM or 1mM. Buffer with 1mM NaCl was used a monovalent salt control/comparator. From GA Working Solutions at 1000mg/L, 10µL was added to appropriate wells. For untreated samples, 10µL buffer was added and assay proceeded as previously.

To test GA-LPS pre-incubation*, P. aeruginosa* LPS and GA were incubated together in 1.5mL eppendorfs at 20 X final concentrations in HEPES-glucose in experiment-specific volumes according to:

Table 2-13. Working solutions prepared for pre-incubation of GA with P. aeruginosa LPS for administration in $DiSC_3(5)$ assays.

Working Solution	Final Concentration
0.2mg/mL LPS	0.01mg/mL LPS
0.2mg/mL LPS + 1000mg/L GA	0.01mg/mL LPS + 50mg/L GA
1mg/mL LPS	0.02mg/mL LPS
1mg/mL LPS + 1000mg/L GA	0.02mg/mL LPS + 50mg/L GA
2mg/mL LPS	0.1mg/mL LPS
2mg/mL LPS +1000mg/L GA	0.1mg/mL LPS + 50mg/L GA

After quenching of $DiSC_3(5)$ fluorescent signal as previously, Working Solutions were added to appropriate wells in 10µL volumes, including wells containing buffer without bacteria, and assay proceeded as previously.

To test background LPS, bacterial suspensions were prepared in 5mM HEPES buffer and *P. aeruginosa* LPS added to concentrations of 0.01, 0.02 and 0.1mg/mL. $DiSC_3(5)$ was added and solutions aliquoted as previously before solutions were allowed to quench for 30mins in the dark. From 1000mg/L GA working solutions, 10µL volumes were added to appropriate wells and assay proceeded as previously.

P. aeruginosa eDNA and GA were incubated together in 1.5mL eppendorfs at 20 X final concentrations HEPES-glucose buffer in experiment-specific volumes according to:

Table 2-14. Working solutions prepared for pre-incubation of GA with eDNA for administration in $DiSC_3(5)$ assays.

Working Solution	Final Concentration
20mg/mL DNA	1mg/mL DNA
20mg/mL LPS + 1000mg/L GA	1mg/mL LPS + 50mg/L GA

After quenching of $DiSC_3(5)$ fluorescent signal as previously, Working Solutions were added to appropriate wells in 10µL volumes, including wells containing buffer without bacteria, and assay proceeded as previously.

2.2.11 Propidium iodide assay for bacterial membrane permeability

Permeabilisation of the bacterial membranes was measured using the fluorescent dye propidium iodide (PI) (Merck/Sigma-Aldrich) as per manufacturer's instructions. Solutions of PI (1mg/mL) were allowed to thaw at room temperature for 30mins. Late-exponential phase bacterial cultures were washed twice and adjusted to an OD_{600} of 0.5 in PBS. Agents to be tested were prepared at 1000 X target concentrations in PBS.

Propidium iodide was added to the bacterial cultures to a in a 1:1000 dilution to a concentration of 1µg/mL. Agents were added to the bacteria-PI suspensions in a 1:1000 dilution to their individual target concentrations. Buffer-PI solutions with/without agents to be tested were also prepared as controls/blanks.

Suspensions were aliquoted to the wells of a black 96-well microtitre plate in 200µL volumes. Fluorescence was measured in a platereader at excitation 544nm, emission 610nm every 30secs for 1hr. Means of appropriate blank wells were subtracted from test wells.

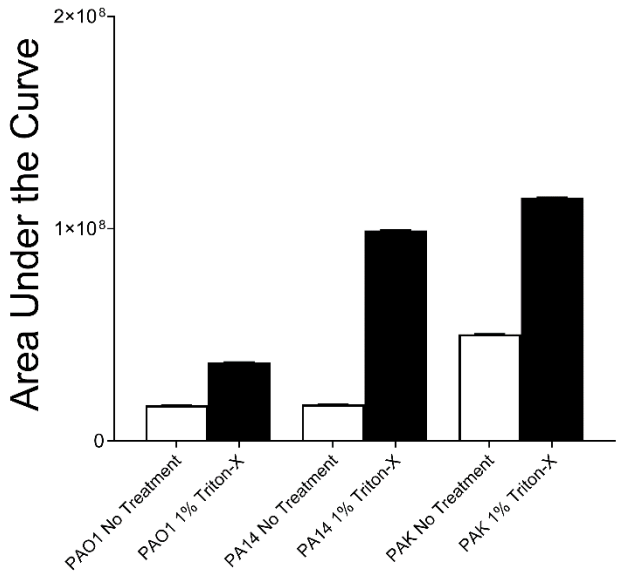


Figure 2-1. Area Under the Curve of propidium iodide signal from P. aeruginosa strains PAO1, PA14 and PAK during treatment with 1% Triton-X.

For all experimental iterations, triplicate wells of each condition and blank were measured.

For comparisons of cationic agents, treatments were prepared in PBS at the following concentrations:

Table 2-15. Concentrations of working solutions of treatments prepared and their final concentrations in the wells of 96-well black microtitre plate after combination with other reagents and/or bacteria.

Working Solution	Final Concentration
6,250mg/L GA	6.25mg/L GA
12,500mg/L GA	12.5mg/L GA
25,000mg/L GA	25mg/L GA
50,000mg/L GA	50mg/L GA
2,000mg/L CST	2mg/L CST
16,000mg/L LL-37	16mg/L LL-37
4,000mg/L TOB	4mg/L TOB

Working solutions were prepared from previously prepared Master Stocks of each agent (**Methods 2.2.2**).

To test the effect of Mg^{++} divalent cations on GA activity, the assay was carried out as previously but with all solutions prepared in 5mM HEPES buffer with $MgSO_4$ at either 0.5mM or 1mM. Buffer with 1mM NaCl was used a monovalent salt control/comparator. From GA Working Solutions at 50000mg/L, 1µL was added, as before. For untreated samples, 1µL buffer was added and assay proceeded as previously.

To test GA-LPS pre-incubation*, P. aeruginosa* LPS and GA were incubated together in 1.5mL eppendorfs at 100 X final concentrations in experiment-specific volumes according to:

Table 2-16. Working solutions prepared for pre-incubation of GA with P. aeruginosa LPS for administration in PI assays.

Working Solution	Final Concentration
1mg/L LPS	0.01mg/mL LPS
1mg/L LPS + 5000mg/L GA	0.01mg/mL LPS + 50mg/L GA
2mg/L LPS	0.02mg/mL LPS
2mg/L LPS + 5000mg/L GA	0.02mg/mL LPS + 50mg/L GA
10mg/L LPS	0.1mg/mL LPS
10mg/L LPS + 5000mg/L GA	0.1mg/mL LPS + 50mg/L GA

Working solutions were diluted 1:100 into adjusted bacterial-PI suspensions. Samples were vortexed briefly and distributed to the wells of a black 96-well plate as previously.

To test background LPS, bacterial suspensions were prepared in PBS and *P. aeruginosa* LPS added to concentrations of 0.01, 0.02 and 0.1mg/mL. PI was added and solutions aliquoted as previously before solutions were allowed to quench for 30mins in the dark. From 50,000mg/L GA working solutions, 10µL volumes were added, as before. For untreated samples, 1µL buffer was added and assay proceeded as previously.

2.2.12 Endotoxin Unit measurement from pre-incubated LPS-GA

Endotoxin Units were measured using a Pierce™ Chromogenic Endotoxin Quant Kit, per manufacturer's instructions. The provided *E. coli* Endotoxin Standard was reconstituted to a concentration of 10EU/mL in the provided Endotoxin-free water and diluted to Standard concentrations of 1, 0.5, 0.25 and 0.1EU/mL. Lyophilized Amebocyte Lysate and Chromogenic Substrate were also reconstituted as directed in Endotoxin-free water. In the wells of a 96-well plate, 50µL of Endotoxin Standard dilutions, blank, and samples was added per well. Amebocyte Lysate Reagent was added, 50µL per well and samples mixed by gently tapping 10 times on the side of the plate. The plate was incubated for 10mins at 37°C. In 100µL volumes, Chromogenic Substrate Solution was added to each well and samples mixed by

gently tapping 10 times on the side of the plate. Plates was incubated 37°C for 6mins. Stop Solution (25% acetic acid) was added in 50µL volumes to each well and samples mixed by gently tapping 10 times on the side of the plate. The optical density was read at 405nm OD_{405}.

Blanked Standards were fitted using simple linear regression (GraphPad Prism) to create a standard curve. Concentrations of samples were interpolated from the standard curve via their blanked OD_{405} results.

P. aeruginosa LPS was incubated at 37°C for 30mins, with and without 50mg/L GA, as was GA without LPS. Samples were diluted 1:10000, to be within the sensitivity range of the kit, before addition to the remaining kit components with triplicate wells for each.

2.2.13 Stimulation of LPS modification

P. aeruginosa were grown overnight in 5mL MHB in 50mL tubes. Bacteria were pelleted at 3500g for 15mins , supernatant removed and pellet resuspended in fresh MHB. The OD_{600} was measured and, in a new sterile 50mL tube, adjusted to OD_{600} 0.05 in MHB. The stimulant to be tested was added and the tube incubated at 37°C with agitation at 200rpm for ~4hrs to allow bacteria to reach mid-log phase.

Reference strains were incubated with No Treatment, 50mg/L GA, 0.5mg/L CST or 16mg/L LL-37. Clinical strains were incubated with No Treatment or 50mg/L GA. Biological replicates of each strain at each condition were performed with the exception of LL-37, which was performed in duplicate due to resource limitation.

After incubation, OD_{600} was measured and ~1 X 10^8 CFU were harvested and placed in a 1.5mL eppendorf and centrifuged at 15000g for 10mins. The supernatant was removed and the pellet was used for RNA extraction (**Methods 2.2.14**) and qRT-PCR (**Methods 2.2.15**). The remaining culture in the 50mL tube was centrifuged at 3500g for 15mins, supernatant

removed and pellet resuspended in 1mL PBS. This cell suspension was transferred to a 1.5mL eppendorf and heat treated at 80°C in a heating block for 1hr. Heat killed cell suspensions were stored at -80°C for MALDI-TOF analysis (**Methods 2.2.16**).

2.2.14 RNA Extraction

Bacterial RNA was extracted from ~1 X 10^8 CFU using Direct-zol RNA Miniprep kit (Zymo) as per manufacturer's instructions. The bacterial pellet was resuspended in 300µL TRI reagent and lysed by mixing with a pipette. An equal volume of 95-100% ethanol was added, mixed and total volume transferred to spin column and centrifuged at 15000g for 30secs. Flow through was discarded and column washed with 400µL RNA Wash Buffer. Column was treated with DNase for 15mins at room temperature. The column was washed with 400µL RNA PreWash and centrifuged at 15000g for 30secs, followed by two washes with 700µL RNA Wash Buffer. The column was transferred to a sterile 1.5mL eppendorf and RNA eluted in 50µL of the provided DNA/RNA-Free water by centrifugation at 15000g for 30secs. RNA was stored at -20°C.

2.2.15 Quantitative Real-Time PCR with reverse transcription

2.2.15.1 Primers

Primers from Lee et al, 2014[159], were used (**Table 2-17**) and were sourced from ThermoFisher Scientific in 100µM concentrations and diluted to 10µM working stocks.

Table 2-17. Primers used in this study, from Lee et al, 2014[159].

Gene	Primer (F/R)	Amplicon size
rpsL	GTGGTGAAGGTCACAACCTG / CCTGCTTACGGTCTTTGACA	135
phoP	TCTACCGGGTCAGCGAATAC / GATCAGGATCGGGAAGGACT	122
pmrA	CACCAGGTGACCCTGTCC / CGTAGAGGCTCTGCTCCAGT	124
cprR	CGCCTGGAAGATCCTTGAGT / CACGTTGAGGGTGTTGCTTT	115
parR	AGAATGGTCTGCAGGTGTGC / CGGCTTGATCACGTAGTCGT	134
arnB	GTTCGTCAGCGACGACAGT / AAACCGGGCTCGATAACTTC	129

2.2.15.2 PCR MasterMix

The KAPA SYBR FAST One-Step kit (KAPA) was used to perform quantitative Real-Time PCR to assess gene expression levels of target genes, as per manufacturer's instructions. The kit encompasses cDNA production from RNA by reverse transcription (RT), polymerase chain reaction (PCR) and SYBR Green fluorescent dye to measure amplification. The provided ROX Low fluorescent dye was also included per manufacturer's instructions, providing a stable, non-reactive baseline for fluorescence measurement.

Reactions were carried out in 20µL volumes:

Table 2-18. qRT-PCR MasterMix.

Reagent	Volume	Final Concentration
MasterMix (10X)	10µL	1X
dUTPs (10mM)	0.4µL	0.2mM
Forward Primer (10µM)	0.4µL	200nM
Reverse Primer (10µM)	0.4µL	200nM
RT Mix (50X)	0.4µL	1X
ROX Low (50X)	0.4µL	1X
PCR-grade water	3µL	
Template RNA	5µL	1pg – 100ng

PCR reagents were allowed to thaw on ice and individual MasterMixes were prepared for each target gene to be tested in the volumes required by scaling up from **Table 2-18**. Three technical replicate wells of each gene for each strain were included.

MasterMix was distributed to the wells of a MicroAmp Fast Optical 96-Well Reaction Plate in 15µL volumes, followed by addition of samples and controls in 5µL volumes.

2.2.15.3 Template RNA

Purified RNA was diluted 1:5 with DNA/RNA-Free water immediately after extraction. Where possible qRT-PCR was carried out on the same day as stimulation/extraction, otherwise stored at -20°C.

2.2.15.4 Control RNA

Five RNA extractions were carried out on *P. aeruginosa* strain PAO1 grown for 4hrs in MHB. These extractions were pooled, diluted 1:5 in DNA-RNA-Free water and aliquoted in 20µL volumes and stored at -20°C. For every qRT-PCR plate which was run, one of these aliquots was thawed on ice and included as the template for amplification of the housekeeping gene *rpsL* (triplicate wells) as a positive control.

2.2.15.5 No Template Control

Each reaction plate also included triplicate wells of *rspL* MasterMix using PCR-grade water as template, as a negative control.

2.2.15.6 Cycling Conditions

Plates were sealed with optically clear MicroAmp PCR seals and run on a QuantStudio 7 Flex (ThermoFisher) real time thermocycler under the following conditions:

Table 2-19. qRT-PCR cycling conditions.

	Temperature	Time	Cycles
Reverse Transcription	42°C	5mins	1
Enzyme Activation	95°C	3mins	1
Denaturation	95°C	10secs	40
Annealing/Extension	60°C	300secs	

2.2.15.7 Data Analysis

The QuantStudio analysis software was used to generate the cycle threshold (Ct) for each well using the fluorescence of SYBR green during cycling.

Relative expression of each gene (ΔCt) was calculated in comparison with the expression of housekeeping gene *rpsL* under the same condition, as follows:

$$\Delta\mathrm{Ct} = Mean\ Ct\ value\ of\ rpsL\ wells - Ct\ value\ of\ test\ gene$$

followed by:

$$2^{-\Delta\mathrm{Ct}}$$

Technical replicates for each gene were calculated and meaned for each plate.

Similarly, expression of each gene under different conditions was assessed relative to the same gene in an untreated control (ΔΔCt).

$$\Delta\Delta\mathrm{Ct} = Mean\ \Delta Ct\ gene\ under\ control\ conditions - \Delta Ct\ of\ gene\ under\ stimulation$$

followed by:

$$2^{-\Delta\Delta\mathrm{Ct}}$$

Technical replicates were again calculated and meaned.

2.2.16 MALDI-TOF

MALDI-TOF processing was kindly carried out by Dr. Gerald Larrouy-Maumus and Jade Pizzato using Dr. Larrouy-Maumus' technique[178]. Briefly, heat killed bacterial suspensions from **Methods 2.2.13** were centrifuged at 17000g for 2mins, supernatant discarded and the pellet was washed three times with 300µl of ultrapure water and resuspended to a density of McFarland 20 as measured using a McFarland Tube Densitometer. A volume of 0.4µl of this suspension was loaded onto the MALDI target plate overlaid with 1.2µl of Norharmane matrix (Sigma-Aldrich) solubilised in chloroform/methanol (90:10 v/v) to a final concentration of 10mg/mL.

The samples were loaded onto a disposable MSP 96 target polished steel BC (Bruker Part-No. 8280800). The bacterial suspension and matrix were mixed directly on the target by pipetting. The spectra were recorded in the linear negative-ion mode (laser intensity 95%, ion source 1 = 10.00kV, ion source 2 = 8.98kV, lens = 3.00kV, detector voltage = 2652V, pulsed ion extraction = 150 ns). Each spectrum corresponded to ion accumulation of 5,000 laser shots

randomly distributed on the spot. The spectra obtained were processed with default parameters using FlexAnalysis v.3.4 software (Bruker Daltonik, Germany).

For each replicate of strain under each condition, the level of L-Ara4N addition to Lipid A was calculated using the Polymyxin Resistance Ratio (PRR) which is the Abundance of Unmodified Lipid A:Abundance of Modified Lipid. The abundance (Area Under the Curve) of unmodified Lipid A was the sum of intensities of native Lipid A (mass-to-charge-ratio [*m*/*z*] at 1404, 1442 and 1462). The abundance of modified Lipid A was the sum of intensities of Lipid A with L-Ara4N attached (*m*/*z* at 1577, 1593, 1708, 1724, 1748, 1763, 1816, 1832, 1879, 1894, 1947 and 1962). Biological replicates were compared.

2.2.17 Biofilm Assay

Biofilm assays were carried out using the crystal violet microtitre plate method of O'Toole, 2010[309], with modifications. The medium used was sterile M9 (**Table 2-20**), supplemented with the following;

Table 2-20. Components of supplements added to M9 in crystal violet biofilm assay from O'Toole, 2010[309].

	Working Solution	Final Concentration
$MgSO_4$	1M	1mM
Glucose	20%	0.2%
Casein hydrosolate	20%	0.5%

Supplements were added to M9 to their Final Concentrations and medium was filter sterilised. *P. aeruginosa* strains were inoculated into 5mL sterilised supplemented M9 by taking a single colony from solid media with a sterile inoculating loop in a 15mL tube. Bacteria were grown overnight at 37°C with agitation at 200rpm.

Bacteria were harvested by centrifugation (3500g for 15mins), the supernatant discarded and pellet resuspended in fresh sterile media. The OD_{600} was measured and the culture adjusted to an OD_{600} of 0.1, as previously (**Methods 2.2.1**). Adjusted cultures were aliquoted to wells of a flat-bottom, clear, sterile, untreated 96-well plate in 100µL volumes with 6 X wells per condition to be tested. Plates were sealed with a breathable seal and incubated statically at 37°C for the required duration.

Once incubation and/or treatments were completed, 96-well plate was submerged in a tub of water to remove unattached cells and plate was blotted onto paper towels. This was repeated. Crystal violet at 0.1% was added to each well in 125µL volumes and incubated at room temperature for 15mins. The plate was washed X 3 times by submerging into a tub of water and blotting onto paper towels. Plates were allowed to dry completely at room temperature. The crystal violet was solubilised from the wells with 95-99% ethanol in 200µL volumes and incubated for 15mins at room temperature. The optical density of the crystal violet was measured at 550nm (OD_{550}). Technical replicate wells were meaned.

2.2.17.1 GA pre-treatment

Adjusted cultures of each strain were incubated with 50mg/L GA for 1hr at 37°C with agitation in 1.5mL eppendorfs. Following incubation, bacteria were pelleted by centrifugation (15000g for 10mins), supernatant removed and pellet resuspend in an equal volume of fresh, sterile media. After vortexing briefly (5secs), cultures were added to the wells of a 96-well plate and incubated statically for the required amount of time.

2.2.17.2 GA treatment of forming biofilms

GA was added to adjusted cultures at 50mg/L and suspensions added directly to the biofilm assay plate and incubated statically for the required amount of time.

2.2.17.3 GA treatment of mature biofilms

After incubation for 24hrs, media was removed from wells without disturbing attached bacteria and replaced with media containing 50mg/L GA at an equal volume. Plates were re-sealed and returned to the 37°C incubator for a further hour.

For 48hr biofilms, media was carefully removed from wells after 24hrs and replaced with fresh media and plate re-sealed and returned to 37°C incubator for a further 24hrs before proceeding as above.

Anaerobic incubation of biofilm plates was performed using AnaeroGen gas packs.

2.2.18 DNA Extraction

Bacterial DNA was extracted from ~1 X 10^8 CFU using NucleoSpin Microbial DNA Mini kit (Macherey-Nagel) as per manufacturer's instructions. The bacterial pellet was resuspended in 100µL BE buffer. The resuspended sample was transferred to a provided MN Bead Tube. Buffer MG (40µL) was added, followed by 10µL Proteinase K and tube sealed. The tube was agitated at 3000rpm for 10mins on a MixMate (Eppendorf). The bead tube was centrifuged for 30secs at 11000g to bring all materials to the bottom of the tube. Buffer MG was added in a volume of 600µL and tube vortexed briefly, followed by centrifugation at 11000g for 30secs. Approximately 500-600µL of supernatant was transferred to a NucleoSpin Microbial DNA column, without disturbing the pellet. The NucleoSpin tube was centrifuged at 11000g for 30secs and column transferred to a fresh collection tube. The column was washed by addition of 500µL Buffer BW and centrifugation at 11000g for 30secs and flowthrough discarded. This wash step was repeated. The column was further centrifuged at 11000g for 30secs to remove residual wash buffer. DNA was eluted by placing the spin column in a fresh nuclease-free 1.5mL tube and addition of 100µL of elution buffer BE. Sample was incubated

at room temperature for 1min followed by centrifugation at 11000g for 30secs. Purified DNA was stored at -20°C. DNA from each strain was eluted in 50µL volumes and pooled together for use as eDNA. Concentration of eDNA was measured on a NanoDrop before appropriate dilution.

2.2.19 Gel retardation assay

To assess direct binding, eDNA and GA were incubated together in a checkerboard with the eDNA concentrations 1, 10 and 50mg/mL and GA in the range 6.25-100mg/L, in 10µL volumes in 0.5mL tubes and incubated at 37°C for 30mins.

Five microlitres of each sample was combined with 1µL 5 X loading dye and loaded onto a 1% agarose gel containing SYBER Safe. The gel was run for 1hr at 80V before visualization in a BioRad ChemiDoc MP.

2.2.20 Cell culture

Cell culture was performed with the kind help of Dr. Matthew Coates using his cell stocks. Immortalised bronchial epithelial cells CFBE41o- expressing either Wild Type or Phe508del CFTR were gown in minimal essential medium (MEM) with phenol red and L-glutamine supplemented with 10% fetal bovine serum (FBS). Cells were detached from growth flask by treatment with 1 X trypsin in PBS (final volume 10mL) for 5mins at 5% CO_2. PBS was removed at replaced with 20mL supplemented MEM, to neutralise trypsin. Media containing detached cells was transferred to a 50mL tube and centrifuged at 1200rpm for 3mins. Supernatant was removed and replaced with 5mL fresh media.

Cells were counted using a haemocytometer and, in a fresh tube, adjusted to $3x10^4$ cells/well for 100µl per well, in the same media. Adjusted cell cultures were aliquoted to the wells of a 96-well plated with 3 X wells per condition to be tested, as technical replicates.

Plates were incubated overnight at 37°C, 5% CO_2. The day before stimulation, media was replaced on cells with MEM without supplementation (starvation media) and returned to 37°C, 5% CO_2 overnight.

After incubation, media was once again removed and replaced with 100µL serum free MEM containing either TNFα (10ng/mL), GA (6.25-100mg/L) or CST (2mg/L); triplicate wells of each conditions were tested for each cell type. Plates were returned to 37°C at 5% CO_2 for a further 24hrs. The following day, cell supernatants were harvested in 50µL volumes into 2 X new 96-well plates; one for IL-6 measurement, one for IL-8 measurement. These aliquot plates were stored at -20°C until ELISAs were performed. Three biological repeats were performed.

2.2.21 Enzyme-linked Immunosorbent Assays

Cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) were measured using sandwich ELISA kits IL-6 DuoSet and CXLC8 DuoSet (R&D Systems). All assays were carried out in 96-well flat bottom high binding ELISA plates (Corning).

The day before the ELISA was to be run, capture antibodies for each ELISA were reconstituted in Reagent Diluent (R&D Systems) and added to appropriate wells of the 96-well plate and incubated overnight at room temperature. In PBS, mouse anti-human IL-6 capture antibody was diluted in 1 ml (for 360 µg/ml) and CXCL8 capture antibody was diluted in 500 µl (for 480 µg/ml), per manufacturer's instructions. After overnight incubation, capture antibody was aspirated and wells washed three times with 200µl of Wash Buffer (0.05% [v/v] Tween® 20 in PBS). Wells were blocked by addition of Reagent Diluent in 100µl volumes and incubated at room temperature for 1hour.

On the day of the ELISA, standard solutions of the provided IL-6 and CXCL8 were reconstituted and serially diluted in 2 X increments in reagent diluent, for a seven point standard curve. For IL-6, a solution of 60pg/mL was prepared and diluted by 50:50 mixtures down to 9.375pg/mL. For CXCL8, a solution of 2000pg/mL was prepared and diluted by 50:50 mixtures down to 31.25pg/mL. In both cases reagent diluent was used as a blank. After blocking was completed, wells were washed 3 X with Wash Buffer. Duplicates of each concentration of standard were added to wells in 100µl volumes. Samples were also added to required wells. Plates were incubated at room temperature for 2hours. Samples and standards were aspirated and wells 3 X washed with Wash Buffer.

Detection antibody for each cytokine was reconstituted in Reagent Diluent, IL-6 to a concentration of 50ng/mL and CXCL8 to 20ng/mL, as per kit instructions. Detection antibodies were added to wells in 200µl volumes and incubated at room temperature for 2hours. Detection antibody were aspirated and wells 3 X washed with Wash Buffer.

Streptavidin horse radish peroxidase (HRP) was diluted at 1:200 in PBS, 100µl was added per well and incubated at room temperature in the dark for 20 minutes. TMB (3, 3', 5, 5'-Tetramethybenzidine) substrate solution and hydrogen peroxide (Colour Reagent A and Colour Reagent B) were removed from the fridge to equilibrate to room temperature. Streptavidin HRP was aspirated and all wells were washed three times with 200µl of Wash Buffer. Substrate reagents A and B were mixed together at a 1:1 ratio just before use, 100µl of mixture was added per well and incubated in the dark for 15mins. the reaction was quenched by the addition of 40 µl of 2N sulphuric acid (H_2SO_4). Immediately after quenching, the OD of each well was measured at 450nm and 540nm in a platereader (FLUOstar Omega).

For each well, OD_{450} results were subtracted from OD_{540} results (correction for plate imperfections) and mean of blank wells subtracted from all sample and standard wells. Standard curve was prepared by plotting the measured results of the standards for each plate performed and fitting using Four Parameter Logistic fit (GraphPad Prism). Sample OD results were interpolated from the standard curve.

Cytokine IL-6 and IL-8 were measured from harvested cell supernatants after treatment with AMPs GA (6.25-100mg/L) or CST (2mg/L) or positive control TNFα (10ng/mL).

2.2.22 Statistical Analyses and data presentation

All analyses performed were non-parametric. Unpaired comparisons of two datasets were performed using Wilcoxon signed rank test and of multiple datasets using analysis of variation (ANOVA) Kruskal-Wallis method, with Dunn's multiple correction (adjusted p values reported). Paired comparisons of two datasets were performed using Mann-Whitney U test and of multiple datasets using analysis of variation (ANOVA) Friedman test method, with Dunn's multiple correction (adjusted p values reported). A significant difference was reported with $p<0.05$. The Area Under the Curve was computed using the trapezoid rule.

Data displayed as biological replicates with medians and 95% confidence intervals shown, unless otherwise stated. Biological replicates comprise means of technical replicates.

All analyses and data presentation were performed in GraphPad Prism version 9.0 or later.

3. Combinations of glatiramer acetate with antibiotics against reference *Pseudomonas aeruginosa* strains

3.1 Introduction

The antibiotic resistance crisis requires urgent action to address the decrease in efficacy of antibiotics due to over-use, non-specific use and poor stewardship. Globally, 1.25million deaths in 2019 were directly attributable to antibiotic resistance infections[310] and in USA in 2017, antibiotic resistance was calculated to have cost 1.9billion dollars[311]. Unless addressed, the crisis is predicted to result in 50million deaths per year by 2050[253]. The dearth of investment in new antimicrobials requires new strategies to be employed.

One of the proposed strategies is the deployment of antimicrobial peptides; the ubiquitous short defence peptides found across all kingdoms of life as part of immune response to infection[216,312]. AMPs, components of innate immunity across the tree of life[203], are commonly cationic and amphipathic and are antimicrobial via these properties; the positive charge allowing binding to negatively charged elements of bacterial membranes followed by insertion and disruption of membranes via hydrophobic interactions[201,202,205]. After binding and insertion AMPs act via 3 mechanisms (i) the detergent-like Carpet model where localised blebbing and sloughing of the membrane results in membrane thinning and weakening (ii) the formation of pores with the Barrel-Stave model where associated peptides insert into the membrane and (iii) the formation of the pores with the Toroidal pore model where peptides associate with the membrane to form pores and the membrane hydrophilic head groups are turned inwards[201,202,205].

Despite investigation for decades, few AMPs are currently in clinical use, primarily to cytotoxicity issues[313–316] at antibacterial concentrations, but the polymyxin antibiotic colistin is the best known, studied and used AMP, including as an inhaled therapy in CF[63,192,197,210,211,317]. One of the reasons for the continued interest in AMPs, in spite of the

relative lack of success in getting them into clinical use , is due to their mechanism of action; the bacterial cell wall is a significant barrier to antibiotic activity and the desire to find "permeabilizers" of bacteria is a long standing one[172,318].

Another route to address antibiotic resistance, and for which AMPs have shown promise, is the restoration of the efficacy of existing antibiotics through co-administration. Often referred to a as antibiotic resistance breakers, they potentiate the action of antibiotics to reduce minimum inhibitory concentrations and/or bring effective concentrations of antibiotics back below breakpoint thresholds[260,261]. *P. aeruginosa* has been shown to be subject to potentiation, including strains from CF and including by AMPs, in vitro[319–321]. Eight percent of antimicrobials in the preclinical pipeline (reviewed in 2020[213]) were potentiators of antibiotic activity.

Glatiramer acetate is a peptide drug used in the treatment of multiple sclerosis[276,282]. It is cationic and amphipathic and, therefore, resembles many naturally occurring cationic AMPs. In MS, GA is immunomodulatory, although its full range of activity is not yet known[276,282]. Of particular interest is GA's ability to rapidly kill human leukocytes which has been shown to occur by polar attachment and membrane disruption in manner very similar to the model of AMP antibacterial activity[277]. Following these observations, GA was investigated as an antimicrobial and was found to kill *P. aeruginosa* in non-dividing suspensions but was less effective against *E. coli* and not active against *S. aureus*[287]. This was extended to a moderate level of killing of clinical strains of *P. aeruginosa* from people with CF.

While that work established the ability of GA rapidly kill *P. aeruginosa*, it is not known what effect GA has on *P. aeruginosa* cells which are actively dividing and in a complex

nutritional media. That half of the clinical strains tested were resistant to GA activity in that static model would indicate that antimicrobial activity of GA may be not be impactful enough for deployment as an antibiotic. However, with the desire to resurrect the activity of exiting antibiotics through the use of antibiotic adjuvants which potentiate their activity and the research showing that AMPs can function as antibiotic resistance breakers in *P. aeruginosa*, this is an avenue of exploration for GA. Antibiotic resistance breakers do not need to be antimicrobial themselves to potentiate antibiotics, for example efflux pump inhibitors[263].

Drug repurposing, where drugs are reinvestigated and reposition from their original approved use, is a growing area of interest[213,260,268]. In response to the antibiotic resistance crisis, the use of drugs already in clinical use is attractive, with the body of research and evidence already in place reducing time and costs for deployment[213,268]. GA has many of the attributes required for repurposing, with a long safety record of minimal side-effects[276,281].

3.2 Hypothesis

I hypothesis that:

Co-administration of GA with commonly used antibiotics in CF makes the antibiotics more effective against *P. aeruginosa*

3.2.1 Aims

To investigate this hypothesis, I will:

- Measure the effect of GA on growth and viability of *P. aeruginosa* strains

- Compare the growth and viability of reference *P. aeruginosa* exposed to antibiotics with and without GA co-treatment
- Calculate whether the activities of GA in combination with each antibiotic result in synergy, additivity or antagonism of the agents in their activities against *P. aeruginosa*

3.3 Results

3.3.1 Glatiramer acetate activity against reference *P. aeruginosa* strains

While work has been published on the effects of GA over short exposure and in non-dividing bacteria[287], the activity of GA against *P. aeruginosa* over longer exposure and during active bacterial growth, in common with conventional susceptibility assays, had not been investigated.

3.3.1 GA minimum inhibitory concentration against reference *P. aeruginosa* strains

Broth microdilutions were carried out on *P. aeruginosa* strains PAO1, PA14 and PAK in a 96-well plate with a range of GA concentrations: 50-1600mg/L. After overnight incubation, under both aerobic and anaerobic conditions, visual inspection of wells showed all broths to be turbid with bacterial growth regardless of the GA concentration, strain or oxygenation (**Figure 3-1**).

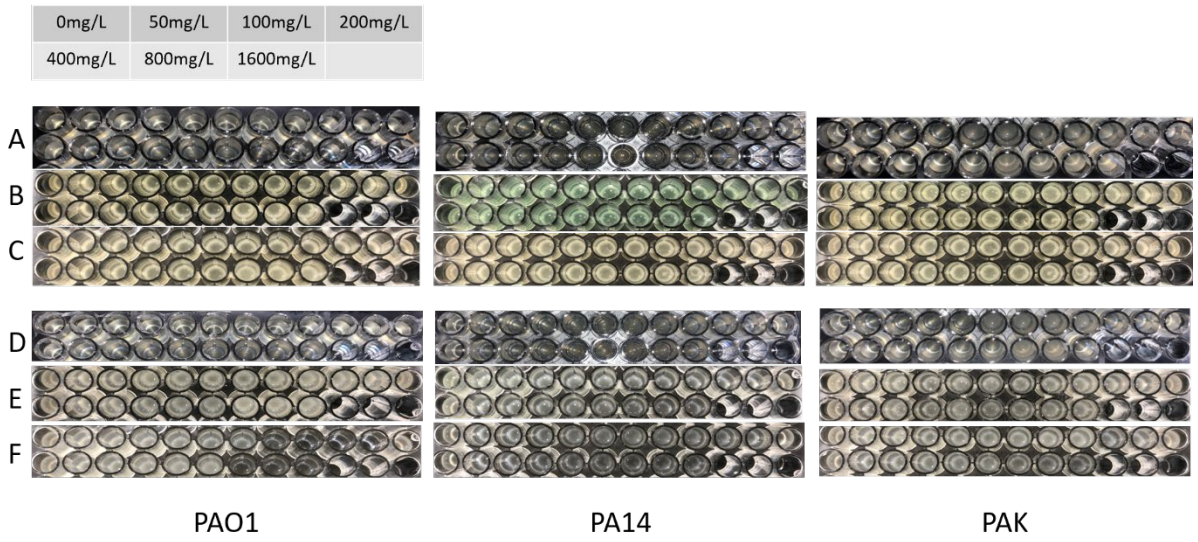


Figure 3-1. Broth microdilutions of P. aeruginosa strains PAO1, PA14 and PAK after overnight treatment with GA at increasing concentrations. Table inset: Layout of GA concentrations in plate wells for each replicate A-F. Triplicate wells of GA concentrations in the range 0-1600mg/L GA. ***A-C*** *Biological replicates incubated aerobically.* ***D-F*** *Biological replicates incubated anaerobically. All wells were turbid on visual inspections and viability was confirmed on LB agar.*

To confirm, 20µL from each well was spotted onto LB agar and incubated overnight. As with broths, spots from all wells produced confluent growth (no individual colonies visible) indicating high levels of bacterial numbers. Uninoculated wells remained sterile. The experiment was repeated on two more occasions with identical results. Growth of all three *P. aeruginosa* strains, up to and including the highest concentration – 1600mg/L GA – indicated that GA does not have a minimum inhibitory concentration comparable with conventional antibiotics and the effects of GA across the concentration gradient tested were indistinguishable via the manner tested. Therefore, the lowest concentration of GA (50mg/L) which had been previously shown[287] to have moderate activity against static *P. aeruginosa* strains was used moving forward.

3.3.1.1 Effect of 50mg/L GA on growth of reference *P. aeruginosa* during overnight incubation

Having determined there was no absolute MIC for GA, any more subtle effects were explored using growth curves. Incubating strains PAO1, PA14 and PAK in MHB with 50mg/L GA did not alter the growth of the bacteria over 18hrs, compared to untreated *P. aeruginosa* under the same growth conditions. Biological replicates, comprised of three technical replicates, were analysed. Plotting the optical density and natural log of the optical density resulted in plots with no observable differences, whether 50mg/L GA was present or absent, for any of the three reference *P. aeruginosa* (**Figure 3-2**).

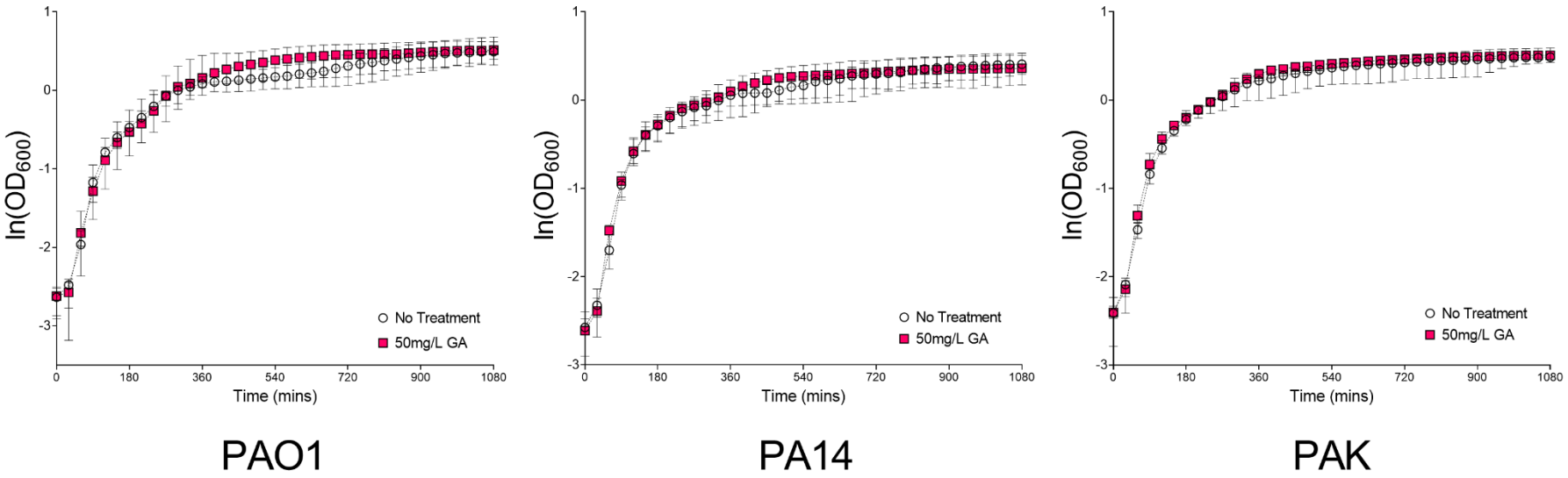


Figure 3-2. Semilog plots of OD_{600} measured every 30mins during overnight growth of P. aeruginosa strains in MHB. Black Circles are No Treatment and Pink Squares are 50mg/L GA treated PAO1 (n=12), PA14 (n=10) and PAK (n=12). Medians and 95% confidence intervals of biological replicates (means of 3 technical replicates).

The Area Under the Curve (AUC) was calculated directly from OD_{600} results. The bacterial growth curves were fitted using Non-linear regression analysis (Logistic Growth) and from these the bacterial Doubling Time, Growth Rate and $^1/_2$max were calculated and compared. Comparing biological replicates showed that the AUC Doubling Time, Growth Rate and $^1/_2$max were not changed by GA for PAO1 (**Figure 3-3**), PA14 (**Figure 3-4**) or PAK (**Figure 3-5**).

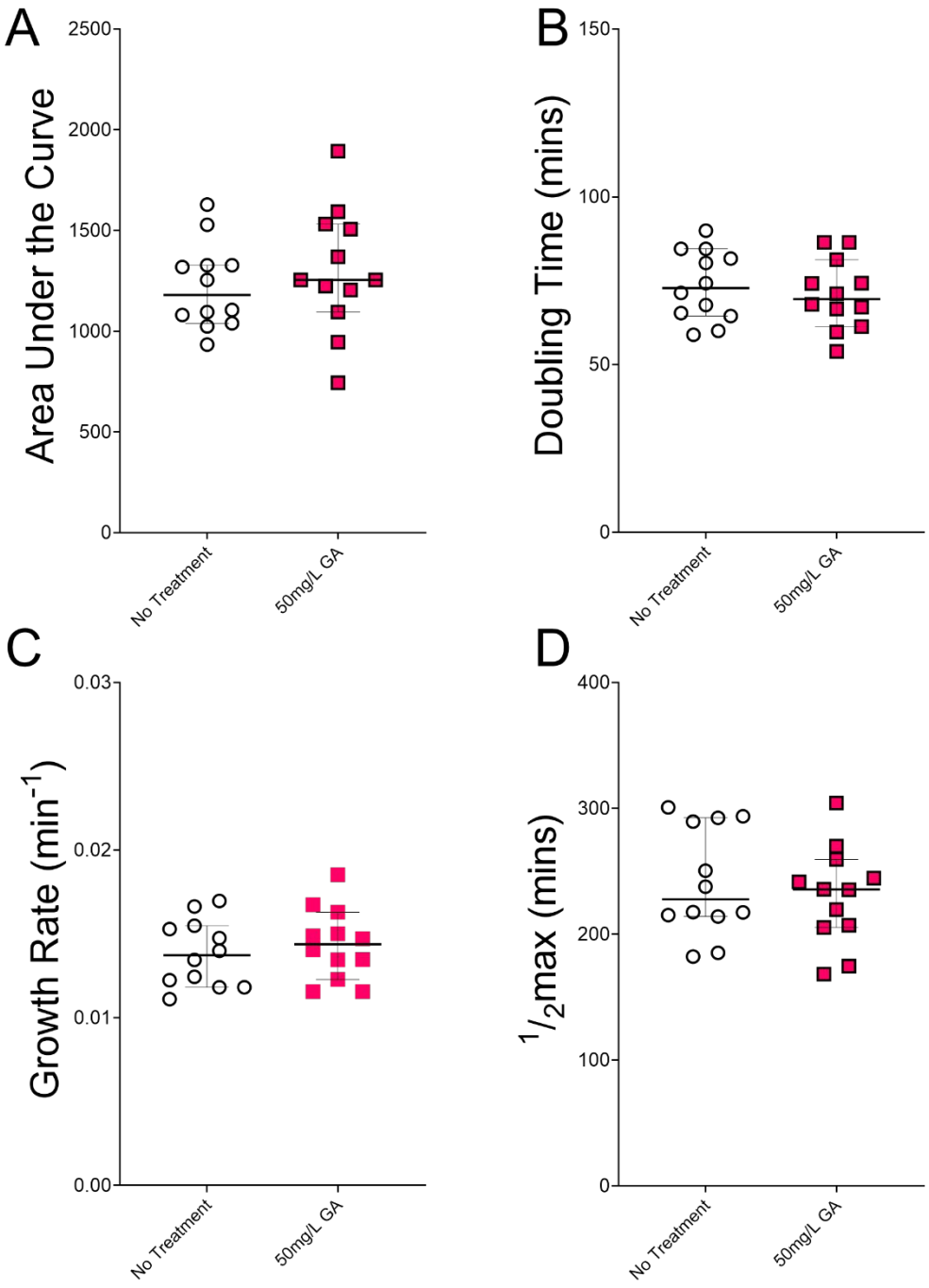


*Figure 3-3. Growth parameters of P. aeruginosa strain PAO1 derived from overnight growth curves (OD_{600}). No Treatment (Black Circles) and 50mg/L GA (Pink Squares). Treatment of PAO1 by 50mg/L GA resulted in no significant change in **A.** AUC of growth curves **B**. PAO1 Doubling Time **C.** PAO1 Growth Rate or **D**. $^1/_2$max, from control PAO1 (Mann-Whitney U test). Points are biological replicates (means of 3 technical replicates) with line at median and 95% confidence intervals (n=12).*

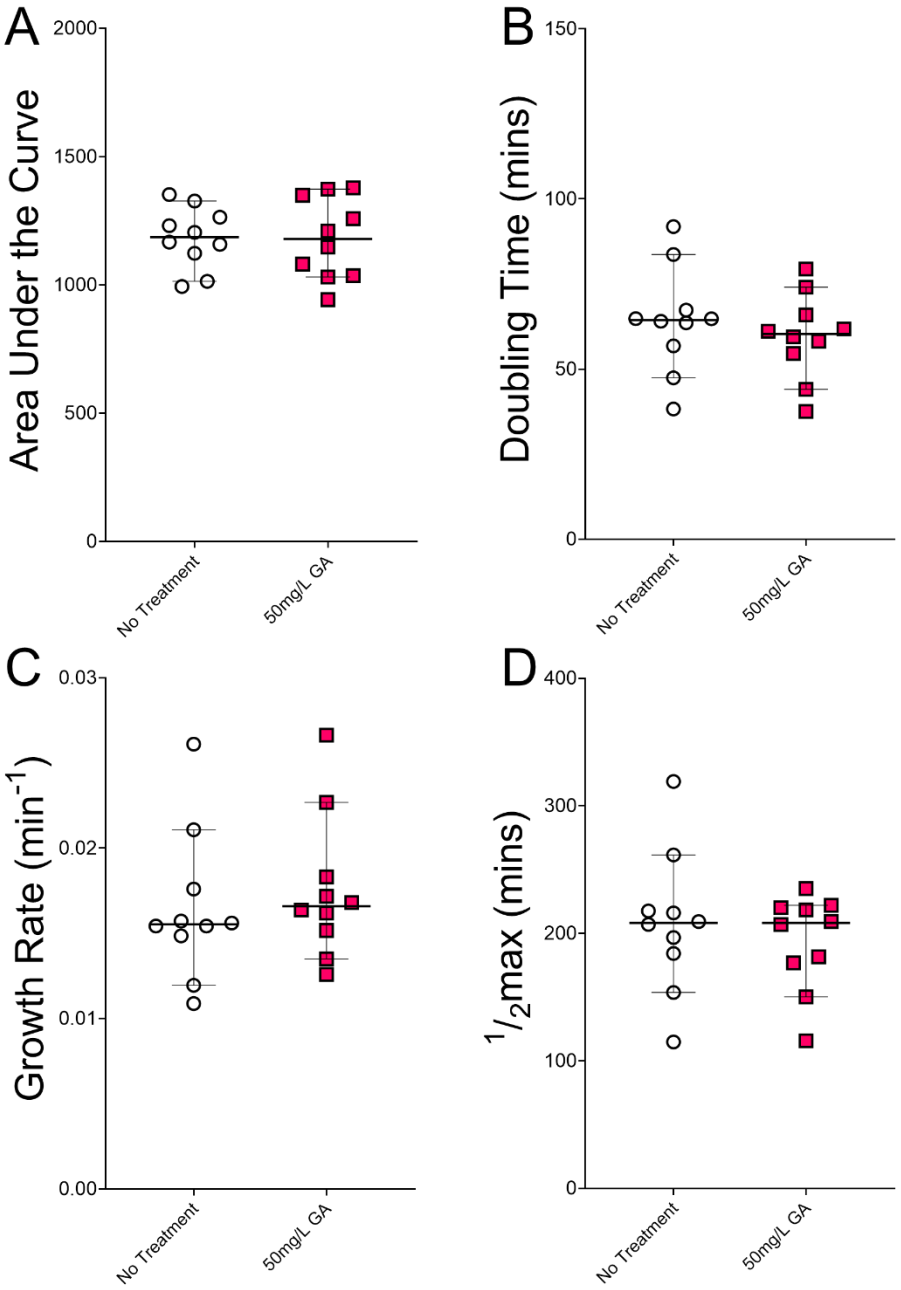


*Figure 3-4. Growth parameters of P. aeruginosa strain PA14 derived from overnight growth curves (OD_{600}). No Treatment (Black Circles) and 50mg/L GA (Pink Squares). Treatment of PA14 by 50mg/L GA resulted in no significant change in **A**. AUC of PA14 growth curves **B**. PA14 Doubling Time **C**. PA14 Growth rate or **D**. $^1/_2$max from control (Mann-Whitney U test). Points are biological replicates (means of 3 technical replicates) with line at median and 95% confidence intervals (n=10).*

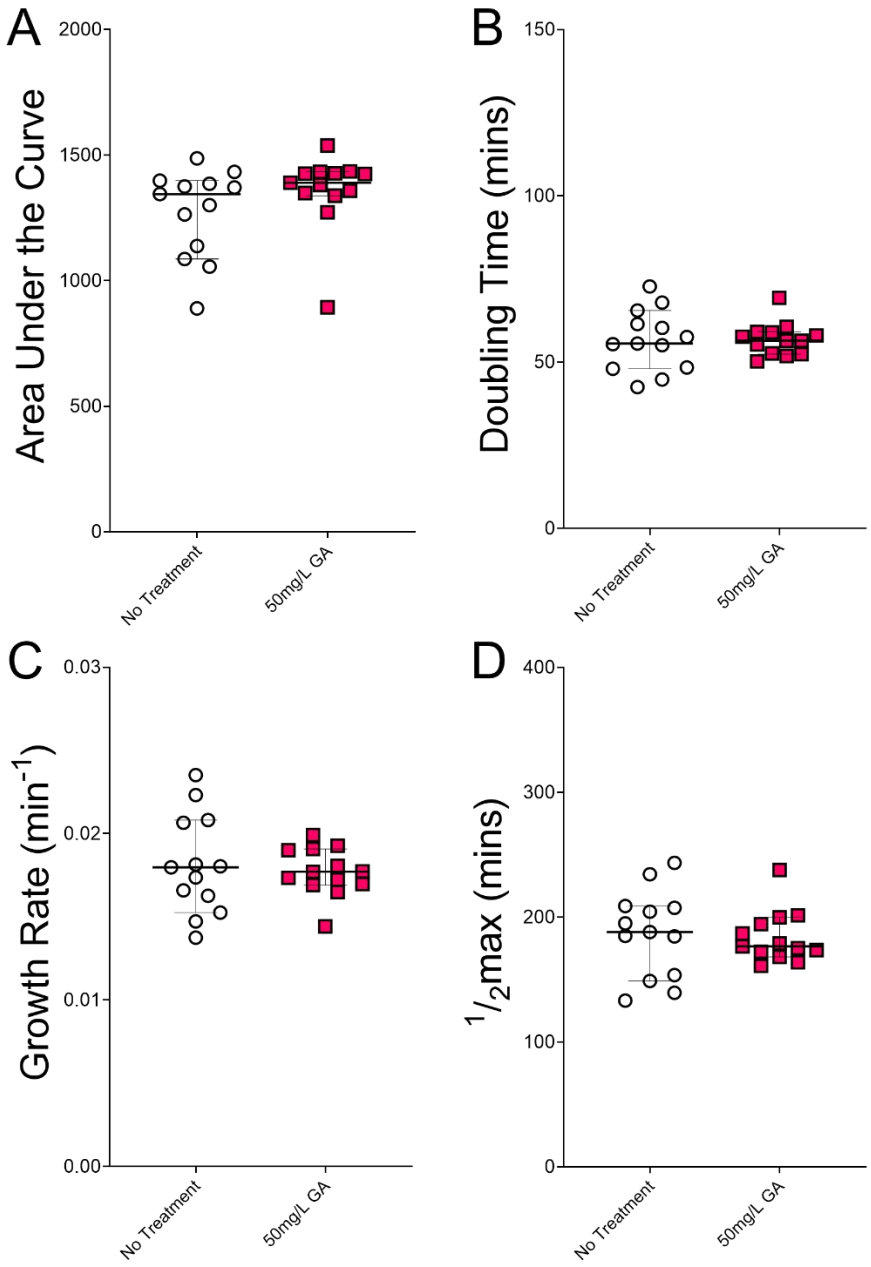


*Figure 3-5. Growth parameters of P. aeruginosa strain PAK derived from overnight growth curves (OD_{600}). No Treatment (Black Circles) and 50mg/L GA (Pink Squares). Treatment of PAK by 50mg/L GA resulted in no significant change in **A.** AUC of growth curves **B**. PAK Doubling Time **C**. PAK Growth rate or **D**. $^1/_2$max from control (Mann-Whitney U test). Points are biological replicates (means of 3 technical replicates) with line at median and 95% confidence intervals (n=13).*

3.3.1.2 Effect of 50mg/L GA on viable colony counts of reference *P. aeruginosa* after overnight incubation

Confirming growth curve OD data, there was no significant difference in colony forming units resulting from overnight exposure to 50mg/L GA for any of the three lab strains (**Figure 3-6**).

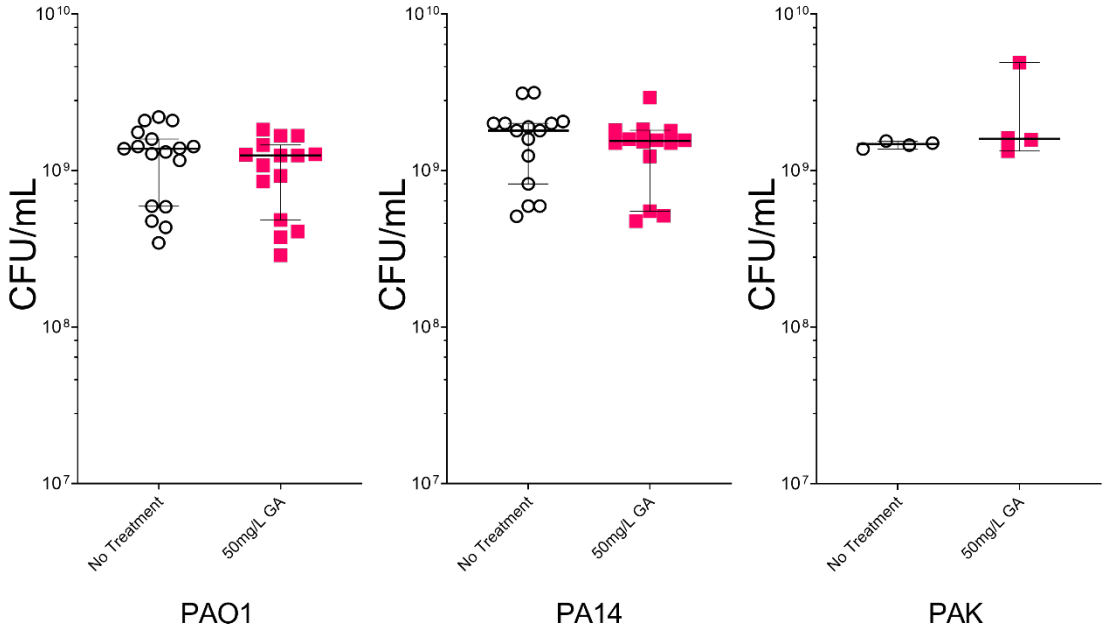


Figure 3-6. Colony forming units (CFU/mL) of P. aeruginosa strains PAO1, PA14 and PAK after overnight growth with No Treatment (Black) or in the presence of 50mg/L GA (Pink) in Mueller-Hinton broth. GA treatment did not change the CFU/mL results of any strain in comparison with control growth (Mann-Whitney U test).Points are means of technical replicates. Lines at median with 95% confidence intervals.

3.3.2 Effect of combining 50mg/L GA with antibiotics from different classes against reference *P. aeruginosa*

Having demonstrated no impact of GA on growth or survival of *P. aeruginosa* reference strains, I sought to explore whether the presence of GA altered the susceptibility of these organisms to a number of antibiotics.

3.3.2.1 Tobramycin

Reference strains were tested for their susceptibilities to the aminoglycoside antibiotic tobramycin (TOB). The EUCAST MIC broth microdilution breakpoint of TOB for *P. aeruginosa* is 4mg/L .

P. aeruginosa PAO1 was treated with increasing of TOB concentrations (0.0625-2mg/L) in log2 increments in order to identify the range of concentrations encompassing it's TOB MIC, to narrow down the concentrations at which GA should be co-administered and to measure the effects of individual concentrations of TOB-alone against PAO1 growth in this model. This screen was carried out using growth curves monitoring OD_{600} overnight. Plotting the OD_{600} and transformed $\ln(OD_{600})$ data allowed for visual comparison of growth at each TOB concentration. As expected, there was a dose response to the TOB concentrations tested, with inhibition of OD_{600} increasing with increased TOB concentration (**Figure 3-7**). These results confirm that PAO1 is sensitive to TOB, according the EUCAST MIC breakpoint of 4mg/L, as the strain showed no growth at the lower concentration of 2mg/L.

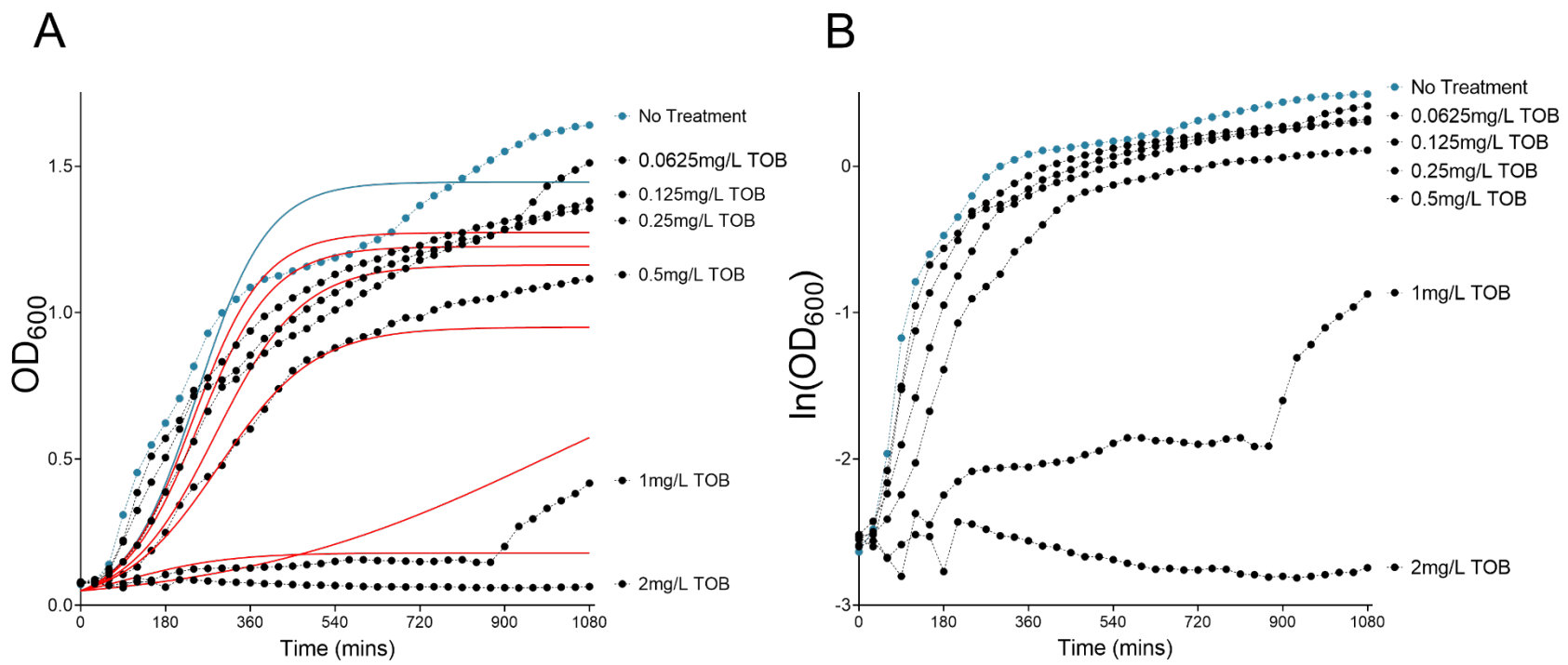


Figure 3-7. Overnight OD_{600} of PAO1 during treatment with TOB (0.0625-2mg/L) measured every 30mins. ***A.*** *OD_{600} (dotted lines) (median values of at least three biological replicates) and fitted curves of non-linear regression (Logistic Growth model) (solid lines) for each concentration tested.* ***B.*** *Transformed OD_{600} (natural log) results (median points).*

As before, the AUCs of the OD_{600} results were calculated and the growth curves were fitted by non-linear regression using the Logistic Growth model. Fitted curves were used to calculate bacterial Doubling Time, Growth Rate and $^1/_2$max. The AUC values for TOB-treated PAO1 decreased in the same dose dependent fashion, indicating inhibition of OD_{600} – as seen in the OD_{600} and Semilog plots of bacterial growth. From 0.0625 to 0.25mg/L TOB the AUCs were comparable to untreated PAO1 (overlapping 95% confidence intervals), at 0.5mg/L TOB the AUC was moderately reduced and from 1 to 2mg/L were even further reduced from control untreated PAO1 (**Table 3-1**).

At the lower TOB concentrations tested (0.0625-0.25mg/L), where little inhibition was seen, the Doubling Times and Growth Rates were comparable to control PAO1 and to each other and to the graphed results of the fitted curves are of Logistic Growth (exponential increase with growth slowing as saturation is approached and, finally, growth stopping). Treatment with 0.5mg/L TOB had a moderate effect on growth curves (but still grew logistically) and this was also reflected in Doubling Time and Growth Rate calculated (**Figure 3-7**, **Table 3-1**).

At the most inhibitory TOB concentrations tested (1-2mg/L), fitted curves of OD_{600} results are no longer logistic in nature (**Figure 3-7A**). As a result the Doubling Time and Growth Rate values derived from these curves did not decrease in a dose-responsive manner. Indeed, the Doubling Times and Growth Rates results calculated for 0.5 and 2mg/L TOB were similar to each other, despite the differences seen the plotted OD_{600}, fitted curves of OD_{600} and Semilog plots of OD_{600}; PAO1 grew to an OD_{600} of 1.12 [95%CI 0.74-1.35] at 0.5mg/L but only 0.06 [95%CI 0.03-0.75] at 2mg/L.

The $^1/_2$max of PAO1 treated with 0.0625-0.25mg/L TOB was comparable to that of the untreated PAO1 (**Table 3-1**). As with the other analyses performed, 0.5mg/L TOB was the concentration at which the results deviated from the control - at this concentration the bacteria did reach half the maximal OD_{600} of PAO1, in all of the replicates tested (n=5). Treatment with 1mg/L TOB resulted in only one of the five replicates performed ever reaching half the maximal OD_{600} of the control and, at 2mg/L, PAO1 never grew to this level (n=5).

Table 3-1. Growth metrics for PAO1 after treatment with TOB (0.0625-2mg/L) derived from overnight growth curves. Medians [95% confidence intervals] (n=5).

	Area Under the Curve	**Doubling Time (mins)**	**Growth Rate (generations min^{-1})**	**$^1/_2$max (mins)**
0mg/L	**1181** [1040-1432]	**72.83** [64.50-84.52]	**0.014** [0.012-0.016]	**227.8** [214.1-292.6]
0.0625mg/L	**1063** [761-1378]	**74.73** [66.56-83.25]	**0.013** [0.012-0.015]	**242.4** [200.2-272.8]
0.125mg/L	**1014** [770-1270]	**79.10** [71.18-87.40]	**0.013** [0.011-0.014]	**245.4** [230.8-315.7]
0.25mg/L	**938.4** [632-1066]	**92.04** [82.89-101.5]	**0.011** [0.0099-0.012]	**298.5** [252.5-356.3]
0.5mg/L	**667.7** [510.2-874.1]	**101.5** [90.50-112.9]	**0.0099** [0.0089-0.011]	**408.5** [251.5-593.5]
1mg/L	**161.1** [101.8-250.7]	**335.1** [233.5-444.7]	**0.0029** [0.0023-0.0043]	**533.1** [-]
2mg/L	**98.89** [36.3-105.6]	**107.2** [12.80-297.1]	**0.0093** [0.0034-0.078]	**-**

These analyses indicate that with high levels of inhibition of bacterial growth, any utility of Doubling Times and Growth Rates was lost and, indeed, the reported results may even be misleading due to the bacterial growth on longer conforming to the Logistic Growth model, which was used to derive these metrics. For this reason, these metrics were no longer considered, going forward. While results for $^1/_2$max times of PAO1 were also derived from

fitted curves, crucially their derivation did not rely on any specific model of growth and potentially misleading results were not produced – if a treatment prevents half the maximal growth level being reached, no value is reported. Indeed, any treatment which prevents the half maximal OD_{600} being achieved is of interest and therefore $^1/_2$max retained some utility. Area Under the Curve data of growth curves appears to be a useful tool for comparison of these data, the values decreasing in the same dose-response manner as the plots.

Strains PA14 and PAK were also screened using growth curves as a high-throughput method to find the concentration range across which TOB was effective against each.

As with PAO1, PA14 response to TOB was dose-dependent with little change from control growth between 0.0625 to 0.25mg/L TOB, moderate inhibition at 0.5mg/L and inhibition at 1 and 2mg/L, from examination of plotted results (**Figure 3-8**). Also as with PAO1, PA14 growth at 1-2mg/L TOB was no longer Logistic. Similarly, the AUCs of the OD_{600} results were comparable to untreated PA14 between 0.0625 and 0.25mg/L TOB and decreased in response to the subsequent increases in TOB (0.5-2mg/L) (**Table 3-3**). Also as with PAO1, PA14 was sensitive to TOB, according to the EUCAST breakpoint for the antibiotic.

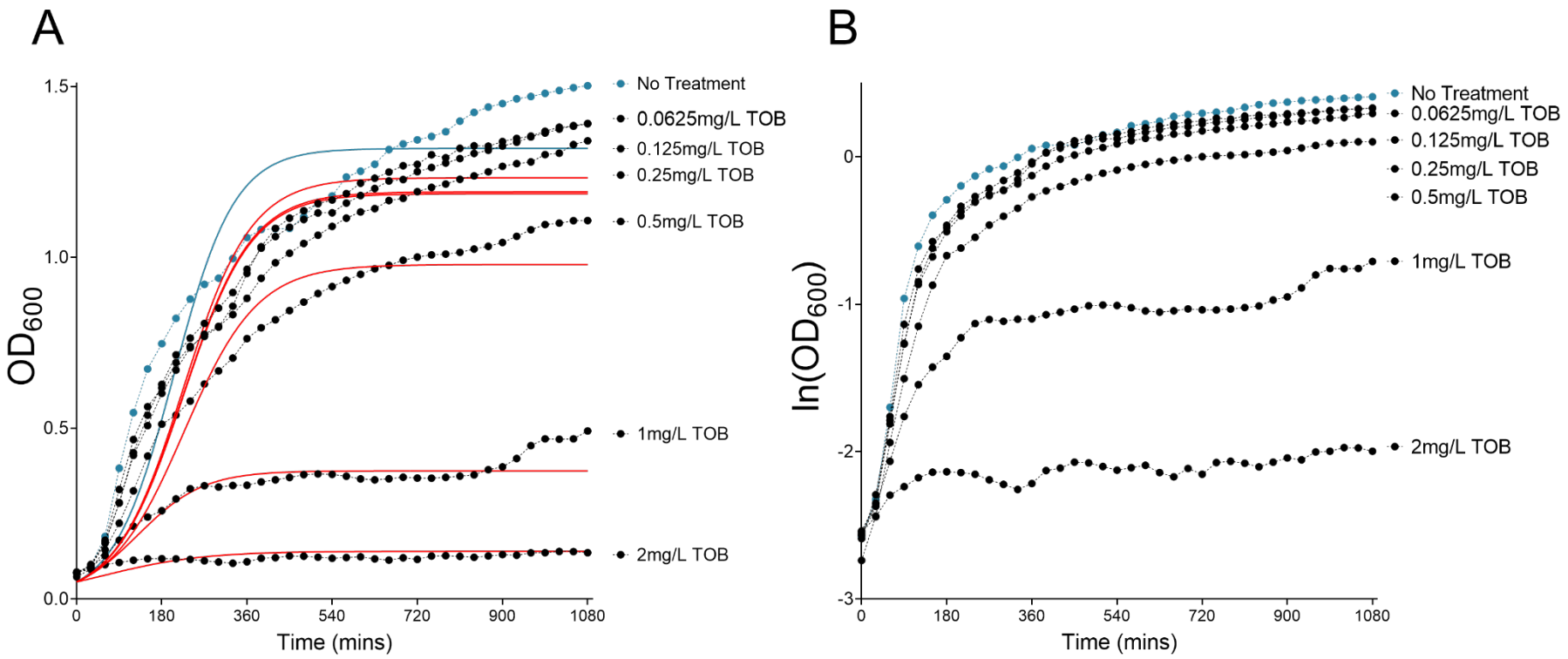


Figure 3-8. Overnight OD_{600} of PA14 during treatment with TOB (0.0625-2mg/L) measured every 30mins. ***A.*** *OD_{600} (dotted lines) (median values of at least three biological replicates) and fitted curves of non-linear regression (Logistic Growth model) (solid lines) for each concentration tested.* ***B.*** *Transformed OD_{600} (natural log) results (median values).*

The $^1/_2$max of PA14 was comparable to untreated whether the strain was treated with any TOB concentration from 0.0625 to 0.5mg/L and all replicates reached the half maximal OD_{600} of untreated PA14 at each of these concentrations (n=3) (**Table 3-3**). Treatment with 1 and 2mg/L TOB prevented PA14 from reaching half maximal growth level, in any of the biological replicates performed (n=3).

Table 3-2. Growth metrics for PA14 after treatment with TOB (0.0625-2mg/L) derived from overnight growth curves. Medians (95% confidence intervals) (n=3).

	Area Under the Curve	**$^1/_2$max (mins)**
0mg/L	**1186** [1014-1327]	**208.1** [153.6-261.5]
0.0625mg/L	**1096** [929.9-1201]	**240.4** [200.1-264.2]
0.125mg/L	**1088** [914.5-1114]	**228.9** [197.5-340.6]
0.25mg/L	**1039** [998.2-1058]	**231.7** [214.4-246.4]
0.5mg/L	**895.3** [782.3-921.9]	**239.2** [224.3-287.4]
1mg/L	**367.4** [261.3-436.1]	**-**
2mg/L	**152.5** [108.8-312.8]	**-**

Strain PAK was the most resistant reference strain to TOB, according to growth curves (**Figure 3-9**). Up to and including 0.5mg/L TOB the AUCs of PAK growth were comparable to the untreated control (**Table 3-4**). At 1mg/L there was moderate inhibition of PAK growth and the AUC was reduced in relation to the control. Unlike the other strains, the results for the two highest concentrations of TOB tested were not dose-responsive for PAK with the OD_{600},

fitted curves and Semilog curves of 4mg/L TOB appearing to show more growth than 2mg/L. While this result was also apparent in the AUCs of these concentrations, with 2mg/L having a numerically lower AUC than 4mg/L, it was noted that these AUC had wide and overlapping 95% confidence intervals; 74.97-780 (n=5) at 2mg/L and 164-763.7 (n=3) at 4mg/L. This implies that the response of PAK to these concentrations of TOB is highly variable at these concentrations and PAK may have an intermediate level of susceptibility to TOB.

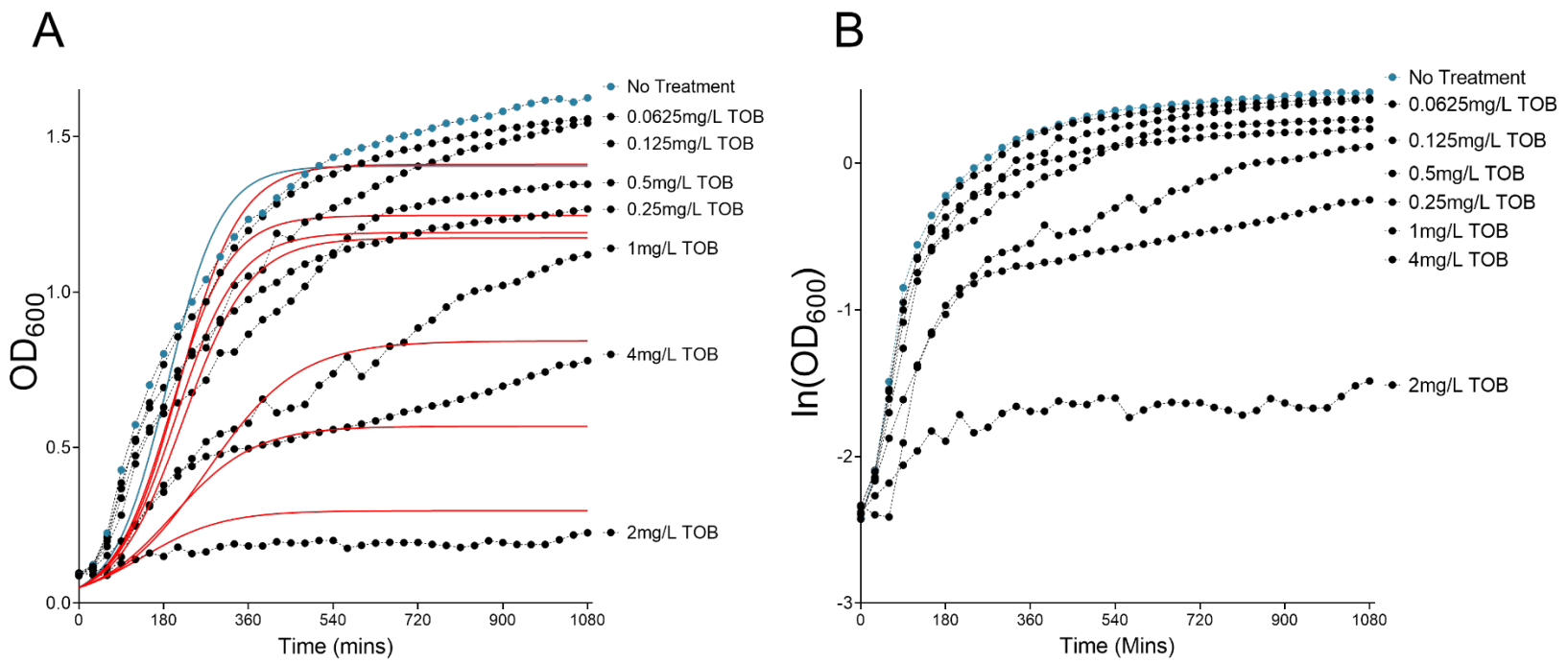


Figure 3-9. Overnight OD_{600} of PAK during treatment with TOB (0.0625-2mg/L) measured every 30mins. ***A.*** *OD_{600} (dotted lines) (median values of at least three biological replicates) and fitted curves of non-linear regression (Logistic Growth model) (solid lines) for each concentration tested.* ***B.*** *Transformed OD_{600} (natural log) results (median values).*

The $^{1}/_{2}$max of PAK was comparable to untreated whether the strain was treated with any TOB concentration from 0.0625 to 0.25mg/L (**Table 3-3**). In contrast with the AUC result, 0.5mg/L did result in a reduced $^{1}/_{2}$max from the control. Treatment with 1mg/L further reduced the $^{1}/_{2}$max of PAK and of the 5 biological replicates performed, 1 did not reach half the maximal growth (**Table 3-3**). Treatment with 2 (n=5) and 4mg/L (n=3) TOB resulted in only one replicate reaching half maximal growth, for each concentration.

Table 3-3. Growth metrics for PAK after treatment with TOB (0.0625-4mg/L) derived from overnight growth curves. Medians (95% confidence intervals) (n≥3).

	Area Under the Curve	**$^{1}/_{2}$max (mins)**
0mg/L	**1345** [1086-1398]	**188.1** [148.8-208.9]
0.0625mg/L	**1213** [845-1308]	**216.7** [195-238.3]
0.125mg/L	**1290** [830.9-1326]	**199.2** [188.4-278.8]
0.25mg/L	**1056** [852.9-1259]	**222.6** [189.2-266.9]
0.5mg/L	**1074** [903.5-1114]	**239.5** [221.5-297.3]
1mg/L	**755.3** [506.6-868.3]	**352.2** [338.6-473.2]
2mg/L	**195.9** [74.97-780]	**384.9** [-]
4mg/L	**574.2** [164-763.7]	**358.8** [-]

3.3.2.1.1 Effect of GA on growth of reference *P. aeruginosa* during TOB treatment

Once the growth of *P. aeruginosa* reference strains had been demonstrated in the presence of different TOB concentrations, I explored the impact of the presence of GA. After

visual inspection of growth curves (**Figure 3-10**) and once variance had been established in growth curve AUCs and 18hr OD_{600} results for the datasets (**Figure 3-11**), two approaches were taken to assess the impact of GA on TOB treatments; the impact of GA on the results of individual TOB concentrations were assessed in pairwise comparisons and the impacts of all treatments were compared to control growth with multiple comparisons.

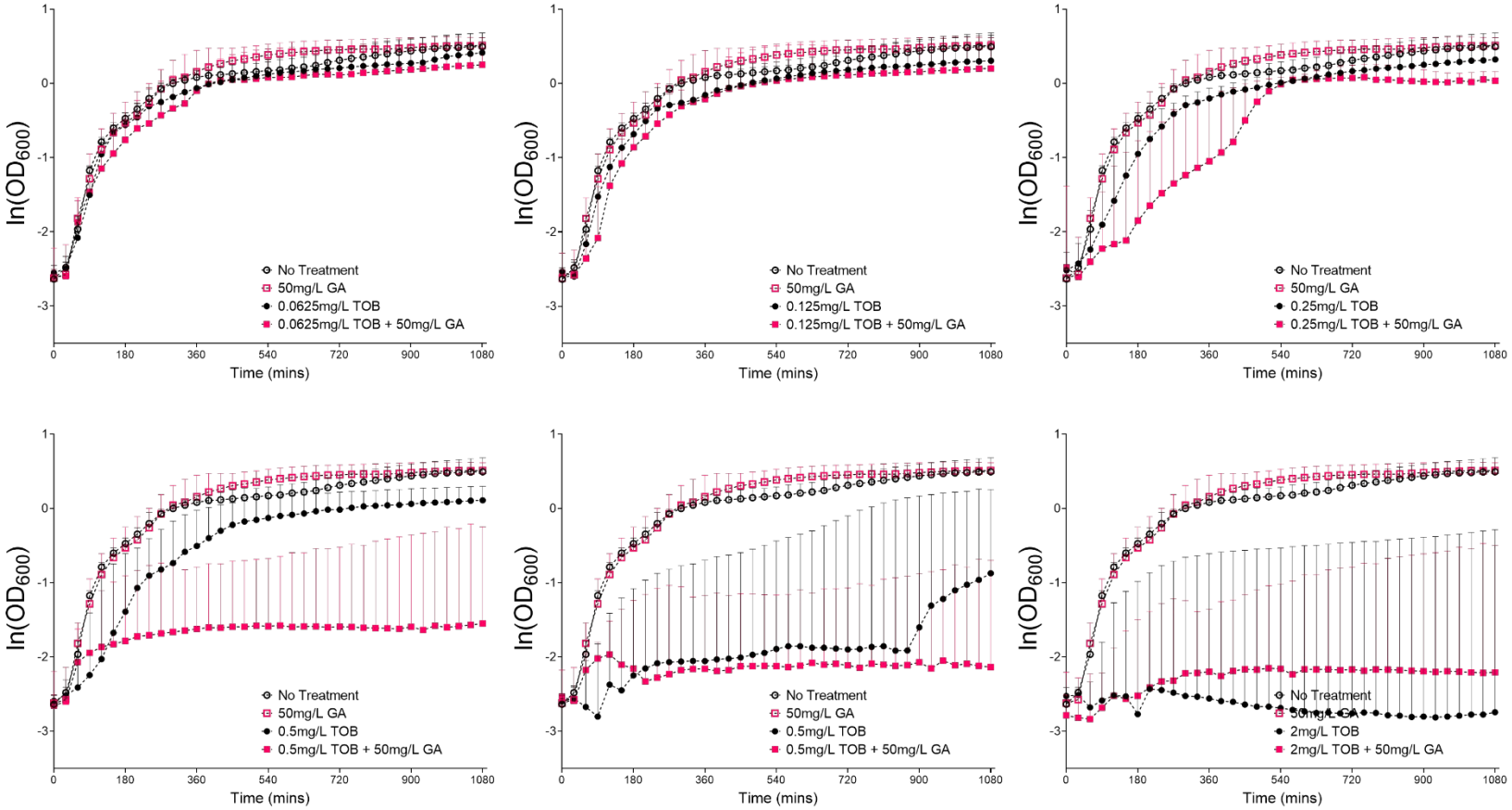


Figure 3-10. Transformed (natural log) OD_{600} growth curves of PAO1 treated with TOB and GA/TOB. OD_{600} measured every 30mins overnight with increasing concentrations of TOB in the absence (black closed circles) and presence of GA (pinks closed squares). Median and 95% confidence intervals of biological replicates (means of technical replicates) (n=5).

For PAO1, visual inspection suggested that at TOB(0.5mg/L) growth curves were different in the presence and absence of GA (**Figure 3-10**). AUC analysis confirmed this was statistically significant (**Figure 3-10**). GA reduced the AUC in the presence of TOB(0.5mg/L) from 798.5 [95%CI 510.2-1057] to 199.2 [95%CI 173.4-565.8] (p=0.03, n=5). At the endpoint of the growth monitoring – 18 hrs – the OD_{600} of PAO1 was reduced from 1.12 [95%CI 0.74-1.35] to 0.21 [95%CI 0.17-0.78] by co-treatment with GA (p=0.02, n=5). The OD_{600} resulting from GA/TOB(0.5mg/L) treated PAO1 was below the PAO1 $^1/_2$max OD_{600} (0.70 [95%CI 0.58-0.85]) and this was the case for all of the biological replicates performed (n=5). Treatment with 0.5mg/L TOB resulted in a $^1/_2$max of 408.5 [95%CI 251.5-593.5] mins.

At TOB concentrations below 0.5mg/L TOB, addition of GA did not result in any additional inhibition of PAO1 growth, according to growth curve results (**Figure 3-10**). PAO1 is not susceptible to TOB between 0.0625 and 0.25mg/L and remained so when GA was present. At concentrations greater than 0.5mg/L TOB inhibition was apparent which was unaffected by the presence of GA.

This impact of GA/TOB(0.5mg/L) was confirmed by multiple comparison of the AUCs of PAO1 across the full range of TOB concentrations tested, with and without GA, compared with control PAO1 growth (**Figure 3-11**). When treated with TOB alone, there was no significant difference from untreated AUCs from 0.0625-0.5mg/L while at concentrations above this (1-2mg/L) AUCS were significantly altered by TOB. In contrast, addition of GA changed this inflection point down to 0.5mg/L and all AUCs from this point and higher were now significantly changed compared with untreated PAO1 (**Figure 3-11**).

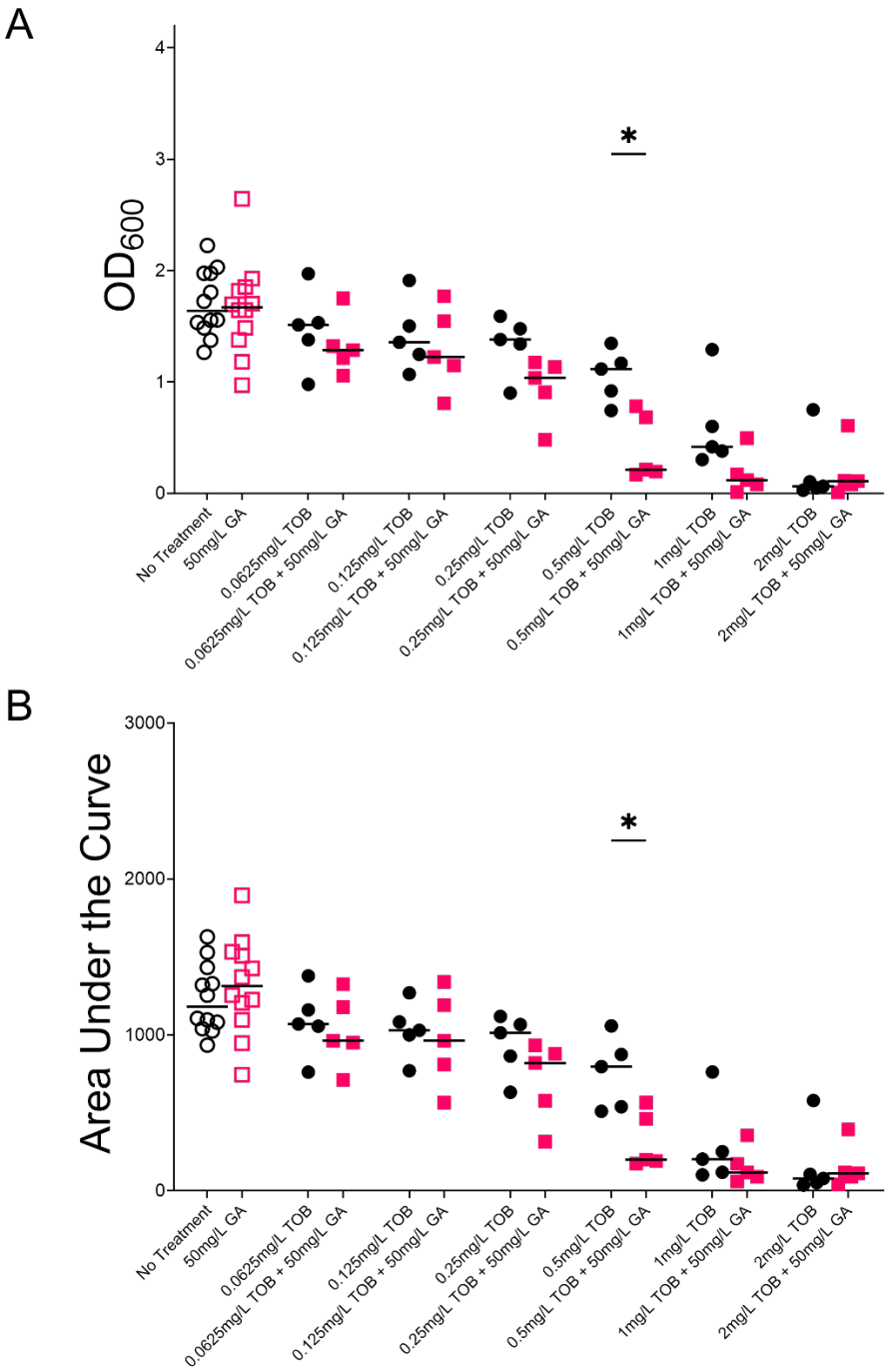


Figure 3-11. Comparisons of growth metrics of P. aeruginosa PAO1 from growth curves after incubation with TOB and GA/TOB. ***A.*** *OD_{600} results of PAO1 at the endpoint of overnight growth (biological replicates with lines at median). At 0.5mg/L TOB the presence of GA caused a significant reduction in OD_{600} compared to TOB only (p=0.0159, n=5) (Mann-Whitney U test). For GA/TOB, all concentrations ≥0.5mg/L TOB were significantly reduced from control PAO1 (p<0.002) whereas for TOB-only significant change from control was only reached at ≥1mg/L TOB (p≤0.0102) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)).* ***B.*** *AUCs of PAO1 overnight growth curves (biological replicates with lines at median). At 0.5mg/L TOB the presence of GA caused a significant reduction in AUCs compared to TOB only at this concentration (p=0.0317, n=5) (Mann-Whitney U test). For GA/TOB, all concentrations ≥0.5mg/L TOB were significantly reduced from control PAO1 (p≤0.0064) whereas for TOB-only significant change from control was only reached at ≥1mg/L TOB (p≤0.0048) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)).*

Plotting the growth curves of GA/TOB of PA14 and comparing them with TOB-treated PA14 indicated that potentiation of the antibiotic may occur at 1mg/L TOB, where the pattern of $\ln(OD_{600})$ was altered when GA was present (**Figure 3-12**).

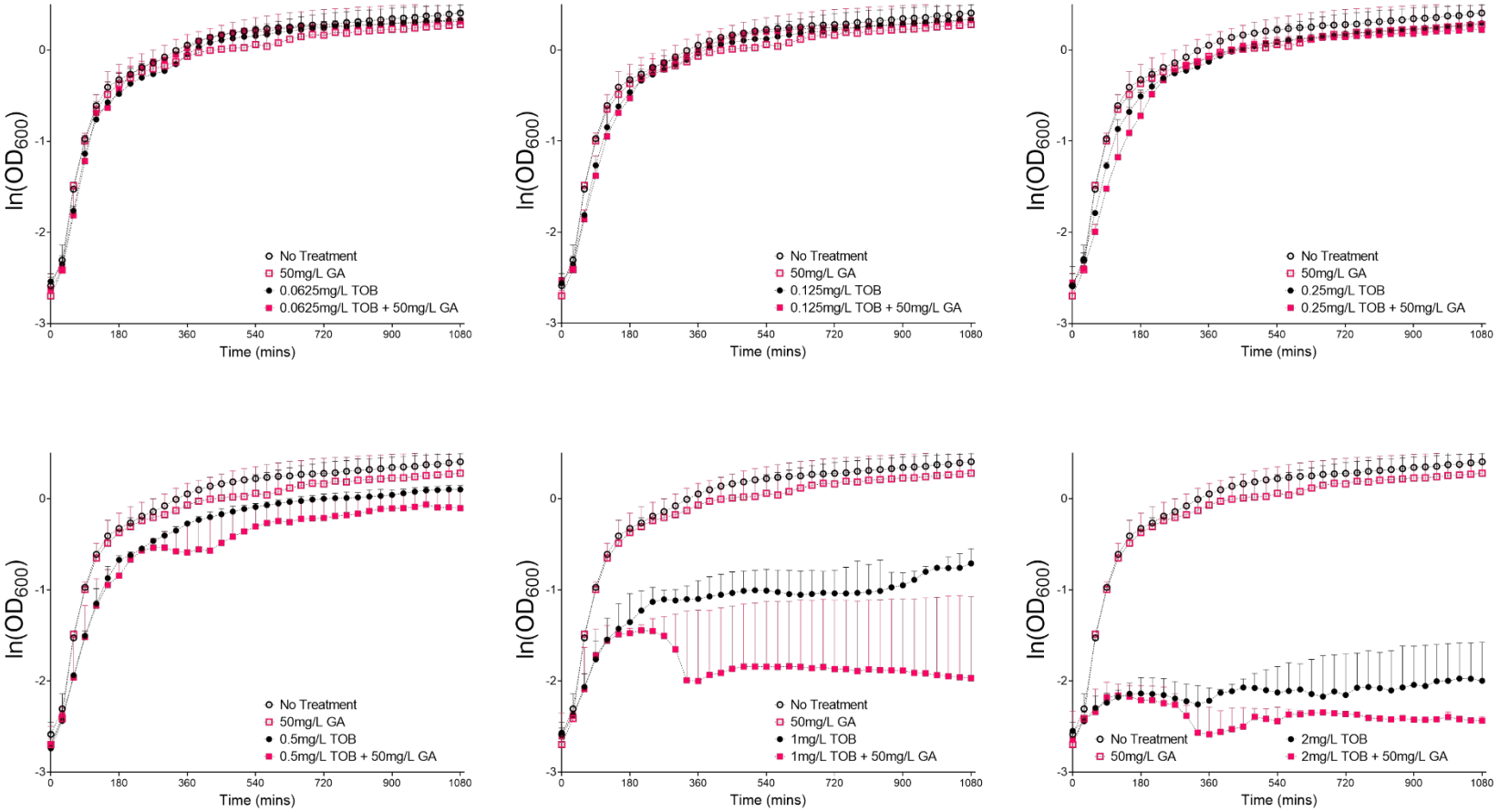


Figure 3-12. Transformed (natural log) OD_{600} growth curves of PA14 treated with TOB and GA/TOB. OD_{600} measured every 30mins overnight with increasing concentrations of TOB in the absence (black closed circles) and presence of GA (pinks closed squares). Median and 95% confidence intervals of biological replicates (means of technical replicates) (n=3).

However, this apparent difference was not statistically significant neither for AUCs nor their final OD_{600} (n=3) (**Figure 3-13**).

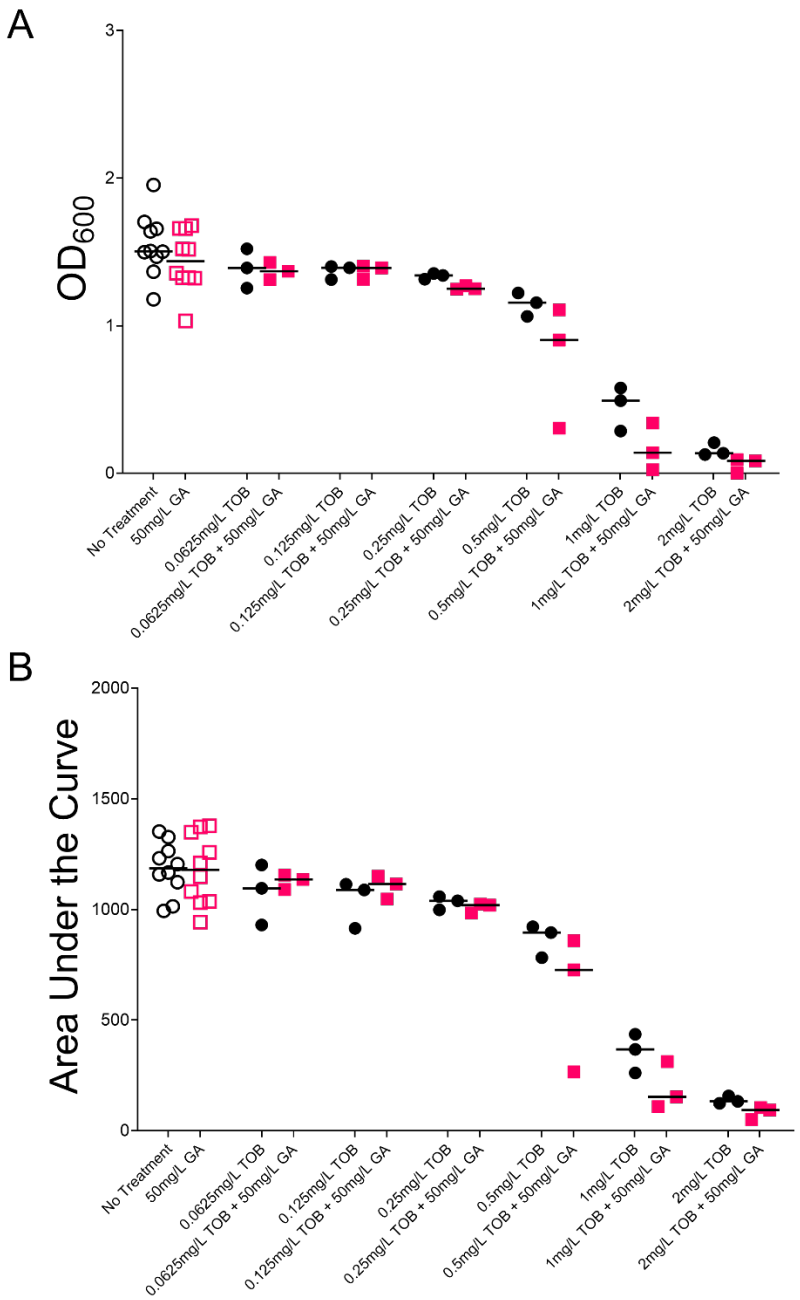


Figure 3-13. Comparisons of growth metrics of P. aeruginosa PA14 from growth curves treated with TOB and GA/TOB. ***A.*** *OD_{600} results of PA14 at the endpoint of overnight growth (biological replicates with lines at median). All concentrations ≥1mg/L TOB were significantly reduced from control PAO1 ($p<0.027$), with presence of GA resulting in a more impactful change at 1mg/L TOB ($p=0.0057$) than this TOB alone (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)).* ***B.*** *AUCs of PA14 overnight growth curves (biological replicates with lines at median). All concentrations ≥1mg/L TOB were significantly reduced from control PA14 whether GA was present of not ($p<0.05$) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)).*

Multiple comparisons to control PA14 concurred with the observations above as TOB at 1 and 2mg/L significantly reduced both the AUCs and OD_{600} and this was the case with or without GA ($p<0.05$, n=3). The $^1/_2$max bacterial concentration (0.66 [95%CI 0.55-0.77]) of PA14 was also not reached by any TOB concentrations >1mg/L, again regardless of GA presence.

The growth curves of PAK indicated that there was potentiation of TOB activity by GA at 1mg/L of the antibiotic (**Figure 3-14**). The AUC of PAK treated with GA/TOB(1mg/L) was significantly reduced compared with 1mg/L TOB ($p=0.03$, n=5). The OD_{600} at the endpoint of the growth curves were similarly significantly reduced by GA at 1mg/L ($p=0.02$, n=5); from 1.12 [95%CI 0.78-1.16] with TOB-only to 0.50 [95%CI 0.32-0.82] with GA/TOB (**Figure 3-15**).

The GA/TOB(1mg/L) treatment prevented PAK from reaching the strain's $^1/_2$max level of growth (0.74 [95%CI 0.59-0.8]) in any of the independent experiments performed (n=5). Whereas in 4 of these 5 replicates, this growth level was reached during PAK treatment with 1mg/L TOB and was 345.4 [95%CI 316.1-473.2] mins (**Figure 3-14**).

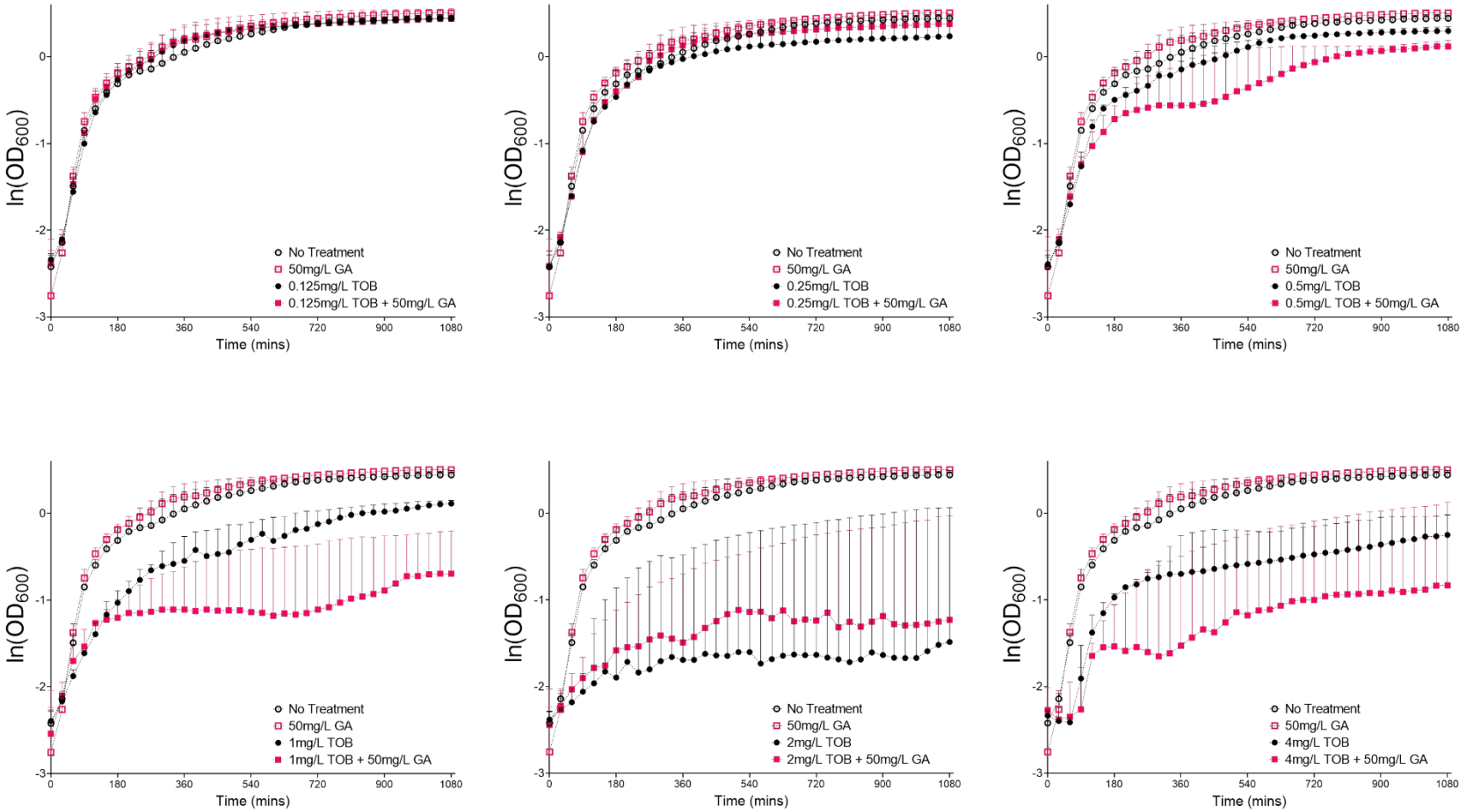


Figure 3-14. Transformed (natural log) OD_{600} growth curves of PAK treated with TOB and GA/TOB. OD_{600} measured every 30mins overnight with increasing concentrations of TOB in the absence (black closed circles) and presence of GA (pinks closed squares). Median and 95% confidence intervals of biological replicates (means of technical replicates) (n=5).

Multiple comparison testing to control PAK also showed 1mg/L to be a potential TOB concentration to be potentiated by GA (**Figure 3-15**). The OD_{600} of GA/TOB(1mg/L) was significantly reduced from that of the untreated PAK while 1mg/L TOB was not, after overnight exposure (p=0.002, n=5). This was not fully mirrored in AUC results where both 1mg/L TOB and GA/TOB(1mg/L) were significantly different from control, although the combination showed a greater effect (p=0.002) than the monotherapy (p=0.05) (n=5).

As with the other strains, addition of GA had no effect on PAK at the lower concentrations tested where the bacteria was resistant to the effects of TOB (0.125-0.5mg/L). Also as with the other *P. aeruginosa* reference strains, at higher concentrations of TOB where the strain is fully susceptible, there was no effect of co-treatments with GA.

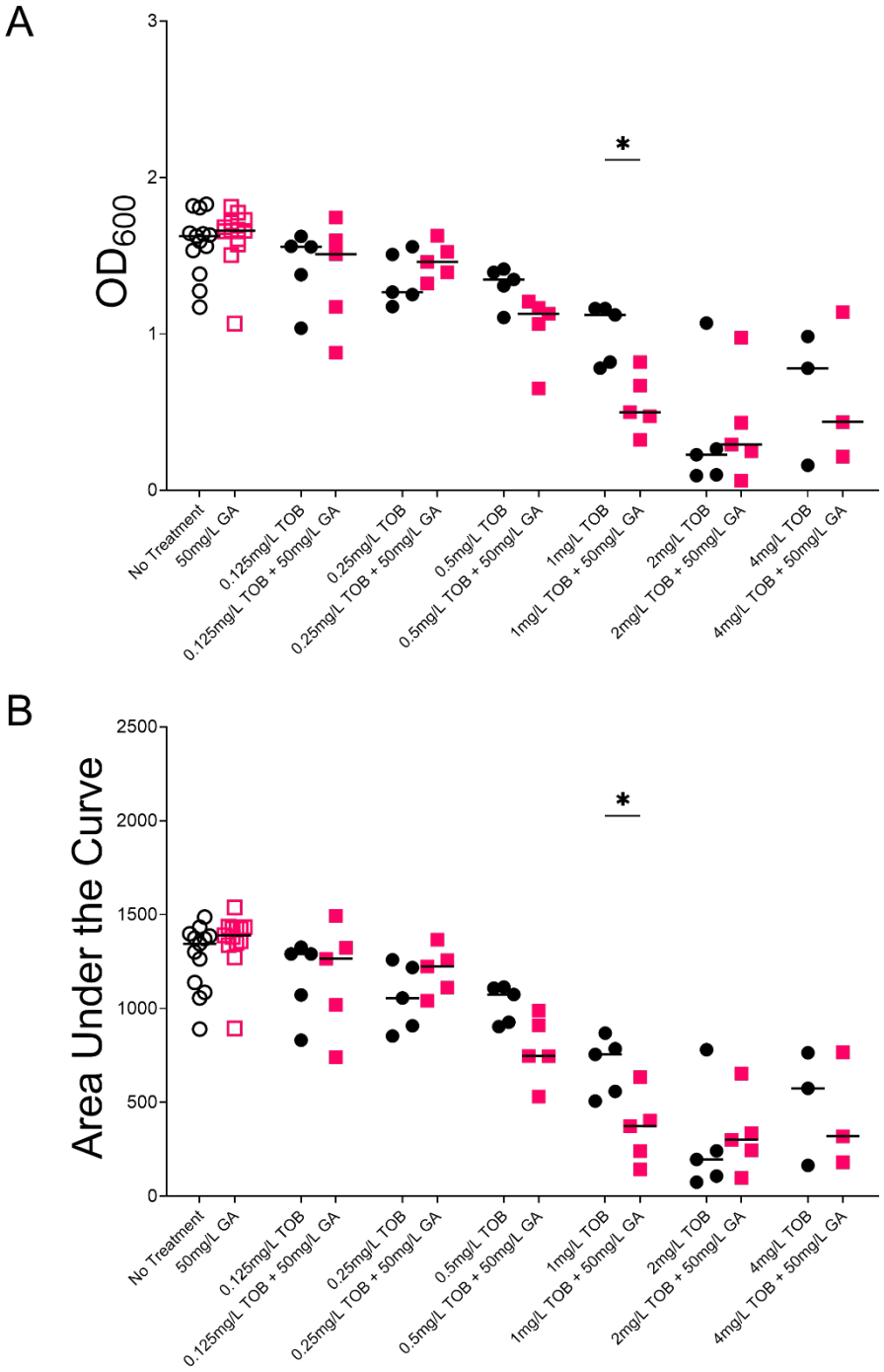


Figure 3-15. Comparisons of growth metrics of P. aeruginosa PAK from growth curves after incubation with TOB and GA/TOB. ***A.*** *OD_{600} results of PAK at the endpoint of overnight growth (biological replicates with lines at median). At 1mg/L TOB the presence of GA caused a significant reduction in OD_{600} compared to TOB only at this concentration (p=0.0159, n=5). For GA/TOB, all concentrations ≥1mg/L TOB were significantly reduced from control PAK (p<0.002) whereas for TOB-only significant change from control was only reached at ≥2mg/L TOB (p≤0.04) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)).* ***B.*** *AUCs of PAK overnight growth curves (biological replicates with lines at median). At 1mg/L TOB the presence of GA caused a significant reduction in AUCs compared to TOB only at this concentration (p=0.0317, n=5) (Mann-Whitney U test). All concentrations ≥1mg/L TOB were significantly reduced from control PAK (p≤0.05) with the exception of 4mg/L TOB which did not reach significance (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)).*

3.3.2.1.2 Effect of GA on viability of reference *P. aeruginosa* during TOB treatment

While growth curves are a useful tool for screening a wide range of different treatments of a bacteria in a high-throughput manner, and can provide strong data on changes over specific time periods, they have some weaknesses. Depending on the instrument involved, optical density can be insensitive, particularly at high and low bacterial concentrations; OD cannot distinguish between live and dead cells; and aggregates/chains/alternate modes of cell morphology can be misleading. For this reason, results of overnight growth were confirmed using viable colony counts of *P. aeruginosa* after treatments with antibiotics and antibiotic and GA combinations.

For strain PAO1, consistent with the growth curve results, the concentration of viable bacteria was dose-responsive to TOB (**Table 3-4**). Numbers of recovered bacteria were comparable to control growth for concentrations between 0.03125 and 0.25mg/L TOB. Inhibition of CFU/mL was increased by increasing TOB concentrations; 0.5mg/L resulted in 67.00 [95%CI 50.00-79.26] % inhibition, 1mg/L in 95.31 [95%CI 92.42-99.98] % inhibition and 2mg/L in 99.99 [95%CI 99.99-100.00] % inhibition.

As with growth curve metrics, CFU/mL results were compared for GA's impact on each TOB concentration and the impacts of treatments across the results for each strain, compared with control results.

In contrast to data obtained from growth curves, viability assays revealed that co-administration of GA led to a significant reduction in PA01 at not only 0.5mg/L TOB ($p=0.03$, $n=5$), but also at 0.25mg/L ($p=0.03$, $n=6$) (**Table 3-4**, **Figure 3-16**).

Below 0.25mg/L, and above 0.5 mg/L, addition of GA did not result in CFUs differing from TOB-only treated PAO1.

*Table 3-4. Colony forming units (CFU/mL) of P. aeruginosa PAO1 after overnight treatment with TOB and GA/TOB at increasing TOB concentrations. * indicates CFU/mL results where significantly fewer colonies were recovered for GA/TOB treatment than TOB treatment at a given TOB concentration (p<0.05) (Mann-Whitney U test). # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3). Median [95% confidence intervals].*

	TOB	**GA/TOB**
0mg/L	**1.38×10^9** [5.92×10^8 - 1.59×10^9]	**1.25×10^9** [4.83×10^8 - 1.16×10^9]
0.03125mg/L	**8.94×10^8** [5.92×10^8 - 1.59×10^9]	**8.13×10^8** [2.93×10^8 - 1.33×10^9]
0.0625mg/L	**6.99×10^8** [2.67×10^8 - 1.22×10^9]	**3.53×10^8** [2.41×10^8 - 1.00×10^9]
0.125mg/L	**6.90×10^8** [3.20×10^8 - 1.17×10^9]	**1.72×10^8** [1.32×10^8 - 3.17×10^8]
0.25mg/L	**3.48×10^8** [1.41×10^8 - 1.00×10^9]	***# 8.54×10^7** [1.38×10^7 - 2.10×10^8]
0.5mg/L	**1.94×10^8** [1.33×10^8 - 2.65×10^8]	***# 1.49×10^7** [1.67×10^6 - 2.85×10^7]
1mg/L	**# 4.72×10^7** [2.41×10^5 – 7.64×10^7]	**# 1.28×10^5** [0 - 2.63×10^6]
2mg/L	**# 3.24×10^3** [0 – 1.86×10^4]	**# 0** [-]

Multiple comparison analysis also indicated the significance of GA/TOB(0.25mg/L) and GA/TOB(0.5mg/L) for PAO1; both of these treatments resulted in significantly fewer bacterial colonies than untreated PAO1 (p<0.05) (**Figure 3-16**). In contrast, TOB monotherapy at these concentrations did not have a significant effect on PAO1 colony numbers. All treatments ≥1mg/L TOB resulted in significant reductions in bacterial colonies, regardless of presence of GA, as would be expected of a dose response relationship. Interestingly, following

GA/TOB(2mg/L) treatment no viable colonies of PAO1 were recovered (n=4) while, although hugely reduced, 2mg/L TOB-only allowed colonies to be enumerated after treatment (3.24 x 10^3 [95%CI 0 – 1.86 x 10^4]) (**Table 3-4**).

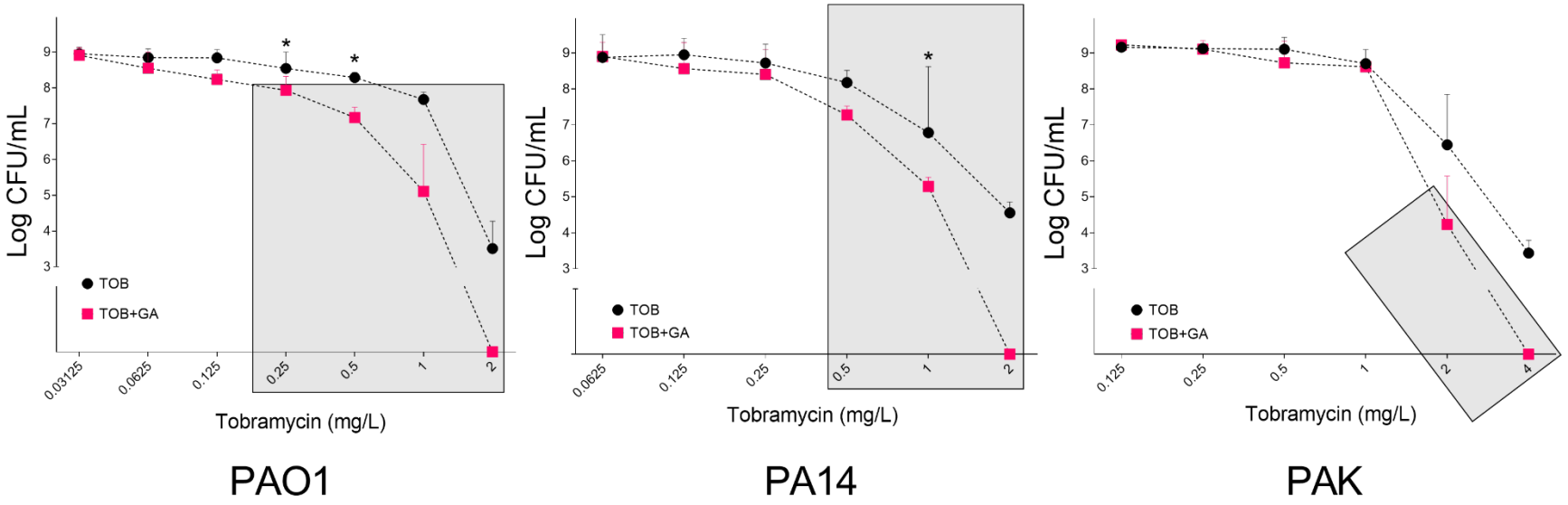


*Figure 3-16. Colony forming units (CFU/mL) of P. aeruginosa PAO1 after overnight treatment with TOB and GA/TOB at increasing TOB concentrations. Medians of biological replicates (with 95% confidence intervals). * indicates concentrations of TOB where addition of GA caused a significant reduction on CFU/mL in a paired test, without vs. with GA. This was the result for PAO1 at 0.25mg/L (p=0.026) and 0.5mg/L (p=0.0286) and for strain PA14 at 1mg/L (p=0.0286) (Mann-Whitney U test). Shaded areas indicates treatments which resulted in significant reductions in CFU/mL compared to control PAO1 under the same growth conditions. For PAO1, GA/TOB resulted in all TOB concentrations ≥0.25mg/L having significantly reduced CFU/mL but for TOB-only significant reductions were only seen at TOB ≥1mg/L. For PA14, CFU/mL were significantly reduced from control at all TOB concentrations ≥0.5mg/L, regardless of GA presence. For PAK, GA/TOB resulted in all TOB concentrations ≥2mg/L having significantly reduced CFU/mL, but no significant change was seen for TOB-only in this test (p>0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values).*

Strain PA14 was similarly dose responsive to TOB with viable colony counts comparable to control PA14 at concentrations 0.0625-0.25mg/L (**Figure 3-16**). Inhibition of PA14 increased with increasing TOB for the remaining concentrations tested; 0.5mg/L resulted in 81.44 [95%CI 71.62-94.19] % inhibition, 1mg/L in 98.81 [95%CI 86.66-99.07] % inhibition and 2mg/L in 99.99 [95%CI 99.99-100.00] % inhibition.

Addition of GA resulted in fewer colonies of PA14 than TOB-only at one specific concentration. At 1mg/L presence of GA significantly reduced the number of viable PA14 colonies after overnight growth compared with TOB alone at the same concentration (p=0.03, n=4) (**Table 3-5**).

*Table 3-5. Colony forming units (CFU/mL) of P. aeruginosa PA14 after overnight treatment with TOB and GA/TOB at increasing TOB concentrations. * indicates CFU/mL results where significantly fewer colonies were recovered for GA/TOB treatment than TOB treatment at a given TOB concentration (p<0.05) (Mann-Whitney U test). # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3). Median [95% confidence intervals].*

	TOB	GA/TOB
0mg/L	**1.80×10^9** [$8.22 \times 10^8 - 2.00 \times 10^9$]	**1.55×10^9** [5.50×10^8 - 1.81×10^9]
0.0625mg/L	**7.55×10^8** [$1.73 \times 10^8 - 3.20 \times 10^9$]	**7.99×10^8** [2.30×10^8 - 2.00×10^9]
0.125mg/L	**8.97×10^8** [$2.50 \times 10^8 - 2.60 \times 10^9$]	**3.68×10^8** [$2.20 \times 10^8 - 1.94 \times 10^9$]
0.25mg/L	**5.30×10^8** [$3.63 \times 10^8 - 1.75 \times 10^9$]	**2.54×10^8** [$2.16 \times 10^8 - 1.25 \times 10^9$]
0.5mg/L	**# 1.51×10^8** [$3.45 \times 10^7 - 3.30 \times 10^8$]	**# 1.91×10^7** [$1.77 \times 10^7 - 3.35 \times 10^7$]
1mg/L	**# 6.10×10^6** [$4.80 \times 10^6 - 4.19 \times 10^8$]	***# 1.94×10^5** [02.05×10^3- 3.50×10^5]
2mg/L	**# 3.58×10^3** [$0 - 7.17 \times 10^4$]	**# 0** [-]

Unlike PAO1, multiple comparison to control growth did not indicate that any GA/TOB concentration was more impactful against control PA14 than TOB alone and all treatments with ≥0.5mg/L TOB caused a significant reduction on CFU/mL vs. untreated ($p<0.05$).

As suggested by growth curves, strain colony counts of PAK also responded to TOB in a dose-dependent manner (**Figure 3-16**). Concentrations of TOB between 0.125 and 1mg/L TOB resulted in viable colony counts comparable to that of the untreated PAK under the same growth conditions. Increasing the concentrations to 2 and 4mg/L caused inhibition of bacterial viability and reduced colonies by 99.81% [95%CI 95.12-99.81] (2mg/L) and 99.99% [95%CI 99.99-100.00] (4mg/L) (**Table 3-6**).

While none of the of TOB concentrations tested resulted in significant changes in the bacterial colony counts for GA/TOB vs. TOB-only in paired tests, further reductions were seen in the CFU/mL with the presence of GA. Co-treatment with GA caused additional reduction in CFU/mL at the highest concentrations of TOB tested. At 2mg/L there was a >2log reduction in CFU/mL by GA/TOB compared with TOB at this concentration and GA/TOB(4mg/L) eliminated bacterial viability (n=3) whereas 4mg/L TOB did not (2.73×10^3 [95%CI 0-6.19×10^3].

*Table 3-6. Colony forming units (CFU/mL) of P. aeruginosa PAK after overnight treatment with TOB and GA/TOB at increasing TOB concentrations. * indicates CFU/mL results where significantly fewer colonies were recovered for GA/TOB treatment than TOB treatment at a given TOB concentration (p<0.05) (Mann-Whitney U test). # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3). Median [95% confidence intervals].*

	TOB	**GA/TOB**
0mg/L	**1.48×10^9** [$1.38 \times 10^9 – 1.55 \times 10^9$]	**1.59×10^9** [$1.33 \times 10^9 – 4.90 \times 10^9$]
0.125mg/L	**1.45×10^9** [$1.19 \times 10^9 – 1.88 \times 10^9$]	**1.69×10^9** [$5.27 \times 10^8 – 1.85 \times 10^9$]
0.25mg/L	**1.33×10^9** [$6.07 \times 10^8 – 1.55 \times 10^9$]	**1.28×10^9** [3.64×10^8 - 2.22×10^9]
0.5mg/L	**1.28×10^9** [$5.30 \times 10^8 – 2.72 \times 10^9$]	**5.33×10^8** [$3.57 \times 10^8 – 2.14 \times 10^9$]
1mg/L	**5.09×10^8** [$4.03 \times 10^8 – 1.25 \times 10^9$]	**4.13×10^8** [$1.50 \times 10^6 – 4.6 \times 10^8$]
2mg/L	**2.80×10^6** [$2.55 \times 10^6 – 7.10 \times 10^7$]	**# 1.71×10^4** [$0 – 3.81 \times 10^5$]
4mg/L	**2.73×10^3** [$0 – 6.19 \times 10^3$]	**# 0** [-]

Comparing treatment with increasing concentrations of TOB, with and without GA, via multiple comparison for the effect on untreated PAK showed significant reductions in CFU/mL after treatment with GA/TOB(2mg/L) and GA/TOB(4mg/L) (p=0.04 and p=0.03, respectively) while these concentrations of TOB did not cause a significant change alone. This result appeared to contrast with the CFU results themselves where the median result of treatment with 4mg/L TOB (2.73×10^3 [95%CI $0 – 6.19 \times 10^3$]) was fewer colonies than GA/TOB(2mg/L) (1.71×10^4 [95%CI $0 – 3.81 \times 10^5$]), but only the latter showed significant difference to control PAK. Examination of the replicate data revealed that the biological repeats of GA/TOB(2mg/L) had a wider range than 4mg/L TOB and resulted in median rank of GA/TOB(2mg/L) lower than 4mg/L (**Table 3-6**).

3.3.2.1.3 Effect of GA on TOB minimum inhibitory concentrations against reference *P. aeruginosa*

The CFU results were used to generate inhibition curves. For both TOB and GA/TOB at each TOB concentration, the percent inhibition of control growth for each biological replicate was calculated. Inhibition percentages were then fitted by non-linear fit and from these curves the point at which the fitted lines cross 50% inhibition (MIC_{50}) and 90% (MIC_{90}) were interpolated.

The replicate data was examined in two way using this approach. Firstly, biological replicate experiments were each fitted separately and MIC_{50} and MIC_{90} results were interpolated for each experiment (**Figure 3-17A**) and, secondly, biological replicates were fitted as a complete dataset and a single overarching MIC_{50} and MIC_{90} (along with error values) was interpolated (**Figure 3-17B**).

For PA01, curves were successfully fitted and MIC values interpolated from the individual biological replicates. However, Prism flagged the majority of the curves, both TOB-only and GA/TOB treatments, as being “ambiguous” in the goodness of their fit meaning the fit was unreliable. This was not unexpected as these curves were generated from single data points at each TOB concentration tested (median inhibition at that concentration), either with or without GA, unlike the grouped data where the biological replicates of inhibition levels at each concentration were used to fit a single curve encompassing all the replicate data at once.

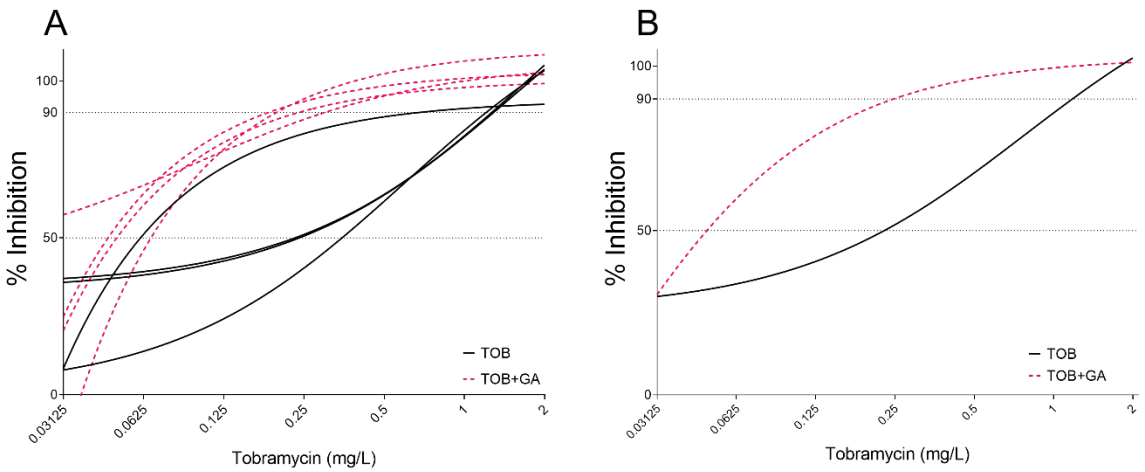


Figure 3-17. Inhibition Curves of P. aeruginosa PAO1. Non-linear regression ([Inhibitor] vs. response (three parameters)) of Inhibition as a percentage of control CFU/mL after overnight incubation with TOB (Black lines) or GA/TOB (Pink lines) at increasing TOB concentrations (0.03125-2mg/L). ***A.*** *Biological replicates fitted and 50% and 90% inhibition interpolated individually.* ***B.*** *Biological replicates fitted and 50% and 90% inhibition interpolated as single group.*

The MIC values generated by each of the two methods was compared using the median and subsequent 95% confidence interval of the individual biological replicates and the single value and error interpolated from the grouped dataset (**Figure 3-17**). The two approaches generated comparable results across the two parameters (MIC_{50} and MIC_{90}) and two conditions (TOB and GA/TOB) being assessed for PAO1. The MIC_{50} of TOB for PAO1 resulting from individual fitted curves was 0.25 [95%CI 0.06-0.35] mg/L while the grouped result for the same data was 0.23 [95%CI 0.10-0.63] mg/L. The PAO1 MIC_{90} from individual curves was 1.24 [95%CI 0.68-1.29] mg/L and for the grouped data was 1.18 [95%CI 0.45-] mg/L TOB (the upper confidence limit of the grouped data could not be calculated by Prism) (**Figure 3-18**).

The MIC values generated for GA/TOB combinations against PAO1 were similarly comparable to each other whether the data was fitted as replicate data or a grouped dataset;

MIC_{50} was 0.05 [95%CI 0.01-0.07] and 0.05 [95%CI 0.01-0.11] and MIC_{90} was 0.22 [95%CI 0.18-0.30] and 0.25 [95%CI 0.09-0.74] mg/L, respectively (**Figure 3-18**)

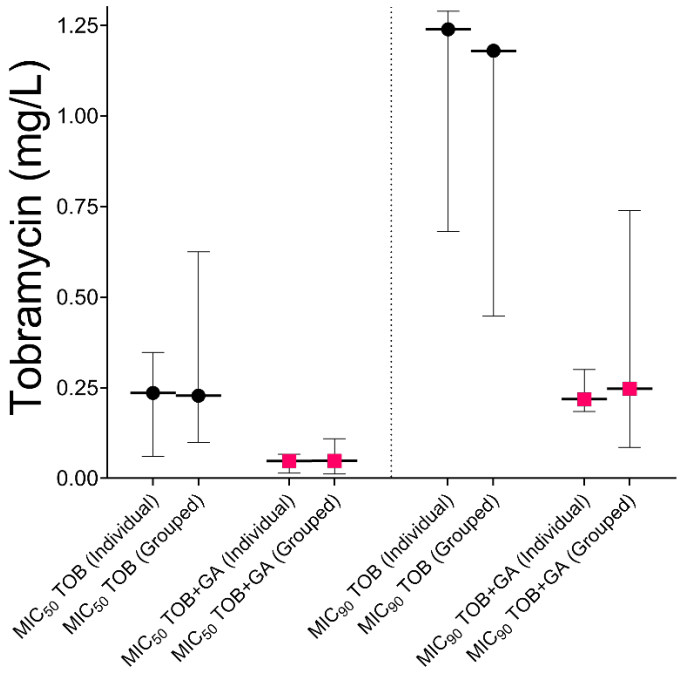


Figure 3-18. MIC_{50} and MIC_{90} values of PAO1 interpolated from biological replicate results using an Individual approach (MIC_{50} and MIC_{90} values interpolated from individual biological replicates – median and 95% confidence intervals) or Grouped approach (biological replicates used to interpolate single MIC_{50} and MIC_{90} values and their 95% confidence intervals). Black points are TOB-only. Pink points are GA/TOB.

The comparable nature of the PAO1 MICs generated using the two methods indicates that this a robust method for generating these values (**Figure 3-21**). It was decided to use a grouped dataset method to interpolate a single value it's error rate for MIC_{50} and MIC_{90} going forward, taking into account as it does replicate data in fitting the inhibition curve, rather than curves generated by single of sets values which had been shown to be prone to ambiguity.

Using these results, PAO1 reached 50% inhibition and 90% inhibition by treatment with lower TOB concentrations when GA was present than when GA was not present (**Figure**

3-19). The MIC_{50} of PAO1 for TOB when administered alone was found to be 0.23 [95%CI 0.10-0.63] mg/L while, in the presence of GA, MIC_{50} was 0.05 [95%CI 0.01-0.11] mg/L. The MIC_{90} was 1.18 [95%CI 0.45-] mg/L for TOB-only treatment and for GA/TOB was 0.25 [95%CI 0.09-0.74] mg/L.

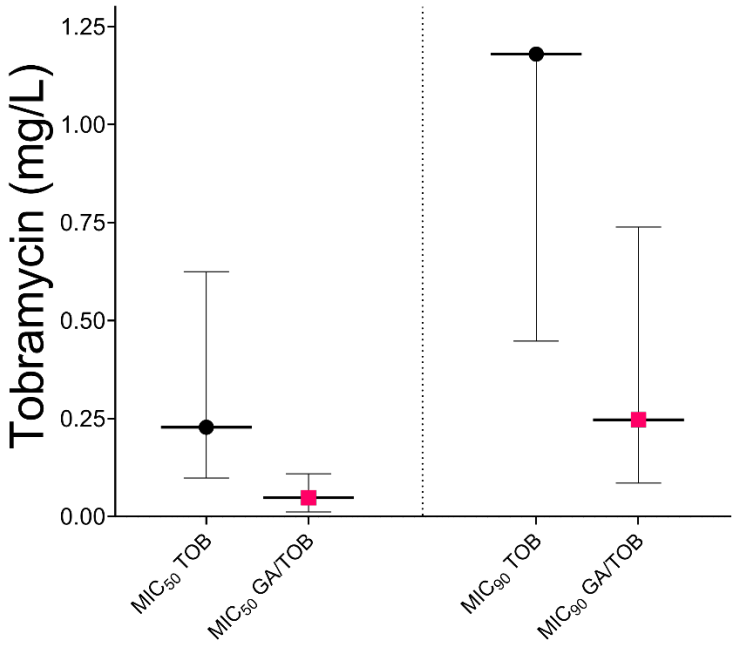


Figure 3-19. MIC_{50} and MIC_{90} results of TOB for P. aeruginosa PAO1 after treatment with TOB-only (Black) or GA/TOB (Pink) (interpolated values from inhibition curves with their 95% confidence intervals).

Using this methodology MIC results for TOB against PA14 and PAK were also derived. When treated with TOB-only the MIC_{50} of PA14 was 0.12 [95%CI -0.26] mg/L and for the GA/TOB combination was 0.04 [95%CI 0.08-] mg/L and the TOB MIC_{90} for TOB-only was 0.86 [95%CI 0.50-] mg/L and for GA/TOB was 0.44 [95%CI 0.24-] mg/L (Figure 3-20). Consistent with the growth curves and CFU/mL results for PAK, this strain was the most resistant to TOB of the reference strains (**Figure 3-20**). The TOB MIC_{50} results for PAK were, for TOB-only

treatment, 0.86 [95%CI 0.51-1.48] mg/L and for GA/TOB was 0.45 [95%CI 0.23-0.86] mg/L. The MIC_{90} was 2.49 [95%CI 1.47-] mg/L with TOB-only and was 1.71 [95%CI 0.83-] mg/L (**Figure 3-20**, **Table 3-7**).

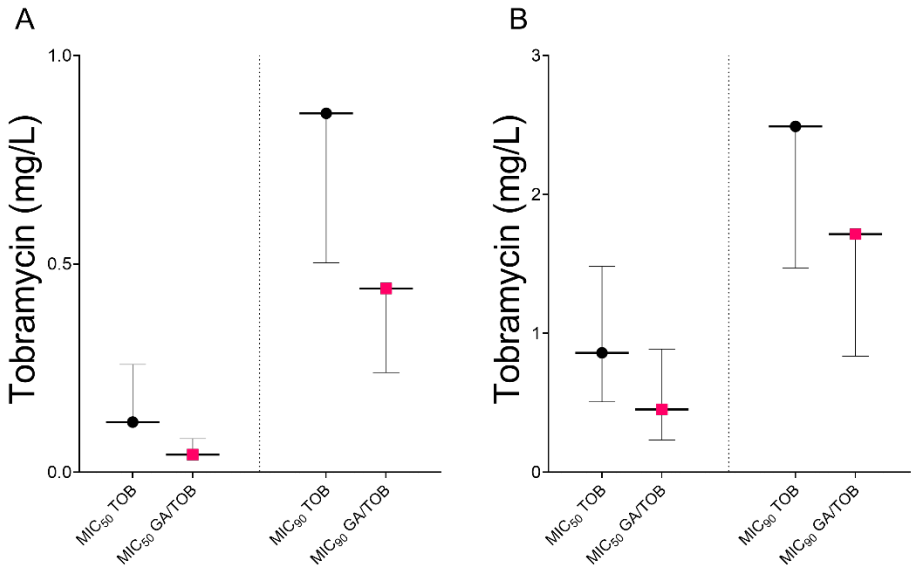


Figure 3-20. MIC_{50} and MIC_{90} results of TOB for P. aeruginosa A. PA14 and B. PAK after treatment with TOB-only (Blank) or GA/TOB (Pink) (interpolated values from inhibition curves with their 95% confidence intervals).

Table 3-7. Minimum Inhibitory Concentrations of TOB against P. aeruginosa strains PAO1, PA14 and PAK without and with GA co-treatment. Interpolated concentration (mg/L TOB) with 95% confidence intervals.

	MIC_{50}		**MIC_{90}**	
	TOB	**GA/TOB**	**TOB**	**GA/TOB**
PAO1	**0.23** [0.10 - 0.63]	**0.05** [0.01 - 0.11]	**1.18** [0.45 -]	**0.25** [0.09 - 0.74]
PA14	**0.12** [- 0.26]	**0.04** [0.08 -]	**0.86** [0.50 -]	**0.44** [0.24 -]
PAK	**0.86** [0.51 - 1.48]	**0.45** [0.23 - 0.86]	**2.49** [1.47 -]	**1.71** [0.83 -]

3.3.2.2 Ceftazidime

Reference strains PAO1 and PA14 were next tested for their susceptibilities to the cephalosporin antibiotic ceftazidime (CAZ) (PAK was not available in the lab at the time of these experiments). The EUCAST MIC CAZ breakpoint for *P. aeruginosa* is 8mg/L in broth microdilution.

Strain PAO1 was resistant to CAZ according to growth curves, with AUCs of OD_{600} results being comparable to control PAO1 at 8mg/L CAZ and above. At all CAZ concentrations tested here (up to 128mg/L) the resulting plots were comparable to untreated PAO1 growth whether OD_{600} or Semilog of OD_{600} (**Figure 3-21**).

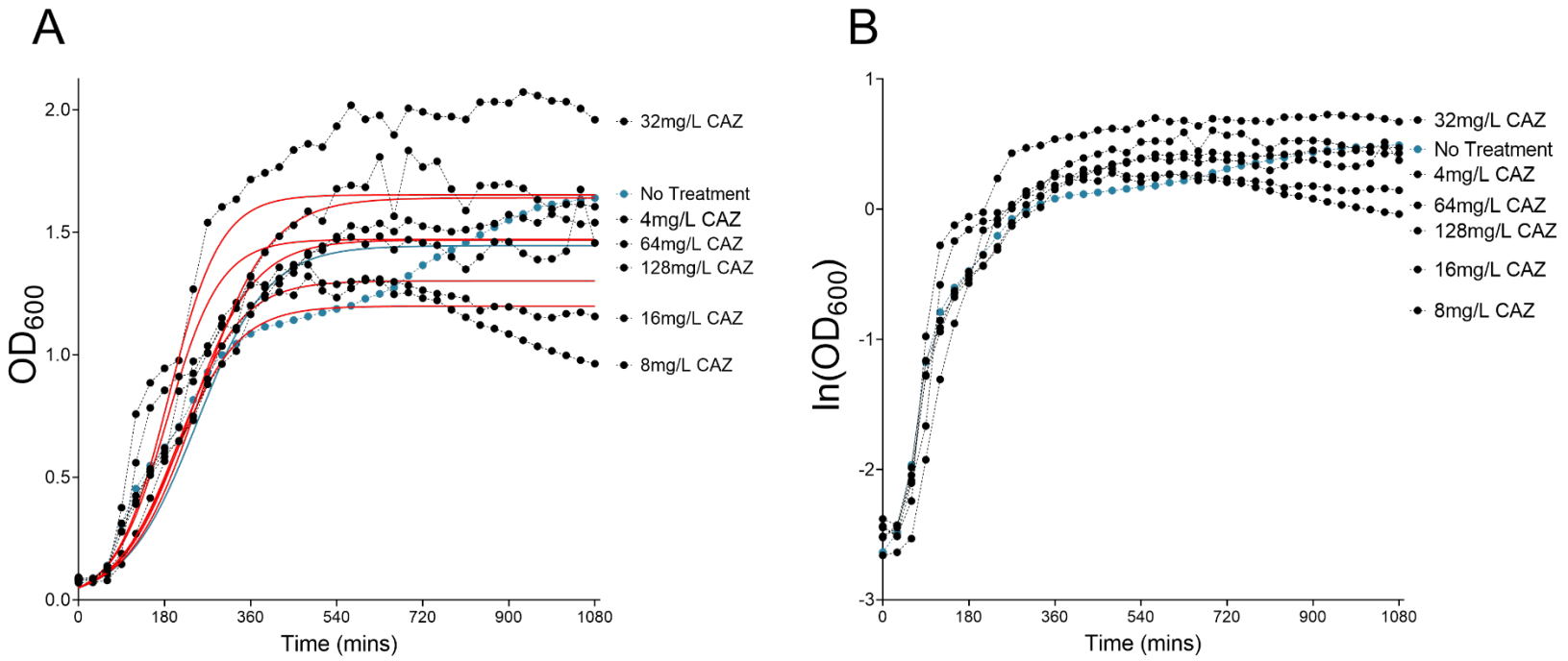


Figure 3-21. Overnight OD_{600} of PAO1 during treatment with CAZ (4-128mg/L), measured every 30mins (median values of at least three biological replicates). ***A****. Red lines are fitted curves of non-linear regression (Logistic Growth model) for each concentration tested.* ***B.*** *Transformed OD_{600} (natural log) results (median values).*

Area Under the Curve and $^1/_2$max results were also similar to control PAO1 across all CAZ concentrations tested (**Table 3-8**).

Table 3-8. Growth metrics for PAO1 after treatment with CAZ (4-128mg/L) derived from overnight growth curves. Medians (95% confidence intervals) (n=3).

	Area Under the Curve	**$^1/_2$max (mins)**
0mg/L	**1181** (1040-1432)	**227.8** (214.1-292.6)
4mg/L	**1396** (961-1762)	**256.8** (223-301.6)
8mg/L	**1091** (866-1202)	**257.4** (197.2-351.6)
16mg/L	**1068** (980.1-1369)	**215.7** (195.8-343.3)
32mg/L	**1786** (788.1-1881)	**171.9** (142.2-367.1)
64mg/L	**1328** (834.4-1793)	**209** (152.4-354.1)
128mg/L	**1257** (968.3-1539)	**226.8** (201.6-299.4)

Strain PA14 was more inhibited by CAZ than PAO1, with a greater disparity noted in plotted growth curves compared with untreated PA14 (**Figure 3-22**). However, this strain did not show strongly inhibited growth in a concentration range which included 4xMIC breakpoint of CAZ (4-32mg/L). A greater level of growth inhibition was suggested by the highest CAZ concentrations tested in a dose-dependent manner – 128mg/L causing more inhibition than 64mg/L.

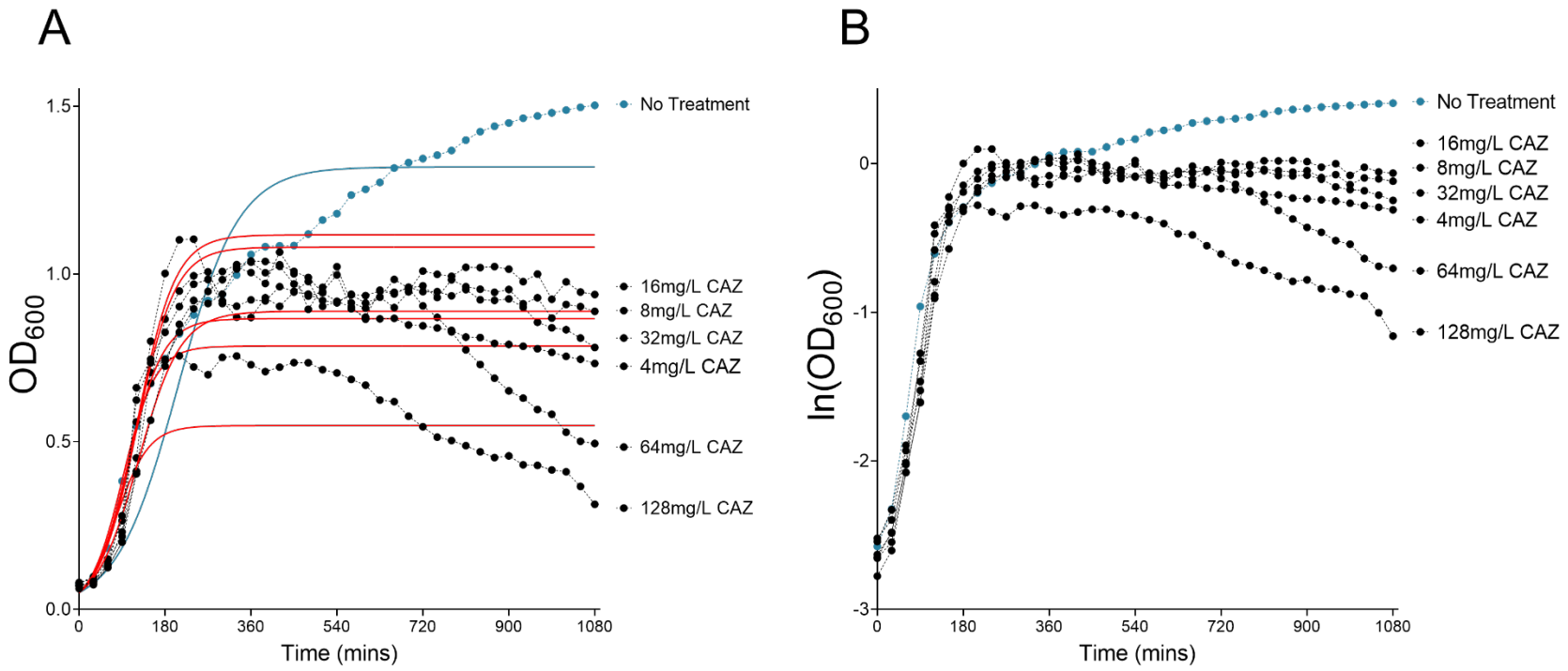


Figure 3-22. Overnight OD600 of PA14 during treatment with CAZ (4-128mg/L), measured every 30mins (median values of at least three biological replicates). ***A****. Red lines are fitted curves of non-linear regression (Logistic Growth model) for each concentration tested.* ***B****. Transformed* OD_{600} *(natural log) results (median values).*

Comparison of AUC and $^1/_2$max results for PA14 growth curves showed growth comparable with control PA14 growth at 4-8mg/L for AUCs and $^1/_2$max at 4-16mg/L CAZ. The lowest AUC and slowest $^1/_2$max were seen at the highest CAZ concentration tested, 128mg/L (**Table 3-9**).

Table 3-9. Growth metrics for PA14 after treatment with CAZ (4-128mg/L) derived from overnight growth curves. Medians (95% confidence intervals) (n=3).

	Area Under the Curve	**$^1/_2$max (mins)**
0mg/L	**1186** (1014-1327)	**208.1** (153.6-261.5)
4mg/L	**858.9** (756.3-1581)	**119.9** (113.2-166.7)
8mg/L	**900.1** (851.9-1338)	**130.1** (108.4-152.2)
16mg/L	**923.2** (634.3-977.3)	**157.3** (148.6-166)
32mg/L	**908.4** (686.4-936.1)	**134.1** (120.5-138.7)
64mg/L	**825.9** (522.9-971.6)	**124.7** (123.5-125.8)
128mg/L	**594.7** (302.8-744.8)	**196.8** (163.4-230.2)

3.3.2.2.1 Effect of GA on growth of reference *P. aeruginosa* during CAZ treatment

Growth curves were performed on the *P. aeruginosa* reference strains in the same CAZ concentration ranges as previously but with addition of 50mg/L GA to each and the resulting growth curves were analysed and compared as before.

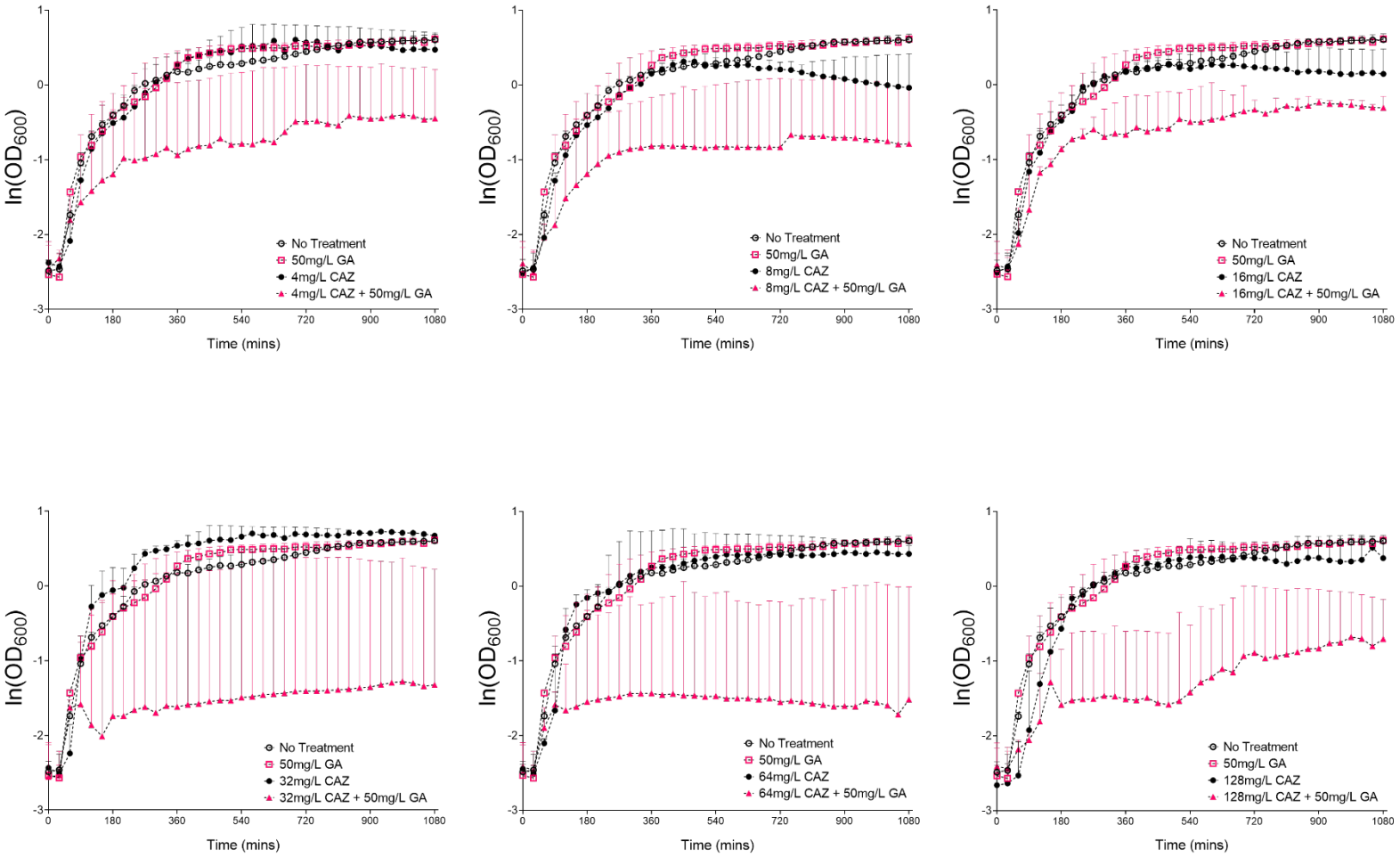


Figure 3-23. Transformed (natural log) OD_{600} growth curves of PAO1 measured every 30mins overnight with increasing concentrations of CAZ in the absence and presence of GA. Median and 95% confidence intervals of biological replicates (means of technical replicates) (n=3).

Growth curves of PAO1 appear to show addition of GA to CAZ had a potentiation effect on the antibiotic at a wide range of CAZ concentrations with combination treatment growth curves deviating from CAZ-only treatment (albeit often with wide confidence intervals) (**Figure 3-23**). However, examining these curves in their entirety using their AUCs did not reveal any significant differences between presence and absence of GA at each individual CAZ concentration. Similarly, while analysis of the variance for PAO1 for all growth tests carried out showed there was a significant change in the AUCs across the experiments ($p=0.003$), no concentration of CAZ significantly changed the growth curves from the untreated PAO1 control, regardless of GA presence (**Figure 3-24**).

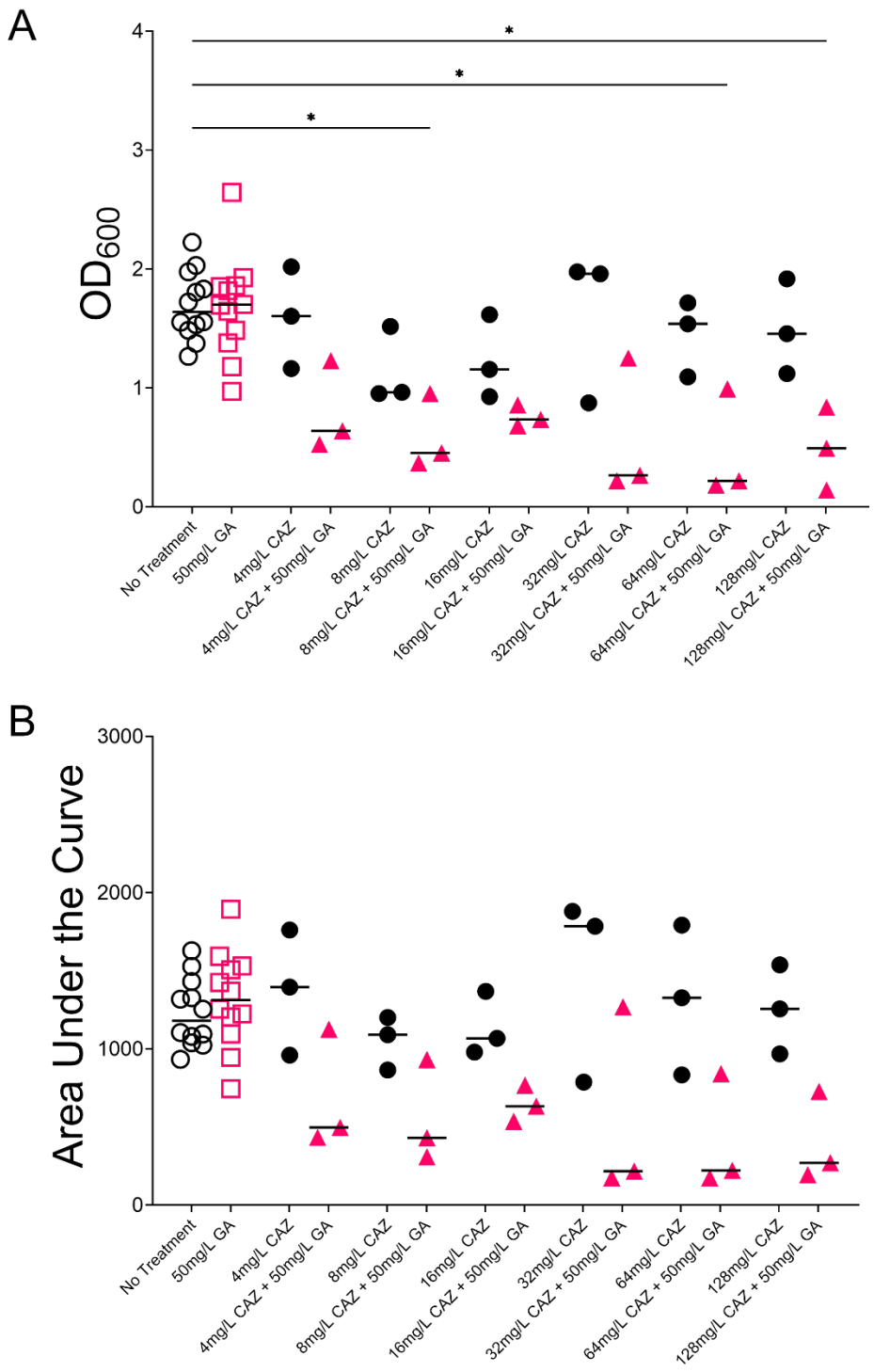


Figure 3-24. Comparisons of growth metrics of P. aeruginosa PAO1 from growth curves after incubation with CAZ and GA/CAZ. ***A****. OD_{600} results of PAO1 at the endpoint of overnight growth (biological replicates with lines at median). There was significant variance across the OD_{600} results of PAO1 after overnight treatment (p=0.003). There was a significant reduction in the OD_{600} from control after incubation with GA/CAZ(8mg/L) (p=0.0414), GA/CAZ(64mg/L) (p=0.0294) and GA/CAZ(128mg/L) (p=0.0187).* ***B****. AUCs of PAO1 overnight growth curves (biological replicates with lines at median). There was significant variance across the AUC results of PAO1 after overnight treatment (p=0.031).*

Unlike AUCs, certain OD_{600} were significantly reduced from control PAO1 OD_{600}. Interestingly, these significant changes all occurred in co-treatments with GA; at GA/CAZ(8mg/L) (p=0.04, n=3), GA/CAZ(64mg/L) (p=0.0295, n=3) and GA/CAZ(128mg/L) (p=0.02, n=3) (**Figure 3-24**). While all of the CAZ-only treatments resulted in $^1/_2$max results for all replicates, this was not the case when GA was present and only 1 of the 3 biological replicates performed reached the half PAO1 maximal growth.

Plotting of growth of PA14 during GA/CAZ treatment showed deviation of growth with co-treatments at specific CAZ concentrations with greater inhibition seen with GA/CAZ(32mg/L), GA/CAZ(64mg/L) and GA/CAZ(128mg/L) vs. their counterparts (**Figure 3-25**). Areas Under the Curve showed significant variance across the dataset (p=0.0002) but no individual CAZ concentration pair showed a significant AUC, with vs. without GA (**Figure 3-26**). There was an apparent difference with the effect of the presence of GA on certain CAZ concentrations and in comparison with the PA14 control growth. At GA/CAZ(32mg/L) (p=0.04, n=3), GA/CAZ(64mg/L) (p=0.01, n=3) and GA/CAZ(128mg/L) (p=0.03, n=3) there was a significant reduction in their AUCs from control PA14, but CAZ-only at these concentrations was not changed from control. This is similar to the result visualised in the transformed growth plots (**Figure 3-25**).

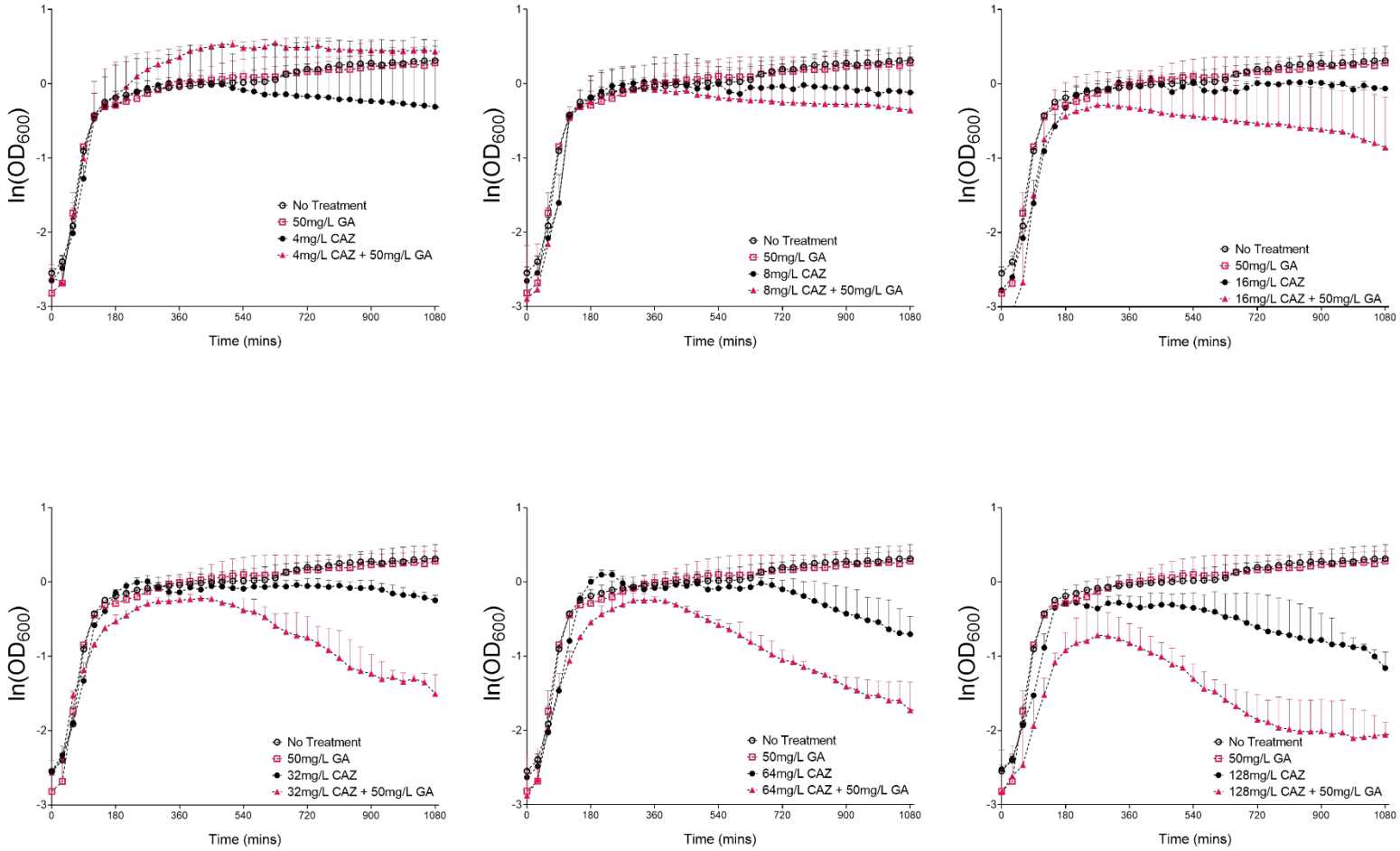


Figure 3-25. Transformed (natural log) OD_{600} growth curves of PA14 measured every 30mins overnight with increasing concentrations of CAZ in the absence and presence of GA. Median and 95% confidence intervals of biological replicates (means of technical replicates) (n=3).

As with AUCs of the entire growth data, GA/CAZ did not significantly reduce the OD_{600} of PA14 vs. their CAZ-only counterpart (**Figure 3-26**). However, GA/CAZ was more effective at changing OD_{600} from control growth at several CAZ concentrations, than the antibiotic alone. These were the same concentrations which showed a significant difference for the AUC results; GA/CAZ(32mg/L) (p=0.009, n=3), GA/CAZ(64mg/L) (p=0.005, n=3) and GA/CAZ(128mg/L) (p=0.0009, n=3). The only concentration of CAZ which caused significant OD_{600} reduction from control PA14 was 128mg/L (p=0.01, n=3).

As with the other measurements, $^1/_2$max results indicated the importance of combining GA with CAZ concentrations ≥32mg/L as none of the co-treatments at these three CAZ concentrations resulted in PA14 reaching half its maximal growth (0.66 [95%CI 0.55-0.77]). In contrast, treatment with 32mg/L CAZ alone had a $^1/_2$max of 134.1 [95%CI 120.5-138.7] mins and 64mg/L CAZ and 128mg/L CAZ each achieved half maximal growth of the control in 2 of 3 biological replicates.

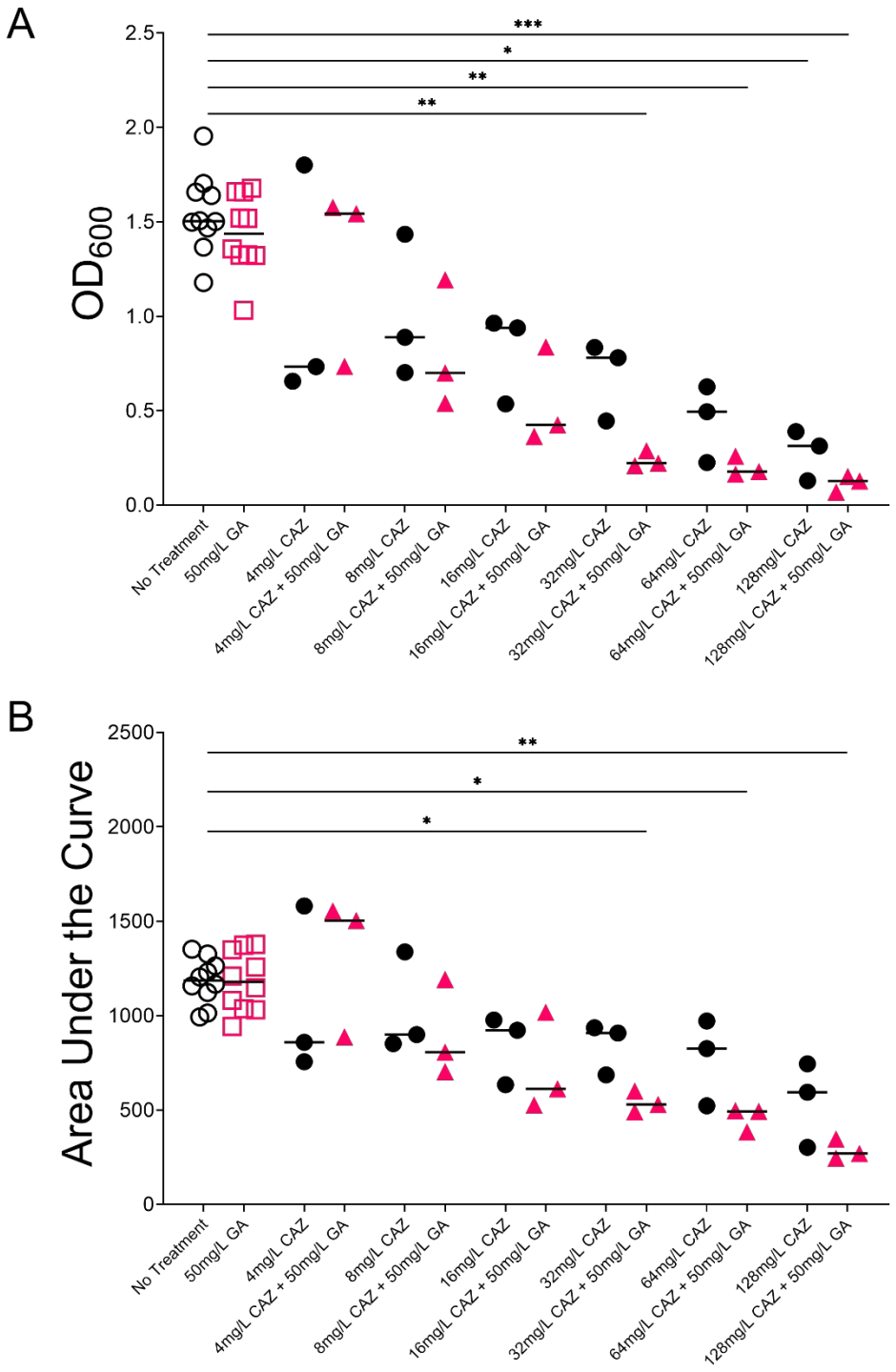


Figure 3-26. Comparisons of growth metrics of P. aeruginosa PA14 from growth curves after incubation with CAZ and GA/CAZ. ***A.*** *OD_{600} results of PA14 at the endpoint of overnight growth (biological replicates with lines at median). There was significant variance across the OD_{600} results of PAO1 after overnight treatment ($p<0.0001$). There was a significant reduction in the OD_{600} from control after incubation with GA/CAZ(32mg/L) ($p=0.009$), GA/CAZ(64mg/L) ($p=0.005$), GA/CAZ(128mg/L) ($p=0.0009$) and 128mg/L CAZ ($p=0.0126$).* ***B.*** *AUCs of PA14 overnight growth curves (biological replicates with lines at median). There was significant variance across the AUC results of PAO1 after overnight treatment ($p=0.0002$). There was a significant reduction in the AUCs from control after incubation with GA/CAZ(32mg/L) ($p=0.0397$), GA/CAZ(64mg/L) ($p=0.0139$), and GA/CAZ(128mg/L) ($p=0.0031$).*

3.3.2.2.2 Effect of GA on viability of reference *P. aeruginosa* during CAZ treatment

Colony counts of PAO1 showed a high yet stable level of inhibition across the CAZ concentrations tested (**Table 3-10, Figure 3-27**). From 8 to 128mg/L CAZ the inhibition of CFU/mL was similar: 94.48 [95%CI 91.67-99.06] % at 8mg/L, 97.33 [95%CI 90.33-99.16] % at 16mg/L, 98.65 [95%CI 92.69-99.61] % at 32mg/L, 96.54 [95%CI 93.77-99.68] % at 64mg/L and 98.11 [95%CI 96.15-99.90] % at 128mg/L. These results are in contrast to the growth curve data, where no inhibition of PAO1 by CAZ was apparent.

*Table 3-10. Colony forming units (CFU/mL) of P. aeruginosa PAO1 after overnight treatment with CAZ and GA/CAZ at increasing TOB concentrations. * indicates CFU/mL results where significantly fewer colonies were recovered for GA/TOB treatment than TOB treatment at a given TOB concentration ($p<0.05$) (Mann-Whitney U test). # indicates CFU/mL results which were significantly reduced from untreated control growth ($p<0.05$) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3). Median[95% confidence intervals].*

	CAZ	GA/CAZ
0mg/L	**1.38×10^9** [5.92×10^8 - 1.59×10^9]	**1.25×10^9** [4.83×10^8 - 1.16×10^9]
4mg/L	**1.09×10^8** [4.25×10^8 - 1.75×10^9]	**3.33×10^6** [2.66×10^6 – 4.00×10^8]
8mg/L	**2.37×10^7** [7.40×10^6 – 7.26×10^8]	***# 8.13×10^5** [1.14×10^4 - 1.90×10^7]
16mg/L	**2.50×10^7** [7.19×10^6 – 1.12×10^8]	****# 5.00×10^5** [7.66×10^4 - 1.10×10^7]
32mg/L	**1.35×10^7** [4.05×10^6 - 1.12×10^8]	***# 1.08×10^6** [7.66×10^4 - 1.10×10^7]
64mg/L	**1.11×10^7** [2.53×10^6 – 8.19×10^7]	***# 5.00×10^5** [2.78×10^5 – 2.88×10^6]
128mg/L	**# 2.29×10^6** [1.31×10^6 – 1.67×10^7]	**# 5.10×10^5** [2.09×10^4 - 1.00×10^6]

Combining GA with CAZ at the previously tested concentrations resulted in addition reductions in CFU/mL results of PAO1. There were significant reductions in the colonies resulting from GA/CAZ compared with CAZ-only treatments at 8mg/L ($p=0.02$, $n=5$), 16mg/L ($p=0.009$, $n=5$), 32mg/L ($p=0.02$, $n=5$) and 64mg/L ($p=0.03$, $n=4$). Interestingly, and as with CAZ-only treatments, increasing concentrations GA/CAZ from 8-128mg/L resulted in similar CFU/mL results (**Table 3-10**).

There were significant reductions in CFU, compared with control PAO1, resulting from GA/CAZ combinations at concentrations of CAZ where the antibiotic alone did not significantly reduce CFU/mL; at GA/CAZ(8mg/L) ($p=0.0002$), GA/CAZ(16mg/L) ($p=0.04$) and GA/CAZ(64mg/L) ($p=0.0002$). There were also significant reductions from control at 32 and 128mg/L whether or not GA was present ($p<0.05$).

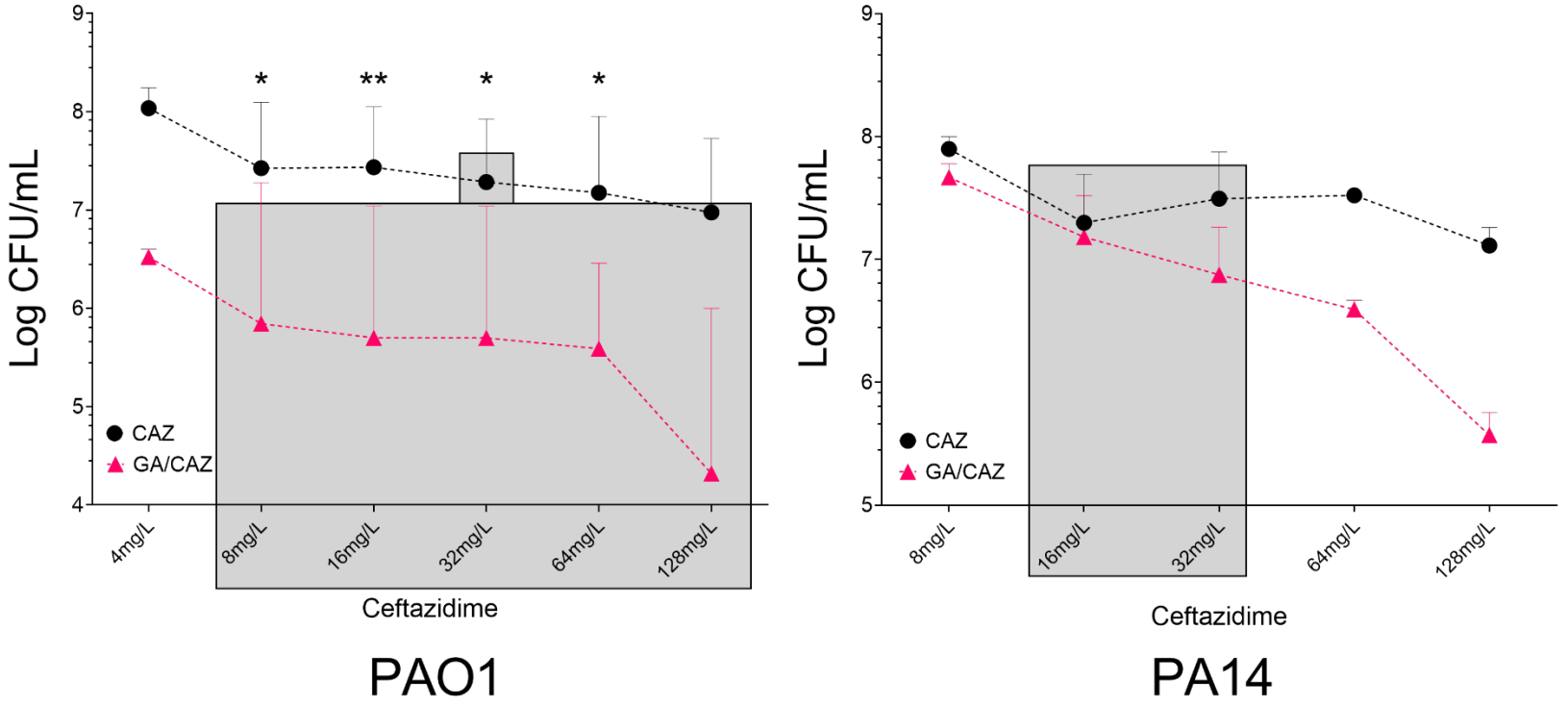


*Figure 3-27. Colony forming units(CFU/mL) of P. aeruginosa PAO1 after overnight treatment with CAZ and GA/CAZ at increasing CAZ concentrations. Medians of biological replicates (with 95% confidence intervals). * indicates concentrations of TOB where addition of GA caused a significant reduction on CFU/mL in a paired test, without vs. with GA. For PAO1 significant reductions in the colonies resulting from GA/CAZ compared with CAZ-only treatments at 8mg/L (p=0.0159, n=5), 16mg/L (p=0.0087, n=5), 32mg/L (p=0.0173, n=5) and 64mg/L (p=0.0317, n=4) (Mann-Whitney U tests). Shaded areas indicates treatments which resulted in significant reductions in CFU/mL compared to control under the same growth conditions. This was the case for PAO1 with GA/CAZ (8-128mg/L) and CAZ (32 and 128mg/L) (p>0.05). For PA14, 16 and 32mg/L CAZ resulted in significant CFU/mL reductions both for GA/CAZ and CAZ-only (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)).*

Colony forming units of PA14 showed a similar stable level of inhibition across the CAZ concentrations tested, as PAO1 had (**Figure 3-27**, **Table 3-11**). Percent inhibition of control PA14 was seen at comparable level across 8-128mg/L CAZ; 96.56 [95%CI 95.00-98.12] % at 8mg/L, 99.12 [95%CI 96.03-99.36] % at 16mg/L, 97.89 [95%CI 96.15-99.62] % at 32mg/L, 98.67 [95%CI 98.50-98.83] % at 64mg/L and 99.51 [95%CI 99.42-99.61] % at 128mg/L.

*Table 3-11. Colony forming units(CFU/mL) of P. aeruginosa PA14 after overnight treatment with CAZ and GA/CAZ at increasing TOB concentrations. * indicates CFU/mL results where significantly fewer colonies were recovered for GA/TOB treatment than TOB treatment at a given TOB concentration (p<0.05) (Mann-Whitney U test). # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥2). Median [95% confidence intervals].*

	CAZ	**GA/CAZ**
0mg/L	**1.80×10^9** [$8.22 \times 10^8 – 2.00 \times 10^9$]	**1.55×10^9** [5.50×10^8 - 1.81×10^9]
8mg/L	**7.55×10^8** [$1.73 \times 10^8 – 3.20 \times 10^9$]	**7.99×10^8** [2.30×10^8 - 2.00×10^9]
16mg/L	**# 8.97×10^8** [$2.50 \times 10^8 – 2.60 \times 10^9$]	**# 3.68×10^8** [$2.20 \times 10^8 – 1.94 \times 10^9$]
32mg/L	**# 5.30×10^8** [$3.63 \times 10^8 – 1.75 \times 10^9$]	**# 2.54×10^8** [$2.16 \times 10^8 – 1.25 \times 10^9$]
64mg/L	**1.51×10^8** [$3.45 \times 10^7 – 3.30 \times 10^8$]	**1.91×10^7** [$1.77 \times 10^7 – 3.35 \times 10^7$]
128mg/L	**6.10×10^6** [$4.80 \times 10^6 – 4.19 \times 10^8$]	**1.94×10^5** [2.05×10^3- 3.50×10^4]

Unlike PAO1, however, addition of GA to CAZ treatments did not result in additional reductions in PA14 CFU/mL at individual CAZ concentrations when compared with and without GA. There were however differences seen in comparisons with control PA14 CFU/mL and there was significant variance across all results (p<0.0001). Treatment with 16 and 32mg/L resulted in significant reductions in CFU/ml whether GA was present or not (p<0.05).

While CAZ at 64 and 128mg/L, with and without GA, also had reduced CFU/mL (**Table 3-11**), the ability to analyse these results was limited by the fact that there were only two biological replicates performed.

3.3.2.3 Colistin

Reference strains PAO1 and PA14 were next tested for their susceptibilities to the polymyxin antibiotic colistin (CST) (PAK was not available in the lab at the time of these experiments). The EUCAST MIC of CST for *P. aeruginosa* is 2mg/L in broth microdilution. The reference strains were found to be sensitive to colistin in this assay.

Growth of PAO1 during treatment with CST was shown to be altered from control growth at all concentrations tested and while the lowest concentration here (0.5mg/L) resulted in an OD_{600} (1.31 [95%CI 0.92-3.14]) comparable to control (1.67 [95%CI 1.38-1.85]) the growth plots of each are distinct. Moderate inhibition is visible during treatment with 1mg/L CST (**Figure 3-28**).

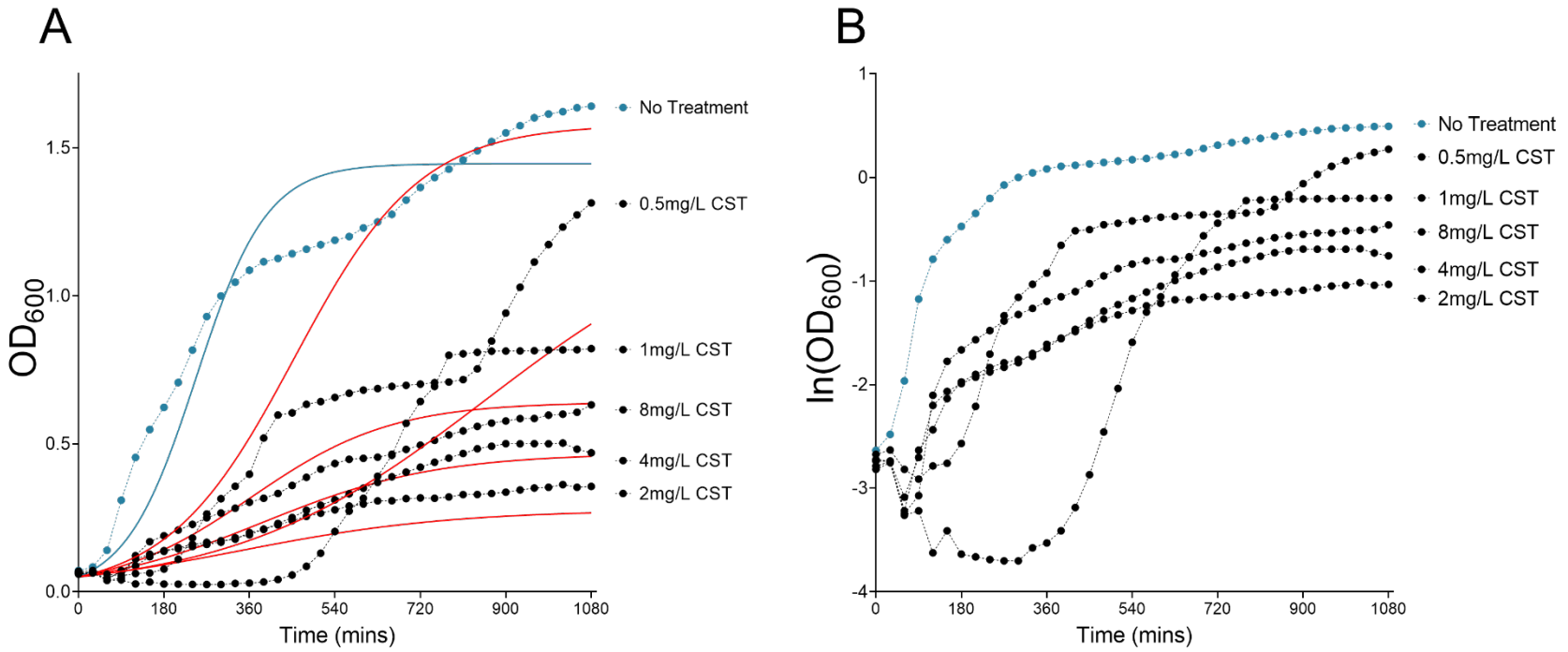


Figure 3-28. Overnight OD_{600} of PAO1 during treatment with CST (0.5-8mg/L) measured every 30mins. A. OD_{600} (dotted lines) (median values of at least three biological replicates) and fitted curves of non-linear regression (Logistic Growth model) (Red lines) for each concentration tested. B. Transformed OD_{600} (natural log) results (median values).

The AUC results for the OD_{600} curves show that CST is inhibitory to PAO1 between 1 and 8mg/L although results within this concentration range are comparable to each other with wide overlapping confidence interval, suggesting the PAO1 response to CST is variable. Similarly the $^1/_2$max OD_{600} of PAO1 was only reached by a single biological replicate at 1, 4 and 8mg/L CST and never at 2mg/L (n=3) (**Table 3-12**).

Table 3-12. Growth metrics for PAO1 after treatment with CST (0.5-8mg/L) derived from overnight growth curves. Medians [95% confidence intervals].

	Area Under the Curve	**$^1/_2$max (mins)**
0mg/L	**1181** [1040 - 1432]	**227.8** [214.1 - 292.6]
0.5mg/L	**571.3** [382 - 2121]	**478.4** [264.4 - 907.1]
1mg/L	**564.1** [19.75 - 568.7]	**707.8** [-]
2mg/L	**281.8** [17.25 - 292.4]	**-**
4mg/L	**335** [229.8 - 387.2]	**1014** [-]
8mg/L	**409.2** [331.6 - 613.5]	**468.3** [-]

Strain PA14 had a dose-response reaction to treatment with CST with moderate inhibition at lower concentrations (0.25-1mg/L) and growth of OD_{600} prevented at higher CST levels (2-8mg/L) with the lowest OD_{600} seen at the highest concentration of the antibiotic (**Figure 3-29**).

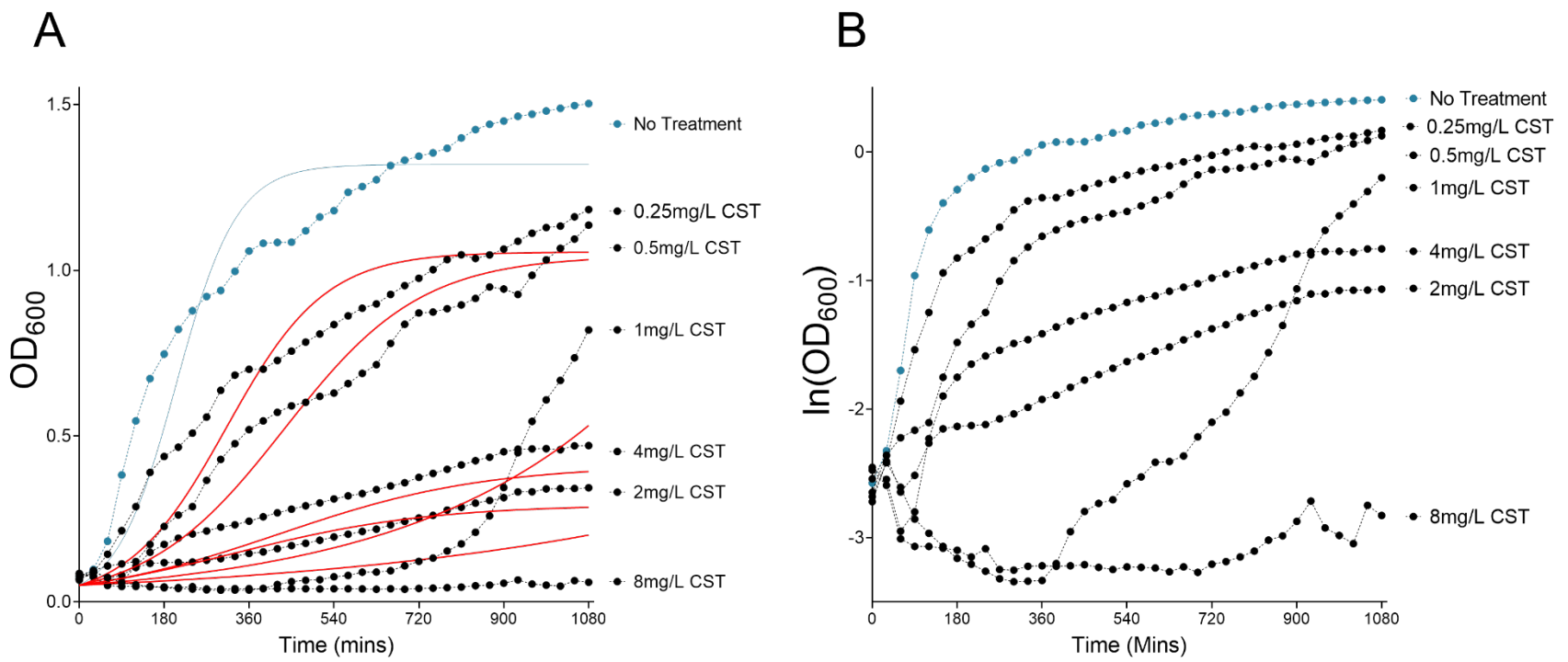


Figure 3-29. Overnight OD_{600} of PAO1 during treatment with CST (0.25-8mg/L) measured every 30mins. ***A.*** *OD_{600} (dotted lines) (median values of at least three biological replicates) and fitted curves of non-linear regression (Logistic Growth model) (Red lines) for each concentration tested.* ***B.*** *Transformed OD_{600} (natural log) results (median values).*

AUCs of PA14 were similarly stratified by CST concentration and a dose-response seen in the results and, as with the curves, 8mg/L causing the greatest inhibition of OD_{600}. Similarly, the $^1/_2$max OD_{600} of PA14 (0.66 [95%CI 0.55-0.77]) was not reached by the strain when treated with any concentration above 2mg/L (**Figure 3-29**, **Table 3-13**).

Table 3-13. Growth metrics for PA14 after treatment with CST (0.25-8mg/L) derived from overnight growth curves. Medians [95% confidence intervals].

	Area Under the Curve	**$^1/_2$max (mins)**
0mg/L	**1186** [1014 – 1327]	**208.1** [153.6 - 261.5]
0.25mg/L	**832.7** [643.5 - 956.7]	**339** [228.9 - 568.2]
0.5mg/L	**664** [525.5 – 913]	**501.1** [392.2 - 833.9]
1mg/L	**184.5** [136.4 - 372.6]	**1172** [877.1 – 1270]
2mg/L	**222.6** [96.21 - 327.7]	**-**
4mg/L	**324.9** [66.8 - 406.1]	**-**
8mg/L	**58.97** [35.47 – 235]	**1282** [-]

3.3.2.3.1 Effect of GA on growth of reference *P. aeruginosa* during CST treatment

Growth curves were performed on the *P. aeruginosa* reference strains in the same CST concentration ranges as previously but with addition of 50mg/L GA to each and the resulting growth curves were analysed and compared as before.

PAO1 growth curves did not suggest potentiation of CST by GA (**Figure 3-30**). Divergence of growth patterns, visualised by plotted transformed OD_{600} was seen in a limited

manner, early in the PAO1 growth in the form of inhibition by CST from which PAO1 recovers. Examination of the AUCs of these growth curves showed that there was variance across the growth of PAO1 ($p<0.0001$) but, in a reversal of results seen for other antibiotics, comparison to control PAO1 showed that only two treatments significantly changed PAO1 AUCs; 2mg/L CST ($p=0.009$, $n=3$) and 4mg/L CST ($p=0.04$, $n=3$) (**Figure 3-30**).

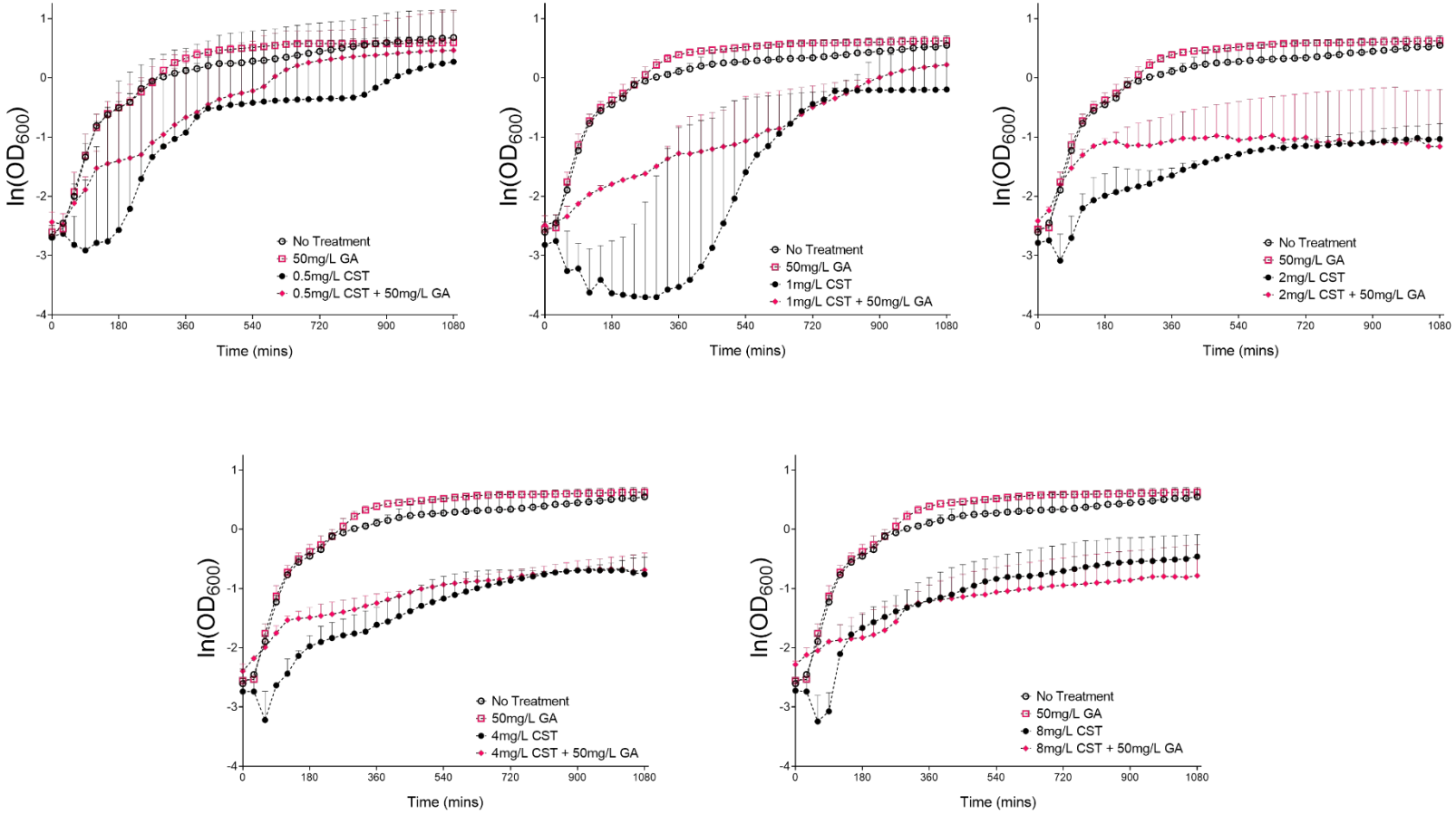


Figure 3-30. Transformed (natural log) OD_{600} growth curves of PAO1 treated with CST and GA/CST. OD_{600} measured every 30mins overnight with increasing concentrations of CST in the absence (black closed circles) and presence of GA (pinks closed squares). Median and 95% confidence intervals of biological replicates (means of technical replicates).

On this occasion, multiple comparison to control PAO1 resulted in significant reduction in OD_{600} at 2mg/L CST in both the absence (p=0.01, n=3) and presence (p=0.04, n=3) of GA. The only other treatment which caused a significant OD_{600} change in this manner was GA/CST(8mg/L) (p=0.04, n=3) (**Figure 3-31**).

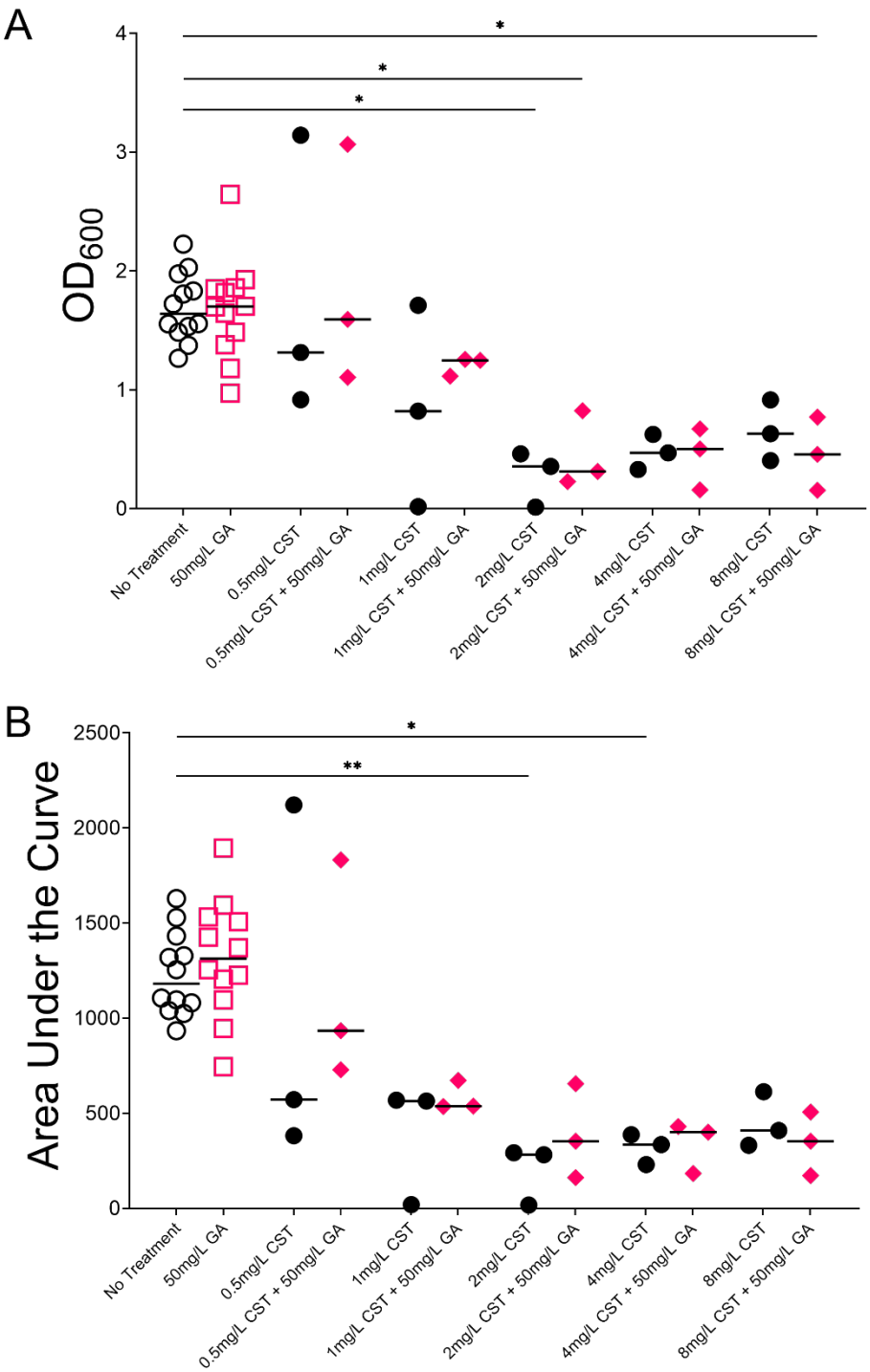


Figure 3-31. Comparisons of growth metrics of P. aeruginosa PAO1 from growth curves after incubation with CST and GA/CST. ***A.*** *OD_{600} results of PAO1 at the endpoint of overnight growth (biological replicates with lines at median). There was significant variance across the OD_{600} results of PAO1 after overnight treatments (p=0.0001). There was a significant reduction in the OD_{600} from control after incubation with both 2mg/L CST in the absence (p=0.0131) and presence of GA (p=0.0394). There was also significant reduction after incubation with GA/CST(8mg/L) (p=0.0394).* ***B.*** *AUCs of PAO1 overnight growth curves (biological replicates with lines at median). There was significant variance across the AUC results of PAO1 after overnight treatment with CST and GA/CST (p<0.0001). There was a significant reduction in the AUCs from control after incubation with 2mg/L CST (p=0.0089) 4mg/L CST (p=0.0426).*

Combining GA/CST was not more effective at inhibiting the growth of PA14 than CST only (**Figure 3-32**). In some cases, analyses indicated that the combination treatment was less effective than CST-only. While comparison to control PA14 showed reduction in AUCs by CST at 1mg/L ($p=0.01$, $n=3$), 2mg/L ($p=0.008$, $n=3$), 4mg/L ($p=0.02$, $n=4$) and 8mg/L ($p=0.002$, $n=3$), this was not the case for GA/CST at these concentrations. Only GA/CST(1mg/L) ($p=0.04$, $n=3$) and GA/CST(4mg/L) ($p=0.04$, $n=3$) significantly reduced overnight growth from control.

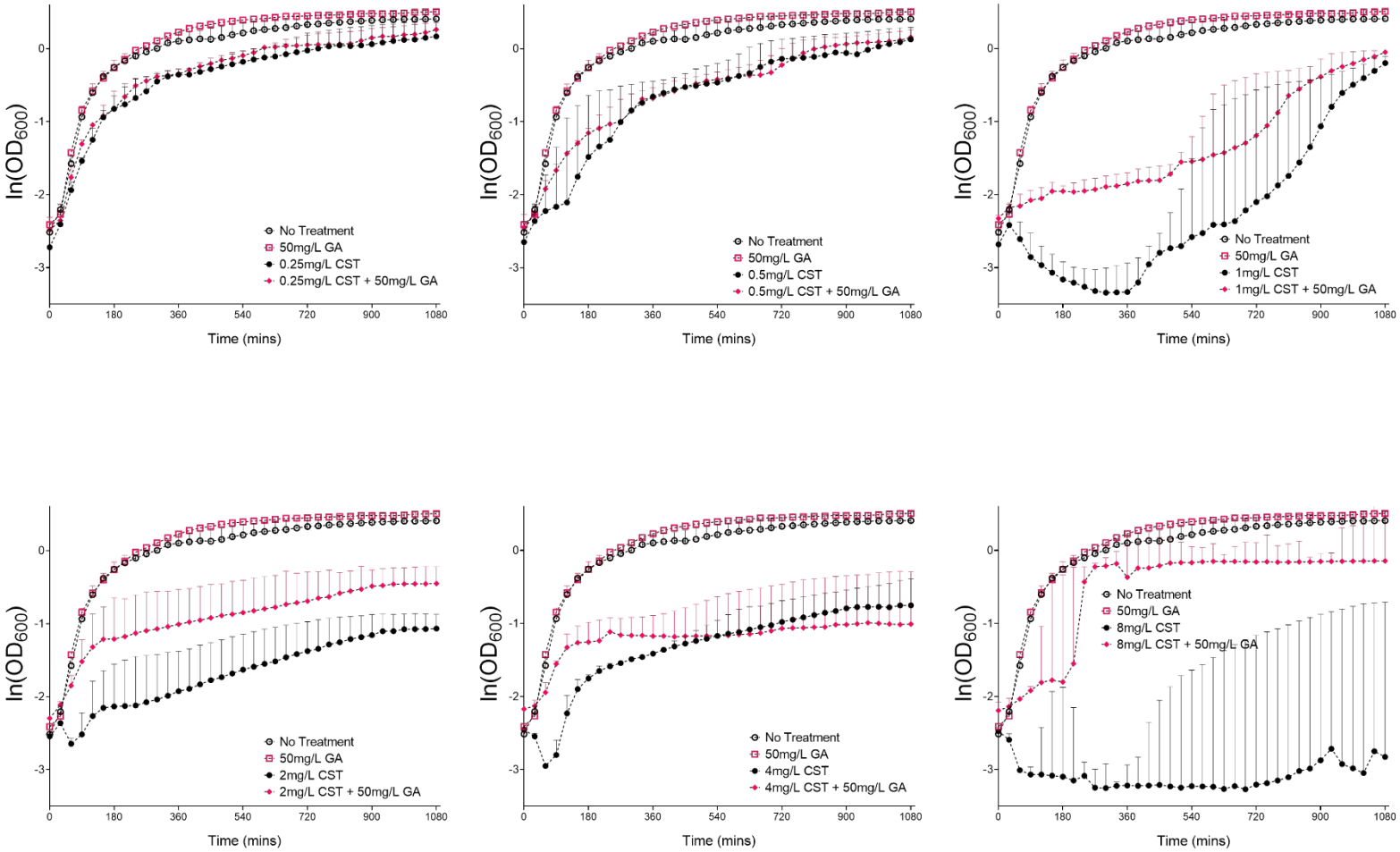


Figure 3-32. Transformed (natural log) OD_{600} growth curves of PA14 treated with CST and GA/CST. OD_{600} measured every 30mins overnight with increasing concentrations of CST in the absence (black closed circles) and presence of GA (pinks closed squares). Median and 95% confidence intervals of biological replicates (means of technical replicates).

As with the AUCs, analysis of OD_{600} results did not show GA/CST to be more effective in inhibiting PA14 than CST alone (**Figure 3-33**). PA14 was significantly changed from control growth by 2 and 4mg/L but this was the case whether GA was present ($p=0.04$ and $p=0.02$, respectively, $n=3$) or absent ($p=0.006$ and $p=0.01$, respectively, $n=3$). At 8mg/L CST caused significant OD_{600} reduction ($p=0.002$, $n=3$) but GA/CST(8mg/L) did not.

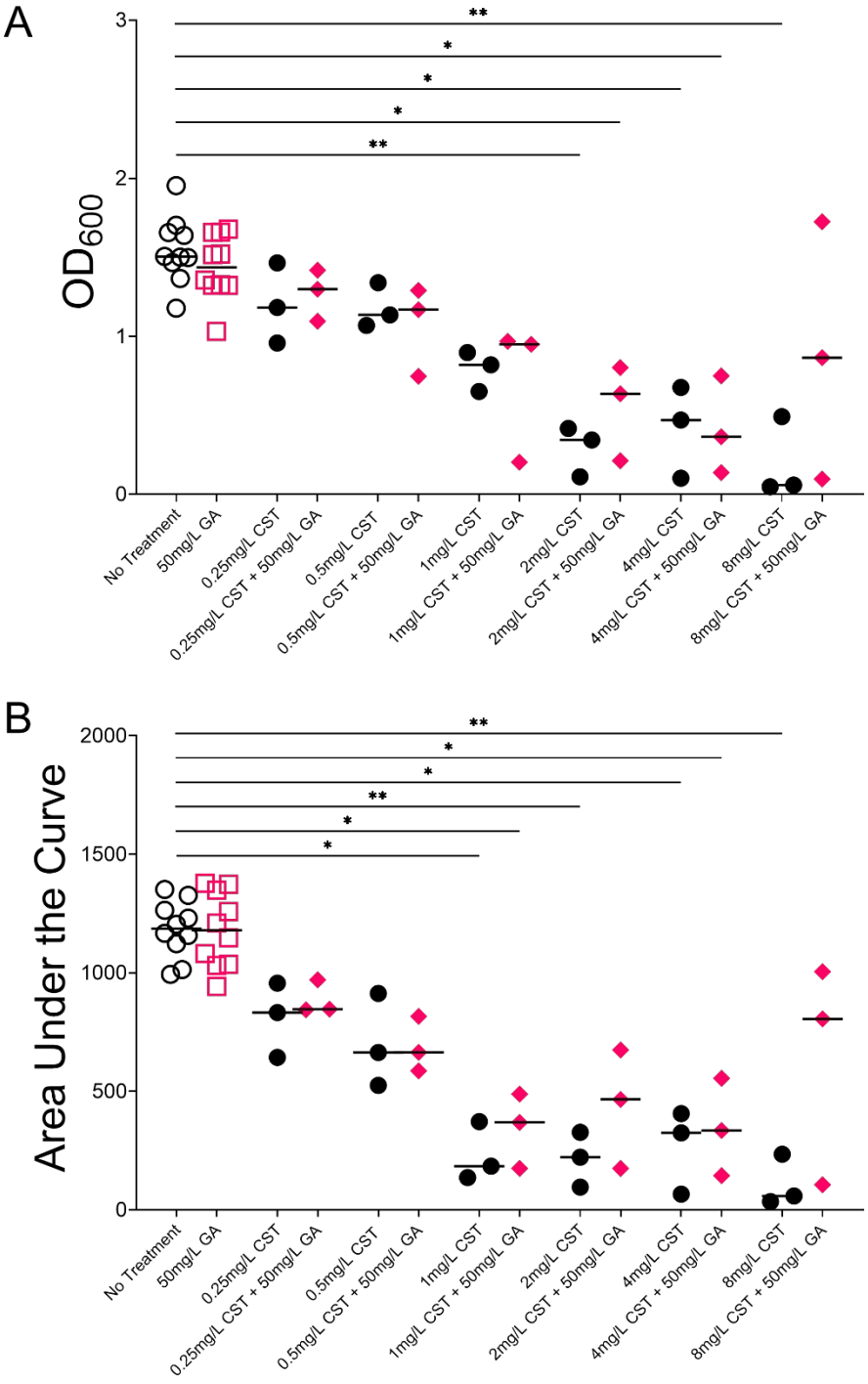


Figure 3-33. Comparisons of growth metrics of P. aeruginosa PAO1 from growth curves after incubation with CST and GA/CST. ***A.*** *OD_{600} results of PA14 at the endpoint of overnight growth with CST and GA/CST (biological replicates with lines at median). There was significant variance across the OD_{600} results of PA14 after overnight treatments ($p<0.0001$). There was a significant reduction in the OD_{600} from control after incubation with CST 2-8mg/L with and without GA ($p<0.05$), with the exception of GA/CST(8mg/L). There was also significant reduction after incubation with GA/CST(8mg/L) ($p=0.0394$).* ***B.*** *AUCs of PA14 overnight growth curves with CST and GA/CST (biological replicates with lines at median). There was significant variance across the AUC results of PA14 after overnight treatment with CST and GA/CST ($p<0.0001$). There was a significant reduction in the AUCs from control after incubation with 1-8mg/L CST and GA/CST at 1 and 4mg/L ($p<0.05$).*

3.3.2.3.2 Effect of GA on viability of reference *P. aeruginosa* during CST treatment

As suggested by growth curves, there was a dose response reaction to CST by PAO1, seen in colony count results. Bacterial numbers were comparable to control PAO1 growth at CST 0.5-2mg/L CST, followed by inhibition at 4mg/L (99.99 [95%CI 99.98-99.99] % inhibition) and at 8mg/L (99.99 [95%CI 99.99-100.00] % inhibition) (**Table 3-14**).

When GA was co-administered with CST there was significant variance across all treatments of PAO1 (p=0.0006) but no difference seen CFU/mL results whether GA was present or not. Each pair of CST concentrations used (with or without GA) was comparable to each other in numerical terms (**Table 3-14**) and no difference was seen between them in paired tests (n=3). In multiple comparison tests, to control, 8mg/L CST was the only concentration at which there was significant reduction in CFUs from untreated and this was the case for CST-only and GA/CST (p=0.02 and p=0.03, respectively) (**Figure 3-34**).

Table 3-14. Colony forming units (CFU/mL) of P. aeruginosa PAO1 after overnight treatment with CST and GA/CST at increasing CST concentrations. # indicates CFU/mL results which were significantly reduced

from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3). Median [95% confidence intervals].

	CST	GA/CST
0mg/L	**1.38×10^9** [5.92×10^8 - 1.59×10^9]	**1.25×10^9** [4.83×10^8 - 1.16×10^9]
0.5mg/L	**2.09×10^9** []	**2.17×10^9** []
1mg/L	**1.53×10^9** [1.46×10^9 - 1.61×10^9]	**1.56×10^9** [1.29×10^9 - 1.83×10^9]
2mg/L	**8.60×10^8** [7.30×10^8 – 2.38×10^9]	**7.28×10^8** [5.80×10^8 – 2.29×10^9]
4mg/L	**4.44×10^4** [1.65×10^4 – 3.10×10^5]	**3.83×10^4** [1.25×10^4 – 5.04×10^4]
8mg/L	**# 1.73×10^3** [0 – 2.75×10^3]	**# 2.00×10^3** [0 – 5.00×10^3]

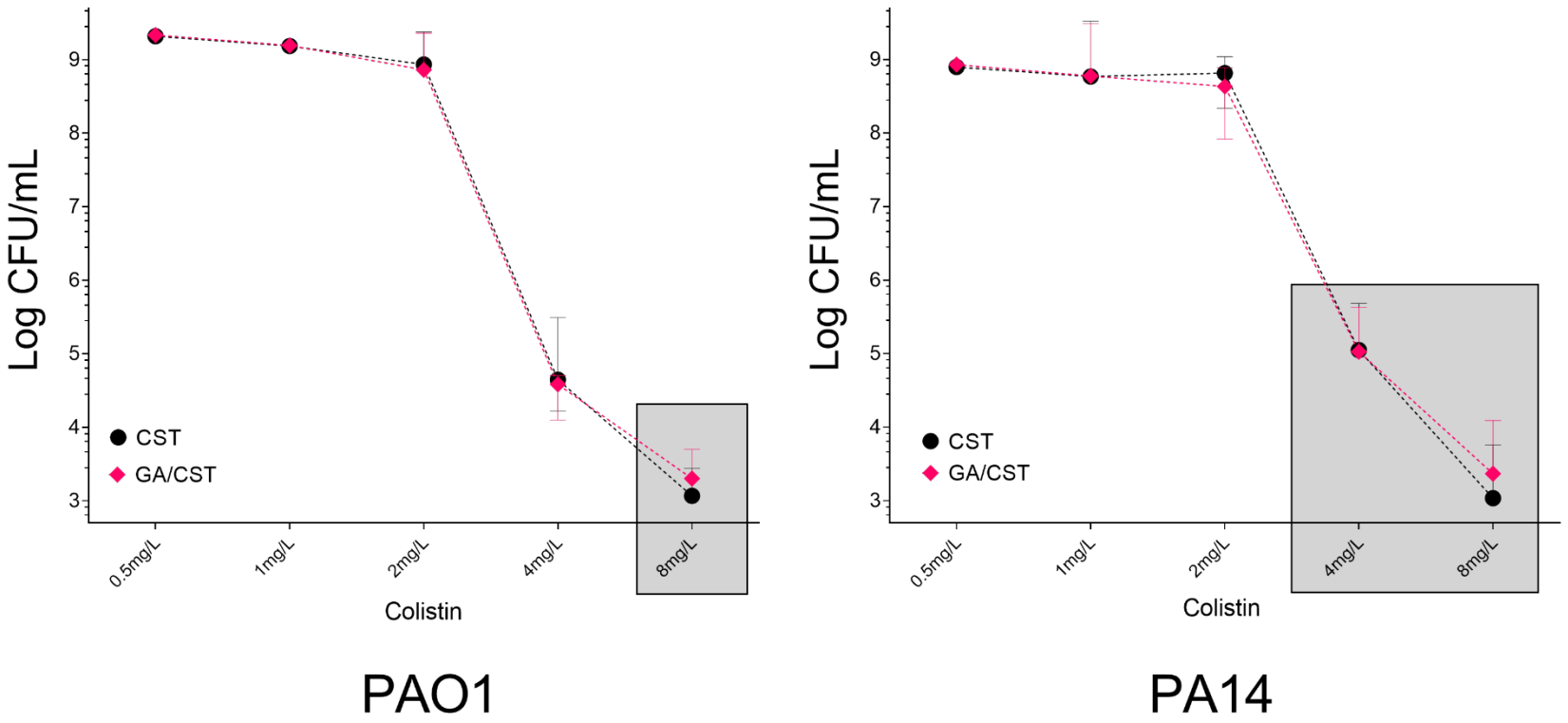


Figure 3-34. Colony forming units (CFU/mL) of P. aeruginosa PAO1 and PA14 after overnight treatment with CST and GA/CST at increasing CST concentrations. Medians of biological replicates (with 95% confidence intervals). Shaded areas indicates treatments which resulted in significant reductions in CFU/mL compared to control PAO1 under the same growth conditions. For PAO1, 8mg/L CST resulted in a significant reduction in CFU/mL in the presence and absence of GA. For PA14, there was a significant reduction in CFU/mL at 4 and 8mg/L CST, also with and without GA ($p>0.05$). (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)).

As with PAO1 and as seen by growth curves, there was a dose response reaction to CST by PA14, in colony count results. Bacterial numbers were comparable to control PAO1 growth at CST 0.5-2mg/L CST, followed by inhibition at 4mg/L (99.99 [95%CI 99.98-99.99] % inhibition) and at 8mg/L (100.00 [95%CI 99.99-100.00] % inhibition) (**Table 3-15**).

When GA was co-administered with CST there was significant variance across all treatments of PAO1 ($p<0.00001$) but no difference seen CFU/mL results whether GA was present or not. Each pair of CST concentrations used (with or without GA) was comparable to each other in numerical terms (**Table 3-15**) and no difference was seen between them in paired tests (n=3). In multiple comparison tests, to control, 4 and 8mg/L CST were the concentrations at which there was significant reduction in CFUs from untreated and this was the case for CST-only and GA/CST. At 4mg/L, p=0.02 and p=0.03, and at 8mg/L, p=0.002 and p=0.002, for CST and GA/CST, respectively.

Table 3-15. Colony forming units (CFU/mL) of P. aeruginosa PA14 after overnight treatment with CST and GA/CST at increasing TOB concentrations. # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3). Median [95% confidence intervals].

	CST	**GA/CST**
0mg/L	**1.80×10^9** [$8.22 \times 10^8 - 2.00 \times 10^9$]	**1.55×10^9** [5.50×10^8 - 1.81×10^9]
0.5mg/L	**7.90×10^8** []	**8.50×10^8** []
1mg/L	**5.85×10^8** [$3.71 \times 10^8 - 8.00 \times 10^8$]	**5.96×10^8** [$4.03 \times 10^8 - 7.90 \times 10^8$]
2mg/L	**7.48×10^8** [$4.50 \times 10^8 - 7.60 \times 10^8$]	**3.50×10^8** [$3.47 \times 10^8 - 5.90 \times 10^8$]
4mg/L	**# 2.68×10^4** [$2.39 \times 10^4 - 2.83 \times 10^5$]	**# 3.38×10^4** [$2.94 \times 10^4 - 2.56 \times 10^5$]
8mg/L	**# 0** [0 - 3.23×10^3]	**# 0** [0 – 6.96×10^3]

3.3.2.4 Ciprofloxacin

Reference strains PAO1 and PA14 were tested for their susceptibilities to the fluroquinolone antibiotic ciprofloxacin (strain PAK was not available in the lab at the time of these experiments). The EUCAST MIC of CIP for *P. aeruginosa* is 0.5mg/L in broth microdilution.

Strain PAO1 had a dose-response reaction to CIP in growth curve with no difference from control seen at the lowest concentrations tested (0.00097-0.00195mg/L), moderate inhibition of the OD_{600} with 0.0039-0.0625mg/L CIP and inhibition from 0.125-1mg/L (**Figure 3-35**).

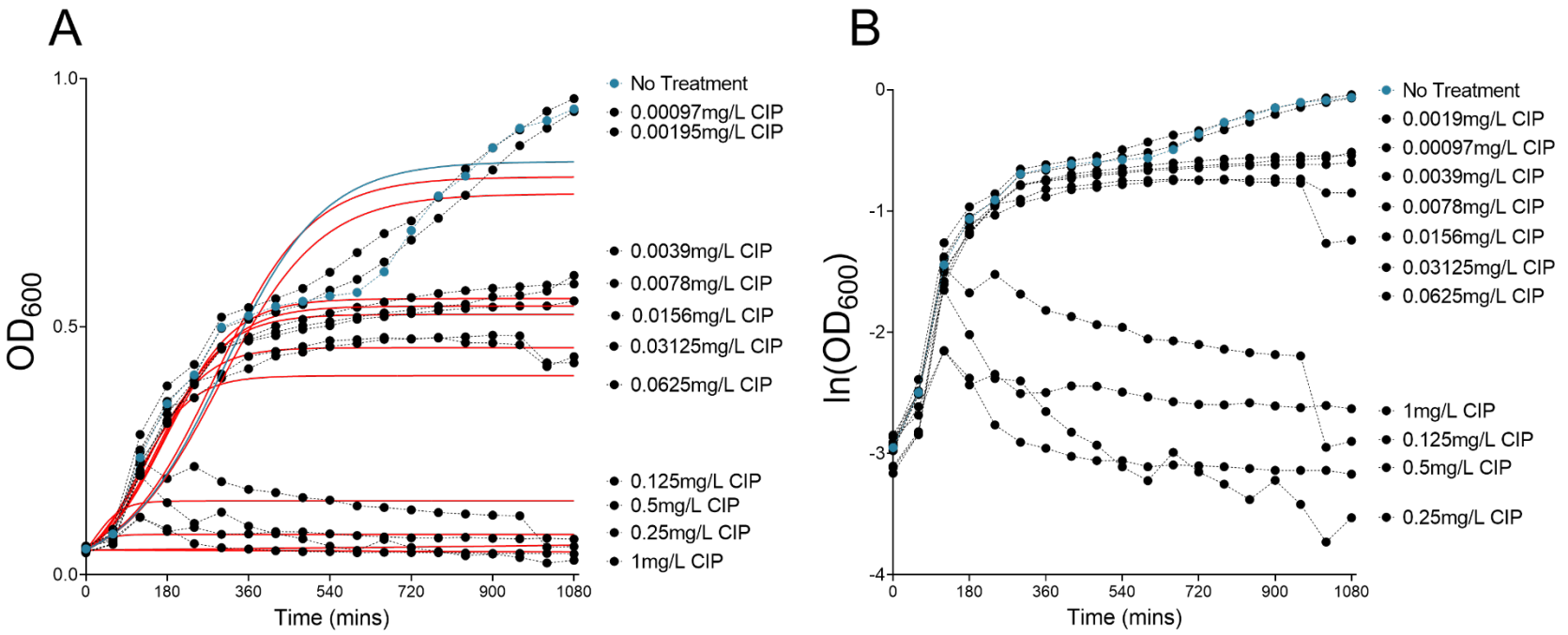


Figure 3-35. Overnight OD_{600} of PAO1 during treatment with CIP (0.00097-1mg/L) measured every 30mins. ***A.*** *OD_{600} (dotted lines) (median values of at least three biological replicates) and fitted curves of non-linear regression (Logistic Growth model) (Red lines) for each concentration tested.* ***B.*** *Transformed OD_{600} (natural log) results (median values).*

A dose response was also apparent in growth curves of PA14 with no inhibition at 0.0039-0.03125mg/L and increase inhibition at 0.0625 and 0.125mg/L CIP (**Figure 3-36**).

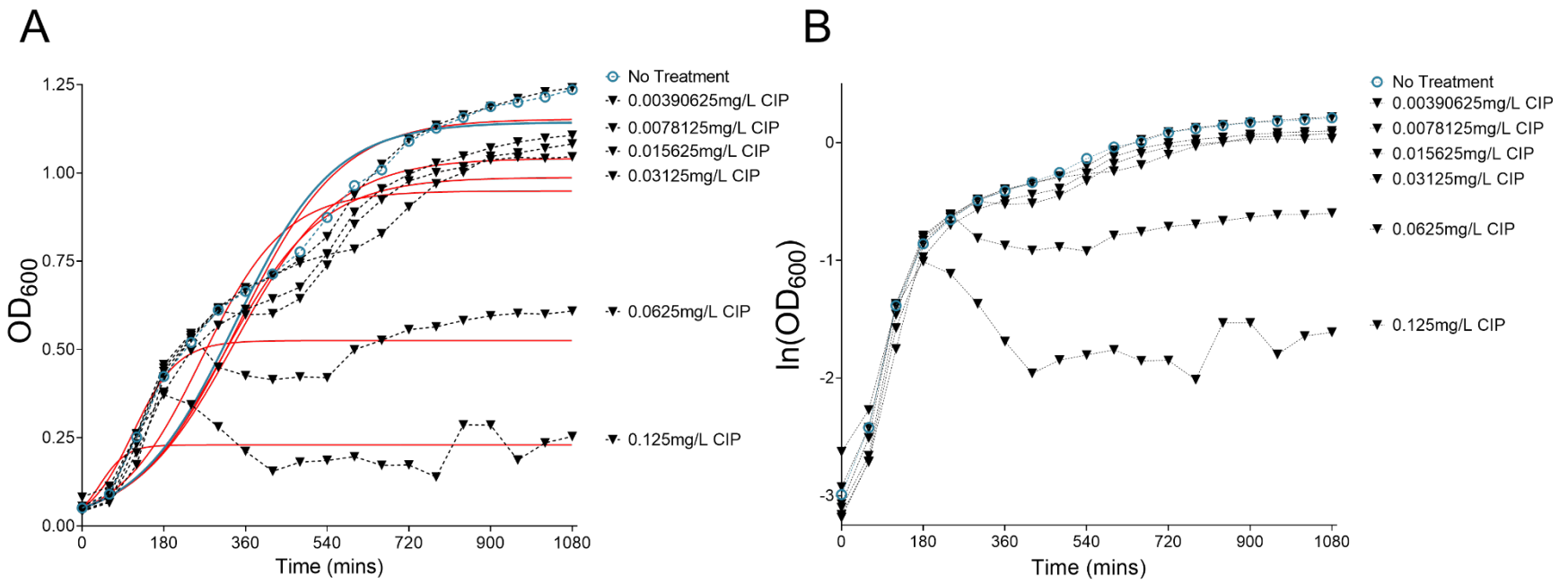


Figure 3-36. Overnight OD_{600} of PA14 during treatment with CIP (0.0039-1mg/L) measured every 30mins. ***A.*** *OD_{600} (dotted lines) (median values of at least three biological replicates) and fitted curves of non-linear regression (Logistic Growth model) (Red lines) for each concentration tested.* ***B.*** *Transformed OD_{600} (natural log) results (median value).*

3.3.2.4.1 Effect of GA on growth of reference *P. aeruginosa* during CIP treatment

Growth curves were performed on the *P. aeruginosa* reference strains in the same CIP concentration ranges as previously but with addition of 50mg/L GA to each and the resulting growth curves were analysed and compared as before.

Inspection of growth curves suggested the CIP was not potentiated by GA for strain PAO1 (**Figure 3-37**).

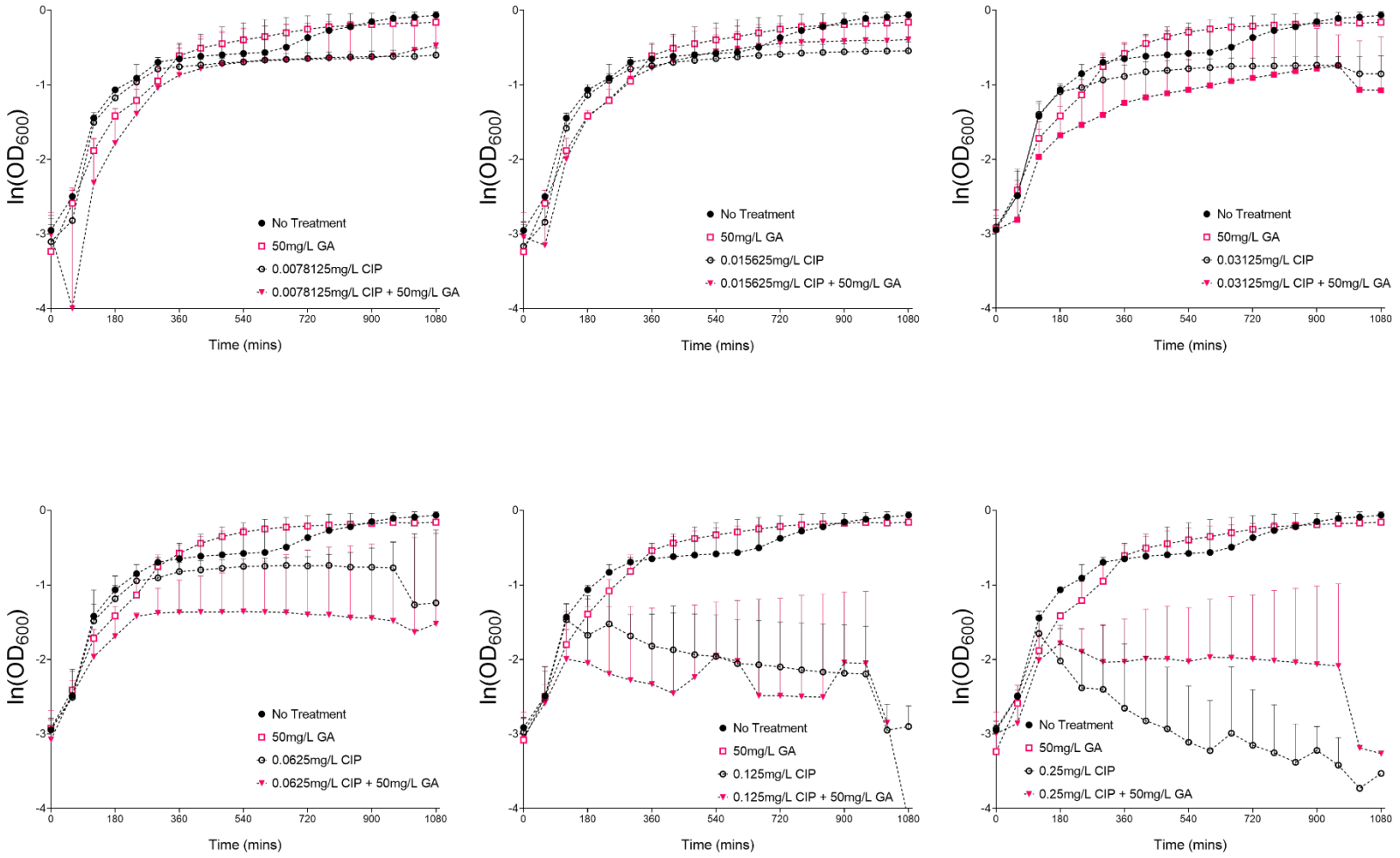


Figure 3-37. Transformed (natural log) OD_{600} growth curves of PAO1 treated with CIP and GA/CIP. OD_{600} measured every 60mins overnight with increasing concentrations of CIP in the absence (black closed circles) and presence of GA (pinks closed squares). Median and 95% confidence intervals of biological replicates (means of technical replicates).

Similarly to PAO1, growth curves of PA14 did not indicate potentiation of CIP by GA (**Figure 3-38**).

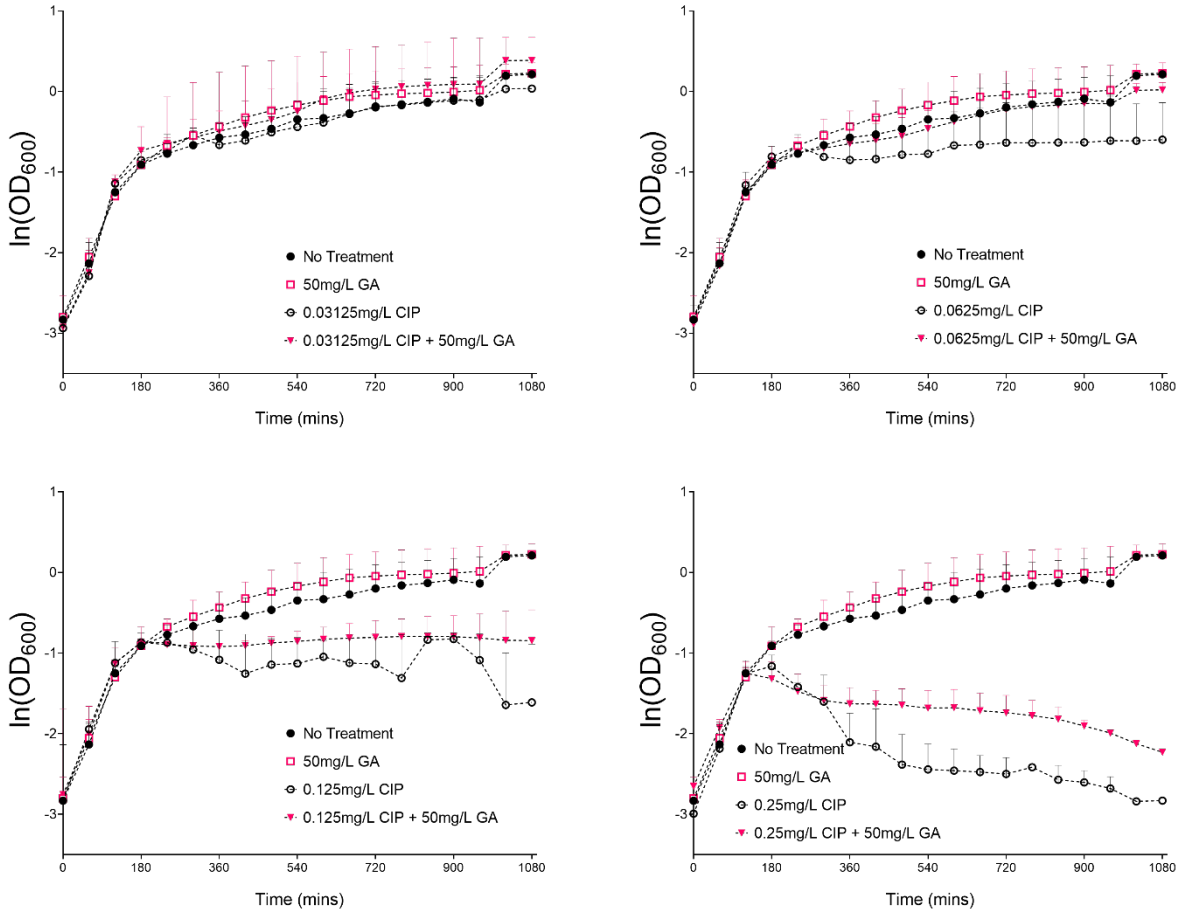


Figure 3-38. Transformed (natural log) OD_{600} growth curves of PA14 treated with CIP and GA/CIP. OD_{600} measured every 60mins overnight with increasing concentrations of CIP in the absence (black closed circles) and presence of GA (pinks closed squares). Median and 95% confidence intervals of biological replicates (means of technical replicates)

3.3.2.4.2 Effect of GA on viability of reference *P. aeruginosa* during CIP treatment

As suggested by growth curves, there was a dose response reaction to CIP by PAO1, seen in colony count results. Bacterial numbers were comparable to control PAO1 growth at 0.03125mg/L CIP. Inhibition was increased by increasing concentration of CIP; 0.0625mg/L resulted in 87.26 [95%CI 84.85-88.55] % inhibition, 0.125mg/L in 99.78 [95%CI 99.67-99.89] % inhibition , 0.25mg/L in 99.99 [95%CI 99.98-99.99] % inhibition and 0.5mg/L in 99.99 [95%CI 99.98-99.99] % inhibition (**Table 3-16**).

No difference seen CFU/mL results whether GA was present or not. Each pair of CIP concentrations used (with or without GA) was comparable to each other in numerical terms (**Table 3-16**) and no difference was seen between them in paired tests (n=3). In multiple comparison tests, to control, all concentrations >0.125mg/L CIP significantly reduced CFU/mL of PAO1 from control growth and this was the case whether GA was also present or not ($p<0.05$) (**Figure 3-39**).

Table 3-16. Colony forming units (CFU/mL) of P. aeruginosa PAO1 after overnight treatment with CIP and GA/CIP at increasing CIP concentrations. # indicates CFU/mL results which were significantly reduced

from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3). Median [95% confidence intervals].

	CIP	GA/CIP
0mg/L	**1.38×10^9** [5.92×10^8 - 1.59×10^9]	**1.25×10^9** [4.83×10^8 - 1.16×10^9]
0.03125mg/L	**7.98×10^8** [2.67×10^8 – 7.89×10^8]	**8.69×10^8** [1.73×10^8 – 8.69×10^8]
0.0625mg/L	**2.66×10^8** [1.63×10^8 – 2.66×10^8]	**6.70×10^7** [3.75×10^7 – 6.70×10^7]
0.125mg/L	**# 4.57×10^6** [1.88×10^6 – 4.57×10^6]	**# 2.71×10^7** [2.94×10^6 – 2.71×10^7]
0.25mg/L	**# 1.85×10^5** [7.04×10^4 – 2.94×10^5]	**# 2.44×10^5** [1.88×10^5 – 3.00×10^5]
0.5mg/L	**# 1.46×10^5** [3.11×10^4 – 2.60×10^5]	**# 1.79×10^4** [0 – 3.57×10^4]

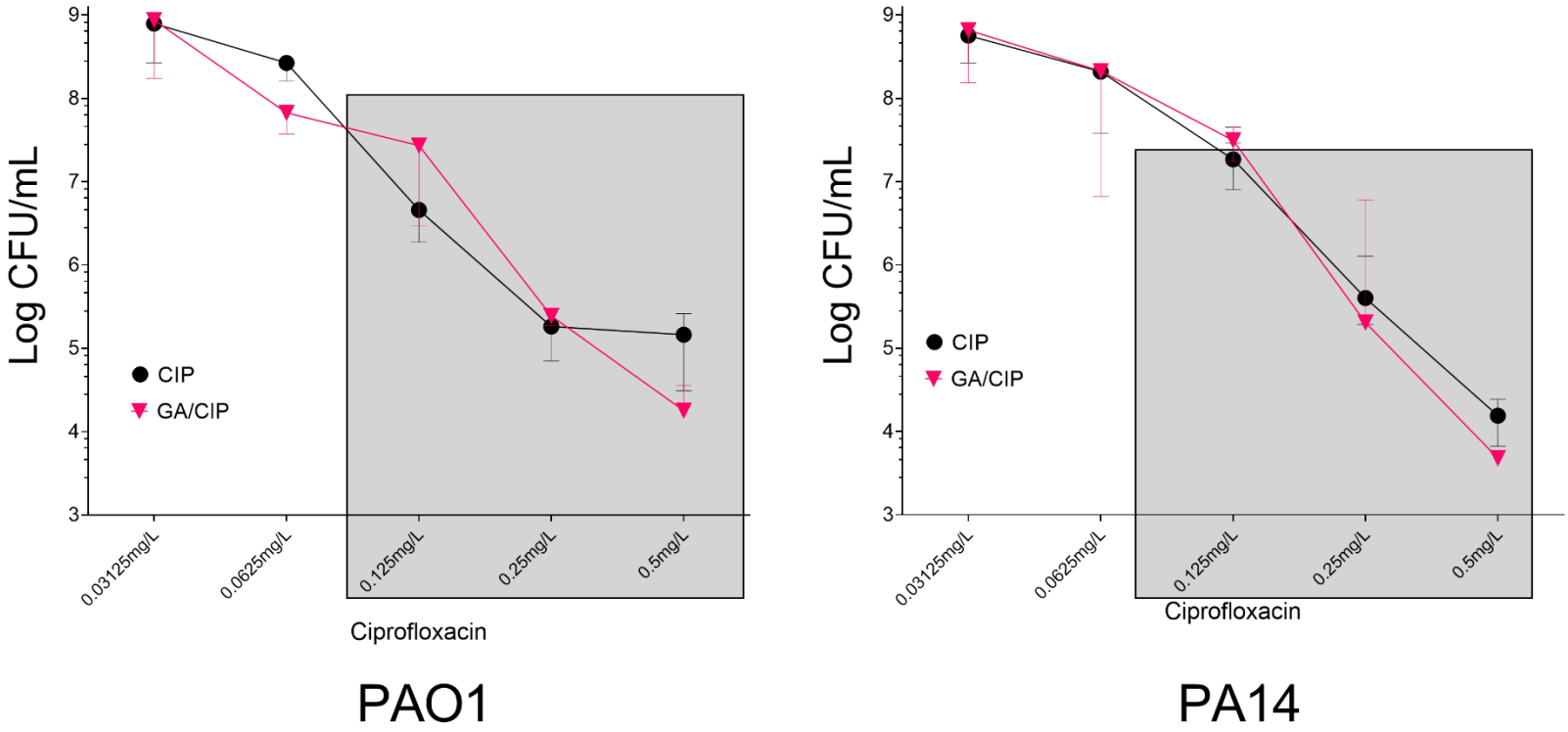


Figure 3-39. Colony forming units (CFU/mL) of P. aeruginosa PAO1 and PA14 after overnight treatment with CIP and GA/CIP at increasing CIP concentrations. Shaded areas indicates treatments which resulted in significant reductions in CFU/mL compared to control PAK under the same growth conditions. For PAO1, all CFU/mL results for 0.125-.5mg/L CIP were significantly reduced in the both the presence and absence of GA (p>0.05) For PA14, all CFU/mL results for 0.125-.5mg/L CIP were also significantly reduced in the both the presence and absence of GA (p>0.05,) with the exception of GA/CIP(0.125mg/L). Kruskal-Wallis test with Dunn's multiple correction (adjuted p values). Points are medians of biological replicates (with 95% confidence intervals).

As with PAO1 and as seen by growth curves, there was a dose response reaction to CIP by PA14, in colony count results (**Figure 3-39**, **Table 3-17**). Bacterial numbers were comparable to control PAO1 growth at CST 0.5-2mg/L CST, Inhibition was increased by increasing concentration of CIP; 0.0625mg/L resulted in 89.55 [95%CI 88.39-97.97] % inhibition, 0.125mg/L in 99.03 [95%CI 98.55-99.55] % inhibition , 0.25mg/L in 99.98 [95%CI 99.93-99.99] % inhibition and 0.5mg/L in 99.99 [95%CI 99.99-99.99] % inhibition (**Table 3-17**).

Each pair of CIP concentrations tested for PA14 (with or without GA) was comparable to each other numerically (**Table 3-17**) and no difference was seen between them in paired tests (n=3). In multiple comparison tests, to control, >0.125mg/L CIP all concentrations caused significant reduction in CFUs from untreated and this was the case for CIP-only and GA/CIP ($p<0.05$). The only exception to this was GA/CIP(0.125mg/L) which did cause a significant reduction from control PA14 CFU/mL results.

Table 3-17. Colony forming units (CFU/mL) of P. aeruginosa PA14 after overnight treatment with CIP and GA/CIP at increasing CIP concentrations # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3). Median [95% confidence intervals].

	CIP	**GA/CIP**
0mg/L	**1.80×10^9** [$8.22 \times 10^8 – 2.00 \times 10^9$]	**1.55×10^9** [5.50×10^8 - 1.81×10^9]
0.03125mg/L	**5.67×10^8** [$2.65 \times 10^8 – 5.67 \times 10^8$]	**6.57×10^8** [$1.53 \times 10^8 – 6.57 \times 10^8$]
0.0625mg/L	**2.09×10^8** [$3.85 \times 10^7 – 2.09 \times 10^8$]	**2.12×10^8** [$6.63 \times 10^6 – 2.12 \times 10^8$]
0.125mg/L	**# 1.85×10^7** [$8.00 \times 10^6 – 2.90 \times 10^7$]	**3.13×10^7** [$1.75 \times 10^7 – 4.50 \times 10^7$]
0.25mg/L	**# 4.00×10^5** [$1.93 \times 10^5 – 1.27 \times 10^6$]	**# 2.04×10^5** [$1.96 \times 10^5 – 6.00 \times 10^6$]
0.5mg/L	**# 1.55×10^4** [$6.63 \times 10^3 – 2.44 \times 10^4$]	**# 4.87×10^3** [$4.68 \times 10^3 – 4.89 \times 10^3$]

3.3.3 Combination effects of GA/antibiotics of reference *P. aeruginosa*

Having shown the various degrees to which GA changes the inhibition of *P. aeruginosa* reference strains in combination with different antibiotics from different classes, the results observed by combining agents experimentally (E_{OBS}) were compared to results that would be expected by the same combination (E_{EXP}), based on their experimental results when used alone. The level of inhibition of the colony count results relative to untreated control of biological replicates (means of technical replicates) were used. E_{EXP} was calculated as being = $E_A + E_B – (E_A X E_B)$ where E_A was 50mg/L GA (as only this concentration was employed) and E_B was each antibiotic examined at each concentration. When a combination showed greater inhibition than would expected by the results seen for each agent alone ($E_{OBS} > E_{EXP}$) the combination was synergistic, when the combination showed comparable results experimentally to expected results ($E_{OBS} = E_{EXP}$) the combination was additive or independent and when the combination effect was less inhibitory than expected ($E_{OBS} < E_{EXP}$) the combination was antagonistic.

3.3.3.1 Tobramycin

At each TOB concentration tested using CFU/mL, the combination effect was tested for PAO1 and it was seen that GA and TOB synergised for the combinations GA/TOB(0.25mg/L) and GA/TOB(0.5mg/L). The E_{OBS} of GA/TOB(0.25mg/L) was 95.14 [95%CI 83.57-96.01] % and the E_{EXP} of 50mg/L GA and 0.25mg/L TOB was 66.91 [95%CI 33.65-81.54] % (n=6). The E_{OBS} of GA/TOB(0.5mg/L) was 99.72 [95%CI 91.74-99.87] % and the E_{EXP} of 50mg/L GA and 0.5mg/L TOB was 83.98 [95%CI 52.74-8450] % (n=4). Therefore $E_{OBS} > E_{EXP}$ for both of these TOB concentrations when GA was present, with non-overlapping confidence intervals. These were the same TOB concentrations where combination treatments caused significant reductions in colony forming units compared to TOB monotherapy (**Figure 3-40**).

For the remainder of the TOB concentrations test with PAO1, their activities were additive/independent of GA ($E_{OBS} = E_{EXP}$) with comparable results and overlapping 95% confidence values seen (**Figure 3-40**).

For strain PA14, synergy was detected at GA/TOB(1mg/L) where the E_{OBS} was 99.99 [95%CI 99.94-100.00] % and the E_{EXP} of 50mg/L GA and 0.25mg/L TOB was 98.98 [95%CI 87.57-99.30] % (n=4). As with PAO1, this was a result that was suggested by the CFU/mL results where this combination of agents resulted in a significant reduction in colonies compared to TOB-only, where a >1log difference in CFU/mL was seen. The remainder of the concentrations of TOB tested were additive with GA according to this analysis.

For strain PAK, synergy was indicated at GA/TOB(2mg/L); E_{OBS} was 100.00 [95%CI 99.97-100.00] % while the E_{EXP} of 50mg/L GA and 2mg/L TOB was 98.34 [95%CI 95.53-99.80] % (n=3). Once again, additivity was the result of the analysis of the remaining TOB concentrations tested.

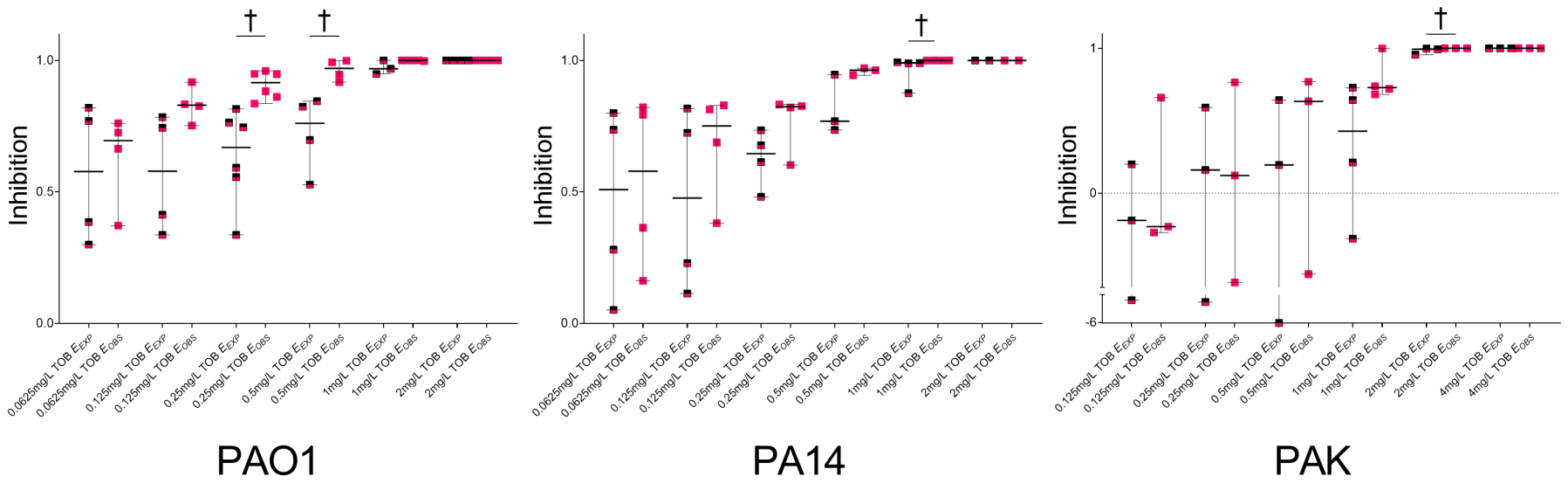


Figure 3-40. Comparisons of the Expected Effect (E_{EXP}) and the Observed Effect (E_{OBS}) of combining GA and TOB in treatment of P. aeruginosa reference strains PAO1, PA14 and PAK. Using inhibition of control growth in the CFU/mL for each strain, E_{EXP} was calculated from the individual effects of GA and TOB and E_{OBS} was the result of the combined GA/TOB experimental results. PAO1 showed synergy between both 0.25mg/L and 0.5mg/L TOB when combined with 50mg/L GA with the combinations resulting in a greater level of inhibition than would be expected from their activities alone (†). The remaining combinations of TOB and GA were additive for PAO1. PA14 showed synergy between 1mg/L TOB and 50mg/L GA with the combination resulting in a greater level of inhibition than would be expected from their activities alone (†). The remaining combinations of TOB and GA were additive for PA14. PAK showed synergy between 2mg/L TOB and 50mg/L GA with the combination resulting in a greater level of inhibition than would be expected from their activities alone (†). The remaining combinations of TOB and GA were additive for PAK. Medians of biological replicates (means of technical replicates) with 95% confidence intervals

3.3.3.2 Ceftazidime

Synergy was seen for PAO1 with the combination treatment of that strains with GA/CAZ(64mg/L) as the E_{OBS} was inhibition of control by 99.92 [95%CI 99.87-100.00] % compared with 96.14 [95%CI 92.00-99.73] % inhibition predicted by results of GA and this concentration of CAZ alone (n=4). The remaining paired results for each CAZ concentration were comparable to each other and therefore indicative of additivity (**Figure 3-41**).

A similar result was seen for strain PA14 and CAZ, with synergy between GA and CAZ indicated at a concentration of 64mg/L also. The experimentally derived inhibition of PA14 by GA/CAZ(64mg/L) was higher at 99.85 [95%CI 99.84-99.85] % than the inhibition predicted by the individual GA and CAZ results 98.98 [95%CI 98.63-99.33] %. However, only two biological replicates were performed at 64mg/L CAZ for PA14 so the full significance of this analysis is not clear. Once again, the remaining CAZ pairs did not indicate synergy at any other CAZ concentration tested, with vs. without GA.

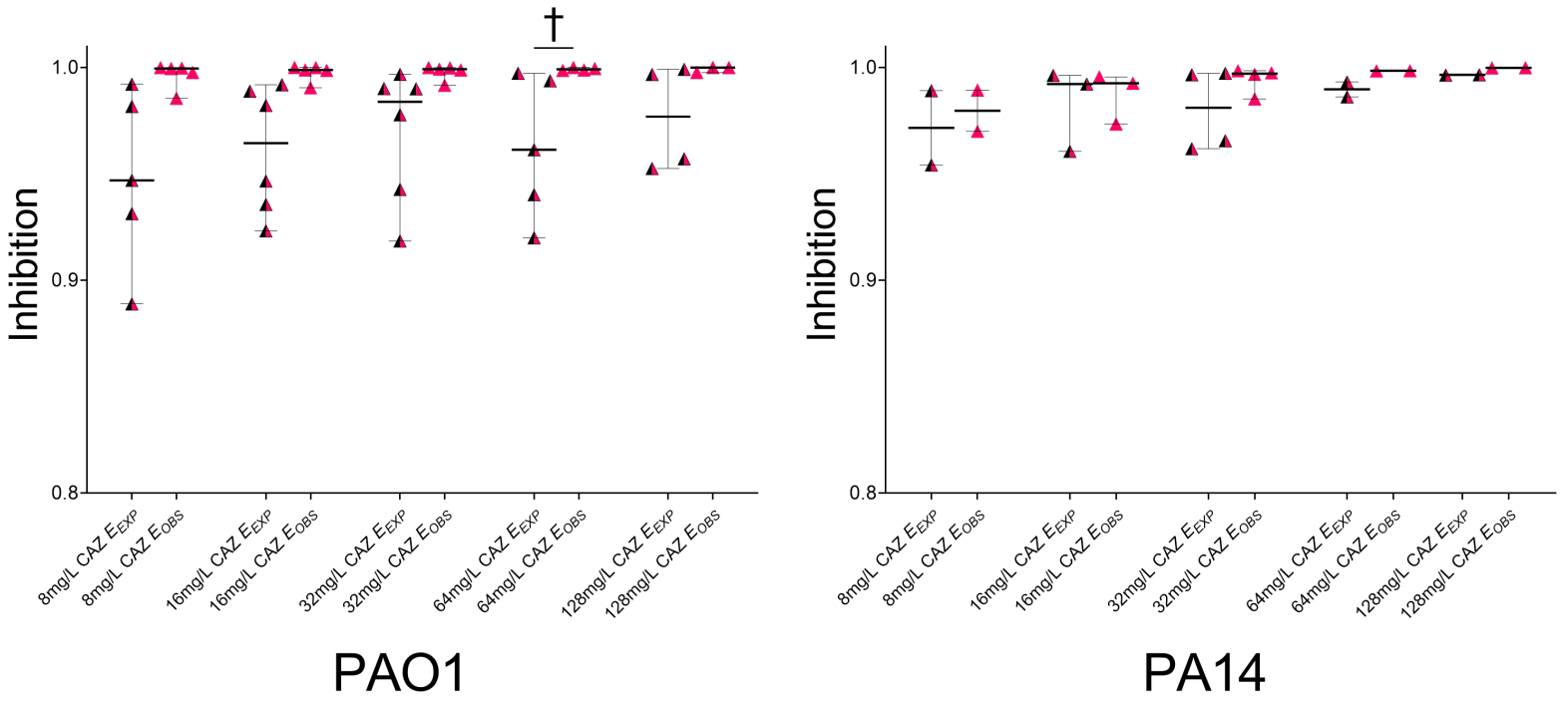


Figure 3-3-41. Comparisons of the Expected Effect (E_{EXP}) and the Observed Effect (E_{OBS}) of combining GA and CAZ in treatment of P. aeruginosa reference strains PAO1 and PA14. Using inhibition of control growth in the CFU/mL for each strain, E_{EXP} was calculated from the individual effects of GA and CAZ and E_{OBS} was the result of the combined GA/CAZ experimental results. PAO1 showed synergy between 64mg/L CAZ and 50mg/L GA with the combinations resulting in a greater level of inhibition than would be expected from their activities alone (†). The remaining combinations of CAZ and GA were additive for PAO1. PA14 showed additivity for the actions of CAZ and GA at all concentrations tested. Only two biological replicates were performed at 64 and 128mg/L CAZ. Medians of biological replicates (means of technical replicates) with 95% confidence intervals

3.3.3.3 Colistin

All concentrations of CST tested (0.5-8mg/L) resulted in independent activity with GA against PAO1. Results for each concentration pair, with or without GA, were very similar to each other with GA/CST causing inhibition at each CST concentration in manner highly comparable to what was predicted (**Figure 3-42**).

The same was the case for PA14, with the experimental outcome revealing highly comparable results with those that were predicted based on the two agents being applied alone (**Figure 3-42**).

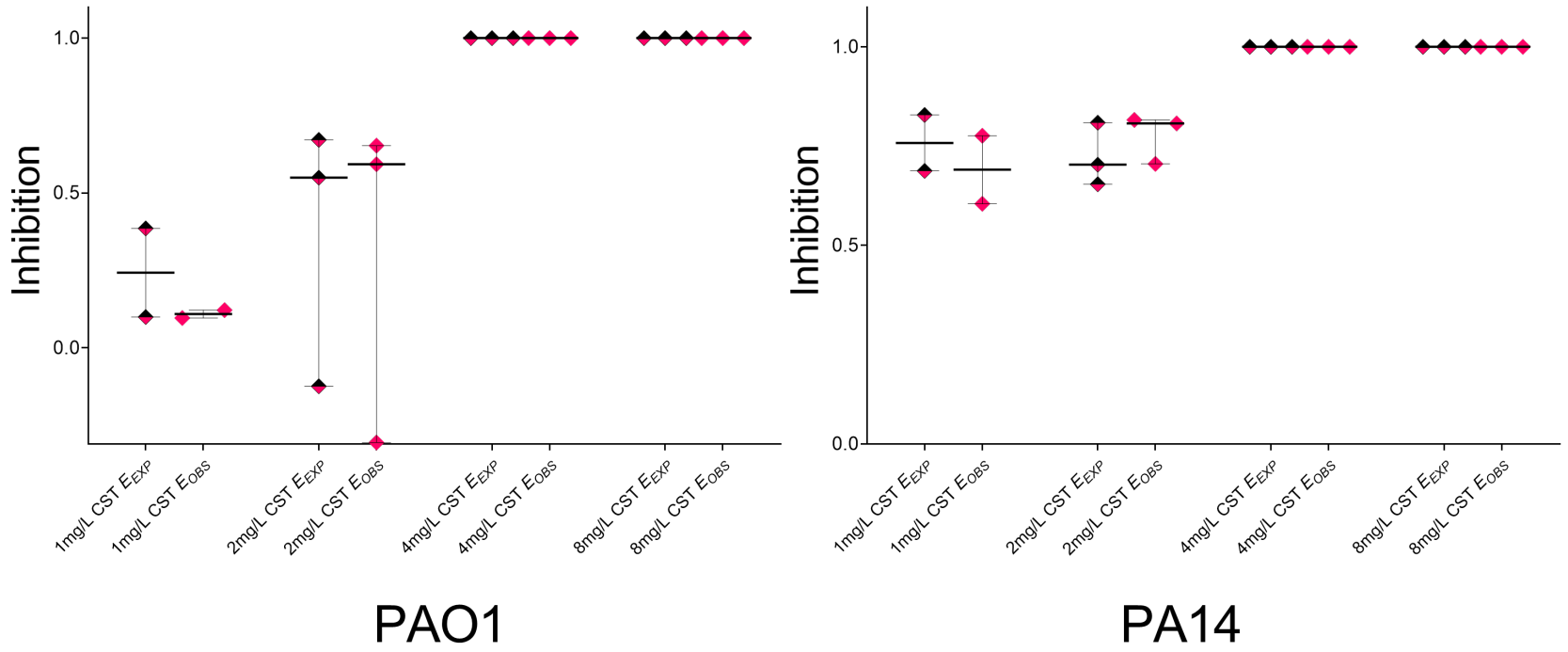


Figure 3-42. Comparisons of the Expected Effect (E_{EXP}) and the Observed Effect (E_{OBS}) of combining GA and CST in treatment of P. aeruginosa reference strains PAO1 and PA14. Using inhibition of control growth in the CFU/mL for each strain, E_{EXP} was calculated from the individual effects of GA and CST and E_{OBS} was the result of the combined GA/CST experimental results. PAO1 additivity for the actions of CST and GA at all concentrations tested. PA14 showed additivity for the actions of CST and GA at all concentrations tested. Medians of biological replicates (means of technical replicates) with 95% confidence intervals

3.3.3.4 Ciprofloxacin

Inhibition results of PAO1 showed that there was synergy between GA and CIP at 0.0625mg/L CIP. The observed inhibition at GA/CIP(0.0625mg/L) was 96.72 [95%CI 96.18-97.37] % while the predicted effect of this combination of agents was 89.83 [95%CI 87.41-89.94] % (n=3). For the remaining concentrations of CIP tested, additivity was seen (**Figure 3-43**).

In contrast to this, PA14 did not show any synergy between GA and CIP and the results of the comparison showed that addition of GA lead to comparable inhibition levels with CIP as were predicted by CIP and GA inhibition measured separately.

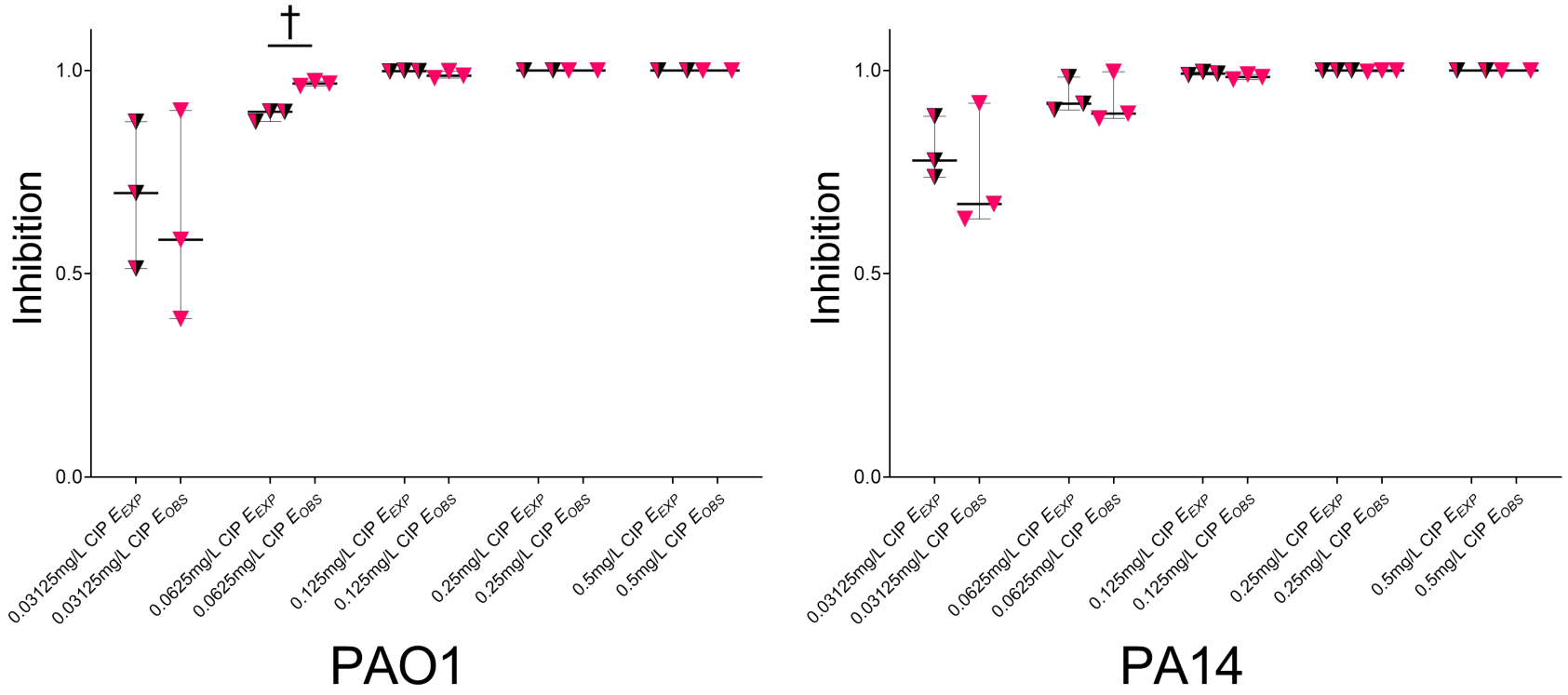


Figure 3-43. Comparisons of the Expected Effect (E_{EXP}) and the Observed Effect (E_{OBS}) of combining GA and CIP in treatment of P. aeruginosa reference strains PAO1 and PA14. Using inhibition of control growth in the CFU/mL for each strain, E_{EXP} was calculated from the individual effects of GA and CIP and E_{OBS} was the result of the combined GA/CIP experimental results. PAO1 showed synergy between 0.0625mg/L CIP and 50mg/L GA with the combinations resulting in a greater level of inhibition than would be expected from their activities alone (†). The remaining combinations of CIP and GA were additive for PAO1. PA14 showed additivity for the actions of CIP and GA at all concentrations tested. Medians of biological replicates (means of technical replicates) with 95% confidence intervals

3.4 Discussion

In the Chapter, I have shown that GA does not have an absolute MIC against *P. aeruginosa* reference strains. Using conventional broth microdilution methodology for MIC-determination, the strains tested were capable of turbid growth overnight up to and including 1600mg/L GA. Treatment of overnight cultures with 50mg/L GA does not alter the growth and viability of *P. aeruginosa* cultures. I have shown that *P. aeruginosa* strain PAO1 is subject to antibiotic potentiation by GA for three classes of antibiotic; an aminoglycoside (tobramycin), a cephalosporin (ceftazidime) and a fluoroquinolone (ciprofloxacin). This was limited in scope to the synergy with ciprofloxacin – a single concentration of the antibiotic. Potentiation was seen more extensively for ceftazidime with significant reductions in viable colonies numbers seen for PAO1 treated with GA/CAZ compared to CAZ and synergy seen, albeit in already inhibited cultures. For tobramycin, evidence of potentiation by GA was found in all analyses of PAO1 growth and viability; significant reductions in overnight growth curves, significant reductions in viable PAO1, reduced MICs and synergy between the two agents. I have shown that the evidence for tobramycin potentiation by GA is not limited to PAO1 but present for all the reference *P. aeruginosa* tested, each resulting in reduced MICs and synergy of activities. Colistin was the only antibiotic tested which was unaltered by GA in its activity for any of the strains tested.

3.4.1 GA antimicrobial activity

While GA has been shown to be capable of killing *P. aeruginosa* cells in very short time-frames (including killing of clinical strains from people with CF, in our hands)[287] the results of GA MIC testing showed that GA is not a potent antimicrobial when administered in a

conventional MIC assay. This is despite testing with very high GA concentrations (1600mg/L) (**Figure 3-1**). Given that a dose-dependent effect was not seen for GA in published data[287], but rather 50mg/L was the optimum concentration tested for anti-pseudomonal activity, this GA concentration was chosen for further experimentation.

Growth and viability analyses of reference *P. aeruginosa* treated with 50mg/L GA overnight further affirmed the lack of GA activity as an antimicrobial in this, classical, broth microdilution context. During this GA treatment none of PAO1, PA14 and PAK showed any deviation from control growth and viability (**Figures 3-2**, **3-3**, **3-4, 3-5** and **3-6**).

3.4.2 Tobramycin

The aminoglycoside tobramycin was the most extensively altered antibiotic by GA and was potentiated by GA for all 3 reference strains – each of PAO1, PA14 and PAK were altered in their susceptibility to TOB when GA was present. Differences in GA/TOB treated cultures from TOB treated cultures were apparent in the growth curves of the bacteria and statistical differences were seen when the curves were analysed; either in direct comparisons of TOB treatments (with vs without GA) or in changes to the TOB concentration where significant differences from control growth were first seen. All of these changes by GA moved the effectiveness of TOB down the concentration gradient (**Figures 3-11**, **3-13** and **3-15**).

Similar effects were seen on viable colonies of the strains, counted post-treatment, with all of the strains showing a significant difference in CFU/mL at a specific concentration of TOB or a change in the inflection point of TOB significance from control growth. Again, these changes were all towards lower concentrations of TOB being effective against *P. aeruginosa* when GA was present. In the CFU results for all reference *P. aeruginosa* with the

highest TOB concentration tested, viable colonies were consistently eliminated when GA was also present but this was not the case for TOB at the same concentration when alone.

Colony counts were a more sensitive tool for the testing of combination therapies compared to monotherapies. While screening concentrations of TOB with growth curves provided a high-throughput method to test ranges of concentrations quickly and efficiently, and was a good evidence base to pursue potential potentiation by GA, colony counting proved to be a more sensitive output. Colony count results revealed a wider range of potentially significant TOB concentrations for each strain tested and a greater level of difference between GA/TOB and TOB treatments of each strain (**Figure 3-16**). For example, growth curves of PAO1 treated with 0.25mg/L TOB did not indicate a significant impact of GA, either by visual inspection or statistical analysis. Yet, colony counts at this TOB concentration resulted in GA significantly reduced colonies for both a pairwise comparison and from control growth (**Table 3-5**).

Using these colony count results to calculate precise, granular MICs (MIC_{50} and MIC_{90}) resulted in reduced TOB MIC values for each strain when GA present. The presence of GA allowed lower concentrations of TOB to inhibit both 50% and 90% of bacterial growth in all the reference strains (**Table 3-7**).

TOB and GA synergise together to kill *P. aeruginosa*. Inhibition of viable colonies of PAO1, PA14 and PAK by combined GA/TOB was greater, than would be expected by the results seen for the two agents alone, for at least one concentration of TOB against each strain (**Figure 3-40**). Tobramycin has been shown to be subject to potentiation by cationic antimicrobials in previous studies on *P. aeruginosa*[320,322,323].

Tobramycin is antimicrobial due to its ability to bind to bacterial ribosomes, disrupt protein synthesis by the cell and kill the bacterium[93]. To have its effect, tobramycin must therefore make its way to the ribosome in the cytoplasm of *P. aeruginosa. P. aeruginosa* is adept at excluding compounds from their cytoplasm, however; primarily, their cells are less permeable than most Gram-negative bacteria. They have fewer channels in their membrane, have a lower membrane polarity and a high level of impermeability resistance[131,324]. Secondly, should a compound overcome this impermeability and make it through the first line of defence and into the cytoplasm, *P*. *aeruginosa* employs a plethora of efflux pumps to remove toxic compounds[102,140,142]. *P. aeruginosa* can also use intrinsic and acquired enzymes which can modify the aminoglycoside an render it inactive[96]. All of these strategies serve the purpose of preventing of aminoglycoside accumulation in the bacterial cytoplasm.

Cationic AMPs are frequently associated with membrane disruption of Gram-negative bacteria and in the context of impermeable cells, like those of *P. aeruginosa*, this property has increased significance. While results here and elsewhere have shown that GA is not a particularly potent anti-pseudomonal, especially in dividing cultures as those used here, that does not mean that GA has no activity in these assays. The activity of GA in disrupting the bacterial membrane may allow tobramycin access to its targets within the bacteria, by breaching the normally impermeable cell wall and/or overwhelming the activity of efflux pumps and aminoglycoside-inactivating enzymes, preventing the cell from removing the antibiotic as quickly as it accumulates. **Chapter 5** will explore the ability of GA to interact with and perturb the cell wall of *P. aeruginosa*.

3.4.3 Ceftazidime

Overall results for potentiation of ceftazidime were more ambiguous than those seen for tobramycin (coupled with the fact that only two strains were available for testing). There was some indication from bacterial growth curves of extra anti-pseudomonal activity of combined treatments compared to ceftazidime-only treatments and statistical reductions in growth metrics were seen in both reference strains tested for GA/CAZ (**Figures 3-24 and 3-26**).

As with tobramycin, colony count results were more sensitive than growth curves and revealed differences between ceftazidime combination treatment and monotherapy. Four concentrations of the antibiotic had significant reductions in PAO1 colonies when GA was present compared to antibiotics alone, in paired comparisons. A dose response was not strongly apparent for ceftazidime against PAO1 in these experiments either with or without GA. This may be biased somewhat by unevenness in number of biological replicates of PAO1 with a higher n at 32mg/L (n=5) than 64mg/L (n=4) or 128 mg/L (n=3), aiding in confirming the statistical difference in the multiple comparison.

For PA14 colonies did not result in any statistical differences for combination treatments, either in paired comparisons or changes from control PA14. As with PAO1, multiple comparisons had an unexpected result with the highest concentrations of ceftazidime tested (64-128mg/L) not being significantly reduced while lower concentrations (16-32mg/L) were (**Figure 3-27**, **Tables 3-10 and 3-11**). And this was despite the CFU/mL showing fewer recovered colonies at 64-128mg/L. However, only two biological replicates were performed at 64-128mg/L while at 16-32mg/L the n=3, confounding the ability to statistically test these results.

Inhibition curves and interpolation of MICs was not performed for ceftazidime due the range of concentrations tested being too narrow. No concentration of ceftazidime was tested which resulted in 0% inhibition or full 100% inhibition. Therefore, inhibition curves and accurate interpolation of 50% and 90% inhibition were not possible.

Synergy between GA and CAZ was indicated for the results of PAO1 treated with 64mg/L CAZ (**Figure 3-41**). No other concentration of ceftazidime tested indicating a reaction other than additivity and PA14 was unaltered for any ceftazidime concentration when GA was present.

Overall, the results for ceftazidime would benefit from addition of PAK and a more consistent and systematic approach with regards to biological replicates and concentration ranges.

3.4.4 Colistin

Results for growth curves of reference *P. aeruginosa* treated with colistin did not indicate potentiation of the antibiotic by GA (**Figures 3-31** and **3-33**). Indeed, at some concentrations tested, the curves suggested the possibility of competition between the two agents with colistin alone resulting in significant reductions not seen for combinations with GA. Examples of AUC results for both strains indicated significant differences, from control, for CST-only treatments that were not significant for GA/CST at the same CST concentrations. However, this split between GA/CST and CST results was not seen in the endpoint OD_{600} for the same growth curves. As AUCs take into account the entirety of the timecourse of the experiment, this may indicate that the contrasts seen are as a result of differences which occur earlier in the experiments but resolve themselves towards the endpoint.

Results of colony counts did not indicate any differences in the effects of administration of colistin alone compared to in combination with GA (**Figures 3-34**, T**ables 3-14** and **3-15**). Neither potentiation nor antagonism was seen in the CFU/mL results, a further indication that competition that may have been indicated by growth curve AUCs was resolved by the end of the incubation period of the experiments.

No synergy or antagonism was seen for combination treatments and predicted and actual inhibition for GA and colistin were highly comparable (**Figure 3-42**). As with CAZ, the concentration range of colistin tested was not wide enough to perform inhibition curves and interpolate MICs, although, with CFU/mL results as comparable as they were at each CST concentration, differences in MICs due to the presence of GA were unlikely.

As both GA and colistin are cationic peptides, they may function in similar ways and have similar cellular targets and therefore no extra effect of having both present was seen over the incubation period. However, early timepoint colony counts would be of interest to elucidate whether or not the indications of early competition/antagonism – seen in growth curves – of the two agents is still apparent.

3.4.5 Ciprofloxacin

There were no strong indications of potentiation of ciprofloxacin by GA in PAO1 or PA14 in growth curves (**Figures 3-37** and **3-38**). Treatments of the strains with the various concentrations of ciprofloxacin were closely matched for both strains, regardless of GA presence, and with regards to both results and statistical analyses of those results. The only exception to this was for PA14 where treatment with 0.125mg/L CIP significantly reduced CFU/mL from control, while GA/CIP(0.125mg/L) did not. However, in paired comparison these

results were not different from each other and the enumerated bacterial numbers are comparable with overlapping confidence intervals (**Figures 3-39**, **Tables 3-16** and **3-17**).

This potential antagonism was not borne out in testing the results from PA14 where no synergy or antagonism was seen for any ciprofloxacin concentration tested (**Figure 3-43**).

Inhibition curves and MICs were not performed for the same reasons as before, although, as with colistin, the level of comparability of the CFU/mL results for CIP and GA/CIP suggest that differences in MIC_{50} and MIC_{90} due to GA were unlikely.

Published observations suggest that GA may be able to bind to *P. aeruginosa* DNA[287], a property of other AMPs[225]. With ciprofloxacin's method of action being to prevent bacterial DNA from uncoiling and replicating, GA also preventing DNA uncoiling by binding to it may mean a crossover of activities of these agents and explain the absence of potentiation.

3.4.6 Limitations of approach

While the method of synergy calculation used here (Bliss Independence)[325] is an appropriate test for synergy between two agents of differing mechanism[326], as GA and tobramycin do, it is not the most common method of synergy testing and may not be the best methodology for other antibiotics tested here. The most common method of antibiotic synergy testing is Fractional Inhibitory Concentration Index (FICI)[327] which uses the chequerboarding of concentrations of two agents to test for synergy, as opposed to the single GA concentration employed here. Despite the difficulty presented by GA not having a conventional MIC – FICI generally tests concentration ranges of two agents spanning susceptibility to resistance breakpoints of each – a chequerboard approach to synergy testing would have been of benefit.

3.4.7 Future directions

Testing the activity of GA alone and in antibiotic combinations in CF relevant media would be of great interest. Media which mimic the conditions of CF sputum, such as artificial sputum medium (ASM) or synthetic CF sputum medium (SCFSM) could be employed for such experiments. Moreover, use of sputum from people with CF would be an even more physiologically relevant model. Such media presents more challenges than bacteriological broths, however, and other approaches to measuring the efficacy of agents would be required, such as fluorescently tagged bacteria (e.g. green fluorescent protein) or metabolic assays such as resazurin.

3.4.8 Discussion of hypothesis

I hypothesised that GA would make antibiotics commonly used in the treatment of *P. aeruginosa* infections in people with CF more effective and, using a range of tests and analyses, have shown this to be the case. Most compellingly, I have shown this for the aminoglycoside tobramycin, where all metrics and analyses used and all strains tested showed potentiation by GA. There was also proof of improved activity of ceftazidime against strain PAO1 but not PA14. I have shown that GA does not potentiate the activity of colistin but that antagonism at early timepoints of GA/CST treatments is worth exploring further. Finally, I have shown that there is some minor evidence of GA potentiation of ciprofloxacin, although this was only apparent for PAO1 for a single concentration in one of tests performed. Some evidence of antagonism of ciprofloxacin by GA was seen in strain PA14 but this is similarly caveated and worth exploring further.

4. Combination of glatiramer acetate with tobramycin against clinical *Pseudomonas aeruginosa* from people with cystic fibrosis

4.1 Introduction

The ability of *P. aeruginosa* strains to operate in wide range of ecological niches is part of the reason for its ubiquity and for the threat it poses. With a large genome encoding the ability to use a variety of nutritional sources and to evade a variety of antimicrobials, *P. aeruginosa* can adapt and colonise quickly[13]. However, opportunistic infection is not the only lifestyle which *P. aeruginosa* adopts – it also has the ability to colonise over much longer timescales. This is particularly pertinent in CF, where *P. aeruginosa* colonisation frequently lasts years and can persist for decades.

The very specific and unique conditions of the CF lung sputum – nebulised antibiotics[117], immune cells[60–62], NETs[328], human AMPs[329], altered pH[69], reactive oxygen species[330], anoxic microenvironments[39,60], polymicrobial communities[42] – lead to specific adaptations and phenotypes of *P. aeruginosa*. Production of the polysaccharide alginate and the switch to a mucoid lifestyle is commonly associated with chronic infection in CF. Alginate is an important component of the biofilm matrix of *P. aeruginosa*, forming and supporting the biofilm structure and playing an important role in the physical barrier protection afforded against antibiotic treatment[126,331,332]. The antibiotic concentrations required to eradicate biofilm-grown *P. aeruginosa* can be orders of magnitude greater than for planktonic cultures[333].

P. aeruginosa have large collections of genes with associated antibiotic resistances. These can involve the regular functioning of systems like the efflux pump systems[142,334], antibiotic deactivating enzymes[96,156], target alteration systems[114,335]. Specific mutations or accumulations of mutations in these systems (and their regulators) can also result in changed phenotypes and increased antibiotic resistance[147,159,188,336]. The selective pressures exerted

on strains in the CF lung can drive the production of these phenotypes and genotypes, including prolonged and repeated exposure to antibiotic treatments[118].

In particular, the resistance of *P. aeruginosa* strains to the aminoglycoside tobramycin, a commonly deployed antibiotic in CF treatment, is mediated by many of these processes. Tobramycin is subject to degradation by genomically encoded enzymes[96]. Tobramycin resistance is mediated by efflux pumps and mutations with efflux systems can increase resistance levels[147]. As a mildly cationic agent which can mediate its own uptake, tobramycin resistance can be mediated by changes to the *P. aeruginosa* outer membrane[94,95,337].

The array of interrelated phenotypes and genotypes that lead to colonisation of the CF lung by *P. aeruginosa* or as a result of colonisation of the CF lung and its selective pressures, means that study of a cohort of strains from the lungs of people with CF was vital in assessing the effect of GA on the efficacy of antibiotics. Given the results from **Chapter 3**, where the impact of GA was greatest on the efficacy of tobramycin, it was decided to test clinical strains with GA-tobramycin combinations.

4.2 Hypothesis

I hypothesis that:

Co-administration of GA with TOB makes TOB more effective against *P. aeruginosa* from people with cystic fibrosis

4.2.1 Aims

To investigate this hypothesis, I will:

- Expand the knowledge and methods of Chapter 3 to a larger cohort of clinical *P. aeruginosa* from people with cystic fibrosis: growth and viability tests, minimum inhibitory concentrations, synergy analyses

4.3 Results

4.3.1 Genomic characteristics of clinical *P. aeruginosa* strains

Table 4-1. Genome G+C contents and sizes of the genomes of each of the clinical P. aeruginosa from people with CF sequenced.

	G+C Content (%)	Length (bp)
RBH750	66.05	6706934
RBH899	66.03	6525788
RBH550	66.18	6928312
RBH294	65.95	6810463
RBH982	66.41	6328544
RBH422	66.11	6690515
RBH519	66.05	6808752
RBH461	66.41	6484943
RBH490	66.79	6207923
RBH065	66.51	6291499
RBH072	65.98	6991588

Nine of the eleven clinical *P. aeruginosa* in the dataset had a unique MLST profiles within the set (**Table 4-2**). The only repeated ST was ST17 (RBH550 and RBH072) which is also the ST of the highly virulent *P. aeruginosa* Clone C. One clinical strain (RBH899) had the same ST (ST310) has the well-studied reference strain PA14 and one strain (RBH461) had the same

ST (ST146) as the virulent CF clone Liverpool Epidemic Strain (LES) B58. No new STs were identified in any of the 11 strains. There was a wide spread in the frequency with which each ST was found on the PubMLST online database (between 7 and 310) and sources of the isolates of each ST (although this data is incomplete on the database)[302].

Table 4-2. MLST profiles of clinical P. aeruginosa from people with CF. Alleles and Sequence Types were derived from whole genome sequences of each strain using PubMLST[302]. Frequency and Source data accessed 03/01/2022 from a total of 8096 isolates.

	MLST alleles	Sequence Type	Frequency in PubMLST	Sources in PubMLST	Common ST
RBH750	19, 5, 12, 11, 11, 4, 14	267	7	sputum, env	
RBH899	4, 4, 16, 12, 1, 6, 3	253	310	blood, BAL, eye, tissue, sputum, env	PA14
RBH550	11, 5, 1, 7, 9, 4, 7	17	89	blood, BAL, tissue, sputum, env	Clone C
RBH294	105, 5, 30, 3, 3, 4, 14	261	9	sputum, UTI, env	
RBH982	6, 68, 20, 11, 4, 4, 7	439	10	env	
RBH422	22, 20, 11, 3, 3 ,3, 7	348	43	blood, tissue, env	
RBH519	28, 5, 36, 3, 3, 13, 7	155	77	blood, BAL, tissue, sputum, UTI, env	
RBH461	6, 5, 11, 3, 4, 23, 1	146	38	sputum	LESB58
RBH490	28, 5, 11, 18, 4, 13, 3	217	13	sputum	
RBH065	17, 22, 5, 11, 3, 86, 19	794	9	sputum, UTI, env	
RBH072	11, 5, 1, 7, 9, 4, 7	17	89	blood, BAL, tissue, sputum, env	Clone C

BAL=bronchoalveolar lavage UTI=urinary tract infection, env=environmental

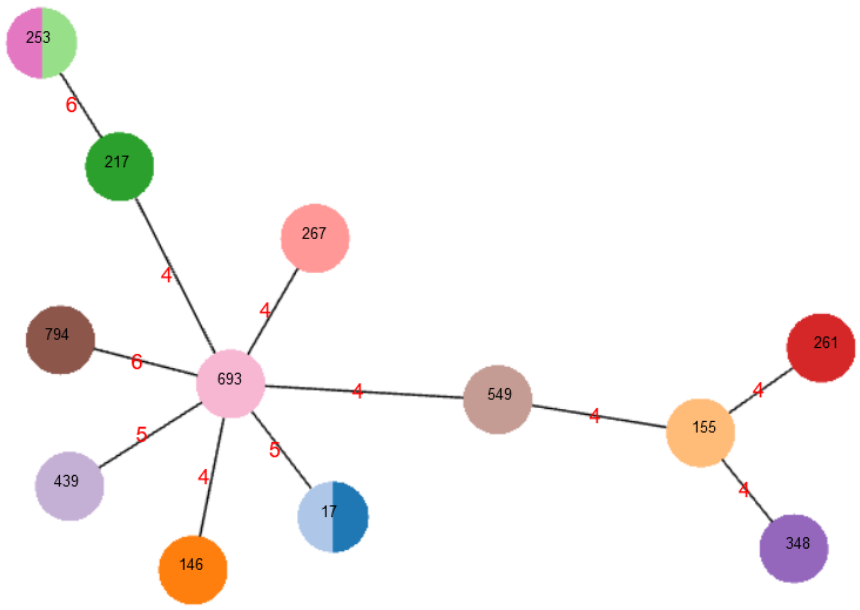


Figure 4-1. Minimum spanning tree of Sequence Types of Multilocus Sequence Types of the 11 clinical P. aeruginosa from people with CF. Nodes labelled with ST. Branches labelled with alleles in common between connected nodes. Reference strains PAO1 (ST549), PA14 (ST253) and PAK (ST693) were included. Tree generated with PHYLOViZ[303,304].

Table 4-3. Genes associated with antibiotic resistance found the 11 clinical P. aeruginosa from people with cystic fibrosis used in this study. Only genes with Perfect and Strict results according the Resistance Gene Identifier (RGI) on The Comprehensive Antibiotic Resistance Database (CARD)[305].

	Gene	Resistance Mechanism	Strains
Aminoglycosides	AAC(6')-Ib9	antibiotic inactivation	RBH072
	ANT(2'')-Ia	antibiotic inactivation	RBH072
	APH(3')-IIb	antibiotic inactivation	All strains
	CpxR	antibiotic efflux regulator	All strains
	EmrE	antibiotic efflux	All strains
	MexCD-OprJ	antibiotic efflux	All strains
	MexXY-OprJ	antibiotic efflux	RBH750, RBH550, RBH294, RBH982, RBH422, RBH519, RBH461, RBH065, RBH072
	MexZ	antibiotic efflux regulator	All strains (RBH422 x 2)
	PmpM	antibiotic efflux	RBH899, RBH550, RBH294, RBH982, RBH422, RBH519, RBH461, RBH490, RBH065, RBH072

Cephalosporins	ArmR	antibiotic efflux regulator	RBH750, RBH899, RBH422, RBH490
	CpxR	antibiotic efflux regulator	All strains
	MexAB-OprM	antibiotic efflux	All strains
	MexXY-OprJ	antibiotic efflux	RBH750, RBH550, RBH294, RBH982, RBH422, RBH519, RBH461, RBH065, RBH072
	MexR	antibiotic efflux regulator	All strains
	MexZ	antibiotic efflux regulator	All strains (RBH422 x 2)
	NalC	antibiotic efflux regulator	All strains
	NalD	antibiotic efflux regulator	All strains
	NfxB	antibiotic efflux regulator	All strains
	OXA-50	beta-lactamase	RBH550, RBH461, RBH065, RBH072
	OXA-488	beta-lactamase	RBH899
	OXA-850	beta-lactamase	RBH750, RBH294, RBH982, RBH422, RBH519, RBH490
	PDC-3	beta-lactamase	RBH750, RBH461, RBH490, RBH065
	PDC-5	beta-lactamase	RBH982, RBH422, RBH519,
	PDC-8	beta-lactamase	RBH550, RBH294, RBH072
	PDC-68	beta-lactamase	RBH899
	SoxR	antibiotic efflux regulator	RBH750, RBH550, RBH294, RBH982, RBH422, RBH519, RBH461, RBH065, RBH072
	YajC	antibiotic efflux regulator	All strains
Peptide Antibiotics	ArmR	antibiotic efflux regulator	RBH750, RBH899, RBH422, RBH490
	BasSR	two-component system	All strains
	CprSR	two-component system	All strains
	MexAB-OprM	antibiotic efflux	All strains
	MexR	antibiotic efflux regulator	All strains
	NalC	antibiotic efflux regulator	All strains
	NalD	antibiotic efflux regulator	All strains
	ParSR	two-component system	All strains
Fluoroquinolones	AdeF	antibiotic efflux regulator	RBH750, RBH899, RBH294, RBH461
	ArmR	antibiotic efflux regulator	RBH750, RBH899, RBH422, RBH490
	CrpP	beta-lactamase	RBH550
	GyrA	resistance mutation	RBH461
	MexAB-OprM	antibiotic efflux	All strains

	MexCD-OprJ	antibiotic efflux	All strains
	MexEF-OprN	antibiotic efflux	RBH750, RBH899, RBH550, RBH294, RBH422, RBH519, RBH461, RBH490, RBH065, RBH072
	MexGHI-OpmD	antibiotic efflux	All strains
	MexR	antibiotic efflux regulator	All strains
	MexS	antibiotic efflux regulator	RBH750, RBH899, RBH550, RBH294, RBH422, RBH519, RBH461, RBH490, RBH065, RBH072
	MexT	antibiotic efflux regulator	RBH750, RBH899, RBH550, RBH294, RBH422, RBH519, RBH461, RBH490, RBH065, RBH072
	MexVW-OprM	antibiotic efflux	All strains
	MexXY-OprJ	antibiotic efflux	RBH750, RBH550, RBH294, RBH982, RBH422, RBH519, RBH461, RBH065, RBH072
	MexZ	antibiotic efflux regulator	All strains (RBH422 x 2)
	NalC	antibiotic efflux regulator	All strains
	NalD	antibiotic efflux regulator	All strains
	NfxB	antibiotic efflux regulator	All strains
	ParE	resistance mutation	RBH490
	PmpM	antibiotic efflux	RBH899, RBH550, RBH294, RBH982, RBH422, RBH519, RBH461, RBH490, RBH065, RBH072
	RsmA	antibiotic efflux regulator	All strains
	SoxR	antibiotic efflux regulator	All strains
	YajC	antibiotic efflux regulator	All strains

4.3.2 Screen of clinical *P. aeruginosa* for TOB active concentration range

As with reference *P. aeruginosa*, clinical strains were screened using growth curves to determine the active concentration range of TOB, to inform the range of TOB concentrations for which colony counts should be performed, with and without GA.

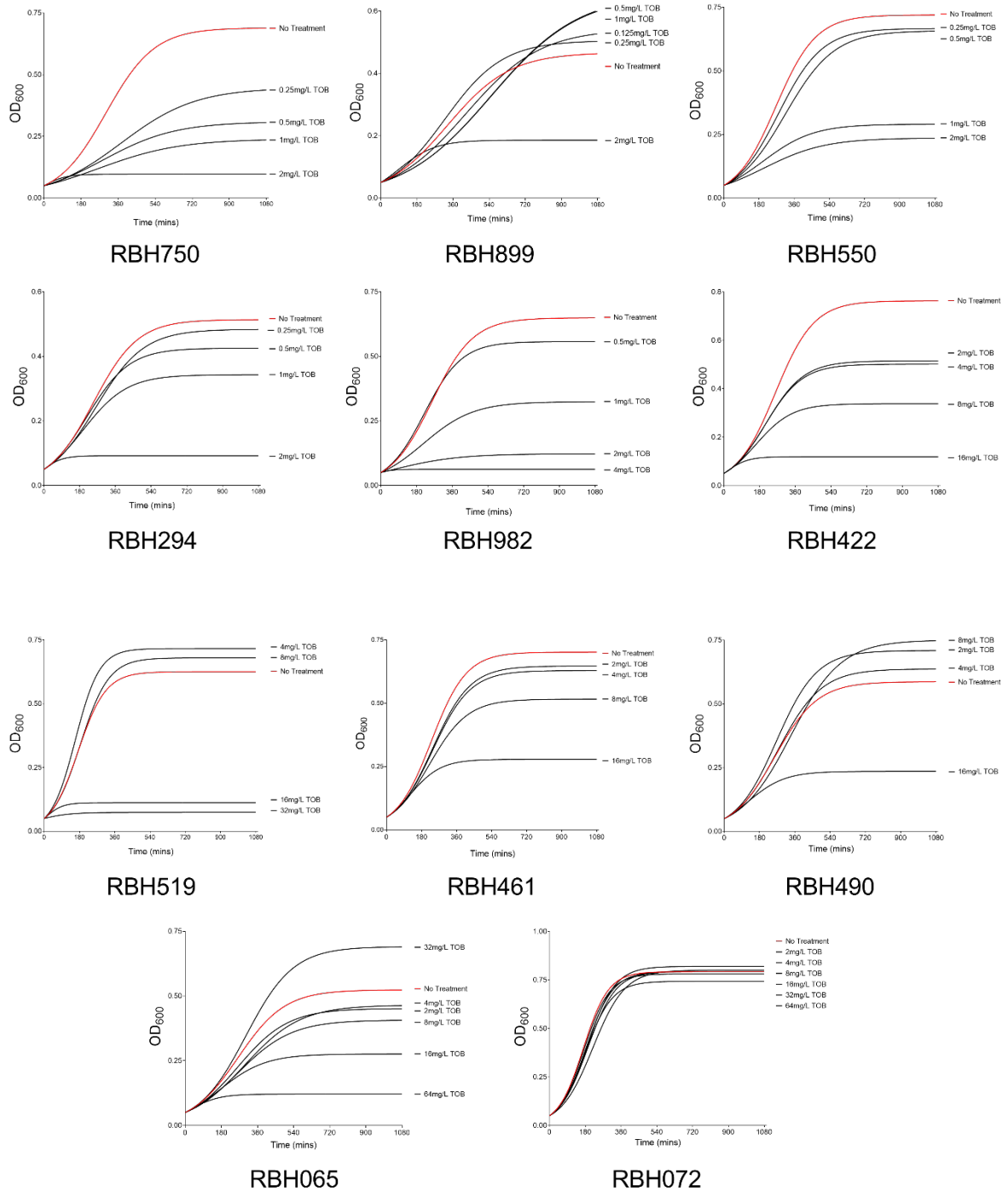


Figure 4-2. Fitted growth curves of clinical P. aeruginosa from people with cystic fibrosis during overnight treatment with TOB. Growth curves were used to screen for the active concentration range of TOB against each strain. Nonlinear regression (Logistic Growth model).

Table 4-4. Concentration ranges of TOB to be tested against each clinical P. aeruginosa strain resulting from growth curve screens.

Strain	TOB (mg/L)
RBH750	0.03125 - 4
RBH899	0.03125 - 2
RBH550	0.125 - 4
RBH294	0.125 - 4
RBH982	0.03125 - 4
RBH422	0.5 - 16
RBH519	0.5 - 16
RBH461	2 - 32
RBH490	0.5 - 16
RBH065	2 - 32
RBH072	32 - 512

4.3.3 Effect of GA on viability of TOB-treated clinical *P. aeruginosa* from cystic fibrosis

Viable colony counts were performed on clinical *P. aeruginosa* after overnight incubation with TOB and GA/TOB to test whether the results seen for GA/TOB in reference *P. aeruginosa* were applicable to the respiratory pathogenic strains. The TOB concentrations used for each strain were guided by the results of the above growth curves.

*Table 4-5. Colony forming units (CFU/mL) of P. aeruginosa strain RBH750 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. * indicates CFU/mL results where significantly fewer colonies were recovered for GA/TOB treatment than TOB treatment at a given TOB concentration (p<0.05) (Mann-Whitney U test). # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).*

	TOB	**GA/TOB**
0mg/L	**1.54×10^9** [$6.13 \times 10^8 – 2.66 \times 10^9$]	**1.45×10^9** [$5.89 \times 10^8 – 2.40 \times 10^9$]
0.03125mg/L	**5.03×10^8** [$4.36 \times 10^8 – 1.22 \times 10^9$]	**4.83×10^8** [$4.50 \times 10^8 – 1.10 \times 10^9$]
0.0625mg/L	**5.50×10^8** [$4.17 \times 10^8 – 1.21 \times 10^9$]	**4.43×10^8** [$2.50 \times 10^8 – 4.63 \times 10^8$]
0.125mg/L	**5.75×10^8** [$5.00 \times 10^8 – 6.87 \times 10^8$]	**3.80×10^8** [$2.67 \times 10^8 – 3.97 \times 10^8$]
0.25mg/L	**4.03×10^8** [$2.19 \times 10^8 – 5.14 \times 10^8$]	**2.47×10^8** [$1.69 \times 10^8 – 3.86 \times 10^8$]
0.5mg/L	**3.43×10^8** [$1.80 \times 10^8 – 5.63 \times 10^8$]	**# 1.24×10^8** [$6.66 \times 10^7 – 2.95 \times 10^8$]
1mg/L	**# 1.56×10^8** [$4.33 \times 10^6 – 3.59 \times 10^8$]	**# 2.64×10^7** [$3.62 \times 10^6 – 1.84 \times 10^8$]
2mg/L	**# 2.45×10^7** [$1.64 \times 10^6 – 3.88 \times 10^7$]	***# 1.57×10^4** [$10 – 1.75 \times 10^5$]
4mg/L	**# 1.96×10^5** [$1.46 \times 10^5 – 2.47 \times 10^5$]	**# 0** [–]

*Table 4-6. Colony forming units (CFU/mL) of P. aeruginosa strain RBH899 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. * indicates CFU/mL results where significantly fewer colonies were recovered for GA/TOB treatment than TOB treatment at a given TOB concentration (p<0.05) (Mann-Whitney U test). # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).*

	TOB	**GA/TOB**
0mg/L	**1.07×10^9** [$3.20 \times 10^8 – 6.00 \times 10^9$]	**1.80×10^9** [$2.72 \times 10^8 – 2.26 \times 10^9$]
0.03125mg/L	**5.64×10^8** [$3.54 \times 10^8 – 7.61 \times 10^8$]	**4.35×10^8** [$3.30 \times 10^8 – 6.27 \times 10^9$]
0.0625mg/L	**5.42×10^8** [$3.25 \times 10^8 – 6.29 \times 10^8$]	**7.00×10^8** [$4.90 \times 10^8 – 7.54 \times 10^8$]
0.125mg/L	**6.67×10^8**	**5.30×10^8**

	[3.63 x 10^8 – 7.23 x 10^8]	[3.13 x 10^8 – 6.96 x 10^8]
0.25mg/L	**4.53 x 10^8**	**4.18 x 10^8**
	[1.96 x 10^8 – 5.54 x 10^8]	[2.12 x 10^8 – 5.00 x 10^8]
0.5mg/L	**2.34 x 10^8**	**1.59 x 10^8**
	[1.78 x 10^8 – 2.60 x 10^8]	[6.45 x 10^7 – 2.13 x 10^8]
1mg/L	**1.74 x 10^8**	**# 4.37 x 10^7**
	[3.63 x 10^7 – 2.06 x 10^8]	[3.45 x 10^7 – 4.48 x 10^7]
2mg/L	**# 1.27 x 10^7**	***# 1.43 x 10^6**
	[6.30 x 10^6 – 1.33 x 10^7]	[2.50 x 10^5 – 4.02 x 10^6]

*Table 4-7. Colony forming units (CFU/mL) of P. aeruginosa strain RBH550 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. **#** indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).*

	TOB	**GA/TOB**
0mg/L	**3.65 x 10^8**	**2.74 x 10^8**
	[2.14 x 10^8 – 1.36 x 10^9]	[1.86 x 10^8 – 1.28 x 10^9]
0.125mg/L	**3.46 x 10^8**	**2.85 x 10^8**
	[1.80 x 10^8 – 4.70 x 10^8]	[2.04 x 10^8 – 2.86 x 10^8]
0.25mg/L	**1.87 x 10^8**	**1.67 x 10^8**
	[1.79 x 10^8 – 5.07 x 10^8]	[1.41 x 10^8 – 2.32 x 10^8]
0.5mg/L	**1.77 x 10^8**	**1.50 x 10^8**
	[1.38 x 10^8 – 2.77 x 10^8]	[3.83 x 10^7 – 2.08 x 10^8]
1mg/L	**3.16 x 10^7**	**5.95 x 10^6**
	[9.00 x 10^6 – 8.32 x 10^7]	[6.50 x 10^5 – 1.35 x 10^8]
2mg/L	**# 1.89 x 10^5**	**# 2.90 x 10^3**
	[8.34 x 10^4 – 2.56 x 10^5]	[0 – 1.59 x 10^5]
4mg/L	**# 0**	**# 0**
	[–]	[–]

*Table 4-8. Colony forming units (CFU/mL) of P. aeruginosa strain RBH294 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. **#** indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).*

	TOB	**GA/TOB**
0mg/L	**4.70 x 10^8**	**5.40 x 10^8**
	[2.68 x 10^8 – 6.50 x 10^9]	[3.43 x 10^8 – 5.90 x 10^9]
0.125mg/L	**3.17 x 10^8**	**3.20 x 10^8**

	[2.39 x 10^8 – 3.77 x 10^8]	[2.19 x 10^8 – 3.57 x 10^8]
0.25mg/L	**2.20 x 10^8**	**2.97 x 10^8**
	[1.30 x 10^8 – 4.58 x 10^8]	[1.69 x 10^8 – 4.14 x 10^8]
0.5mg/L	**2.65 x 10^8**	**2.25 x 10^8**
	[1.25 x 10^8 – 2.77 x 10^8]	[1.73 x 10^7 – 2.54 x 10^8]
1mg/L	**1.25 x 10^8**	**1.07 x 10^8**
	[1.19 x 10^8 – 2.21 x 10^8]	[1.07 x 10^8 – 1.86 x 10^8]
2mg/L	**6.43 x 10^6**	**# 7.18 x 10^5**
	[5.83 x 10^6 – 7.75 x 10^7]	[6.50 x 10^5 – 8.04 x 10^7]
4mg/L	**# 2.60 x 10^5**	**# 1.83 x 10^5**
	[4.70 x 10^4 – 6.10 x 10^5]	[1.39 x 10^3 – 1.83 x 10^5]

Table 4-9. Colony forming units (CFU/mL) of P. aeruginosa strain RBH982 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).

	TOB	**GA/TOB**
0mg/L	**2.66 x 10^8**	**1.55 x 10^8**
	[1.67 x 10^8 – 3.25 x 10^8]	[1.32 x 10^8 – 2.11 x 10^8]
0.03125mg/L	**2.58 x 10^8**	**1.58 x 10^8**
	[1.73 x 10^8 – 2.97 x 10^8]	[1.44 x 10^8 – 2.12 x 10^8]
0.0625mg/L	**3.20 x 10^8**	**2.25 x 10^8**
	[2.64 x 10^8 – 3.56 x 10^8]	[1.46 x 10^8 – 2.50 x 10^8]
0.125mg/L	**1.86 x 10^8**	**9.83 x 10^7**
	[6.50 x 10^7 – 2.27 x 10^8]	[4.64 x 10^7 – 1.78 x 10^8]
0.25mg/L	**1.45 x 10^8**	**1.36 x 10^8**
	[6.93 x 10^7 – 3.41 x 10^8]	[3.60 x 10^7 – 3.06 x 10^8]
0.5mg/L	**7.36 x 10^7**	**2.71 x 10^7**
	[7.40 x 10^7 – 3.39 x 10^8]	[2.29 x 10^7 – 6.94 x 10^7]
1mg/L	**4.82 x 10^7**	**1.58 x 10^5**
	[4.33 x 10^7 – 1.65 x 10^8]	[2.75 x 10^4 – 6.61 x 10^7]
2mg/L	**5.70 x 10^6**	**# 1.17 x 10^3**
	[3.93 x 10^6 – 7.54 x 10^7]	[0 – 8.23 x 10^5]
4mg/L	**# 1.83 x 10^4**	**# 0**
	[2.63 x 10^3 – 1.36 x 10^7]	[0 – 3.00 x 10^3]

Table 4-10. Colony forming units (CFU/mL) of P. aeruginosa strain RBH422 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. # indicates CFU/mL results which were significantly

reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).

	TOB	GA/TOB
0mg/L	**6.62×10^8** [$2.42 \times 10^8 – 1.46 \times 10^9$]	**6.11×10^8** [$1.80 \times 10^8 – 1.33 \times 10^9$]
0.5mg/L	**5.39×10^8** [$4.14 \times 10^8 – 7.00 \times 10^8$]	**4.50×10^8** [$1.90 \times 10^8 – 4.79 \times 10^8$]
1mg/L	**6.30×10^8** [$2.86 \times 10^8 – 6.47 \times 10^8$]	**2.30×10^8** [$1.63 \times 10^8 – 3.47 \times 10^8$]
2mg/L	**2.42×10^8** [$2.25 \times 10^8 – 5.79 \times 10^8$]	**6.00×10^7** [$1.21 \times 10^6 – 7.20 \times 10^7$]
4mg/L	**1.44×10^6** [$2.57 \times 10^6 – 1.75 \times 10^8$]	**4.43×10^5** [$10 – 8.10 \times 10^5$]
8mg/L	**3.21×10^5** [$10 – 5.54 \times 10^5$]	**# 0** [–]
16mg/L	**# 0** [$0 – 1.93 \times 10^3$]	**# 0** [–]

Table 4-11. Colony forming units (CFU/mL) of P. aeruginosa strain RBH519 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).

	TOB	GA/TOB
0mg/L	**2.25×10^9** [$9.50 \times 10^8 – 2.79 \times 10^9$]	**1.67×10^9** [$9.00 \times 10^8 – 2.73 \times 10^9$]
0.5mg/L	**1.86×10^9** [$7.04 \times 10^8 – 3.20 \times 10^9$]	**1.71×10^9** [$7.27 \times 10^8 – 3.09 \times 10^9$]
1mg/L	**6.40×10^8** [$4.87 \times 10^8 – 2.31 \times 10^8$]	**2.00×10^8** [$2.73 \times 10^7 – 4.07 \times 10^8$]
2mg/L	**5.70×10^8** [$3.63 \times 10^8 – 1.65 \times 10^9$]	**2.07×10^7** [$4.50 \times 10^6 – 2.57 \times 10^8$]
4mg/L	**5.47×10^8** [$1.97 \times 10^8 – 1.64 \times 10^9$]	**7.15×10^7** [$1.67 \times 10^6 – 2.30 \times 10^8$]
8mg/L	**1.25×10^9** [$3.93 \times 10^6 – 1.95 \times 10^9$]	**5.36×10^7** [$1.64 \times 10^5 – 2.25 \times 10^8$]
16mg/L	**# 0** [$0 – 5.33 \times 10^4$]	**# 0** [–]

Table 4-12. Colony forming units (CFU/mL) of P. aeruginosa strain RBH461 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).

	TOB	**GA/TOB**
0mg/L	**2.26×10^8** [1.94×10^8 – 3.08×10^8]	**1.73×10^8** [9.00×10^8 – 2.73×10^9]
2mg/L	**1.91×10^8** [5.23×10^7 – 8.50×10^8]	**1.65×10^8** [3.17×10^7 – 6.23×10^8]
4mg/L	**1.68×10^8** [3.87×10^7 – 7.56×10^8]	**1.44×10^8** [3.33×10^7 – 7.58×10^8]
8mg/L	**5.12×10^7** [2.68×10^7 – 1.47×10^8]	**1.75×10^7** [4.21×10^6 – 2.56×10^7]
16mg/L	**1.40×10^7** [4.73×10^6 – 3.05×10^7]	**# 4.17×10^5** [0 – 1.50×10^7]
32mg/L	**# 0** [–]	**# 0** [–]

*Table 4-13. Colony forming units (CFU/mL) of P. aeruginosa strain RBH490 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. * indicates CFU/mL results where significantly fewer colonies were recovered for GA/TOB treatment than TOB treatment at a given TOB concentration (p<0.05) (Mann-Whitney U test). # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).*

	TOB	**GA/TOB**
0mg/L	**7.56×10^8** [5.80×10^8 – 1.33×10^9]	**5.97×10^8** [4.00×10^8 – 1.89×10^9]
0.5mg/L	**7.96×10^8** [7.23×10^8 – 1.90×10^9]	**5.54×10^8** [4.50×10^8 – 1.38×10^9]
1mg/L	**7.35×10^8** [4.67×10^8 – 1.50×10^8]	**3.73×10^8** [1.73×10^8 – 1.50×10^9]
2mg/L	**6.36×10^8** [4.40×10^8 – 7.50×10^8]	**3.50×10^8** [2.07×10^6 – 3.93×10^8]
4mg/L	**4.83×10^8** [2.75×10^8 – 1.94×10^9]	*** 8.50×10^3** [4.90×10^3 – 3.83×10^5]
8mg/L	**5.23×10^5** [2.15×10^5 – 3.30×10^8]	**# 0** [0 – 7.13×10^3]
16mg/L	**# 0** [–]	**# 0** [–]

Table 4-14. Colony forming units (CFU/mL) of P. aeruginosa strain RBH065 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).

	TOB	GA/TOB
0mg/L	**2.19×10^8**	**1.31×10^8**
	[1.56×10^8 – 4.07×10^8]	[3.20×10^7 – 1.83×10^9]
2mg/L	**1.63×10^8**	**1.38×10^8**
	[1.25×10^8 – 2.18×10^8]	[3.25×10^7 – 1.96×10^8]
4mg/L	**1.40×10^8**	**1.63×10^8**
	[1.14×10^8 – 2.96×10^8]	[2.78×10^7 – 2.29×10^8]
8mg/L	**5.57×10^7**	**3.11×10^7**
	[4.97×10^7 – 1.14×10^8]	[6.14×10^6 – 3.47×10^7]
16mg/L	**1.43×10^8**	**# 2.14×10^7**
	[8.50×10^6 – 1.83×10^8]	[2.79×10^5 – 4.10×10^7]
32mg/L	**# 7.66×10^5**	**# 0**
	[2.46×10^5 – 1.29×10^6]	[–]

Table 4-15. Colony forming units (CFU/mL) of P. aeruginosa strain RBH072 from a person with cystic fibrosis after overnight incubation with TOB or GA/TOB. # indicates CFU/mL results which were significantly reduced from untreated control growth (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Median [95% confidence interval] of biological replicates (n≥3).

	TOB	GA/TOB
0mg/L	**1.73×10^8**	**1.27×10^8**
	[1.44×10^8 – 2.13×10^8]	[1.07×10^8 – 1.55×10^8]
32mg/L	**8.15×10^7**	**6.98×10^7**
	[6.36×10^7 – 1.89×10^8]	[4.53×10^7 – 1.10×10^8]
64mg/L	**8.25×10^7**	**5.45×10^7**
	[7.63×10^7 – 1.32×10^8]	[4.52×10^7 – 6.80×10^7]
128mg/L	**9.00×10^7**	**3.25×10^7**
	[6.21×10^7 – 2.77×10^7]	[2.72×10^6 – 3.93×10^7]
256mg/L	**3.84×10^7**	**# 1.97×10^7**
	[1.83×10^7 – 7.75×10^7]	[2.00×10^4 – 3.73×10^7]
512mg/L	**# 2.30×10^4**	**# 0**
	[0 – 2.46×10^7]	[0 – 1.00×10^4]

Colony count results for each clinical strain were analysed using two approaches (once variance had been established); pairwise comparison of CFU/mL results of TOB treatments, without vs with GA (Mann-Whitney U test) and multiple comparison to untreated control CFU/mL results (Kruskal-Wallis test with Dunn's multiple correction).

Results of these analyses indicated that there were four strains for which presence of GA resulted in a significant reduction in CFU/mL results at a strain specific TOB concentration (**Figure 4-2**, **Table 4-17**).

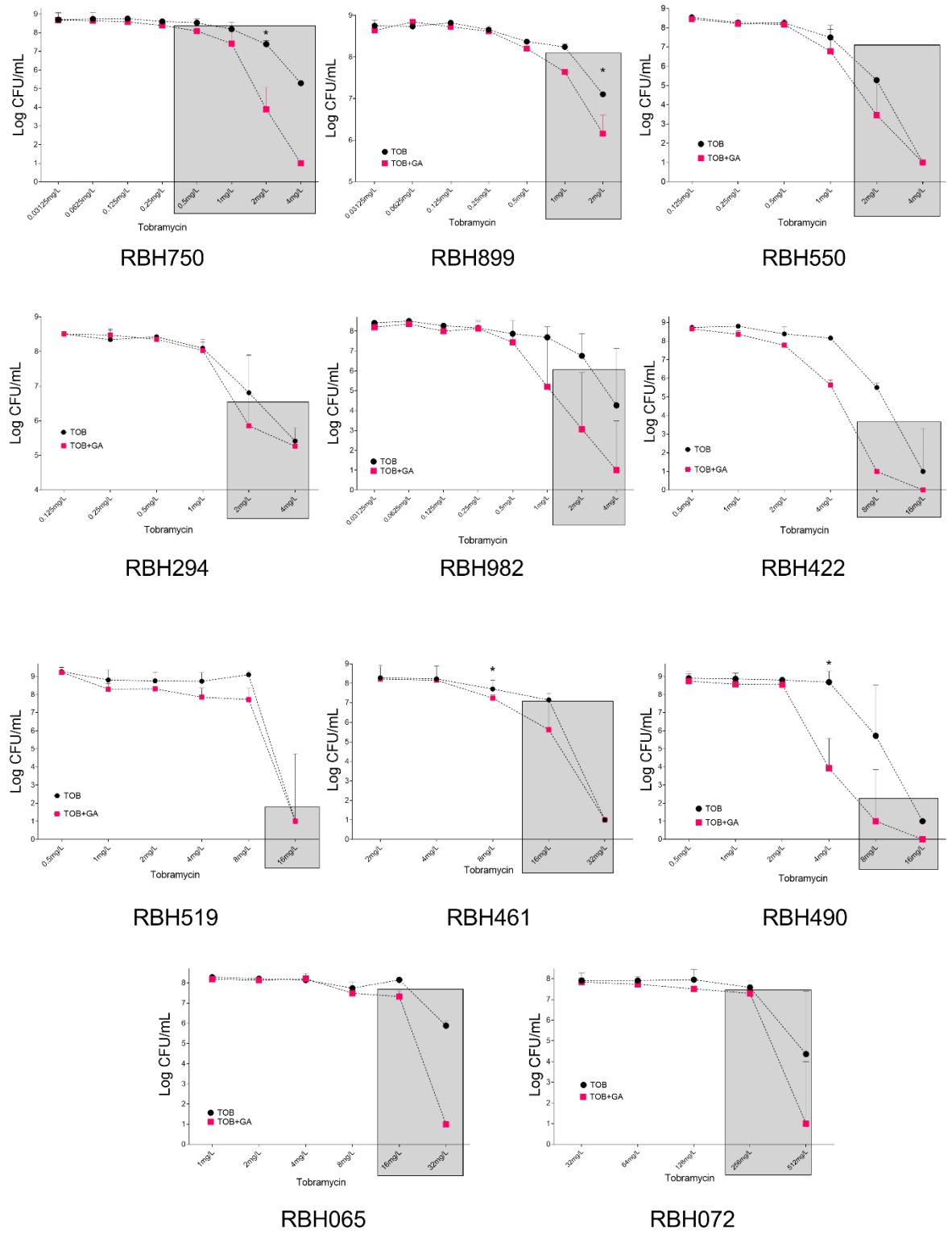


Figure 4-3. Colony forming units (CFU/mL) of P. aeruginosa from people with cystic fibrosis after overnight incubation with TOB or GA/TOB. ***** *indicates CFU/mL results where significantly fewer colonies were recovered for GA/TOB treatment than TOB treatment at a given TOB concentration; for RBH750 at 2mg/L TOB (p=0.035), for RBH899 at 2mg/L TOB (p=0.029), for RBH461 at 8mg/L TOB (p=0.028) and for RBH490 at 4mg/L TOB (p=0.036) (Mann-Whitney U test). Shaded Areas indicate CFU/mL results which were significantly reduced from untreated control growth of a given strain (p<0.05) (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). Points are medians of biological replicates with 95% confidence intervals (n≥3).*

Results of multiple comparison analyses showed that there were 7 clinical strains for which presence of GA resulted in a significant reduction in CFU/mL, compared to No Treatment, at a lower TOB concentration than was seen for TOB-only treatment (**Figure 4-3**, **Table 4-18**).

Table 4-16. Clinical P. aeruginosa strains from people with cystic fibrosis which had significant reductions in their CFU/mL results at a given TOB concentration when GA was present (Mann-Whitney U tests).

Strain	TOB Concentration	p value
RBH750	2mg/L	0.035
RBH899	2mg/L	0.029
RBH461	8mg/L	0.028
RBH490	4mg/L	0.036

Table 4-17. Clinical P. aeruginosa strains from people with cystic fibrosis which had significant reductions in their CFU/mL results from their untreated counterparts at a lower TOB concentration for GA/TOB than TOB only (Kruskal-Wallis test with Dunn's multiple correction (adjuted p values)). p value shown is the significant difference from untreated growth at the lowest TOB concentration where p<0.05.

Strain	TOB → GA/TOB	p value
RBH750	1mg/L → 0.5mg/L	0.03
RBH899	2mg/L → 1mg/L	0.028
RBH294	4mg/L → 2mg/L	0.048
RBH982	4mg/L → 2mg/L	0.008
RBH422	16mg/L → 8mg/L	0.019
RBH461	32mg/L → 16mg/L	0.009
RBH490	16mg/L → 8mg/L	0.021
RBH065	32mg/L → 16mg/L	0.033
RBH072	512mg/L → 256mg/L	0.005

4.3.4 Inhibition curves and MICs

As with reference *P. aeruginosa*, the CFU/mL results from clinical strains were used to create inhibition curves for TOB and for GA/TOB for each strain tested. From these curves,

the MIC_{50} and MIC_{90} for TOB in the absence and presence of GA were interpolated for each strain.

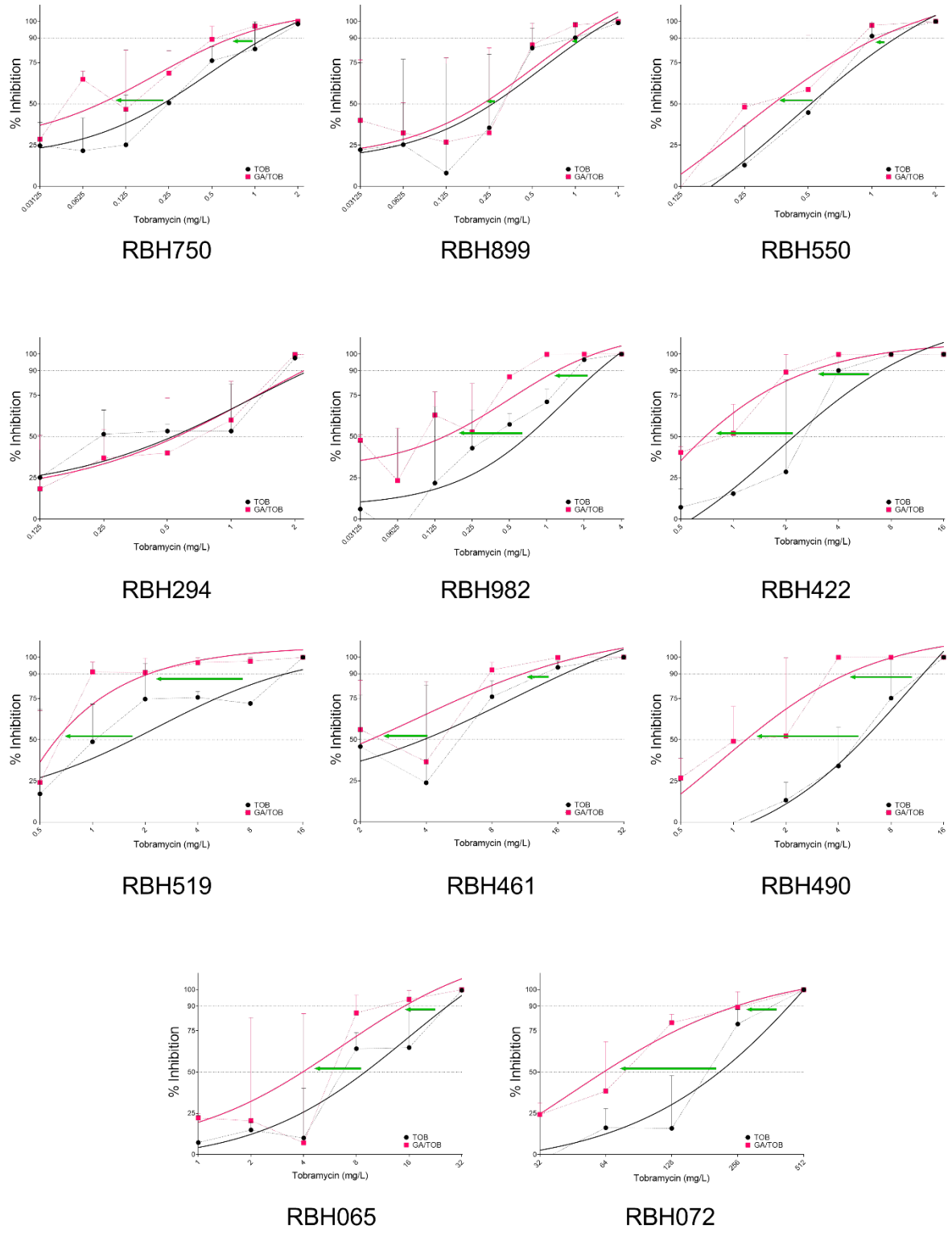


Figure 4-4. Inhibition curves of P. aeruginosa strains from people with cystic fibrosis after treatment with TOB-only and GA/TOB. Points are percentage inhibition of control CFU/mL resulting from GA/TOB treatment (Pink points) or TOB (Black points) (medians with 95% confidence intervals). Solid lines are curves fitted by Non-linear regression ([Inhibition] vs response (three parameters)). TOB minimum inhibitory concentrations (MIC_{50} and MIC_{90}) were interpolated from inhibition curves for GA/TOB (Pink) and TOB (Black).

On a group basis, presence of GA resulted in significant reductions in both MIC_{50} (p=0.002) and MIC_{90} (p=0.001) of TOB against clinical *P. aeruginosa* strains from CF (**Figure 4-4**, **Table 4-19**). The median MIC_{50} of the clinical strains was reduced from 1.69 [95%CI 0.26-8.97] mg/L to 0.62 [95%CI 0.15-3.94] mg/L (p=0.002). This represents a 2.72 [95%CI 1.18-4.66]-fold reduction in the MIC_{50} values. The median MIC_{90} of the clinical strains tested was also reduced, from 7.00 [95%CI 1.18-26.50] mg/L to 2.21 [95%CI 0.99-15.03] mg/L (p=0.001). This a fold reduction of 1.74 [95%CI 1.16-2.72] in this MIC_{90} concentration and crosses the breakpoint MIC concentration of TOB (4mg/L) from resistant to sensitive (**Figure 4-5**).

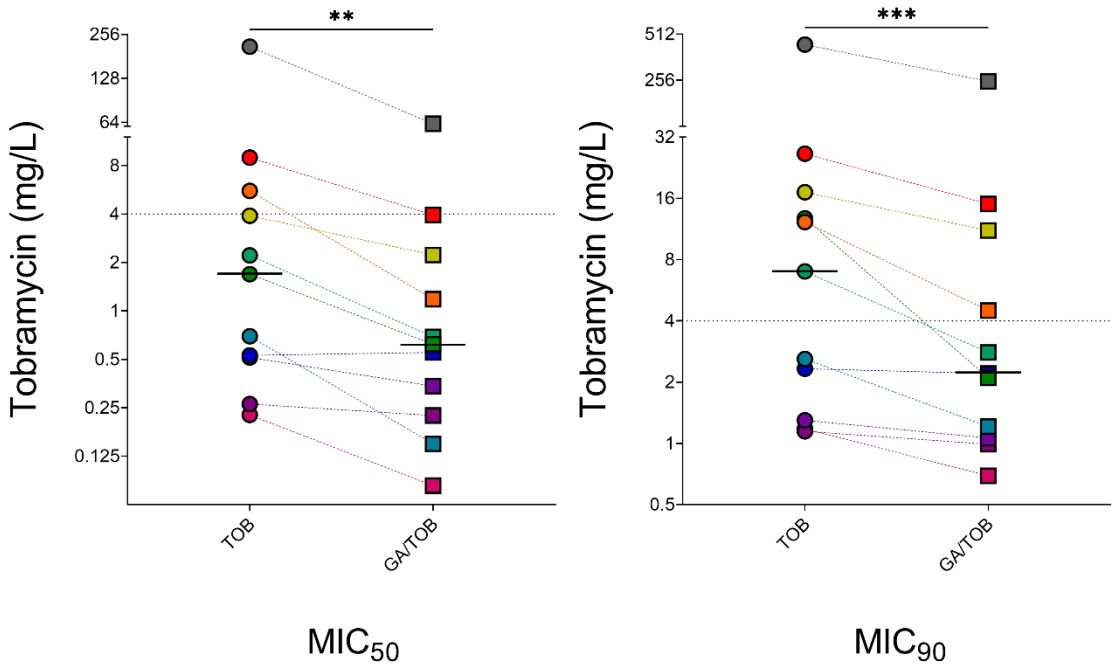


Figure 4-5. MIC_{50} and MIC_{90} values of TOB of clincal P. aerugionsa strains from CF (n=11) in the presence and absence of GA. The MICs of TOB were significantly reduced by the co-adminstration of GA; for MIC_{50} p=0.002 and for MIC_{90} p=0.001. Lines at median.

Table 4-18. TOB minimum inhibitory concentrations of clinical P. areuginosa strains from CF in the absence and presence of GA.

	MIC_{50}			MIC_{90}	
	TOB	**GA/TOB**		**TOB**	**GA/TOB**
RBH750	**0.23** [0.15 - 0.35]	**0.08** [0.04 - 0.13]		**1.18** [0.81 -]	**0.69** [0.44 – 1.41]
RBH899	**0.26** [0.12 - 0.56]	**0.22** [0.10 – 0.46]		**1.15** [0.55 -]	**0.99** [0.50 -]
RBH550	**0.51** [0.33 – 0.78]	**0.34** [0.22 - 0.54]		**1.30** [0.81 –]	**1.06** [0.60 -]
RBH294	**0.53** [0.30 – 0.95]	**0.55** [0.32 – 0.97]		**2.33** [1.37 –]	**2.21** [1.32 –]
RBH982	**0.70** [0.35 – 1.38]	**0.15** [– 0.36]		**2.60** [1.30 –]	**1.21** [0.53 –]
RBH422	**2.21** [1.50 – 3.31]	**0.69** [0.58 – 0.83]		**7.00** [4.39 –]	**2.8** [2.00 – 3.97]
RBH519	**1.69** [– 4.67]	**0.62** [– 0.92]		**12.76** [3.11 –]	**2.10** [1.00 –]
RBH461	**3.90** [– 7.69]	**2.23** [– 4.13]		**17.16** [9.11 –]	**11.12** [5.43 –]
RBH490	**5.57** [3.67 – 8.18]	**1.18** [0.76 – 1.98]		**12.23** [8.49 –]	**4.50** [2.27 –]
RBH065	**8.97** [5.17 – 15.14]	**3.94** [– 8.05]		**26.50** [15.78 –]	**15.03** [6.98 –]
RBH072	**211.19** [125.06 – 333.84]	**62.46** [49.21 – 82.47]		**438.32** [288.05 –]	**251.49** [179.36 –]
Median	**1.69** [0.26 – 8.97]	**0.62** [0.15 – 3.94]		**7.00** [1.18 – 15.03]	**2.21** [0.99– 15.03]

4.3.5 Synergy analysis

As with reference strains, the E_{OBS} and the E_{EXP} were compared for each clinical strain to test whether the combination of GA/TOB was synergistic ($E_{OBS} > E_{EXP}$), additive/independent ($E_{OBS} = E_{EXP}$) or antagonistic ($E_{OBS} < E_{EXP}$). Six of the eleven strains tested showed synergy for at least one concentration of TOB (**Figure 4-6**, **Table 4-20**). The reactions for the remainder of tests was additivity/independent activity and no antagonism was seen.

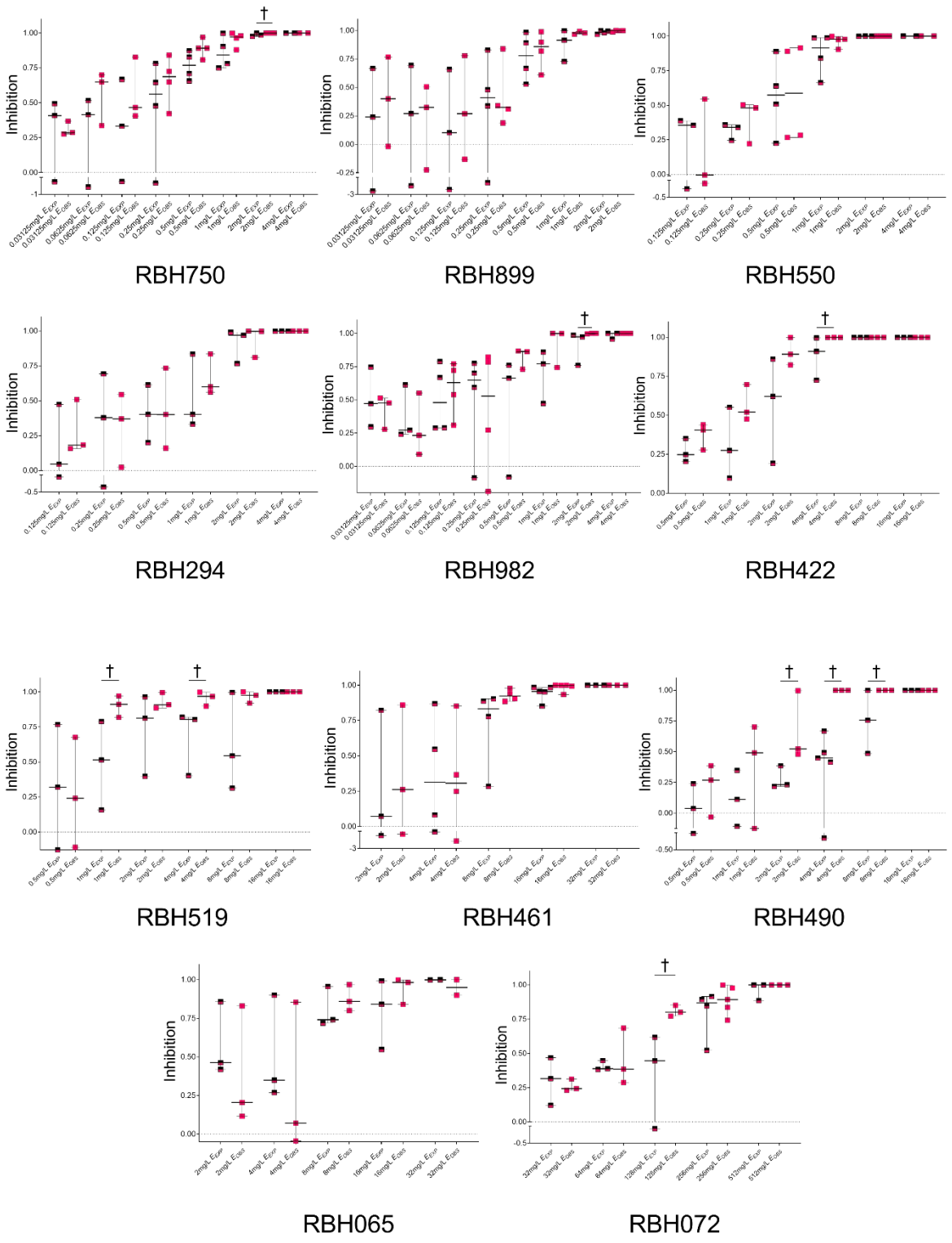


Figure 4-6. The Expect (E_{EXP}) and Observed (E_{OBS}) effects of combining TOB and GA in treatment of P. aeruginosa from people with cystic fibrosis. Inhibition was calculated relative to control growth for each biological replicate performed of each strain. † indicates concentrations of TOB where synergy between GA and TOB was seen ($E_{OBS} > E_{EXP}$). Six clinical strains had a least one TOB concentration where GA and TOB synergised together. Points are biological replicates with lines at median with 95% confidence intervals.

Table 4-19. Clinical P. aeruginosa from people with cystic fibrosis for which synergy was seen for the activities of GA and TOB and the TOB concentrations at which that synergy occurred.

Strain	TOB Concentration
RBH750	2mg/L
RBH982	2mg/L
RBH422	4mg/L
RBH519	1mg/L
	4mg/L
RBH490	2mg/L
	4mg/L
	8mg/L
RBH072	128mg/L

4.4 Discussion

In this Chapter, I have shown that GA potentiates the activity of tobramycin against clinical strains of *P. aeruginosa* from people with cystic fibrosis. I have shown that GA significantly reduces the MIC_{50} and MIC_{90} of tobramycin against clinical strains. I have shown that GA synergises with tobramycin for the majority of clinical strains tested.

Using the approaches of **Chapter 3**, which showed that GA and tobramycin synergise together in their activity against reference *P. aeruginosa* reducing the MICs of these strains, the results in this chapter show that these observations were also applicable to clinical respiratory pathogens. Across the group of 11 clinical *P. aeruginosa*, GA significantly reduced the MIC_{50} and MIC_{90} values of tobramycin ($p<0.01$), indicating that GA makes tobramycin a more effective antibiotic treatment than the aminoglycoside is on its own (**Figure 4-5**). The MIC values were reduced 2.72 [95%CI 1.18-4.66]-fold for the MIC_{50} and 1.74 [95%CI 1.16-2.72]-fold for the MIC_{90} (**Figure 4-5**, **Table 4-19**).

Over half of the clinical strains tested (6 out of 11) showed greater experimental levels of inhibition by GA/TOB than the predicted inhibition for GA and tobramycin for those strains (**Figure 4-6**). This indicates, that for this cohort of strains GA and tobramycin synergised together for at least one tobramycin concentration. In these 6 strains, the remainder of tobramycin concentrations tested were additive with GA and for the other 5 strains, which did not show any synergy, additivity of tobramycin and GA was the only result seen. No antagonism between tobramycin and GA was seen in any of the clinical strains tested.

There was a diverse range of *P. aeruginosa* strains examined here as seen in MLST profiles of the strains; only one ST was present more than once (ST89). Represented within

the panel were strains which shared STs with highly virulent clones of *P. aeruginosa*; ST253 (PA14)[23], ST38 (Liverpool Epidemic Strain 58)[338] and ST89 (Clone C)[339]. There was a broad range of antibiotic susceptibilities present, ranging from pan-susceptible to multidrug resistant (**Table 4-1**). And while the resistance determinants frequently associated with *P. aeruginosa* antibiotic resistance were broadly present, some strains showed additions and losses to the repertoire (**Table 4-4**).

No relationship was seen between the susceptibility of strains to tobramycin and potentiation tobramycin by GA. Three of the strains which showed synergy between GA and tobramycin were tobramycin-sensitive and three were tobramycin-resistant, according to clinical lab testing (**Table 4-1**).

The clinical strain which showed the greatest level of potentiation of tobramycin by GA (RBH490) was a tobramycin-resistant strain which showed synergy between GA and tobramycin at three difference concentrations and had the largest change in MIC_{50} of any strain tested (from 5.57 [95%CI 3.67 – 8.18] to 1.18 [95%CI 0.76 – 1.98] mg/L) (**Table 4-19**). This was one of only two of the clinical strains analysed found to lack the MexXY-OprJ efflux system from its genome (**Table 4-4**). While it may be able to compensate for this in another way to produce tobramycin resistance, addition of GA could overwhelm its other aminoglycoside defences and allow accumulation of tobramycin. The only other strain which lacked this efflux system was the tobramycin-sensitive strain RBH899.

The two strains which were unaltered in their MICs for tobramycin in the presence of GA (RBH899 and RBH294) were both tobramycin sensitive (**Table 4-19**). Neither of these strains showed synergy between GA and tobramycin.

The strain with the highest tobramycin MICs in this panel (RBH072) was predicted to harbour genes for two aminoglycoside inactivating enzymes (AAC(6')-Ib9 and ANT(2'')-Ia) neither of which were found in any of the other clinical strains (**Table 4-4**). This may explain this strain's ability to grow at tobramycin concentrations ≥128mg/L so it was notable that this strain had reduced tobramycin MICs with GA and showed synergy between GA and tobramycin. This indicates that GA is not only capable of overcoming impermeability and antibiotic efflux (for which this strain also has genes), but can impact enzyme-mediated tobramycin resistance.

The extension of the resistance breaking properties of GA to a clinical cohort of strains is important as the properties which allow *P. aeruginosa* to persist in the CF lung and the phenotypes produced by long-term colonisation of the CF lung are varied and distinct from non-CF strains (such as the reference strains). It was of great interest to see that the observations of GA/TOB synergy and MIC reductions in reference strains were applicable to the majority of the clinical strains despite the difference between these strains including a wide range antibiotic susceptibilities, varied genomics and differences in the resistance genes encoded.

4.4.1 Limitations of approach

The approach taken to calculate precise MIC values for each clinical strain (allowing for two MIC metrics to two decimal places per strain) is a labour intensive one and therefore limited the total number of strains it was feasible to test (n=11). A broader approach to MIC testing, such as standard broth microdilution testing via visual inspection of growth, would allow for a larger number of strains to tested.

Selection of strains to test using specific known properties that may affect GA activity e.g. known AMP-resistance SNPs/mechanisms might have been beneficial.

4.4.2 Future directions

Results presented in **Chapter 3** indicate that some additivity and/or synergy may be present for the combination of GA and CAZ. An expansion of these results into clinical *P. aeruginosa* could expand and clarify those observations.

4.4.3 Discussion of hypothesis

My hypothesis was that the potentiation observations seen for tobramycin by GA in reference *P. aeruginosa* would also be applicable to clinical strains of *P. aeruginosa* from people with cystic fibrosis. I have shown this to be the case with significant reductions on tobramycin MICs across the panel of 11 strains tested and synergy between GA and tobramycin in the majority of strains.

5. Mechanisms of action of glatiramer acetate

5.1 Introduction

Our previous published work reported the ability of GA to kill *P. aeruginosa* cells after a short exposure (30mins) in a non-dividing medium (PBS)[287]. While this and other work established GA's antimicrobial effect – resulting in cell death via Live/Dead staining and viable colony counts – neither the mechanism of action nor the kinetics of GA activity have been elucidated.

As previously discussed, the structure of GA is similar to that of cationic antimicrobial peptides[277], which have frequently been shown to associate with and cause perturbations to the membranes of bacteria[202,205,206,340]. The cell wall of Gram-negative bacteria, including *P. aeruginosa*, consists of two phospholipid bilayers with a space between them which contains a layer of peptidoglycan. The cell wall provides containment and protection for the cytoplasmic contents and processes of the bacterium and, as such, the integrity of the cell wall is vital for bacterial survival[26].

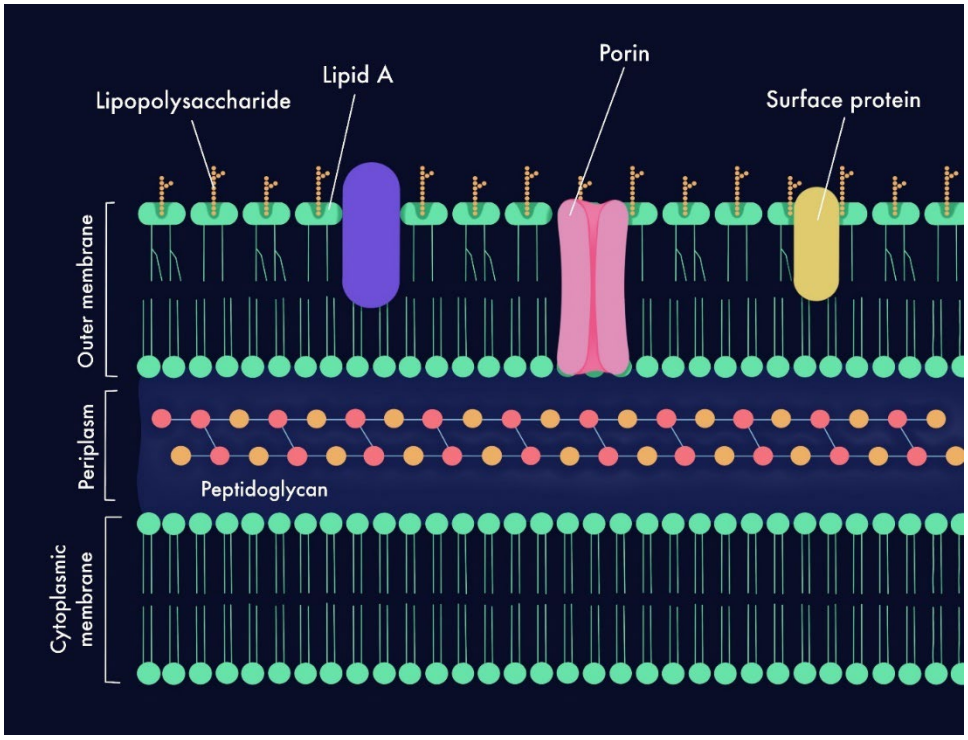


*Figure 5-1. Gram-negative bacterial cell wall. The cell wall consist of three layers; the outer membrane, the Periplasm and the cytoplasmic membrane. The **Outer Membrane** is lipid bilayer with hydrophobic fatty acid tails at centre and hydrophilic head groups on the outer edges. Embedded within the Outer Membrane are lipopolysaccharides. The **Periplasm** is a gel-like liquid region between the two membranes containing peptidoglycan. The **Cytoplasmic Membrane** is the innermost leaflet of the cells wall and is also a lipid bilayer. Created by Colette Healy© (@colettehealydesign).*

The outer membrane is a symmetrical bilayer of phospholipids, each molecule consisting of a hydrophilic head and a hydrophobic tail; mirrored phospholipids join tail-to-tail with head groups at the top and bottom of the layer. Embedded within the outer membrane, protruding into the extracellular environment are lipopolysaccharides. Bound to the outer membrane by the Lipid A portion and further containing a core oligosaccharide and a variable O polysaccharide, LPS chains provide further protection to cell wall. The cytoplasmic membrane is also a lipid bilayer, with hydrophilic outer layer and a hydrophobic interior. The space between the two membranes, known as the periplasmic space, is an aqueous gap containing a peptidoglycan layer[26].

Damage to one or multiple levels of the bacterial cell wall can prove lethal to the cell but, even in the absence of lethality, membrane damage can allow secondary stressors or agents to take effect. While the specific areas targeted, mechanisms of action and outcomes may vary, many antimicrobial peptides have been shown to exert their activity on the cell wall. This includes the so-called "antibiotic-of-last-resort" colistin, a member of the polymyxin group of AMPs[103].

Outer membrane active AMPs like colistin can disrupt the outer membrane through interactions with LPS[197,224]. Cytoplasmic membrane active AMPs against *P. aeruginosa* have also been descrbed[341,342]. Resistance breaking AMPs with membrane activity have been studied and bacterial "permeabilizers" have long been desired in antimicrobial treatment[261,318].

Investigations of integrity of Gram-negative bacterial membranes are often carried out using fluorescent dyes which exhibit different fluorescence levels for intact membranes compared with perturbed membranes. Integrity of the outer bacterial membrane can be measured with the probe NPN which fluoresces weakly in solution but more strongly if bound to hydrophobic regions[224]. Such regions include the hydrophobic tails of the phospholipids of the outer membrane which are not ordinarily exposed in intact membranes. With NPN also unable to cross the intact membrane, increased NPN fluorescence is a marker of outer membrane disruption.

The probe $DiSC_3(5)$ is frequently used to test for the integrity of bacterial cytoplasmic membranes. This dye fluoresces in solution but translocates to the lipid bilayer and binds to polarised membranes and when bound, its fluorescence is quenched and the signal decreases. Depolarisation of the cytoplasmic membrane releases $DiSC_3(5)$ and fluorescence is restored and therefore increased fluorescent signal is a marker of depolarisation[308].

Propidium iodide is a DNA-binding intercalator which displays increased fluorescence when it becomes bound. However, PI is excluded by intact bacterial membranes and therefore increased fluorescent signal indicates that PI has accessed intracellular DNA and that the bacterial cell wall has been permeabilised.

5.2 Hypothesis

I hypothesise that:

Glatiramer acetate will rapidly kill *P. aeruginosa* as a result of disruption of its bacterial membranes

5.2.1 Aims

To investigate this hypothesis, I will:

- Assess the impact of GA on *P. aeruginosa* strains during short exposures
- Test the effect of GA on *P. aeruginosa* membranes including GA dosing and comparison with other cationic agents

- Contrast and compare the effects of GA on *P. aeruginosa* cell membranes with that on other species of bacteria from CF samples

5.3 Results

5.3.1 Effects of GA on reference *P. aeruginosa* strains

5.3.1.1 Bacterial viability

The effect of short exposure (30mins) to 50mg/L GA has been described previously, in a non-dividing culture (PBS), however data was only collected at a single timepoint of 30mins. Here, the *P. aeruginosa* reference strains PAO1, PA14 and PAK were tested for their viability over 60mins during exposure to 50mg/L GA in a non-dividing mode (minimal media M9).

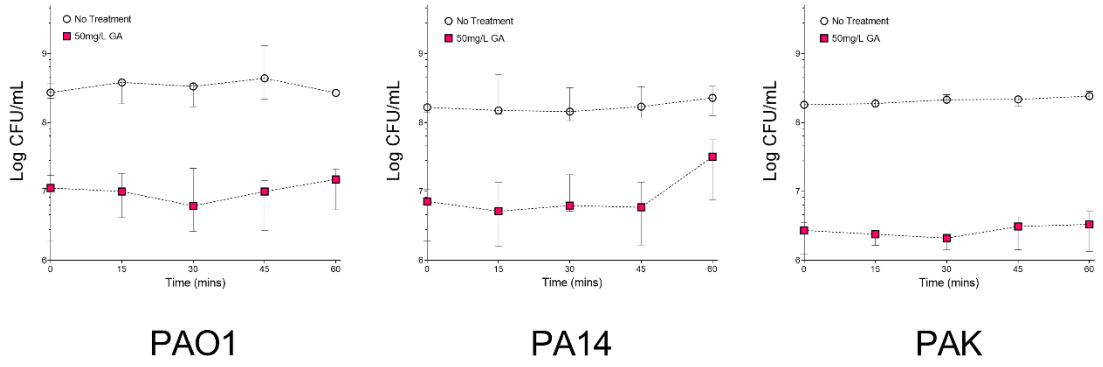


Figure 5-2. Colony forming units (CFU/mL) of P. aeruginosa reference strains PAO1, PA14 and PAK in M9 broth during treatment with 50mg/L GA. Bacteria were harvested and colonies counted every 15mins over 60mins. There was significant variance across the results for each strain – PAO1 (p=0.0098), PA14 (p=0.0093), PAK (p=0.0040) – however no difference was seen at any individual timepoint for any strain. Kruskal-Wallis with Dunn's multiple correction. Points are medians of biological replicates with 95% confidence intervals.

The CFU/mL of a the three reference strains each showed significant variance in their CFU/mL when measured every 15mins over 1hr – for PAO1 p=0.0098, for PA14 p=0.0093, for PAK p=0.0040 – indicating GA has some affect in this model. However, GA treatment did not significantly reduce bacterial numbers at any timepoint for any strain (**Figure 5-2**, **Table 5-5**).

Table 5-1. Colony forming units (CFU/mL) of P. aeruginosa reference strains PAO1, PA14 and PAK in M9 broth during treatment with 50mg/L GA. Bacteria were harvested and colonies counted every 15mins over 60mins. Median vaules [95% confidence intervals] (n=3).

	0min		**15mins**		**30mins**		**45mins**		**60mins**	
	No Treatment	50mg/L GA	No Treatment	50mg/L GA	No Treatment	50mg/L GA	No Treatment	50mg/L GA	No Treatment	50mg/L GA
PAO1	**$2.73*10^8$** [$2.25*10^8$ – $3.75*10^8$]	**$1.13*10^7$** [$1.94*10^6$ – $1.73*10^7$]	**$3.38*10^8$** [$1.88*10^8$ – $3.95*10^8$]	**$1.00*10^7$** [$4.18*10^6$ – $1.83*10^7$]	**$3.36*10^8$** [$1.70*10^8$ – $3.69*10^8$]	**$6.18*10^6$** [$2.64*10^6$ – $2.17*10^7$]	**$4.40*10^8$** [$2.19*10^8$ – $1.30*10^9$]	**$1.00*10^7$** [$2.73*10^6$ – $1.44*10^7$]	**$2.70*10^8$** [$2.58*10^8$ – $2.82*10^9$]	**$1.50*10^7$** [$5.42*10^6$ – $2.13*10^7$]
PA14	**$1.67*10^8$** [$1.40*10^8$ – $1.77*10^8$]	**$7.17*10^6$** [$1.91*10^6$ – $1.08*10^7$]	**$1.50*10^8$** [$1.33*10^8$ – $5.00*10^8$]	**$5.17*10^6$** [$1.61*10^6$ – $1.36*10^7$]	**$1.44*10^8$** [$1.07*10^8$ – $3.21*10^8$]	**$6.23*10^6$** [$5.17*10^6$ – $1.75*10^7$]	**$1.70*10^8$** [$1.17*10^8$ – $3.32*10^8$]	**$5.89*10^6$** [$1.67*10^6$ – $1.36*10^7$]	**$2.29*10^8$** [$1.25*10^8$ – $3.36*10^8$]	**$3.19*10^6$** [$7.50*10^6$ – $5.63*10^7$]
PAK	**$1.82*10^8$** [$1.68*10^8$ – $1.95*10^8$]	**$2.73*10^6$** [$1.23*10^6$ – $3.58*10^6$]	**$1.89*10^8$** [$1.79*10^8$ – $2.23*10^8$]	**$2.40*10^6$** [$1.67*10^6$ – $2.43*10^6$]	**$2.15*10^8$** [$2.12*10^8$ – $2.54*10^8$]	**$2.11*10^6$** [$1.44*10^6$ – $2.42*10^6$]	**$2.18*10^8$** [$1.75*10^8$ – $2.21*10^8$]	**$3.11*10^6$** [$1.43*10^6$ – $4.30*10^6$]	**$2.43*10^8$** [$2.25*10^8$ – $2.85*10^8$]	**$3.35*10^6$** [$1.35*10^6$ – $5.13*10^6$]

5.3.1.2 Effect of GA on membranes of reference *P. aeruginosa*

5.3.1.2.1 Outer bacterial membrane

The effect of 50mg/L GA on the outer bacterial membrane of *P. aeruginosa* reference strains PAO1, PA14 and PAK was tested using the fluorescent dye 1-*N*-phenylnaphthylamine (NPN) which fluoresces when bound to the inner regions of the outer membrane, areas only accessible to the dye during membrane disruption.

For all three reference strains, 50mg/L GA resulted in significant ($p<0.0001$) increases in mean NPN Uptake Factor over 15mins compared with untreated cultures (**Figure 5-3**; $n\geq16$).

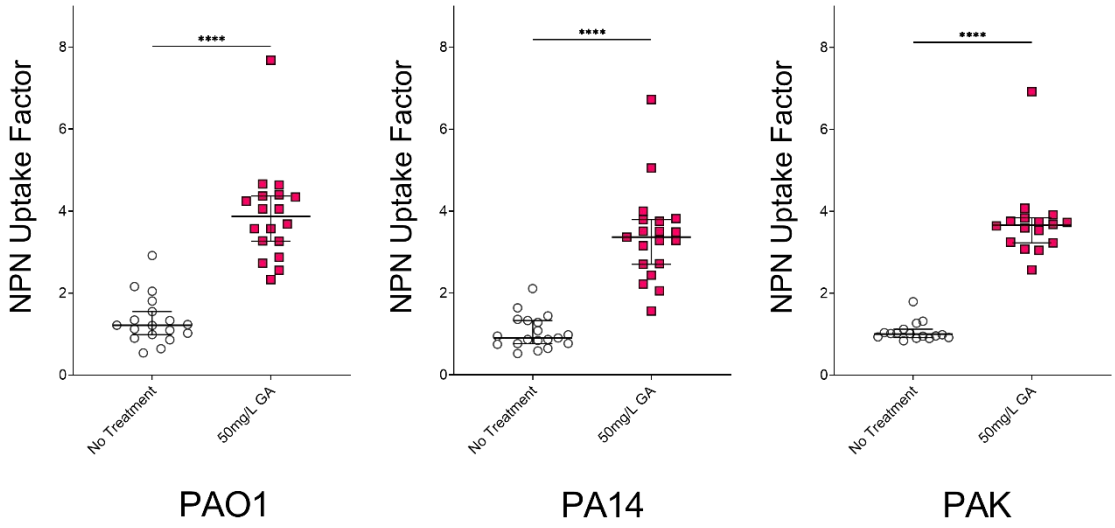


Figure 5-3. Mean NPN Uptake Factor over 15mins of P. aeruginosa PAO1, PA14 and PAK during treatment with 50mg/L GA. Black circles are No Treatment and pink squares are 50mg/L GA. Points are biological replicates with line at median and 95% confidence intervals. For all strains p<0.0001 (Mann-Whitney U test).

5.3.1.2.2 Bacterial cytoplasmic membrane

The effect of 50mg/L GA on the cytoplasmic membranes of *P. aeruginosa* strains PAO1, PA14 and PAK was measured using the fluorescent dye 3,3'-dipropylthiadicarbocyanine

Iodide ($DiSC_3(5)$). The dye integrates into the intact cytoplasmic membrane and, once bound, ceases fluorescing. Any subsequent depolarisation of the membrane results in the dye being released and fluorescence increasing.

After the fluorescent signal of the dye was allowed to quench (**Figure 5-4A**), addition of 50mg/L GA resulted in significant mean increase in fluorescence over 15mins for each reference strain compared to their own No Treatment suspensions. All three strains resulted in $p<0.0001$ ($n=17$) (**Figure 5-4**).

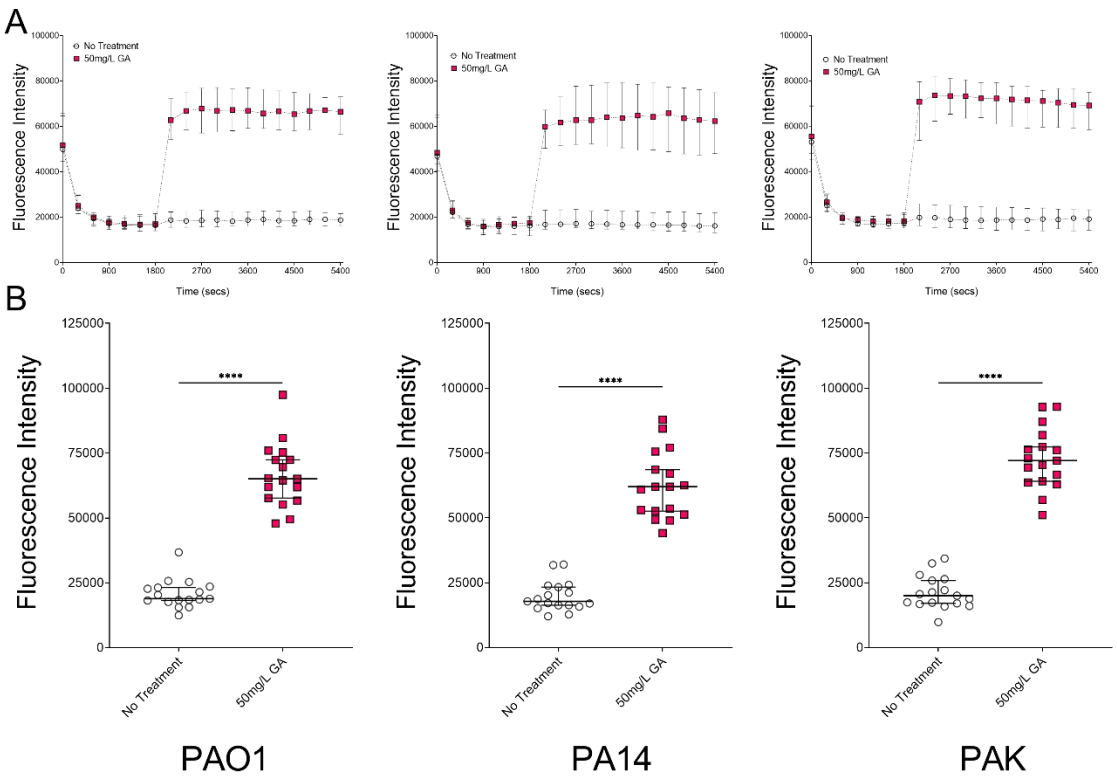


Figure 5-4. Intensity of $DiSC_3(5)$ fluorescence released by P. aeruginosa PAO1, PA14 and PAK over 60mins after treatment with 50mg/L GA. $DiSC_3(5)$ was allowed to quench for 30mins followed by addition of buffer (No Treatment) or GA to a final concetration of 50mg/L ***A****. Timecourse of $DiSC_3(5)$ assay. Either buffer (Black circles) or GA (Pink squares) were added at 30min timepoint.* ***B.*** *Mean intensity of $DiSC_3(5)$ fluorescence over 15mins. For all strains GA significantly increased $DiSC_3(5)$ fluorescence ($p<0.0001$) (Mann-Whitney U test). Medians and 95% confidence intervals of biological replicates.*

5.3.1.2.3 Bacterial cell permeability

The permeability of *P. aeruginosa* cells of PAO1, PA14 and PAK was tested using the dye propidium iodide (PI), a DNA binding dye which cannot cross intact membranes and so can only reach intracellular DNA and fluoresce in the event of permeabilised membranes, allowing PI to pass across.

The AUCs of PI fluorescence over 60mins were compared for No Treatment and GA treated cells. For all three reference strains GA resulted in a significant increase in AUCs during treatment. Unlike outer membrane disruption and cytoplasmic depolarisation, permeability of PAO1 cells was, while significantly increased ($p=0.0007$, $n=19$), not increased as significantly as cells of PA14 and PAK (both $p<0.0001$, $n\geq18$) (**Figure 5-5**).

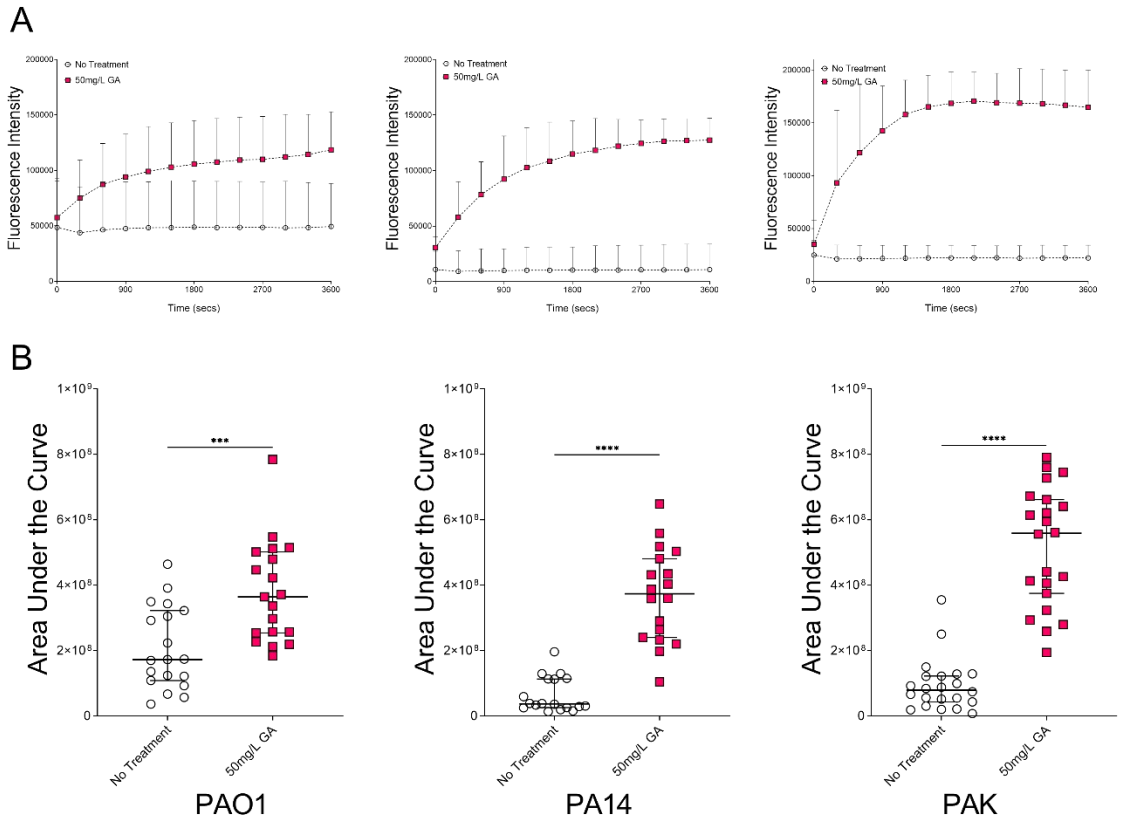


Figure 5-5. Fluorescene intesnity of propidium iodide for P. aeruginosa PAO1, PA14 and PAK over 60mins during incubation with 50mg/L GA. ***A.*** *Timecourse of PI during No Treatment (Black circles) and GA treatment (Pink squares).* ***B.*** *AUCs of PI fluorescence intensity over 60mins. GA resulted in a significant increase in PI AUCs for all strains (PAO1 p=0.0007. PA14 and PAK p<0.0001) (Mann-Whitney U test). Points are biological replicates with line at median and 95% confidence intervals.*

5.3.1.3 Effect of variations of GA concentrations on membranes of reference *P. aeruginosa*

Having noted the ability of 50mg/L GA to disrupt the membranes of and permeabilise reference *P. aeruginosa* strains, the activity of GA doses in log2 increments in the range 6.25-100mg/L was measured.

5.3.1.3.1 Bacterial viability

In contrast to 50mg/L, no decline in bacterial viability was seen in reference strains during treatment with either 25mg/L or 100mg/L (**Figure 5-6**).

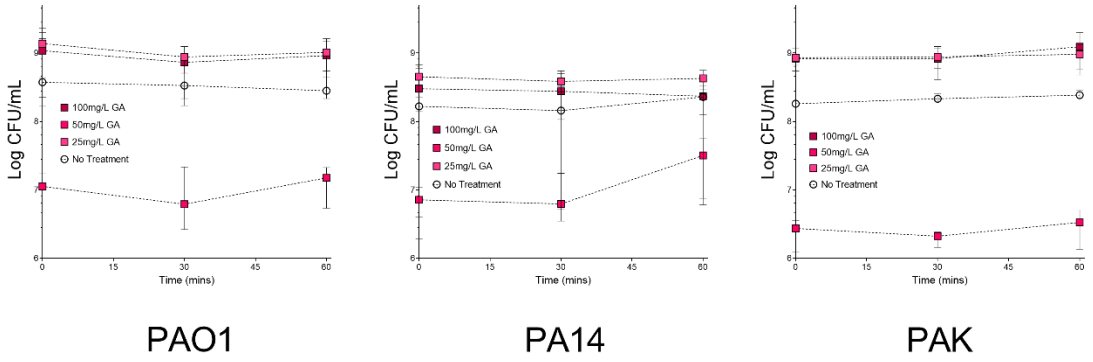


Figure 5-6. Colony forming units (CFU/mL) of P. aeruginosa reference strains PAO1, PA14 and PAK in M9 broth during treatment with 25, 50 and 100mg/L GA.

5.3.1.3.2 Outer bacterial membrane

A dose dependent effect was seen for GA against reference strains of *P. aeruginosa* in its disruptive effect on the outer membrane, via NPN uptake. In multiple comparison, comparing with controls under the same conditions, there was a significant increase in the uptake of NPN by the PAO1 and PA14 outer membranes by the GA at 50mg/L ($p<0.05$) and by all three reference strains at 100mg/L GA ($p<0.01$) (**Figure 5-7**). None of the reference strains had a significant increase in NPN uptake with GA from 6.25-25mg/L, in multiple comparison tests (**Figure 5-8**).

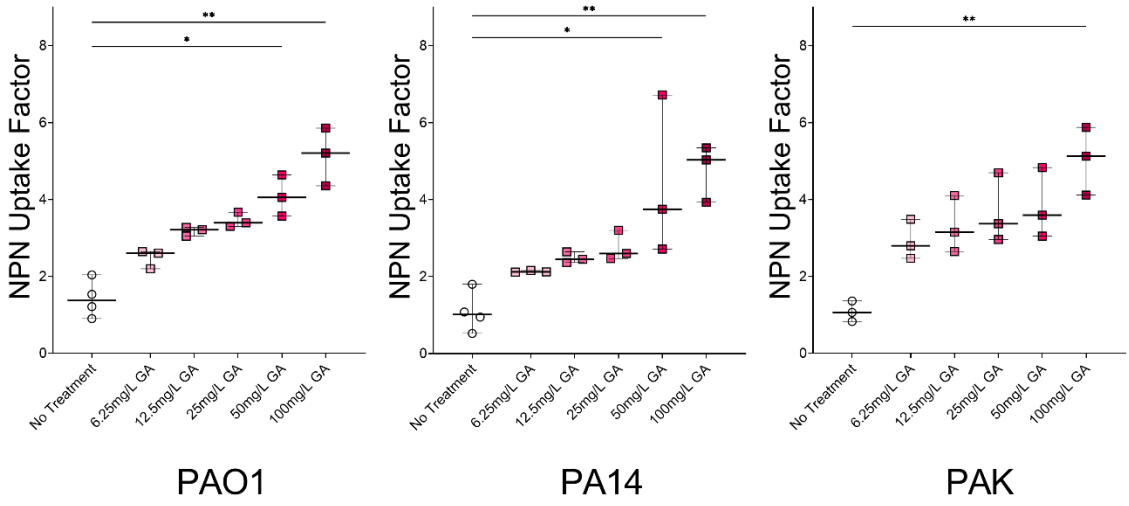


Figure 5-7. Mean NPN Uptake Factor over 15mins of P. aeruginosa PAO1, PA14 and PAK during treatment with 6.25-100mg/L GA. The GA concetrations tested resulted in significant variance in NPN Uptake Factor for each strain tested (p<0.0001). For PAO1 GA at 50mg/L (p=0.0182) and 100mg/L (p=0.0021) significantly increased uptake of NPN from No Treatment. For PA14 GA at 50mg/L (p=0.0109) and 100mg/L (p=0.0037) significantly increased uptake of NPN from No Treatment. For PAK GA at 100mg/L (p=0.005) significantly increased uptake of NPN from No Treatment. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

5.3.1.3.3 Bacterial cytoplasmic membrane

The cytoplasmic membrane of *P. aeruginosa* reference strains was depolarised by GA in a dose dependent manner, over the first 15mins of treatment. Comparison to control $DiSC_3(5)$ fluorescence of PAO1 and PA14 showed significant increases in dye release with concentrations of GA 50 and 100mg/L ($p<0.05$) (**Figure 5-8**).

Multiple comparisons did not result in any significant changes for PAO1 and PA14 when treated with GA 6.25-25mg/L. None of the GA concentrations had a significant effect on PAK cytoplasmic membrane polarisation (**Figure 5-8**).

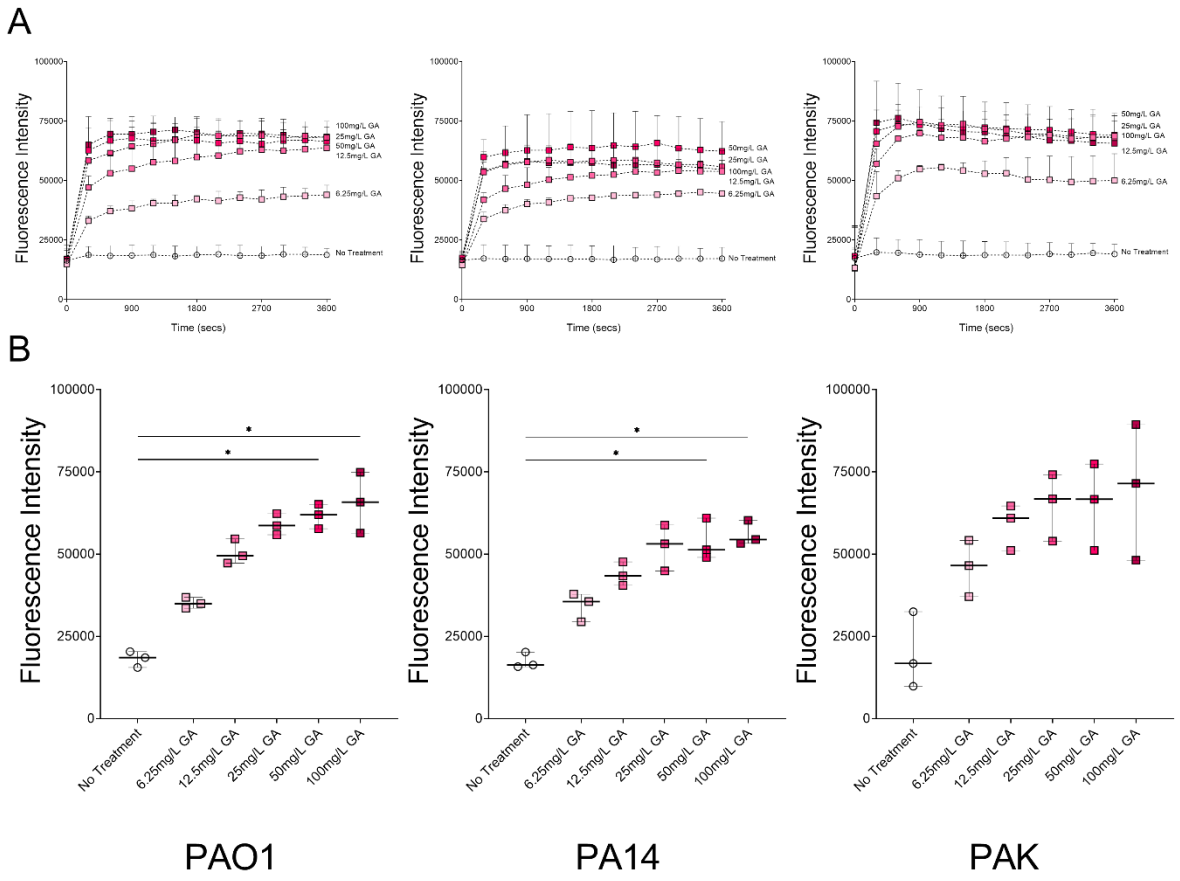


Figure 5-8. A. Intensity of DiSC$_3$(5) fluorescence released by P. aeruginosa PAO1, PA14 and PAK over 60mins after addition of GA in the concetration range 6.25-100mg/L (median and 95% confidence intervals of biological replicates at each timepoint). B. Mean intensity of DiSC$_3$(5) fluorescence over 15mins released by P. aeruginosa PAO1, PA14 and PAK after addition of 6.25-100mg/L GA. For PAO1 GA at 50mg/L (p=0.0295) and 100mg/L (p=0.00111) significantly increased release of DiSC$_3$(5) from No Treatment. For PA14 GA at 50mg/L (p=0.0372) and 100mg/L (p=0.0111) significantly increased release of DiSC$_3$(5) from No Treatment. For PAK GA no concentration of GA significantly increased release of DiSC$_3$(5) from No Treatment. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

5.3.1.3.4 Bacterial cell permeability

Permeabilisation of *P. aeruginosa* strains by GA concentrations from 6.25-100mg/L was measured (**Figure 5-9**). Strains PA14 and PAK each showed significant increases in fluorescence of PI over 60mins when treated with 50mg/L compared with their untreated counterparts ($p<0.05$) and PAK PI AUC was also significantly increased by 100mg/L ($p=0.04$).

None of the other concentrations tested significantly altered permeability of PA14 and PAK and none of the concentrations tested resulted in a significant change in PAO1 (**Figure 5-9**).

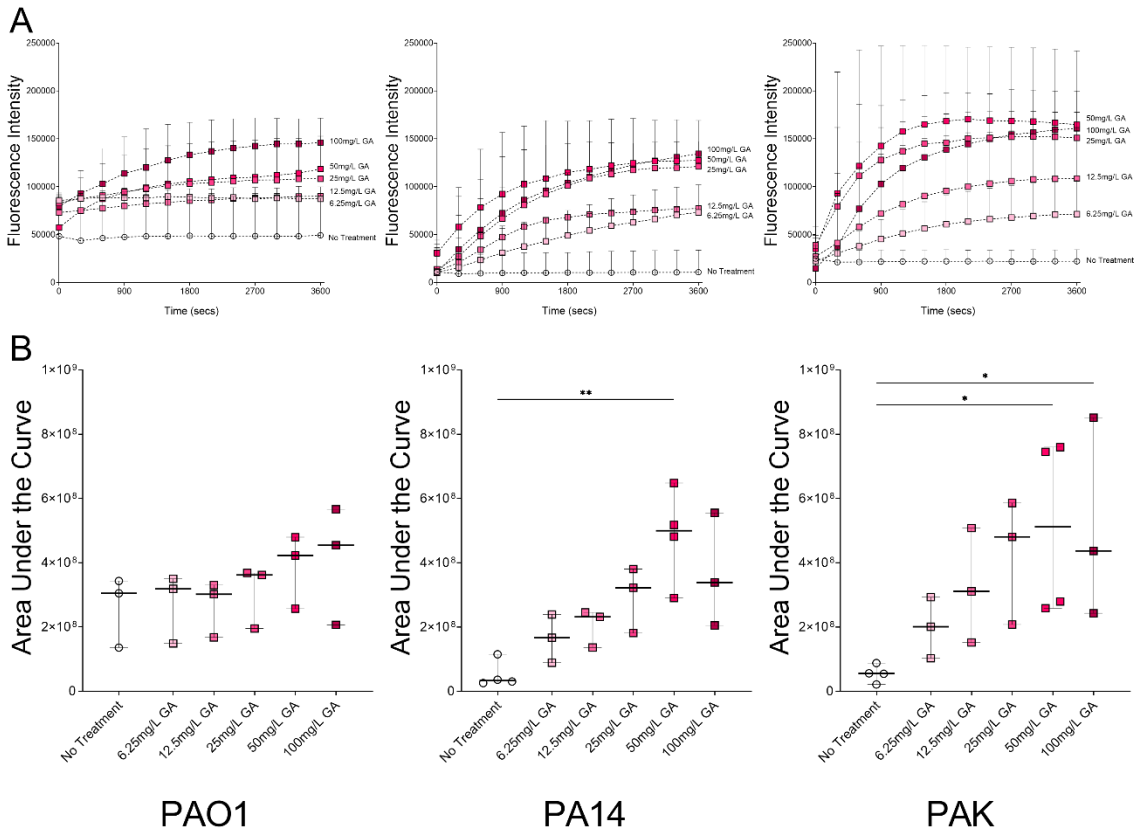


Figure 5-9. A. Intensity of PI fluorescence of P. aeruginosa PAO1, PA14 and PAK over 60mins with treatments of GA in the concetration range 6.25-100mg/L (median and 95% confidence intervals of biological replicates at each timepoint). B. AUCs of PI fluorescence over 60mins of P. aeruginosa PAO1, PA14 and PAK during treatment with 6.25-100mg/L GA. For PAO1 GA no GA concentration significantly increased AUCs of PI from No Treatment. For PA14 GA at 50mg/L (p=0.0033) significantly increased AUCs of PI from No Treatment. For PAK GA at 50mg/L (p=0.0206) and 100mg/L (p=0.0441) significantly increased AUCs of PI from No Treatment. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

5.3.1.4 Comparison of GA activity on bacterial membranes with other antimicrobial agents

The ability of 50mg/L GA to perturb reference *P. aeruginosa* membranes and permeabilise the cells was compared with membrane disrupting peptides CST and LL-37, as well as the mildly cationic aminoglycoside TOB. For the antibiotics, their EUCAST breakpoint MIC – CST (2mg/L) and TOB (4mg/L) – was selected for comparison while for LL-37 a previously published MIC was used (16mg/L)[343].

5.3.1.4.1 Outer bacterial membrane

Treatment with GA resulted in significant increases in NPN uptake by the outer membranes of each of the 3 reference strains in multiple comparisons with other membrane disrupting agents ($p<0.01$) (**Figure 5-10**). This was also the case for treatment with CST ($p<0.01$).

Additionally, strain PAK had a significant increase in NPN uptake resulting from LL-37 treatment ($p<0.05$), showing all three AMPs tested significantly affected PAK. No strains were altered by TOB treatment (**Figure 5-10**).

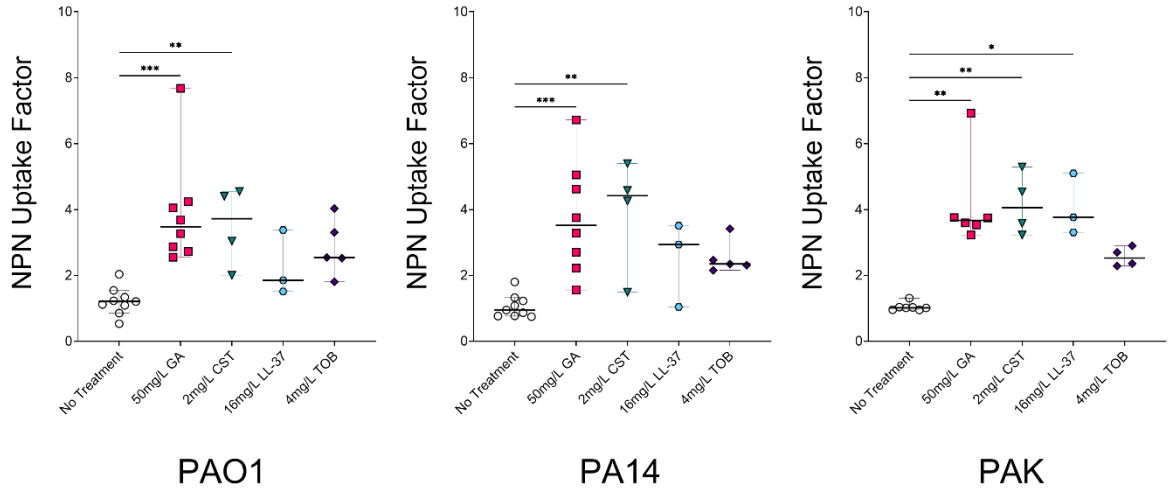


Figure 5-10. Mean NPN Uptake Factor over 15mins of P. aeruginosa PAO1, PA14 and PAK during treatment with 50mg/L GA, 2mg/L CST, 16mg/L LL-37 and 4mg/L TOB. For PAO1 50mg/L GA (p=0.0004) and 2mg/L CST (p=0.0073) significantly increased uptake of NPN from No Treatment. For PA14 GA at 50mg/L (p=0.0008) and CST at 2mg/L (p=0.0067) significantly increased uptake of NPN from No Treatment. For PAK GA at 50mg/L (p=0.0022), CST at 2mg/L (p=0.0057) and LL-37 at 16mg/L (p=0.0106) significantly increased uptake of NPN from No Treatment. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

5.3.1.4.2 Bacterial cytoplasmic membrane

As with NPN Uptake Factor, there was significant increase in $DiSC_3(5)$ release by cytoplasmic membranes seen for all three reference strains during treatment with GA (**Figure 5-11**).

LL-37 treatment of strains PA14 and PAK also resulted in significant dye release by the strains ($p<0.05$). PAO1 was only significantly affected by GA and treatment with CST and TOB did not significantly alter the cytoplasmic membrane of any strain (**Figure 5-11**).

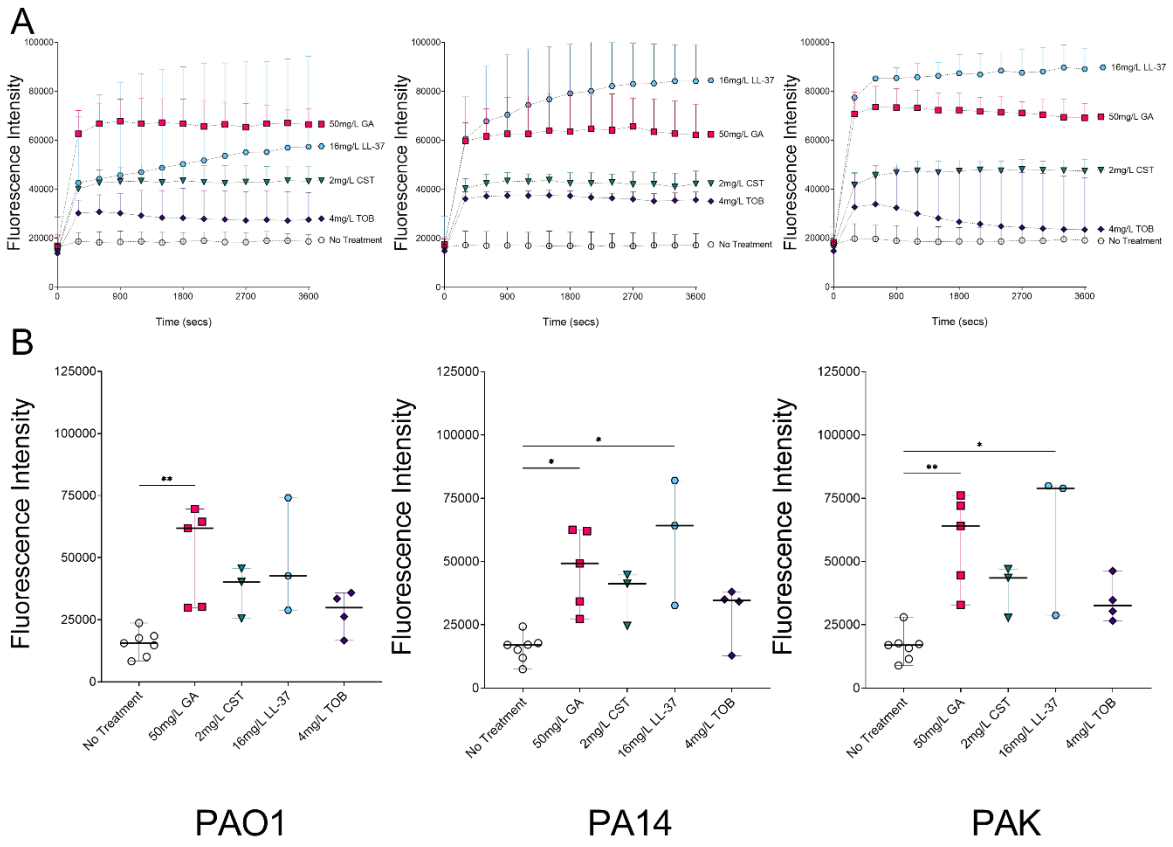


Figure 5-11. A. Intensity of DiSC$_3$(5) fluorescence released by P. aeruginosa PAO1, PA14 and PAK over 60mins after addition of 50mg/L GA, 2mg/L CST, 16mg/L LL-37 and 4mg/L TOB (median and 95% confidence intervals of biological replicates at each timepoint). B. Mean intensity of DiSC$_3$(5) fluorescence over 15mins released by P. aeruginosa PAO1, PA14 and PAK after addition of 50mg/L GA, 2mg/L CST, 16mg/L LL-37 and 4mg/L TOB. For PAO1 GA at 50mg/L (p=0.0038) significantly increased release of DiSC$_3$(5) from No Treatment. For PA14 GA at 50mg/L (p=0.012) and LL-37 16mg/L (p=0.0121) significantly increased release of DiSC$_3$(5) from No Treatment. For PAK GA at 50mg/L (p=0.004) and LL-37 16mg/L (p=0.0113) significantly increased release of DiSC$_3$(5) from No Treatment. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

5.3.1.4.3 Bacterial cell permeability

Permeability of strains PA14 and PAK was significantly increased by both GA and LL-37 in multiple comparisons ($p<0.05$) (**Figure 5-12**). Neither strain was significantly altered by CST or TOB.

None of the agents tested significantly permeabilised PAO1 at the concentrations tested (**Figure 5-12**).

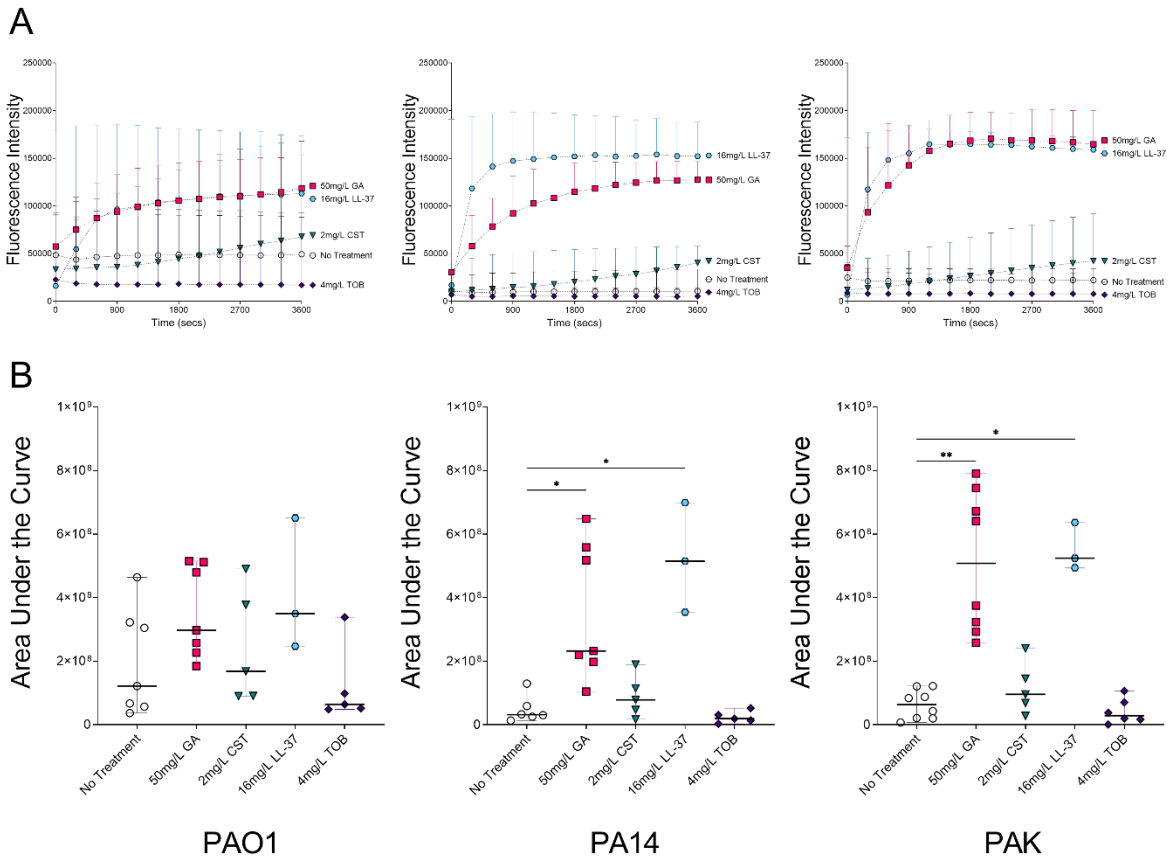


Figure 5-12. A. Intensity of PI fluorescence of P. aeruginosa PAO1, PA14 and PAK over 60mins after addition of 50mg/L GA, 2mg/L CST, 16mg/L LL-37 and 4mg/L TOB (median and 95% confidence intervals of biological replicates at each timepoint). B. AUCs of PI fluorescence intensity over 60mins of P. aeruginosa PAO1, PA14 and PAK during treatment with 50mg/L GA, 2mg/L CST, 16mg/L LL-37 and 4mg/L TOB. For PAO1 no treatment significantly increased PI AUCs from No Treatment. For PA14 GA at 50mg/L (p=0.0228) and LL-37 16mg/L (p=0.0322) significantly increased PI AUCs from No Treatment. For PAK GA at 50mg/L (p=0.0026) and LL-37 16mg/L (p=0.0474) significantly increased PI AUCs from No Treatment. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

5.3.1.5 Assessment of GA activity on bacterial membranes when co-administered with TOB

Given the results reported earlier (**Chapters 3** and **4**) on the potentiation of TOB by the presence of GA in overnight cultures, the combined effects of the two agents were assessed for any additive or inhibitory action on the membranes of reference *P. aeruginosa*.

5.3.1.5.1 Outer bacterial membrane

GA resulted in significantly increased uptake of NPN by all of the reference strains, in keeping with earlier results ($p<0.01$) (**Figure 5-13**). Also as reported above (**Figure 5-10**), TOB at 4mg/L did not have a significant impact on the NPN Uptake Factor of any of these strains. When the two agents were administered in combination at these concentrations, there was also a significant increase in NPN uptake within the range of, and not statistically different from, the GA alone ($p\leq0.05$) (**Figure 5-13**).

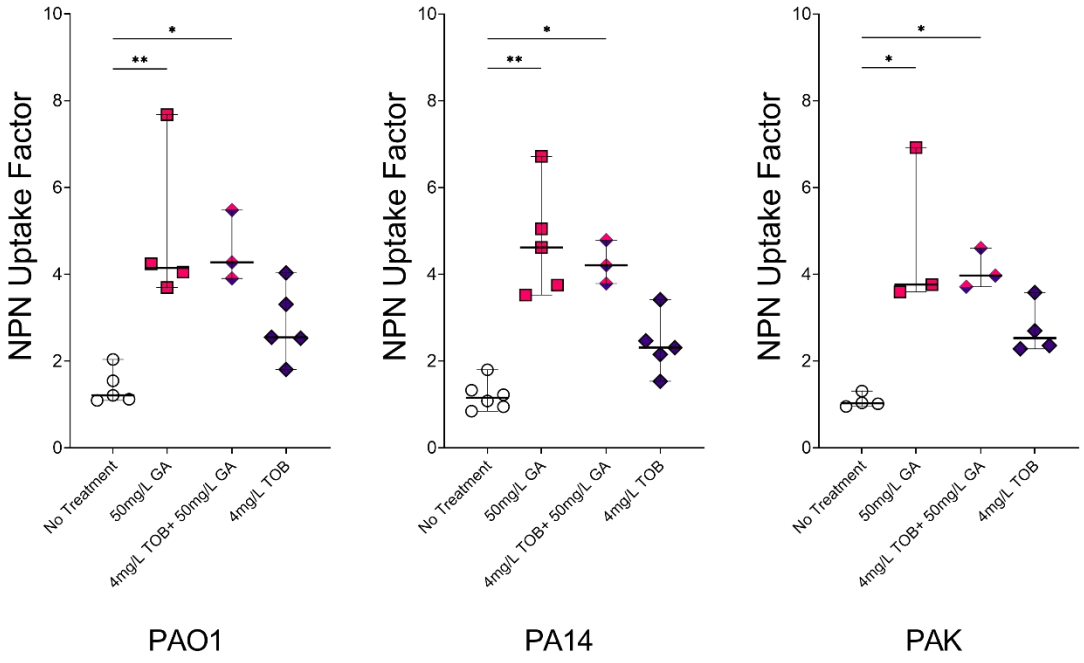


Figure 5-13. Mean NPN Uptake Factor over 15mins of P. aeruginosa PAO1, PA14 and PAK after treatment with 50mg/L GA, 4mg/L TOB and 4mg/L TOB + 50mg/L GA combined. For PAO1 50mg/L GA (p=0.0071) and the combination of 4mg/L TOB + 50mg/L GA (p=0.0102) significantly increased uptake of NPN from No Treatment. For PA14 GA at 50mg/L (p=0.0014) and the combination of 4mg/L TOB + 50mg/L GA (p=0.0101) significantly increased uptake of NPN from No Treatment. For PAK GA at 50mg/L (p=0.0171) and the combination of 4mg/L TOB + 50mg/L GA (p=0.0124) significantly increased uptake of NPN from No Treatment. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

5.3.1.5.2 Bacterial cytoplasmic membrane

As was reported previously (**Figure 5-11**), release of $DiSC_3(5)$ was significantly increased by GA from no treatment ($p<0.05$) while TOB did change the fluorescence intensity of $DiSC_3(5)$ measured during TOB treatment, for all refence strains. In common with the NPN results seen for the reference strains, the increase in $DiSC_3(5)$ release over 15mins of treatment by the combination of 4mg/L TOB + 50mg/L GA retained the significant increase seen with GA-only treatment, for each of PAO1, PA14 and PAK ($p<0.05$) (**Figure 5-14**).

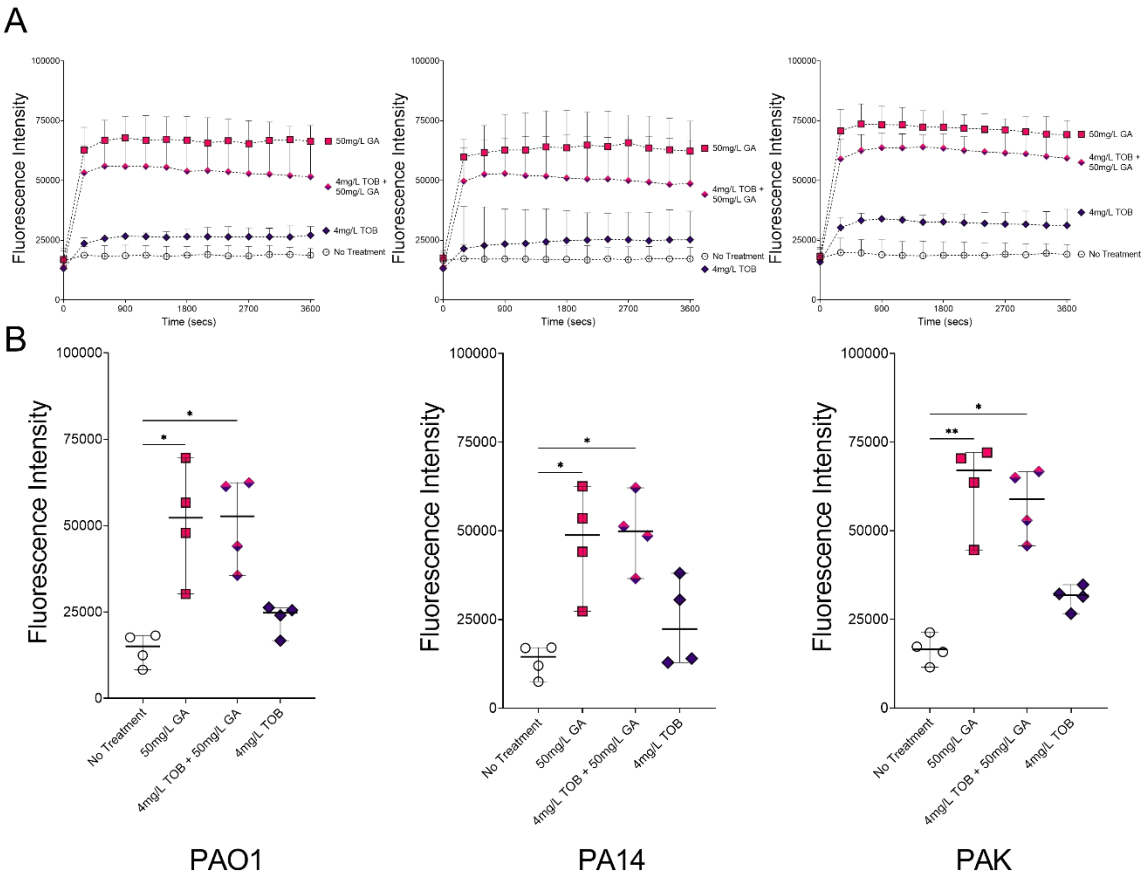


Figure 5-14. ***A****. Intensity of DiSC$_3$(5) fluorescence released by P. aeruginosa PAO1, PA14 and PAK over 60mins after addition of 50mg/L GA, 4mg/L TOB or 4mg/L TOB + 50mg/L GA together (median and 95% confidence intervals of biological replicates at each timepoint).* ***B.*** *Mean intensity of DiSC$_3$(5) fluorescence released over 15mins by P. aeruginosa PAO1, PA14 and PAK after addition of 50mg/L GA, 4mg/L TOB or 4mg/L TOB + 50mg/L GA together. For PAO1 GA at 50mg/L (p=0.0143) and combined 4mg/L TOB + 50mg/L GA (p=0.0143) significantly increased release of DiSC$_3$(5) from No Treatment. For PA14 GA at 50mg/L (p=0.0347) and and combined 4mg/L TOB + 50mg/L GA (p=0.028) significantly increased release of DiSC$_3$(5) from No Treatment. For PAK GA at 50mg/L (p=0.0054) and and combined 4mg/L TOB + 50mg/L GA (p=0.0143) significantly increased release of DiSC$_3$(5) from No Treatment. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.*

5.3.1.5.3 Bacterial cell permeability

As reported above (**Figure 5-12**), PI fluorescence was significantly increased by GA from controls in multiple comparisons for strains PA14 and PAK ($p<0.05$), but not PAO1, and 4mg/L TOB did not significantly affect the permeability of any of the reference strains (**Figure 5-15**). In contrast with NPN uptake and DiSC$_3$(5) release, the presence of 4mg/L TOB with

50mg/L GA led to loss of the impact observed with GA alone in PA14 and PAK, and PI values which were not significantly different from the untreated controls suggesting that permeabilisation of PA14 and PAK bacterial cells by GA may be inhibited in the presence of the antibiotic.

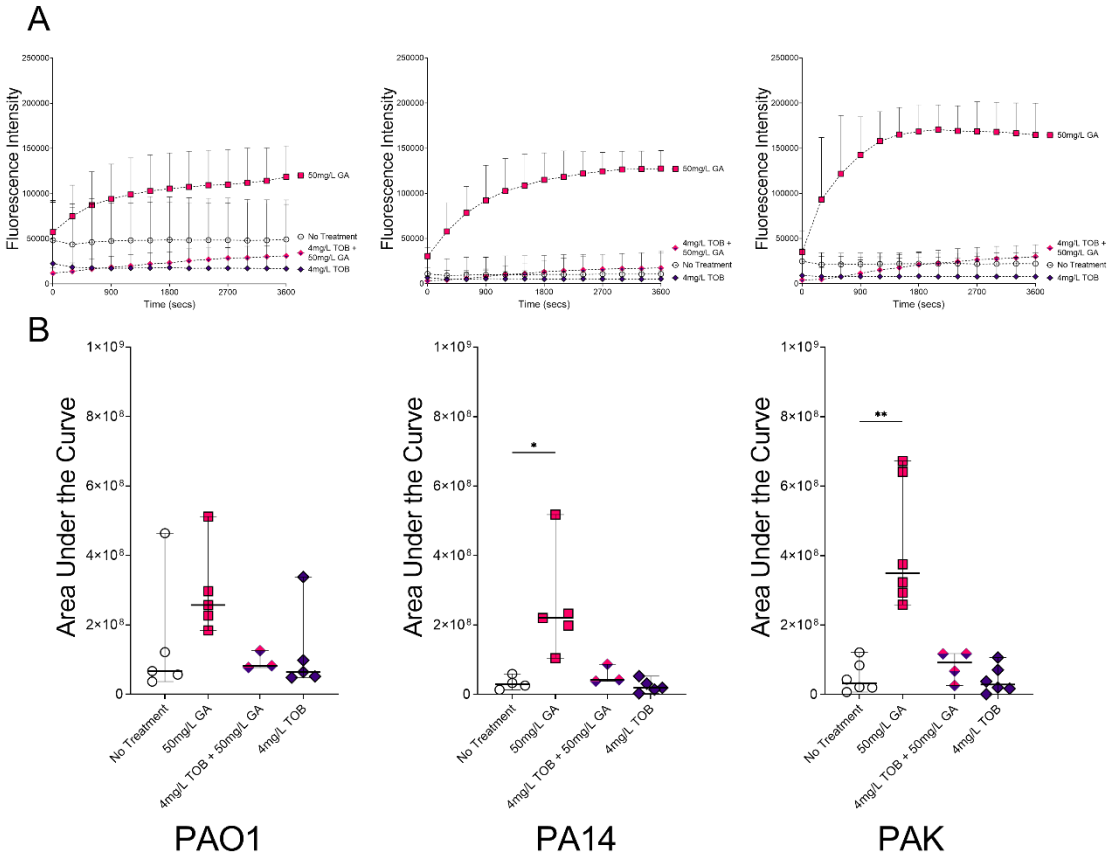


Figure 5-15. A. Intensity of PI fluorescence of P. aeruginosa PAO1, PA14 and PAK over 60mins after addition of 50mg/L GA, 4mg/L TOB or 4mg/L TOB + 50mg/L GA combined (median and 95% confidence intervals of biological replicates at each timepoint). B. AUCs of PI fluorescence intensity over 60mins of P. aeruginosa PAO1, PA14 and PAK during treatment with 50mg/L GA, 4mg/L TOB or 4mg/L TOB + 50mg/L GA combined. For PAO1 no treatment significantly increased PI AUC from No Treatment. For PA14 GA at 50mg/L (p=0.0294) significantly increased PI AUCs from No Treatment. For PAK GA at 50mg/L (p=0.0087) significantly increased PI AUCs from No Treatment. Neither 4mg/L TOB alone nor 4mg/L TOB combined with 50mg/L resulted in significant change from No treatment suspensions of any reference strain. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

5.3.2 Comparison of GA activity on bacterial membranes of *P. aeruginosa* from people with CF with other antimicrobial agents

As with reference strains PAO1, PA14 and PAK, the effect of GA on the membranes of clinical strains of *P. aeruginosa* from people with CF was tested. The same panel of clinical strains as **Chapter 4** was used (**Table 4-1**).

5.3.2.1 Bacterial viability

Across the 11 clinical strains tested there was a significant reduction of colonies in GA-treated suspensions in M9 after 30mins (p=0.02) and 60mins (p=0.001) incubation (**Figure 5-16**, **Table 5-6**).

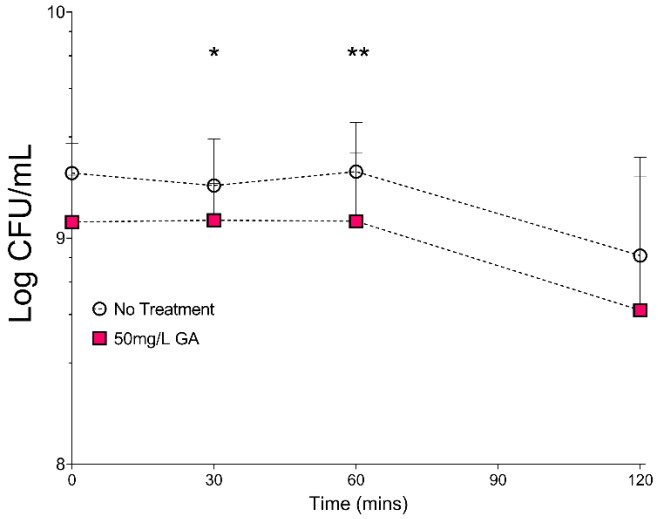


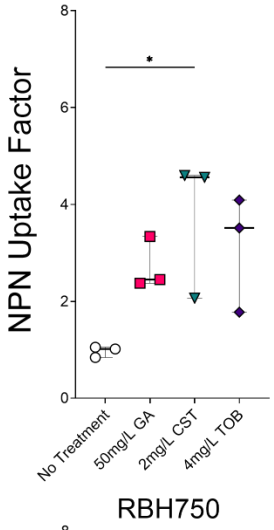
Figure 5-16. Colony forming units (CFU/mL) of P. aeruginosa from people with CF in M9 broth during treatment with 50mg/L GA. There was a significant reduction in colonies in GA treated suspensions after 30mins (p=0.0176) and 60mins (p=0.001) incubation (Wilcoxon test). Points are medians with 95% confidence intervals.

Table 5-2. Colony forming units (CFU/mL) of P. aeruginosa from people with CF in M9 broth during treatment with 50mg/L GA. There was a significant reduction in colonies in GA treated suspensions after 30mins (p=0.0176) and 60mins (p=0.001) incubation (Wilcoxon test). Median vaules with 95% confidence intervals (n=11).

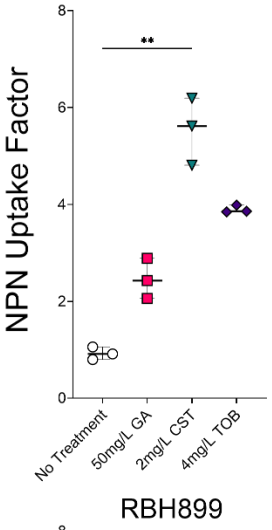
	No Treatment	50mg/L GA
0mins	**1.94 X 10^9** 3.25 X 10^8 – 2.83 X 10^9	**1.18 X 10^9** 1.66 X 10^8 – 3.15 X 10^9
30mins	**1.71 X 10^9** 2.75 X 10^8 – 2.83 X 10^9	*** 1.20 X 10^9** 5.00 X 10^6 – 2.38 X 10^9
60mins	**1.97 X 10^9** 5.19 X 10^8 – 4.33 X 10^9	**** 1.18 X 10^9** 3.00 X 10^7 – 2.61 X 10^9
120mins	**8.38 X 10^8** 2.66 X 10^8 – 3.21 X 10^9	**4.80 X 10^9** 7.51 X 10^6 – 2.00 X 10^9

5.3.2.2 Outer bacterial membrane

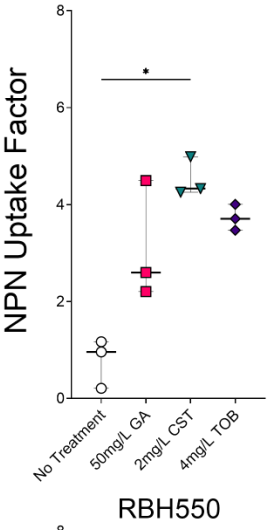
Of the 11 clinical *P. aeruginosa* tested, only one strain had a significant increase in its NPN uptake resulting from GA treatment (**Figure 5-17**). For strain RBH982, GA was the only agent tested which significantly increased its NPN Uptake Factor from No Treatment (p=0.02). Treatment with CST was the most impactful agent tested against the outer membranes of clinical strains with 6 strains have a significant increase in NPN uptake ($p<0.05$). One *P. aeruginosa* clinical strain was significantly impacted by TOB treatment (RBH072, p=0.01). Two of the clinical strains tested were unaltered by any of the treatments tested; RBH294 and RBH461 (**Figure 5-17**).



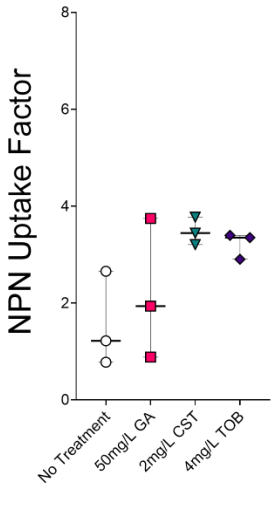
RBH750



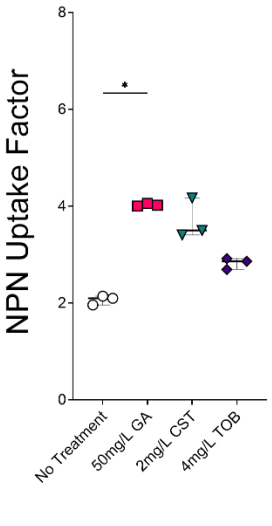
RBH899



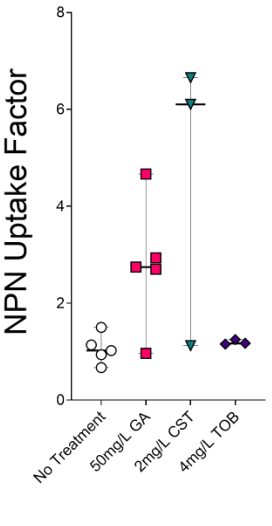
RBH550



RBH294



RBH982



RBH422

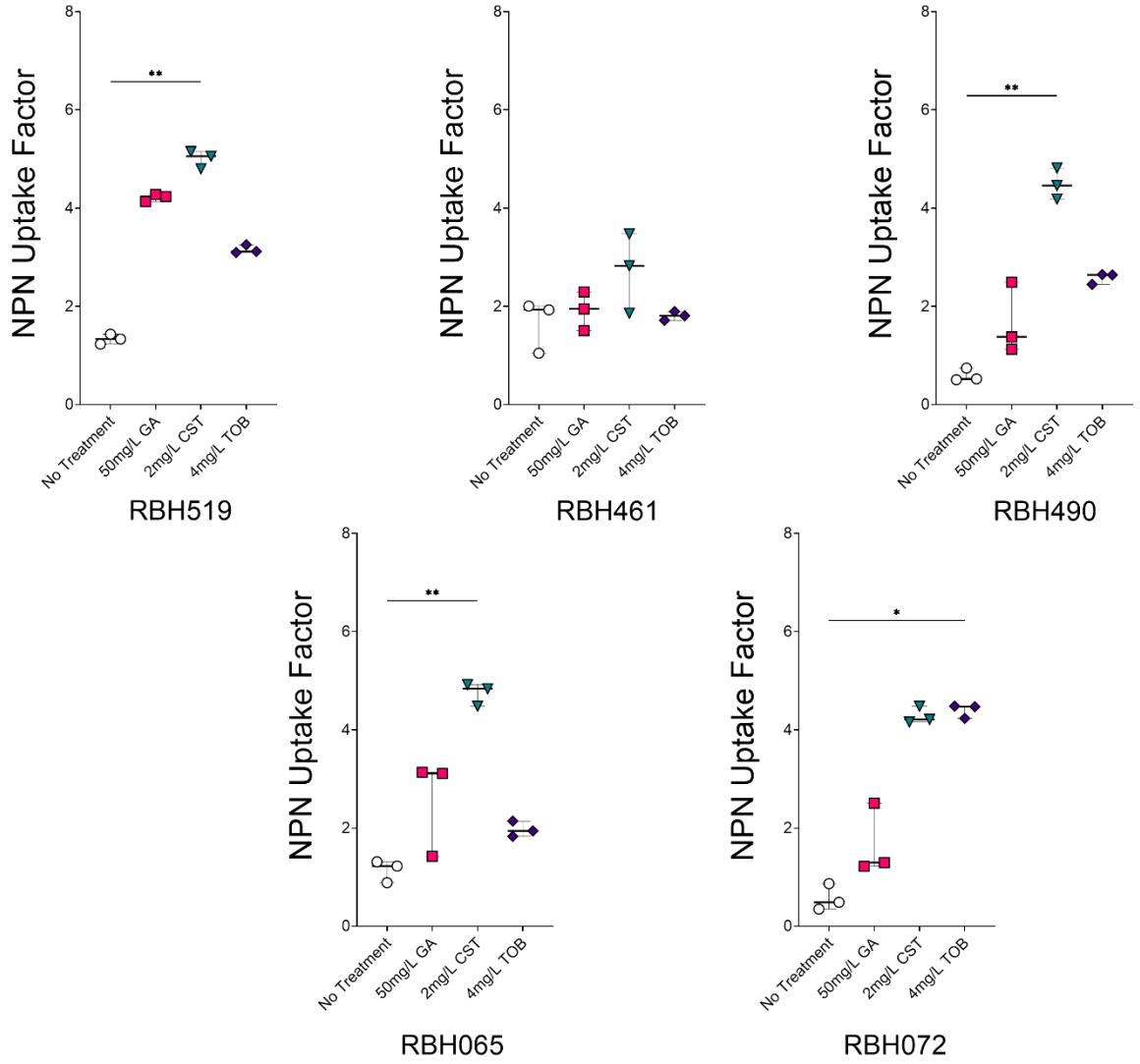


Figure 5-17. Mean NPN Uptake Factor over 15mins from the outer membranes of clinical P. aeruginosa from people with CF during treatment with 50mg/L GA, 2mg/L CST and 4mg/L TOB. GA resulted in significant increases in NPN uptake from No Treatment suspensions for strain RBH982 (p=0.0197). CST resulted in significant uptake in strains RBH750 (0.0382), RBH899 (0.0067), RBH550 (0.0139), RBH490 (0.0067), RBG519 (0.0067) and RB065 (0.0067). TOB resulted in significant uptake in strains RBH072 (0.0139). Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are mean NPN Uptake Factor over 15mins of independent experiments with lines at median and 95% confidence intervals.

Comparison across the results of all clinical *P. aeruginosa* strains tested showed that all the agents applied resulted in an increase in NPN Uptake Factor, to varying degrees (**Figure 5-18**). GA resulted in a significant increase in uptake of NPN (p=0.03) which was less impactful than CST (p<0.0001) and comparable to TOB (p=0.03).

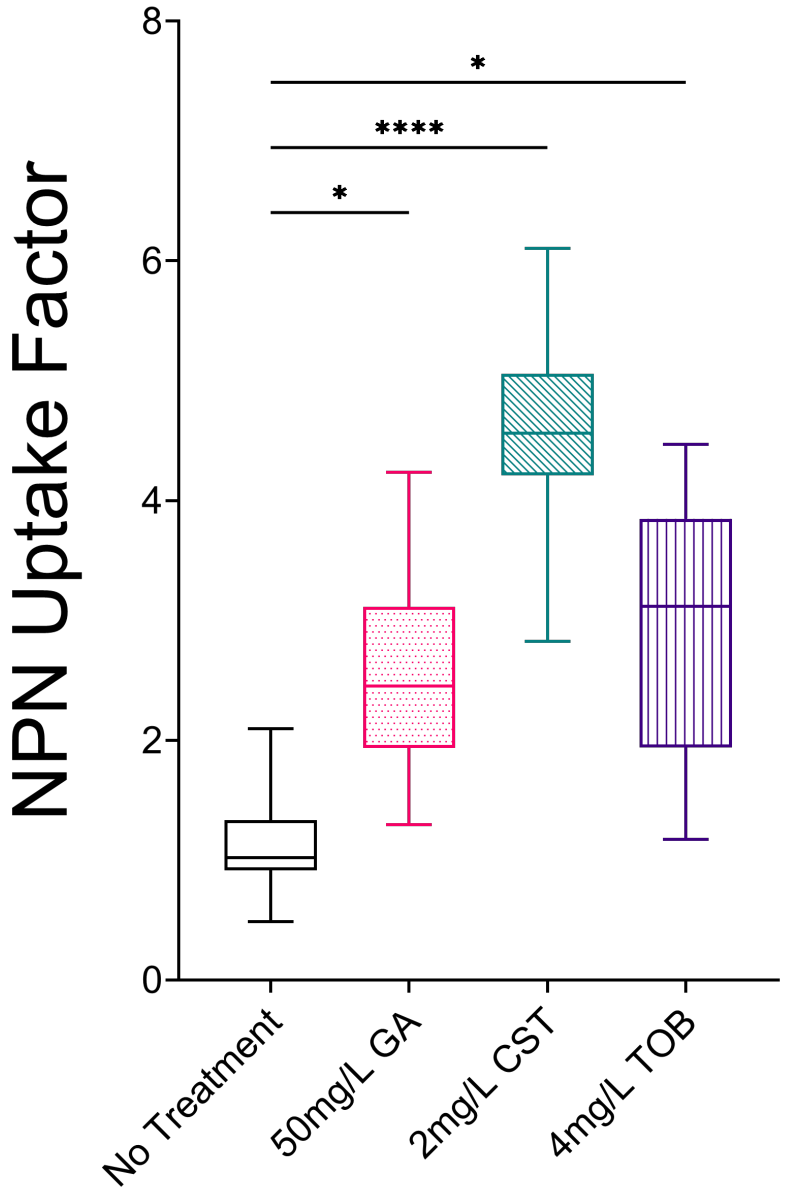


Figure 5-18. Median NPN Uptake Factor over 15mins from the outer membranes of clinical P. aeruginosa from people with CF during treatment with 50mg/L GA, 2mg/L CST and 4mg/L TOB. Compared with No Treatment, 50mg/L GA (p=0.0247), 2mg/L CST (<0.00001) and 4mg/L TOB (p=0.0247) all resulted in significantly increased dye uptake by outer membranes. Friedman test with Dunn's mutlipte correction (adjuted p values). Box and Whisker plots with lines at median and 5-95% percentiles.

5.3.2.3 Bacterial cytoplasmic membrane

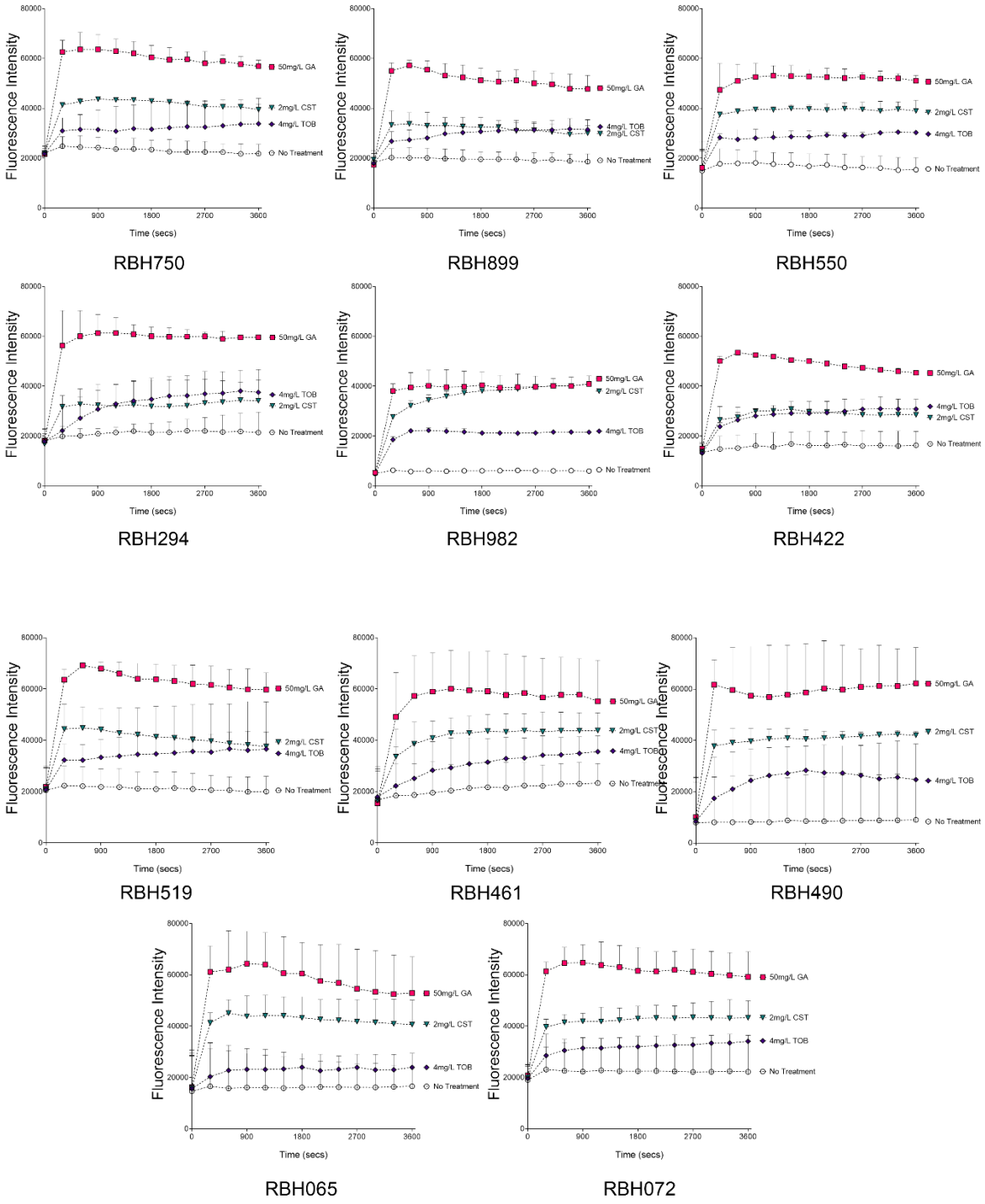
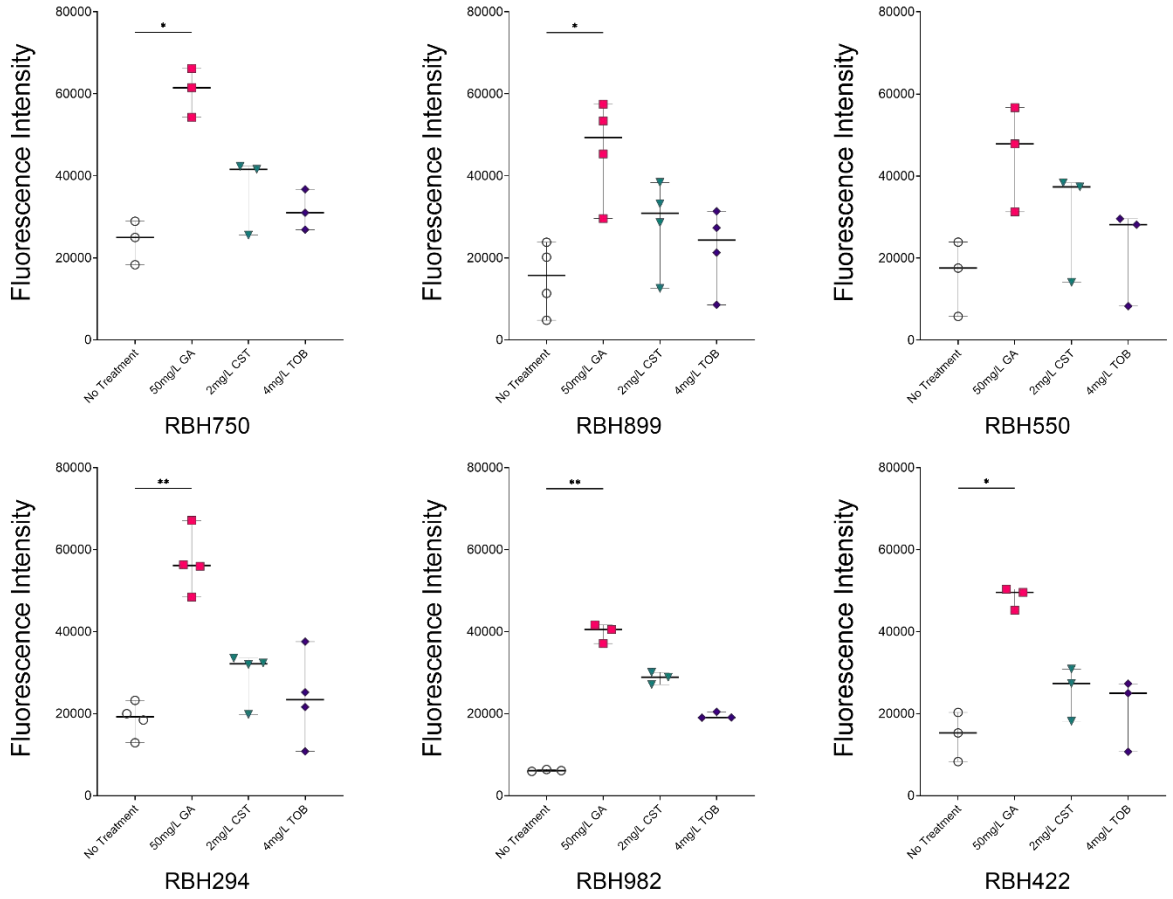


Figure 5-19. Release of $DiSC_3(5)$ over 60mins by cytoplasmic membranes of 11 P. aeruginosa from people with CF during treatment with 50mg/L GA, 2mg/L CST of 4mg/L TOB. Points are means of at least three biological replicates with median and 95% confidence intervals shown.

Of the 11 clinical strains tested, 50mg/L GA significantly increased $DiSC_3(5)$ release in 9 of them, in multiple comparisons of $DiSC_3(5)$ fluorescence over 15mins, compared with No Treatment for each strain ($p<0.05$) (**Figure 5-19**).



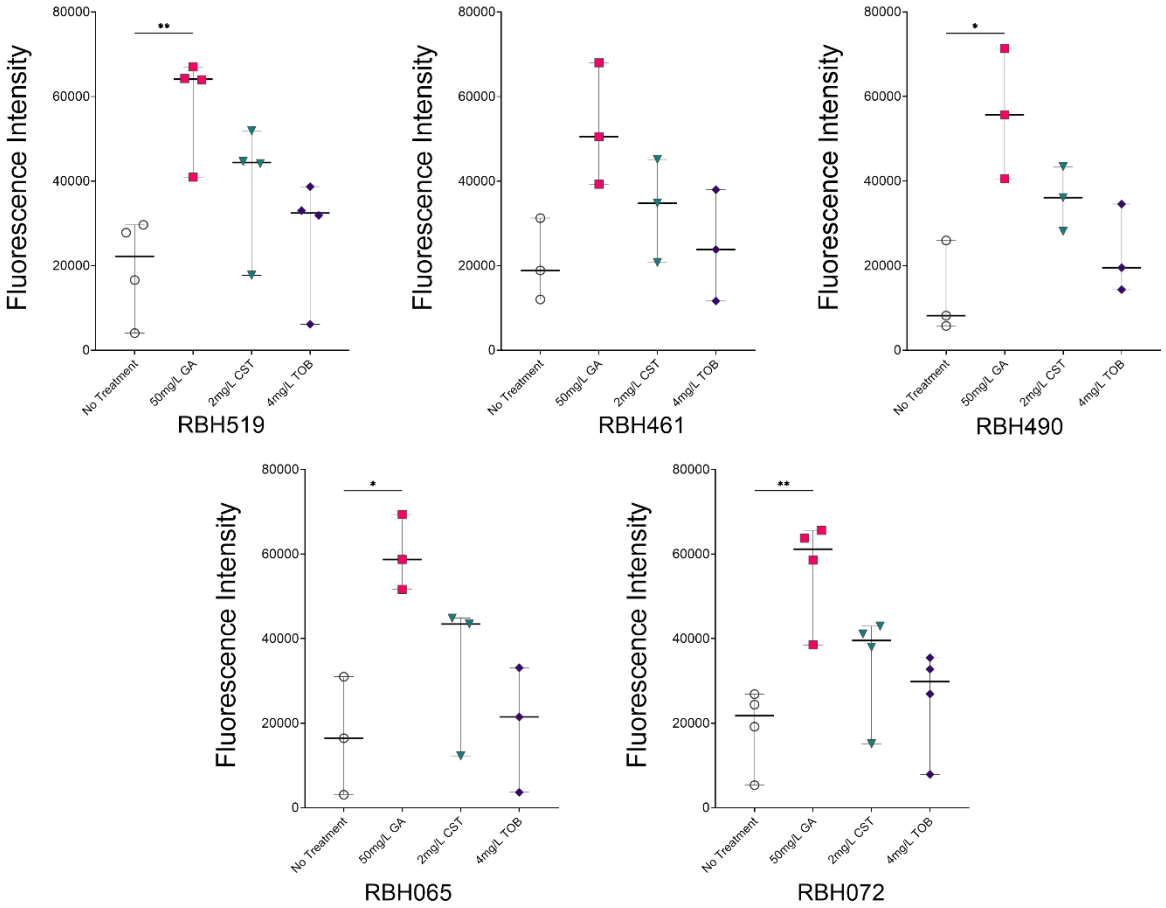


Figure 5-20. Mean DiSC$_3$(5) release over 15mins from the cytoplasmic membranes of clinical P. aeruginosa from people with CF during treatment with 50mg/L GA, 2mg/L CST and 4mg/L TOB. GA resulted in significant increases in DiSC$_3$(5) release from No Treatment suspensions for strains RBH750 (p=0.0139), RBH899 (p=0.0113), RBH294 (p=0.007), RBH982 (p=0.0067), RBH422 (p=0.0197), RBH490 (p=0.0197), RBH519 (p=0.0089), RBH065 (p=0.0382) and RBH072 (p=0.0089). CST and TOB treatments did have a significant impact on any individual strain in these tests. Points are mean DiSC$_3$(5) fluorescence over 15mins of independent experiments with lines at median and 95% confidence intervals.

Examining the results across all 11 strains tested showed that GA also significantly increased DiSC$_3$(5) release, from untreated controls, for this group of strains overall ($p<0.0001$) (**Figure 5-21**). For the grouped data, CST also resulted in a significant increase compared with No Treatment for clinical strains ($p=0.0008$). As with reference strains TOB did not have a significant impact on DiSC$_3$(5) release for the clinical *P. aeruginosa*.

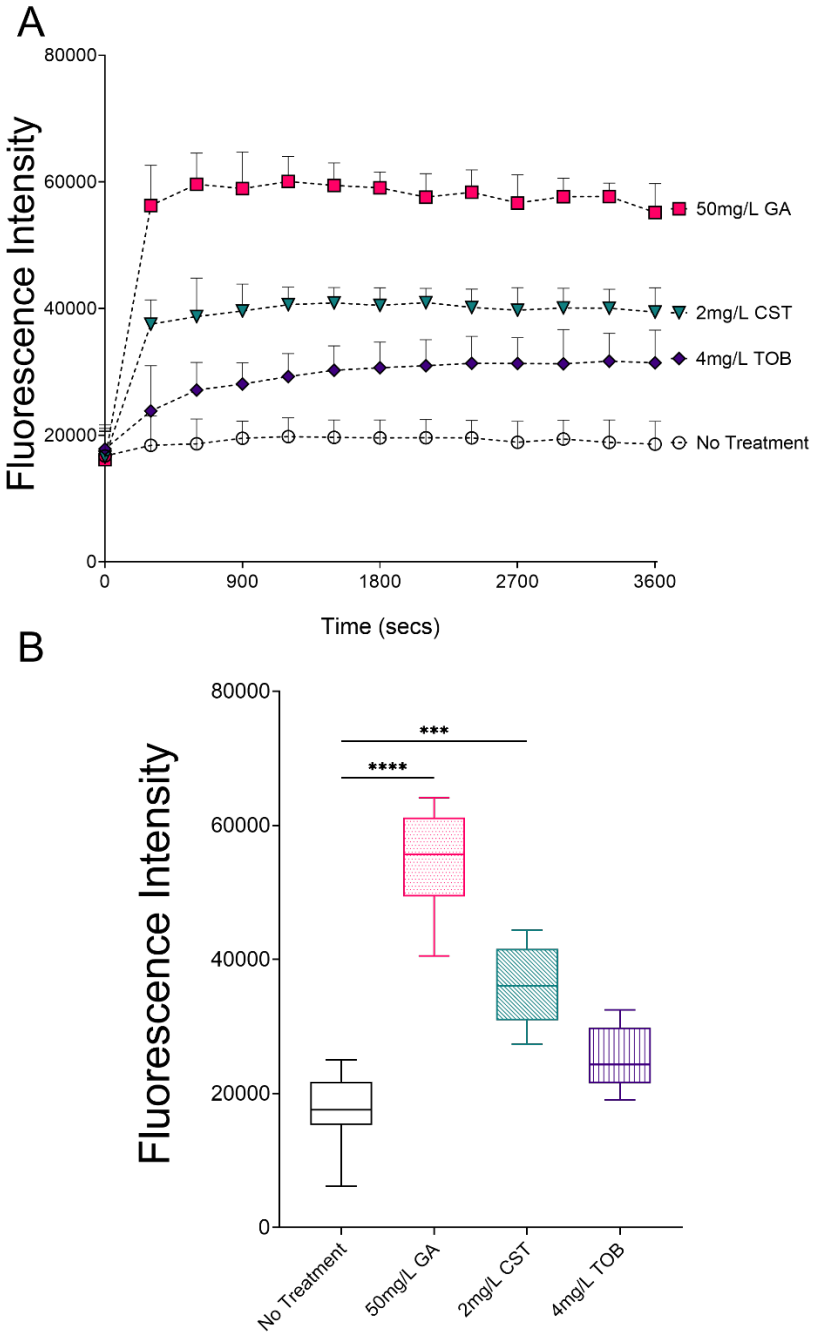


Figure 5-21. A. Release of $DiSC_3(5)$ over 60mins by cytoplasmic membranes of 11 P. aeruginosa from people with CF during treatment with 50mg/L GA, 2mg/L CST of 4mg/L TOB. Points are medians of each clinical strain with median and 95% confidence intervals shown. B. Median $DiSC_3(5)$ release over 15mins from the cytoplasmic membranes of clinical P. aeruginosa from people with CF during treatment with 50mg/L GA, 2mg/L CST and 4mg/L TOB. Compared with No Treatment, 50mg/L GA ($p<0.00001$) and 2mg/L CST ($p=0.0008$) resulted in significantly increase dye release from cytoplasmic membranes while 4mg/L TOB did not. Friedman test with Dunn's mutlipte correction (adjuted p values). Box and Whisker plots with lines at median and 5-95% percentiles.

5.3.2.4 Bacterial cell permeability

The permeability of clinical strains was measured for each strain during GA, CST and TOB treatment over 60mins (**Figure 5-22**).

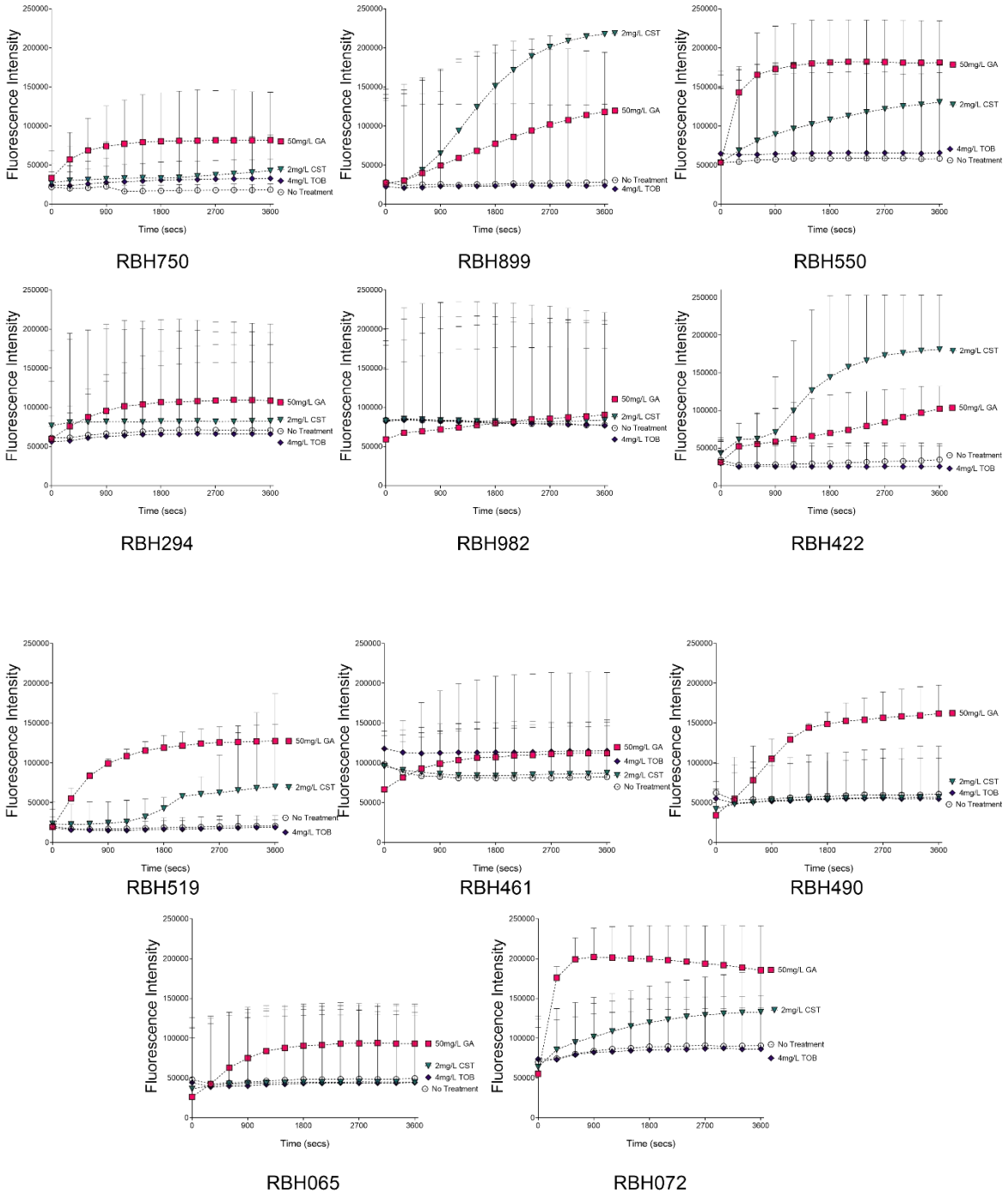
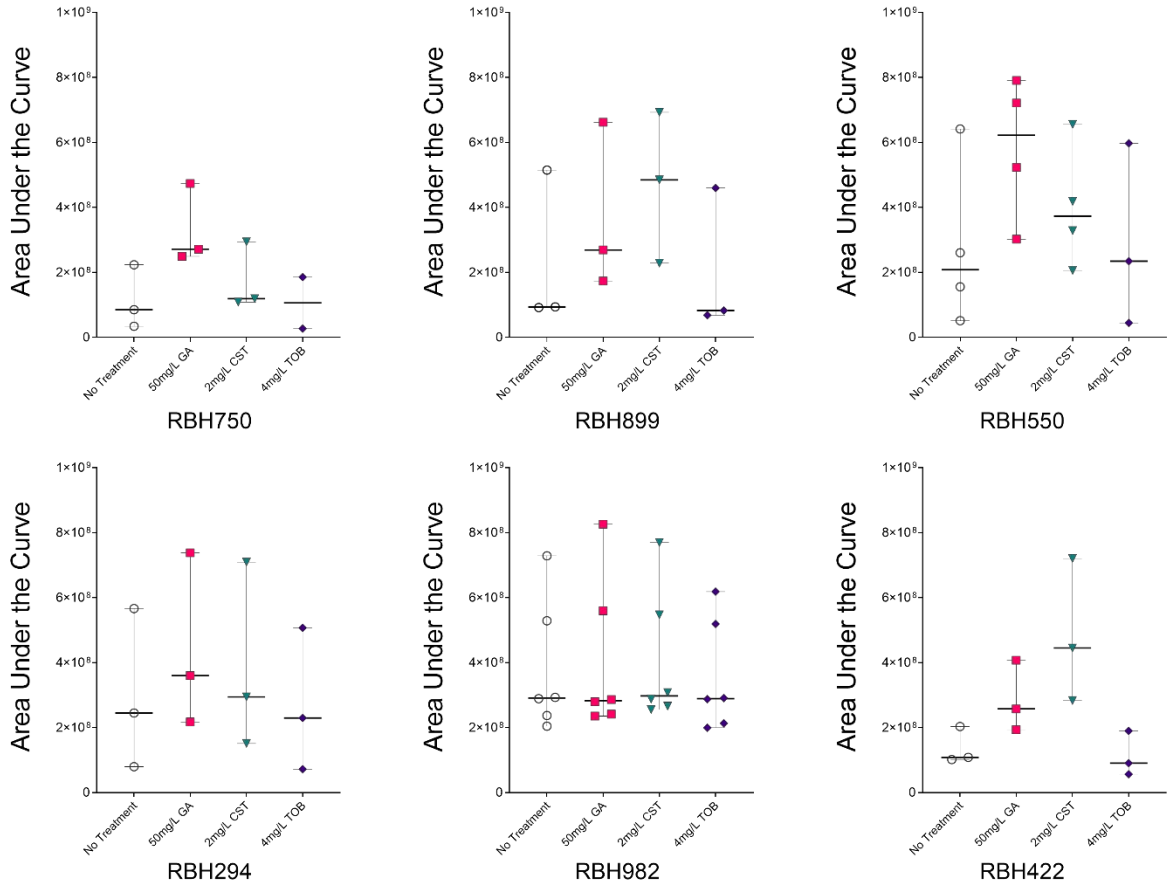


Figure 5-22. Fluorescence of PI over 60mins by 11 P. aeruginosa from people with CF during treatment with 50mg/L GA, 2mg/L CST of 4mg/L TOB. Points are means of at least three biological replicates with median and 95% confidence intervals shown.

In multiple comparison analyses of each strain, comparing with untreated suspensions, the AUCs of PI fluorescence over 60mins did not show any significant difference for any individual strain for any treatment, including GA (**Figure 5-23**).


Area Under the Curve
1×10⁹
8×10⁸
6×10⁸
4×10⁸
2×10⁸
0
No Treatment
50mg/L GA
2mg/L CST
4mg/L TOB
RBH750
RBH899
RBH550
RBH294
RBH982
RBH422

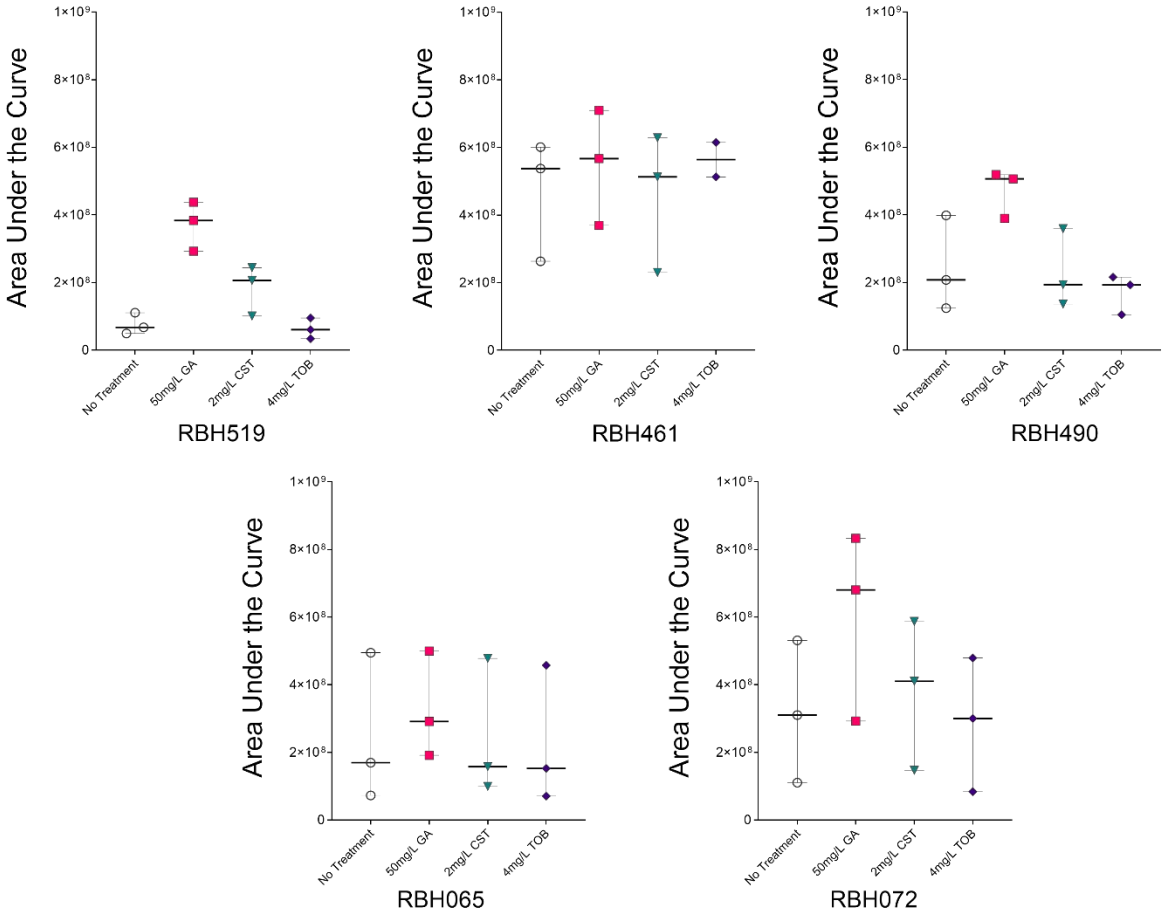


Figure 5-23. AUCs of PI fluorescence over 60mins by 11 P. aeruginosa from people with CF during treatment with 50mg/L GA, 2mg/L CST of 4mg/L TOB. No differences were seen for any strain between the treatment groups. Points are AUCs of independent experiments with lines at median and 95% confidence intervals shown.

When compared as a group of clinical strains, a significant increase was seen in AUCs of GA treated strains, increased from No Treatment (p=0.0247) (**Figure 5-24**). This was not the case for either CST or TOB over this group of strains in the same comparison.

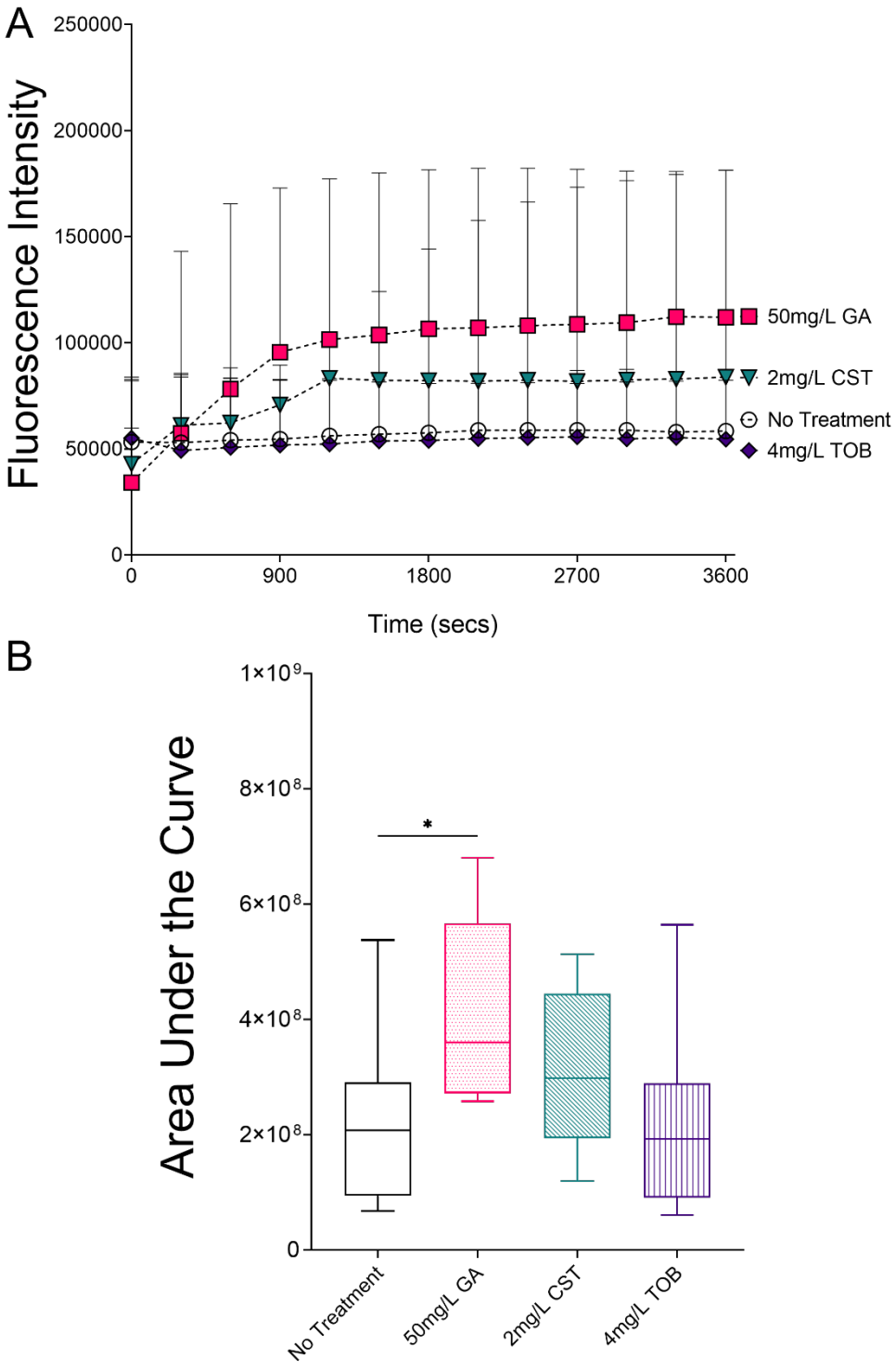


Figure 5-24. A. Fluorescence of PI over 60mins by 11 P. aeruginosa from people with CF during treatment with 50mg/L GA, 2mg/L CST of 4mg/L TOB. Points are medians of each clinical strain with median and 95% confidence intervals shown. B. Median AUCs of PI fluorescence over 60mins from clinical P. aeruginosa from people with CF during treatment with 50mg/L GA, 2mg/L CST and 4mg/L TOB. Compared with No Treatment, 50mg/L GA (p=0.0247)resulted in significantly increase dye release from cytoplasmic membranes while2mg/L CST and 4mg/L TOB did not. Box and Whisker plots with lines at median and 5-95% percentiles.

5.3.3 Effects of GA on the membranes of non-*Pseudomonas* CF pathogenic bacterial species

To compare and contrast the effects of GA seen with *P. aeruginosa* strains, examples of other common pathogens isolated from people with CF were also tested for membrane integrity during treatment with 50mg/L GA. One example each of *S. aureus*, *B. cepacia* and *A. xylosoxidans* were chosen (**Table 5-1**).

5.3.3.1 Bacterial outer membrane

As with *P. aeruginosa,* strains' uptake of NPN by the outer membrane was used to measure disruption resulting from GA treatment, with the exception of *S. aureus* which, as a Gram-positive does not have targets for NPN binding. This was employed as a negative control for the assay and, as expected the NPN Uptake Factor was not increased by GA (**Figure 5-25**).

For *B. cepacia* there was also no difference seen in NPN uptake between untreated and GA treated suspensions. This was also to be expected with *Burkholderia* species containing natural resistance to antimicrobial peptides[344]. Similarly, *A. xylosoxidans*, a species which has been shown to be more resistant to AMPs than *P. aeruginosa*[345], showed no change from untreated when GA was administered (**Figure 5-25**).

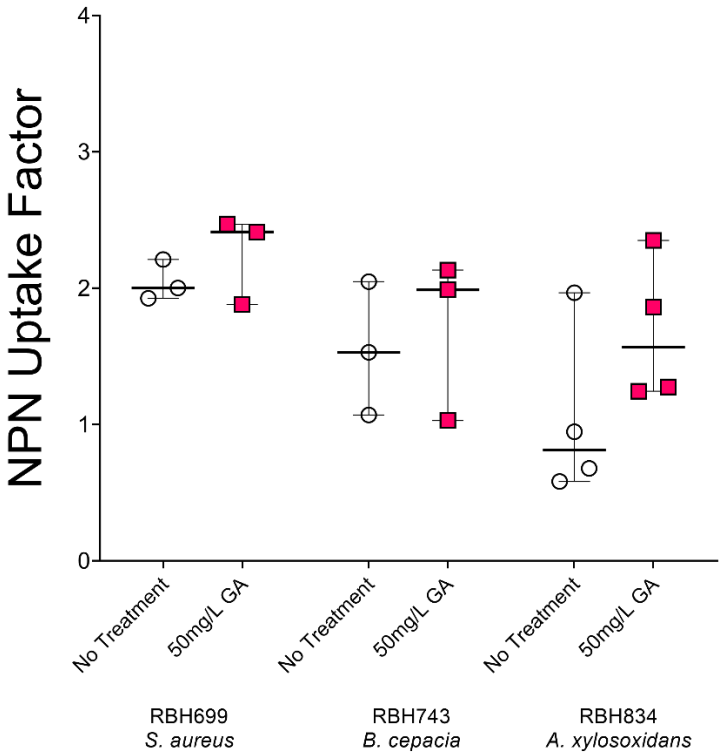


Figure 5-25. NPN Uptake Factor of clinical strains of S. aureus, B. cepacia and A. xylosoxidans during treatment with 50mg/L GA. No change was seen in NPN Uptake Factor for any of the species tested when treated with GA. (Mann-Whitney U test). Points are biological replicates with lines at median and 95% confidence intervals.

5.3.3.2 Bacterial cytoplasmic membrane

While a moderate increase in $DiSC_3(5)$ release was seen for each species tested on administration of 50mg/L GA, comparisons of fluorescence over the 15mins post-treatment did not reach statistical significance on these relatively small group sizes for any of the species (**Figure 5-26**).

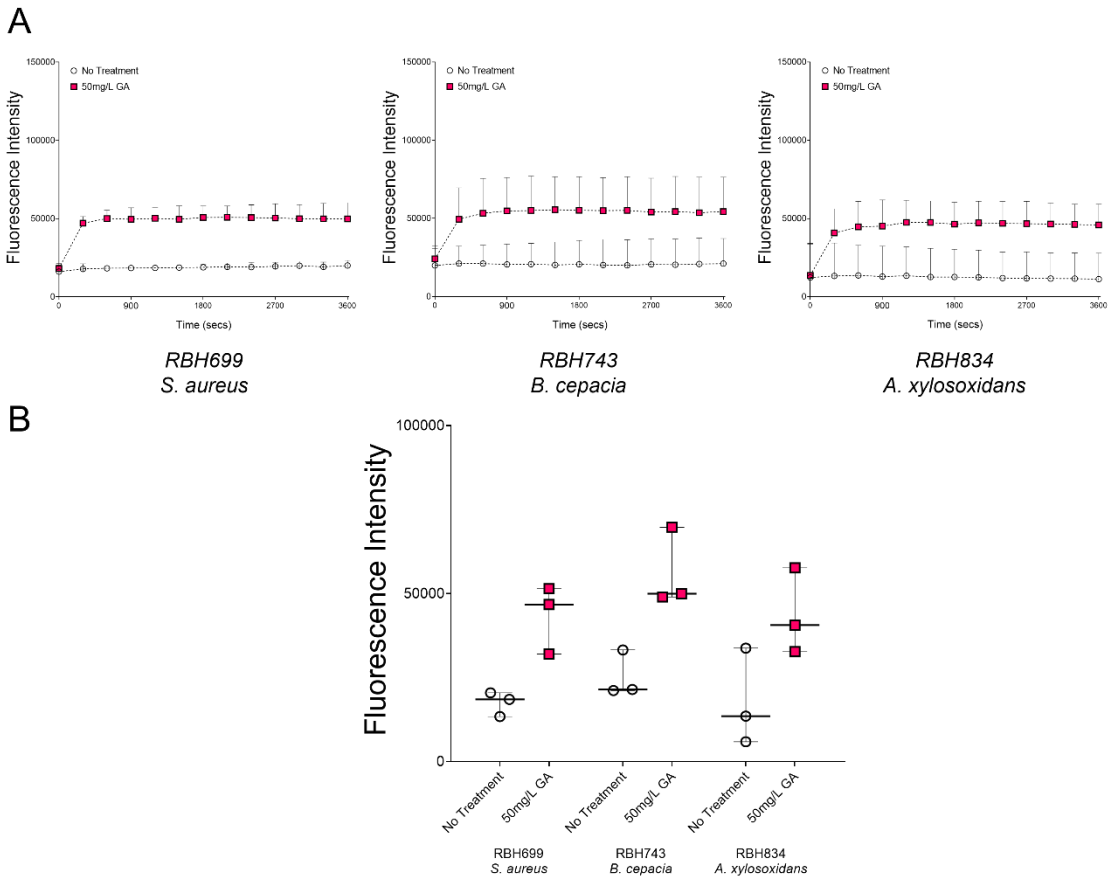


Figure 5-26. A. Intensity of DiSC$_3$(5) fluorescence released over 60mins by clinical strains of S. aureus, B. cepacia and A. xylosoxidans during treatment with 50mg/L GA (median and 95% confidence intervals of biological replicates at each timepoint). B. Mean intensity of DiSC$_3$(5) fluorescence over 15mins released by clinical strains of S. aureus, B. cepacia and A. xylosoxidans during treatment with 50mg/L GA. No significant difference resulted from 50mg/L GA treatment for any of the species tested. (Mann-Whitney U test). Points are biological replicates with lines at median and 95% confidence intervals.

5.3.3.3 Bacterial cell permeability

As with the uptake of NPN and release of DiSC$_3$(5), treatment with 50mg/L did not significantly increase PI fluorescence by any bacterial species tested here, as compared using AUCs of fluorescence intensity over 60mins (**Figure 5-27**).

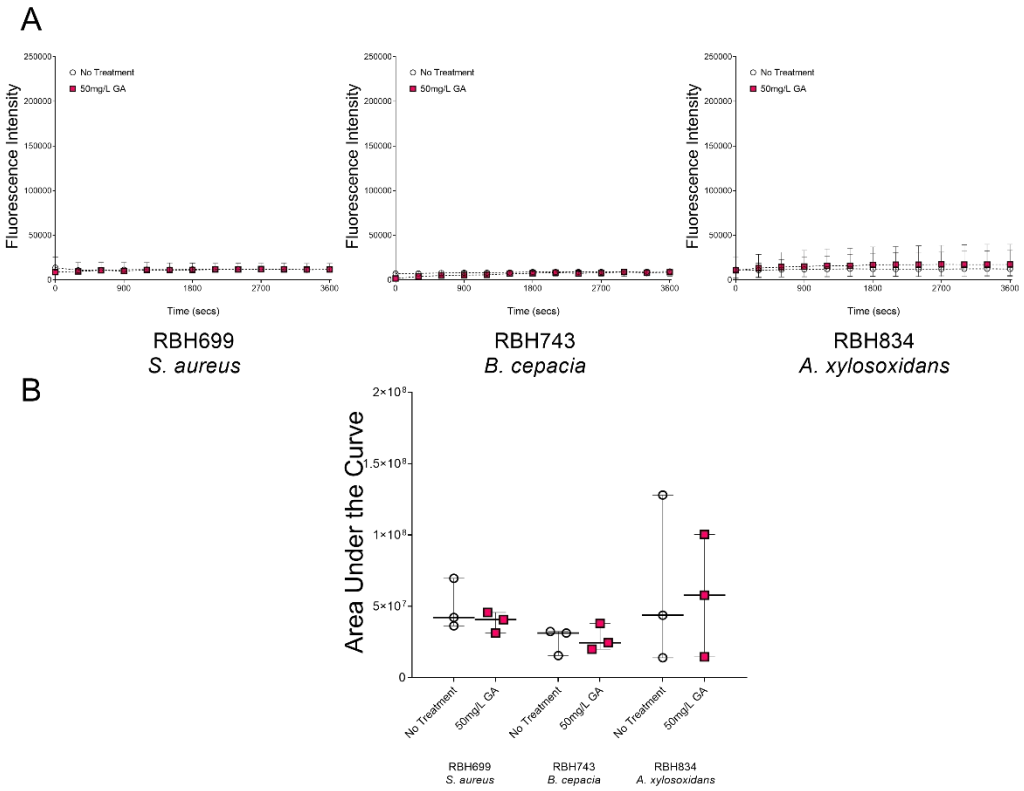


Figure 5-27. A. Intensity of PI fluorescence by clinical strains of S. aureus, B. cepacia and A. xylosoxidans during treatment with 50mg/L GA over 60mins (median and 95% confidence intervals of biological replicates at each timepoint). B. AUCs of PI fluorescence of by clinical strains of S. aureus, B. cepacia and A. xylosoxidans during treatment with 50mg/L GA over 60mins. No significant difference was seen in AUCs of any of the species tested during treatment with GA. (Mann-Whitney U test). Points are biological replicates with lines at median and 95% confidence intervals.

5.4 Discussion

In this chapter, I have demonstrated the rapid killing of reference *P. aeruginosa* by GA. This is an update on the published data about GA killing, which had shown cell death and a decrease in cell numbers after 30mins of GA treatment. The results here are consistent with those previous observations but I have shown that this reduction in viable cells occurs at timepoint 0mins. I have shown that GA is a membrane disruptor of *P. aeruginosa* – both reference and clinical strains. In comparisons with other membrane disrupting agents (including cationic AMPs), GA was the only agent tested which significantly altered the integrity of the outer membrane, the cytoplasmic and the permeability of all three reference strains. And, in group comparisons with CST and TOB, GA was the only agent which significantly altered the same membrane integrity assays of clinical *P. aeruginosa* from people with CF. In contrast, I have shown, that clinical examples of other CF pathogenic bacterial species – *S. aureus*, *B. cepacia* and *A. xylosoxidans* – were unaffected by GA in their membrane integrities.

5.4.1 Bacterial killing

GA has been shown to kill *P. aeruginosa* cells rapidly and results here confirm this and show that GA killing of *P. aeruginosa* is even more rapid than previously reported. Indeed, viable colonies counts of each of the reference strains were decreased at timepoint 0mins, even without any incubation period of the cells with GA (**Figure 5-2**). These cell numbers did not show a further decline over the continued treatment period. The immediate activity of GA was essentially uncapturable with this methodology, as the entirety of the decrease in viable colonies occurred in the manipulation time required to add GA to cell suspensions and

then remove the GA-containing media. This is a property of cationic AMPs which has been reported previously[340].

5.4.2 Membrane integrity of reference *P. aeruginosa*

There were significant increases in all the markers of membrane disruption used in this study when reference strains were treated with 50mg/L GA; GA has the ability to disrupt the outer membrane, depolarise the cytoplasmic membrane and permeabilise the cell wall.

Treatments with 50 and 100mg/L GA were the only concentrations which caused significant increases in membrane perturbations, in multiple comparison tested on GA log2 titrations (**Figure 5-7**, **Figure 5-8 and Figure 5-9**). There were two exceptions to this – depolarisation of the PAK cytoplasmic membrane and the permeability of PAO1 – where no significant changes for any individual GA concentration were seen.

These results for 100mg/L are somewhat in contrast with results shown here for bacterial killing. GA at 100mg/L was as effective at causing membrane perturbations in reference strains as 50mg/L, despite this concentration being lees effective at killing bacteria (**Figure 5-6**).

Compared with other cationic agents, GA was the only treatment which consistently resulted in significant increases in membrane perturbations (**Figures 5-10**, **Figure 5-11** and **Figure 5-12**). GA at 50mg/L significantly disrupted the outer membranes and depolarised the cytoplasmic membranes of all three reference strain and significantly increase permeability of PA14 and PAK. The only exception was PAO1 which did not show a significant increase in permeability for any agent tested. It was noted that PAO1 had a higher PI signal in untreated suspensions than either PA14 or PAK, making significant increases less likely. It is unclear

whether PAO1 was naturally more permeable than the other strains or whether this was an assay artifact e.g. autofluorescence of PAO1 at the wavelengths used for PI.

Colistin at the EUCAST breakpoint concentration of 2mg/L (at which all of the reference strains are susceptible) resulted in outer membrane disruption of all strains while LL-37 caused depolarisation and permeabilisation of PA14 and PAK at the concentration tested. Tobramycin was not an effective agent of membrane disruption for any strains despite being tested at 4mg/L (EUCAST breakpoint concentration) a concentration much higher than that to which they were susceptible in **Chapter 3**.

Strain PAO1 was the most significantly potentiated by GA for treatment with antibiotics; it had the largest drop in tobramycin MICs, synergised with tobramycin at 2 concentrations and synergised with three different classes of antibiotic (**Chapter 3**). The results here showing that PAO1 has the least increase in permeabilisation by GA of any of the reference strains may indicate that permeabilisation of *P. aeruginosa* cell walls by GA is the least important factor in the mechanism of antibiotic potentiation by GA.

In contrast, PA14 was significantly and consistently permeabilised by GA and yet only showed synergy with one antibiotic tested; tobramycin. And strain PAK was the strain with the highest impact of GA on permeabilisation but was the reference strain with the lowest fold change in its tobramycin MIC values during co-treatments with GA (PAK was only tested with tobramycin).

*Table 5-3. Comparison of GA synergy results (Chapter 3) with each antibiotic tested and membrane disruption by GA for reference P. aeruginosa. ✓ indicates antibiotics where synergy with GA was seen. ✘ indicates antibiotics where synergy with GA was not seen. Significance of the change resulting from treatment with 50mg/L GA on the outer membrane (OM), cytoplasmic membrane (CM) and cell wall permeability (CWP) in multiple comparison tests performed in **Section 5.4.2.3**. * p<0.05, ** p<0.01, *** p<0.001.*

	TOB	CAZ	CST	CIP		OM	CM	CWP
PAO1	✓	✓	✘	✓		***	**	ns
PA14	✓	✘	✘	✘		***	*	*
PAK	✓	-	-	-		**	**	**

It was surprising to observe the elimination of cell wall permeabilisation in the reference strains when GA and tobramycin were administered in tandem – propidium iodide results were not merely reduced by combination but returned to levels comparable with untreated suspensions (**Figure 5-15**). This was not the case for either outer or cytoplasmic membrane disruption, were combining GA and tobramycin had results similar to those seen for GA alone in each strain.

The mechanism underlying this observation is unclear. If GA and tobramycin were to bind directly, a similar inhibition of GA activity would be expected for the other membrane assays but this was not observed (**Figures 5-13** and **5-14**). Also, if GA and tobramycin were to bind together and the effect of binding were to be inhibitory to one or both agents, the extensive level of potentiation seen for co-treatments of GA and tobramycin in **Chapters 3** and **4** seems unlikely.

With permeabilisation of the cells driven by GA treatment – tobramycin does not cause any membrane disruption alone – the implication of this result is that tobramycin is preventing the action of GA in these assays. Aminoglycosides have been shown to be capable

of self-promoted uptake by *P. aeruginosa* cells due to their cationic nature and outer membrane binding[95]. It may be that high level of tobramycin administered in this assay is performing a similar function as divalent cations, competing with GA for anionic targets on the bacterial cell surface and diminishing some of its activity as a result.

This also potentially provides further evidence that full cell wall permeabilisation by GA is not the primary mechanism of potentiation of antibiotics by GA and increases the relevance of outer membrane disruption and cytoplasmic membrane depolarisation.

5.4.3 Membrane integrity of clinical *P. aeruginosa*

As a group, clinical *P. aeruginosa* strains reacted in a similar manner to GA as reference strains had with significant increases in outer membrane disruption, cytoplasmic membrane depolarisation and cell wall permeabilisation (**Figures 5-18**, **5-20** and **5-22**). On a strain-by-strain basis, however, clinical strain reaction to GA was more heterogenous than reference strains' (**Figures 5-17**, **5-19** and **5-21**).

Only one clinical strain (RBH982) had significantly increased outer membrane disruption resulting from GA treatment in multiple comparisons, whereas all reference strains had (**Figure 5-17** and **5-9**). At the other end of the spectrum of responses to GA on clinical outer membranes was a strain (RBH461) which was not only non-significant in its reaction to GA but has a comparable level of NPN uptake for GA and untreated suspensions (NPN Uptake Factor 1.95 [95%CI 1.51-2.29] and 1.93 [95%CI 1.05-2.00], respectively) (**Figure 5-17**). This underlines the heterogeneity of the response of clinical strains to GA treatment of their outer membranes.

Colistin was a more effective outer membrane disruptor than GA with 6 of the 11 strains showing significant disruption by colistin and the group NPN Uptake Factor being

higher and more impactful than GA (**Figure 5-18**). Only 1 of the 11 strains tested was colistin-resistant (RBH422) according to clinical lab results, and this strain's outer membrane was not significantly altered by any treatment including GA and colistin. Tobramycin also had a significant overall impact on the group of clinical strains, with a similar impact as seen for GA (**Figure 5-18**). One clinical strain was strain even had a significant increase in membrane disruption by tobramycin. Unexpectedly, this was the clinical strain (RBH072) with the highest tobramycin MIC – as mentioned previously this strain has genes for aminoglycoside inactivation enzymes which are unique in this panel of strains (**Table 4-4**) and may therefore be more reliant on this tobramycin-resistance mechanism than impermeability.

The cytoplasmic membranes of clinical *P. aeruginosa* were more extensively altered by GA than any other agent. This was seen both on the impact on a group basis and in individual strains where 9 of the 11 strains tested were significantly depolarised by GA (**Figures 5-19** and **5-20**). It was noted that the two strains which were not significantly impacted by GA in these experiments were both strains which had not displayed synergy between GA and tobramycin, one of which had had one of the lowest MIC changes of any strain tested (RBH899) (**Table 4-19**). However, the strain with least MIC changes and no synergy (RBH294) was significantly depolarised by GA in these assays.

Colistin treatment also resulted in significant depolarisation across the group of clinical *P. aeruginosa*, although not in any individual strain. Tobramycin was not an impactful depolarising agent of the 11 strains.

There was significant permeabilisation of clinical strain cell walls by GA, as group but no individual strain was significantly permeabilised. Neither colistin nor tobramycin

significantly permeabilised clinical strains, either on an individual or group basis (**Figure 5-22** and **5-23**).

*Table 5-4. Comparison of tobramycin synergy results (Chapter 4) with significance of change to the outer membrane (OM), cytoplasmic membrane (CM) and cell wall permeability (CWP) resulting from 50mg/L GA in Section 5.3.2. * p<0.05, ** p<0.01, *** p<0.001, **** p≤0.0001.*

	TOB Synergy	OM	CM	CWP
RBH750	✓		*	
RBH899			*	
RBH550				
RBH294			**	
RBH982	✓	*	**	
RBH422	✓		*	
RBH490	✓		*	
RBH519	✓		**	
RBH461				
RBH065			*	
RBH072	✓		**	
Grouped		*	*****	*

Taking these results across all the *P. aeruginosa* strains tested (both reference and clinical), and comparing with previous results (**Chapters 3** and **4**), suggest that permeabilisation of *P. aeruginosa* cell walls by GA was the least important aspect of it mechanism of potentiation of antibiotics. Strain PAO1 showed the widest range of antibiotic potentiation of reference strains but the least change in its permeabilisation by GA and no individual clinical strain was permeabilised by GA, including those which had shown GA-tobramycin synergy.

In contrast with this, the most consistent property of GA was the ability to depolarise *P. aeruginosa* cytoplasmic membranes. In comparisons with other antimicrobial agents, GA

significantly increased depolarisation in 12 of the 14 strains tested, and the only 2 strains not significantly impacted in these assays did not show GA-tobramycin synergy. This points to the ability of GA to depolarise *P. aeruginosa* being an important aspect of GA activity and potentiation.

There was a clear divide between reference and clinical strains in the results for outer membrane disruption where reference strain membranes appear to be much more susceptible to GA activity than clinical strains, on a strain-by-strain basis. Only one clinical strain showed significant outer membrane disruption by GA while each of the reference were disrupted. The lack of impact on the outer membranes of clinical strains, including those which displayed GA-tobramycin synergy, points to GA disruption of outer membranes not being as important a mechanism of GA potentiation (**Table 5-9**).

Interestingly this split was also seen for GA killing activity and may point to GA having distinct mechanisms of action; outer membrane disruption leading to rapid cell death and cytoplasmic membrane depolarisation leading to antibiotic potentiation.

While these assays are informative about the ways in which GA is acting on the *P. aeruginosa* cells, they are limited in their timeframe – the longest exposure measured in these experiments was 60mins. This allows conclusions to be drawn about what GA does to the bacterial membranes upon initial contact but does not indicate whether some or all of these effects are long-lasting. Further investigations are required to examine prolonged exposure to GA its effects on the bacterial cell and the relationship to antibiotic potentiation.

A clinical example of each of the respiratory pathogen *S. aureus*, *B. cepacia* and *A. xylosoxidans* were used as comparators and controls for membrane integrity assays and the effects of GA. *S. aureus* has previously been shown not to be susceptible to the effects of

GA[287] in the same manner as *P. aeruginosa* and additionally, as a Gram-positive, does not have targets for the NPN assay. *B. cepacia* are naturally resistant to the effects of cationic AMPs[344] and *A. xylosoxidans* are less susceptible to AMPs than *P. aeruginosa*[345], so serve as Gram-negative pathogenic comparators for the effects of GA.

As expected there were no significant changes in any of the integrity assays for any of the examples of the species tested (**Figure 5-25**, **5-26** and **5-27**). It was noted, however, that of the three integrity assays performed, an increase in signal was seen in cytoplasmic membrane depolarisation. While the increases were not significant, it was the only example of a result where the biological replicates were higher (with non-overlapping confidence intervals) for all the 3 species (**Figure 5-26**).

5.4.4 Limitations of approach

The membrane perturbation assays employed here to decipher the mechanisms of action of GA give an insight into GA activity against the Gram-negative bacterial membranes. They do not fully elucidate the precise mechanism(s) of GA active i.e. which specific mode of AMP-membrane interaction(s) occur; the Carpet model, Barrell-Stave formation, Toroidal pore formation. For this an approach such as high powered microscopy would be required and would give an insight into the specific mechanisms of GA antimicrobial activity and/or antibiotic potentiation.

5.4.5 Future directions

Experiments on the activity of GA over longer timecourses than those performed here would be of interest. Deciphering which, if any, of the membrane disruption effects of GA is the longest lasting would give in insight as to which (if any) of its properties is most important

for the potentiation of antibiotics, given that those GA-antibiotic co-administrations experiments occur in overnight exposures, rather than in the short-term.

It would also be of interest to further the explore the observations that (although non-significant) an increase in fluorescent signal for cytoplasmic membrane depolarisation was seen in non-pseudomonal clinical strains tested. *S. aureus* would be of particular interest for GA-antibiotic potentiation experiments, given that, as a Gram-positive species, any potentiation would be happening exclusively as a result of the activity of GA against the cytoplasmic membrane.

5.4.6 Discussion of hypothesis

I have shown that GA does kill *P. aeruginosa* cells extremely rapidly, more rapidly than had been shown previously. In fact the killing *P. aeruginosa* by GA occurs so rapidly it was not possible to capture the decline in cells numbers, which had already occurred before the treatment could be stopped. Not only was the killing of the bacteria uncapturable, it was also apparently complete, with further incubation time in the presence of GA not resulting in further cells number declines. GA does disrupt the membranes of *P. aeruginosa*, as measured by membrane integrity assays, and both group-specific and strain-specific effects of GA were seen for reference and clinical *P. aeruginosa*.

6. Interactions of glatiramer acetate with *Pseudomonas aeruginosa* lipopolysaccharides

6.1 Introduction

Lipopolysaccharides are the outermost part of the *P. aeruginosa* cell, protruding from the outer membrane into the environment. As such, they represent the first point of contact with, and first line of defence against, antimicrobials. *P. aeruginosa* LPS comprises three domains: Lipid A which embeds the LPS in the outer membrane of the bacteria, the core oligosaccharide and the outermost O-antigen which is the most variable region of *P. aeruginosa* LPS, both intra- and inter-strain[28,346].

The polar and hydrophobic nature of LPS provides protection to the outer membrane reducing its permeability. As well as this, *P. aeruginosa* employ different strategies to modify LPS both as prophylaxis against and in reaction to targeting by antimicrobials[335]. *P. aeruginosa* can use environmental divalent cations to bind individual LPS chains together, creating LPS-LPS bridges. This serves two purposes for the bacterium; the bound together LPS moieties create a physical barrier to the membrane and the positively charged ions binding to the negatively charged LPS neutralises the overall charge of the bacterial cell reducing the ability of antimicrobials to bind and attack the cell wall[32,170,174]. In *P. aeruginosa*, magnesium (Mg^{++}) and calcium (Ca^{++}) ions are the preferred divalent cations for LPS stabilisation in this manner[134,175].

P. aeruginosa can also modify their LPS structures in response to environmental stimuli or in response to attack by antimicrobials[335]. This involves addition of positively charged moieties and serves a similar purpose as divalent cations in reducing the negative charge of the cell and thereby reducing binding opportunities for antimicrobials. *P. aeruginosa* can added a number of molecules to its LPS including phosphoethanolamine (PEtN)[194], decanoic acid species (C12:0)[347], palmitic acid (C16:0)[348] but the best studied modification is the

addition of L-4-aminoarabinose (L-Ara4N)[102,114,176]. Synthesis, transport and addition of L-Ara4N is through the *arn* operon (*arnBCADTEF*)[176,177] and happens in response to AMPs in *P. aeruginosa*[34,114,178,191,198,199,239].

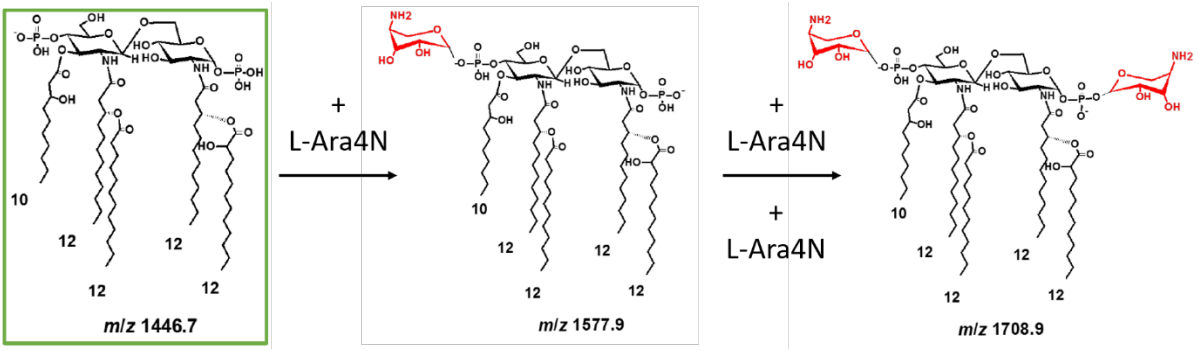


Figure 6-1. Modification of P. aeruginosa Lipid A (m/z 1446.7) by addition of one (m/z 1577.9) or two (m/z 1708.9) molecules of L-4-aminoarabinose.

The addition of L-Ara4N is mediated by a variety of Two Component Systems (TCS) in *P. aeruginosa,* including PhoPQ, PmrAB, CprRS and ParRS[102,192,197]. Each of these systems comprises a sensor protein, which takes in environmental signals, and a response regulator, which passes those signal(s) on to the process which the individual TCS regulates. The TCSs involved in LPS modification in *P. aeruginosa* have been shown to respond to both deficiencies in environmental cations and to cationic AMPs, indicating the link between LPS chain modification, levels of divalent cations and presence of cationic AMPs.

Different AMPs have been shown to target and bind to LPS chains with variable potential outcomes for the bacterium: (i) a lethal effect when an agent causes terminal membrane damage via sheer forces and stress as an AMP inserts between the outer membrane embedded LPS chains (ii) self-mediated uptake when an AMP gives itself access to the

bacterial membrane and other cellular targets and (iii) potentiation of a secondary agent, allowing it increased access to its targets.

The binding of AMPs to LPS is also of interest due the reported ability of LPS to aggregate and sequester AMPs and thereby inactivate them[288,349]. This would be of concern in the potentially LPS rich environment of the CF sputum[36–38,350]. The corollary of this is that aggregation and sequestration would be bilateral and colistin has been proposed as a neutraliser of LPS and its immunogenic effects[351].

6.2 Hypothesis

I hypothesise that:

GA targets *P. aeruginosa* LPS and strains of *P. aeruginosa* react to this targeting by modifying their LPS structures to protect against GA activity

6.2.1 Aims

To investigate this hypothesis, I will:

- Test the effects of divalent cations and *P. aeruginosa* LPS on GA activity
- Asses the *P. aeruginosa* response to GA treatment for known mechanisms of AMP resistance in *P. aeruginosa*

6.3 Results

6.3.1 Effect of divalent cations on the activity of GA

Cations of magnesium (Mg^{++}) are most commonly exploited by *P. aeruginosa* to perform LPS chain cross-linking and Mg^{++} was therefore tested for its protective ability against the effects of GA. The effects of 50mg/L GA on the outer membranes of *P. aeruginosa* reference strains- as have been described earlier in this thesis- were compared to those seen when different levels of magnesium divalent cations Mg^{++} were present in the assay buffer, in the form of $MgSO_4$. Monovalent cations of sodium (Na^+), in the form of NaCl were included as a comparator/control.

6.3.1.1 Effect of divalent cations on GA induced outer membrane disruption

For each of the 3 *P. aeruginosa* reference strains, neither the presence of $MgSO_4$ nor NaCl had an effect on the uptake of NPN in control, No Treatment suspensions. Multiple comparison tests to the previously reported NPN Uptake Factors of strains with No Treatment (**Results 4.3.1.2.1**, **Figure 4.2**) revealed no changes to the results of No Treatment for any of the strains when 1mM NaCl, 0.5mM $MgSO_4$ or 1mM $MgSO_4$ was present, indicating that the presence of these salts did not affect the integrity of the outer membrane or sensitivity of the NPN assay.

Magnesium cations at 1mM were protective against the membrane disrupting activity of GA for all 3 strains tested. The NPN Uptake Factor was significantly reduced in the presence of 1mM $MgSO_4$ compared with GA activity in buffer only suspensions ($p<0.05$). A lower level of $MgSO_4$ and NaCl did not alter the uptake of NPN (**Figure 6-2**).

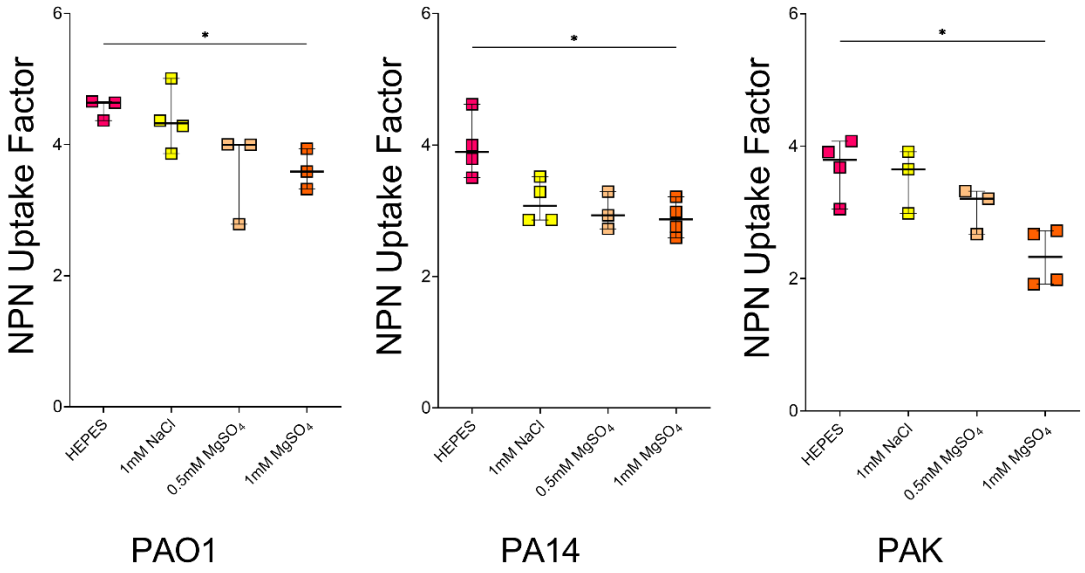


Figure 6-2. Mean NPN Uptake Factor over 15min exposure to 50mg/L GA in HEPES buffer supplemented with 1mM NaCl, 0.5mM $MgSO_4$ and 1mM $MgSO_4$ by P. aeruginosa PAO1, PA14 and PAK. Presence of 1mM $MgSO_4$ resulted in significant reductions if the NPN Uptake Factor of all strains tested: PAO1 (p=0.048), PA14 (p=0.022) and PAK (p=0.018). Neither 0.5mM $MgSO_4$ nor 1mM NaCl resulted in a significant changes for any strain, in the same comparisons. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

6.3.1.2 Effect of divalent cations on GA induced cytoplasmic membrane depolarisation

As with outer membrane disruption measurements, the addition of NaCl and $MgSO_4$ to the assay buffer did not alter the No Treatment results for any of the reference strains. For each strain, multiple comparison to No Treatment in assay buffer from previous experiments (**Results 4.3.1.2.2**, **Figure 4.3**) resulted in no significant differences when salts were present, indicating that the presence of salts did not affect the integrity of the cytoplasmic membrane or sensitivity of the $DiSC_3(5)$ assay.

No significant difference was seen in membrane depolarisation for any of the reference *P. aeruginosa* treated with GA when salts were present (**Figure 6-3**). For each strain, comparison of the results of biological replicates with salts present, to the previously reported

effects of GA (**Results 4.3.1.2.2, Figure 4.3**), resulted in no significant variance and no significant change from GA activity in the absence of cations. This indicated that Mg^{++} was not protective against the depolarisation activity of GA, at the levels tested.

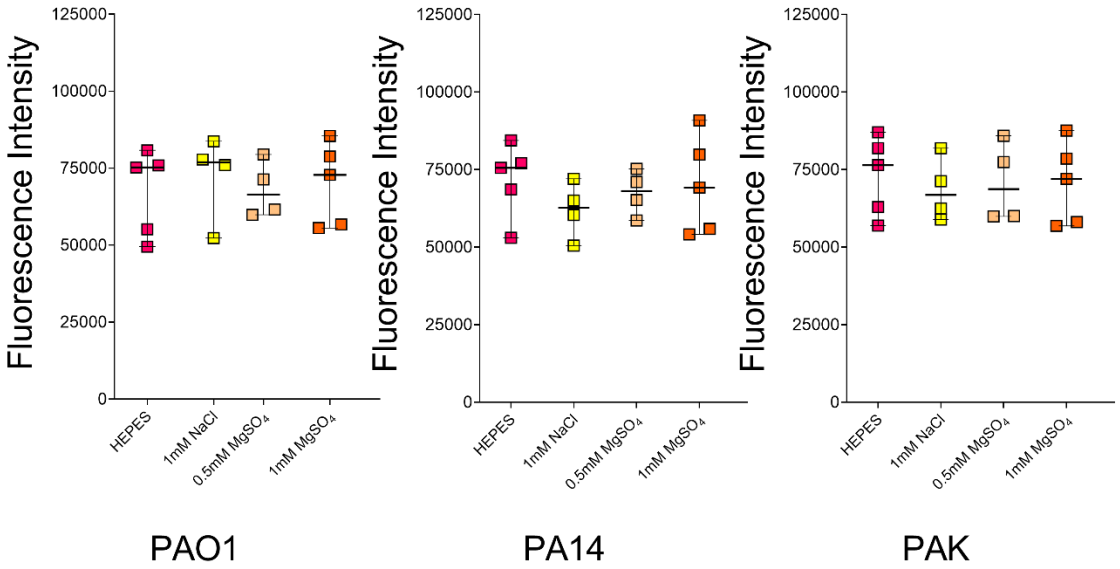


Figure 6-3. Mean intensity of $DiSC_3(5)$ fluorescence over 15mins after addition of 50mg/L GA in HEPES buffer supplemented with 1mM NaCl, 0.5mM $MgSO_4$ and 1mM $MgSO_4$ by P. aeruginosa PAO1, PA14 and PAK. None of the salts tested resulted in a significant change in fluorescence intensity for any of the strains tested, compared to the results seen in un-supplemented suspensions. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

6.3.1.3 Effect of divalent cations on GA induced cell wall permeabilisation

As with previous assays, the permeabilisation assay was unaffected by salts and there was no change in the results seen in No Treatment controls when salts were present or absent from the buffer. Multiple comparisons of untreated bacterial suspensions, with the various salts present, to previously reported controls (**Results 4.3.1.2.3**, **Figure 4.4**) resulted in no significant differences for any of the reference strains.

For all three strains tested, presence of 1mM NaCl did not affect the ability of GA to permeabilise the bacterial membrane. As part of multiple comparisons of each strain to the previously reported permeabilisation results, there was no significant difference between GA activity with and without 1mM NaCl (**Figure 6-4**).

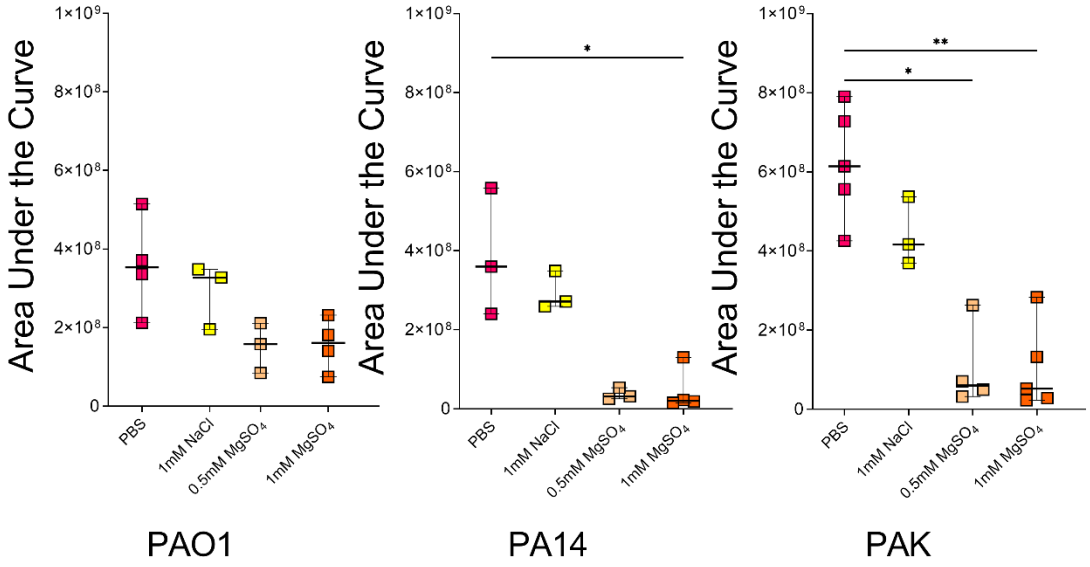


Figure 6-4. AUCs of PI fluorescence over 60mins exposure to 50mg/L GA in PBS supplemented with 1mM NaCl, 0.5mM $MgSO_4$ and 1mM $MgSO_4$ by P. aeruginosa PAO1, PA14 and PAK. For PAO1, comparisons to GA activity in PBS resulted in no significant changes in AUCs with any of the salts tested. For PA14, comparisons to GA activity in PBS resulted in a significant reduction in AUCs when 1mM $MgSO_4$ (p=0.0026) were present but was unchanged by 0.5mM $MgSO_4$ 1mM NaCl. For PAK, comparisons to GA activity in PBS resulted in a significant reduction in AUCs when 0.5mM $MgSO_4$ (p=0.014) and 1mM $MgSO_4$ (p=0.0005) were present but was unchanged by 1mM NaCl. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

Divalent cations of Mg^{++} were protective of the cell wall permeability of PAK at both concentrations tested and of PA14 at the higher concentration tested (1mM $MgSO_4$), significantly reducing the GA permeabilisation of each ($p<0.05$) (**Figure 6-4**). In contrast, PAO1 was not significantly less permeabilised by GA at either $MgSO_4$ concentration tested.

6.3.2 Effect of *P. aeruginosa* LPS on the activity of GA

Having noted the ability of divalent cations to protect *P. aeruginosa* against some aspects of GA's activity and, in the knowledge of the role of divalent cations in cross-linking LPS chains of Gram-negative bacteria and protecting against AMP activity, the impact of GA's direct interactions with *P. aeruginosa* LPS were examined. *P. aeruginosa* LPS and GA were incubated together at 37°C for 30mins before administration to bacterial suspensions to test whether GA and LPS bind together and GA is sequestered by LPS, preventing its activity. In separate experiments, LPS was present in the background during assays. Physiologically relevant concentrations of 0.01, 0.02mg/L[37,38] and the supra-physiological concentration 0.1mg/L LPS were tested with 50mg/L GA.

6.3.2.1 Effect of LPS on GA induced outer membrane disruption: LPS-GA pre-incubation

Untreated suspensions of PAO1, PA14 and PAK with LPS added (which had also been incubated at 37°C for 30mins in parallel with LPS-GA mixtures in the same buffer) were compared to untreated controls from previous NPN assays without LPS. No differences were seen for any of the strains when biological replicates were compared in multiple comparison analyses, indicating that the presence of different concentrations of LPS did not affect the strains or the assay as performed.

For all three reference strains of *P. aeruginosa*, there was an LPS dose-dependent reduction in the uptake of NPN by outer membranes resulting from GA treatment after pre-incubation of LPS-GA (**Figure 6-5**). Multiple comparisons of each strain, compared with GA-treatment in the absence of LPS, showed that at the highest concentration of LPS tested

(0.1mg/mL), pre-incubation resulted in a significant decrease in the NPN Uptake Factor for every strain ($p<0.01$) (**Figure 6-5**).

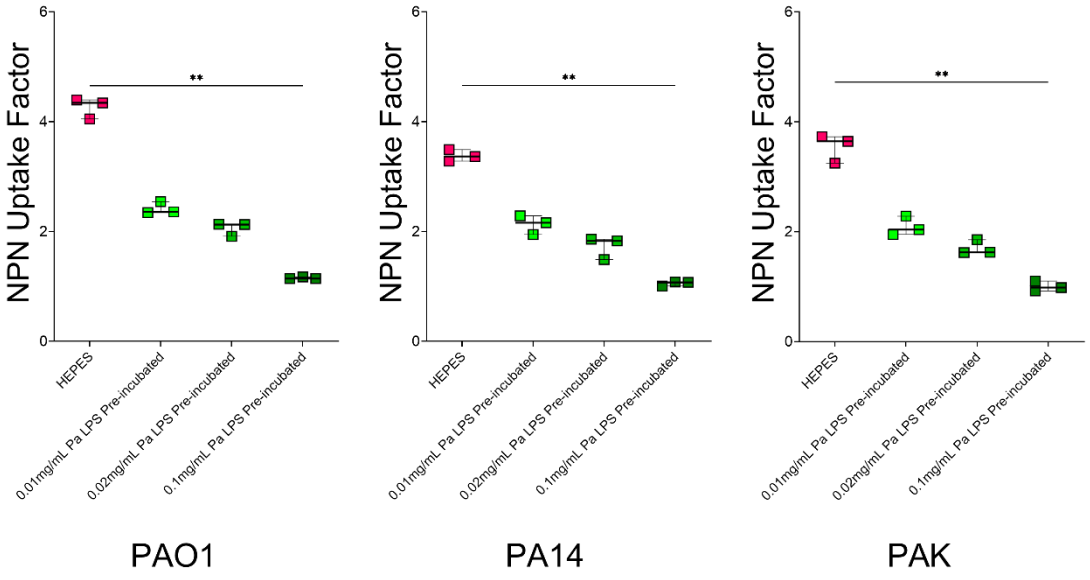


Figure 6-5. Mean NPN Uptake Factor of P. aeruginosa PAO1, PA14 and PAK over 15min exposure to 50mg/L GA pre-incubated with 0.01mg/mL, 0.02mg/mL or 0.1mg/mL P. aeruginosa LPS. For strain PAO1, uptake of NPN was significantly reduced by pre-incubations with 0.1mg/mL LPS (p=0.007). For strain PA14, uptake of NPN was significantly reduced by pre-incubations with 0.1mg/mL LPS (p=0.007). For strain PAK, uptake of NPN was significantly reduced by pre-incubations with 0.1mg/mL LPS (p=0.007). Pre-incubation with 0.01 and 0.02mg/mL LPS did not affect the ability of GA to disrupt the outer membranes of any of the reference strains. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

At a concentrations of 0.01 and 0.02mg/mL, pre-incubation of GA-LPS did not result in a significant change in uptake of NPN by any of the reference strains.

6.3.2.2 Effect of LPS on GA induced outer membrane disruption: Background LPS

To further test for GA-LPS interactions, GA was administered to suspensions of reference *P. aeruginosa* in an LPS background, at the same LPS concentrations as tested previously.

As with pre-incubation experiments, when LPS was administered in the background of the NPN assays no change was seen in the NPN Uptake factor of No Treatment replicates, via multiple comparison to No Treatment samples without LPS. This was the case for all strains and all LPS concentrations, indicating that background LPS does not affect the assay.

For GA treated samples, background LPS exerted a dose-dependent effect on the activity of GA as measured by NPN uptake by outer membranes (**Figure 6-6**). For all three strains the highest concentration of background LPS (0.1mg/mL) resulted in significant reductions in NPN Uptake Factor during GA treatment, compared to GA treatments in the absence of LPS ($p<0.05$) (**Figure 6-6**).

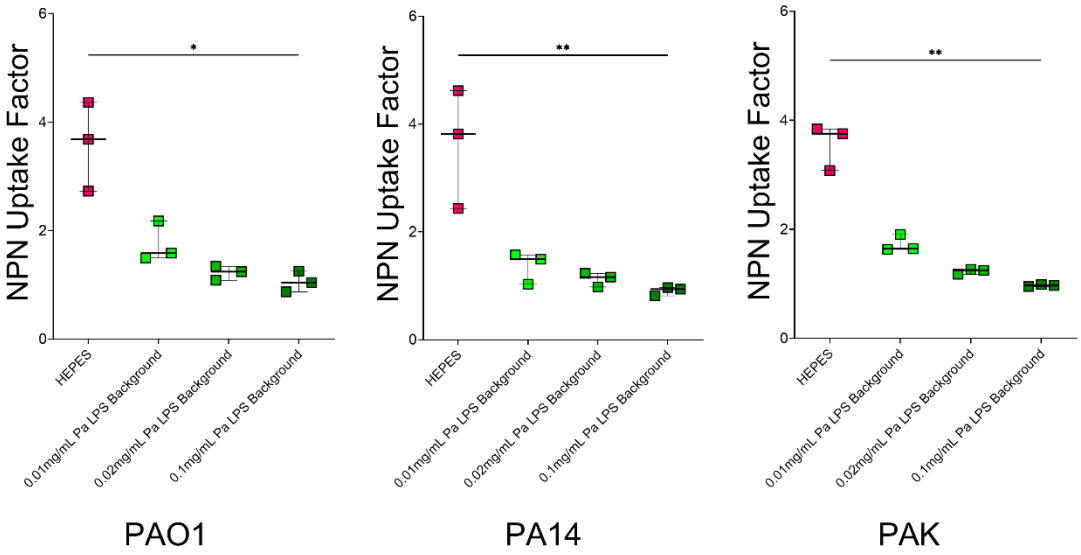


Figure 6-6. Mean NPN Uptake Factor of P. aeruginosa PAO1, PA14 and PAK over 15min exposure to 50mg/L GA in a background of 0.01mg/mL, 0.02mg/mL or 0.1mg/mL P. aeruginosa LPS. For strain PAO1, uptake of NPN was significantly reduced in a background of 0.1mg/mL LPS (p=0.014). For strain PA14, uptake of NPN was significantly reduced in a background of 0.1mg/mL LPS (p=0.007). For strain PAK, uptake of NPN was significantly reduced in a background of 0.1mg/mL LPS (p=0.007). Background concentration of 0.01 and 0.02mg/mL LPS did not affect the ability of GA to disrupt the outer membranes of any of the reference strains. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

As with pre-incubation, a background of 0.01 and 0.02mg/mL did not result in a significant change in the NPN Uptake Factor of GA-treated suspensions of any of the reference strain, when compared with GA activity in suspensions without LPS (**Figure 6-2**).

6.3.2.3 Effect of LPS on GA induced cytoplasmic membrane depolarisation: LPS-GA pre-incubation

The effect of pre-incubation with 0.01, 0.02 and 0.1mg/mL *P. aeruginosa* LPS on the activity of GA on cytoplasmic membranes of PAO1, PA14 and PAK was compared to GA activity in LPS-free suspensions.

In contrast with both NPN assays and $DiSC_3(5)$ assays with salts present, presence of LPS in $DiSC_3(5)$ assays resulted in significant changes to the fluorescent signal of the assay in *untreated* samples. For all three refence strains the combination of the fluorescent dye, bacteria and 0.1mg/mL LPS resulted in a significantly increased mean fluorescence over 15mins, compared with the same suspensions in the absence of LPS ($p<0.05$). This was not the case for 0.01 or 0.02mg/mL LPS. This significant change in fluorescent signal resulting from presence of 0.1mg/mL LPS meant that the fluorescent signal was no longer a comparable metric to test the activity across concentrations of LPS.

For this reason, the relative changes in fluorescence GA Treatment:No Treatment were calculated from the same 15min exposure data for each strain. This was done for all concentrations of LPS, despite 0.01 and 0.02 mg/mL LPS not having a significant impact in the manner in which 0.1mg/mL does, in order preform multiple comparisons across the effects of different LPS concentrations. The same was ratio was calculated for the previously reported results for GA treatment in LPS absent suspensions.

Comparisons of the ratios resulting from GA treatment showed that no concentration of LPS tested had an impact on the activity of GA on the cytoplasmic membranes of reference strains, after pre-incubation with GA (**Figure 6-7**).

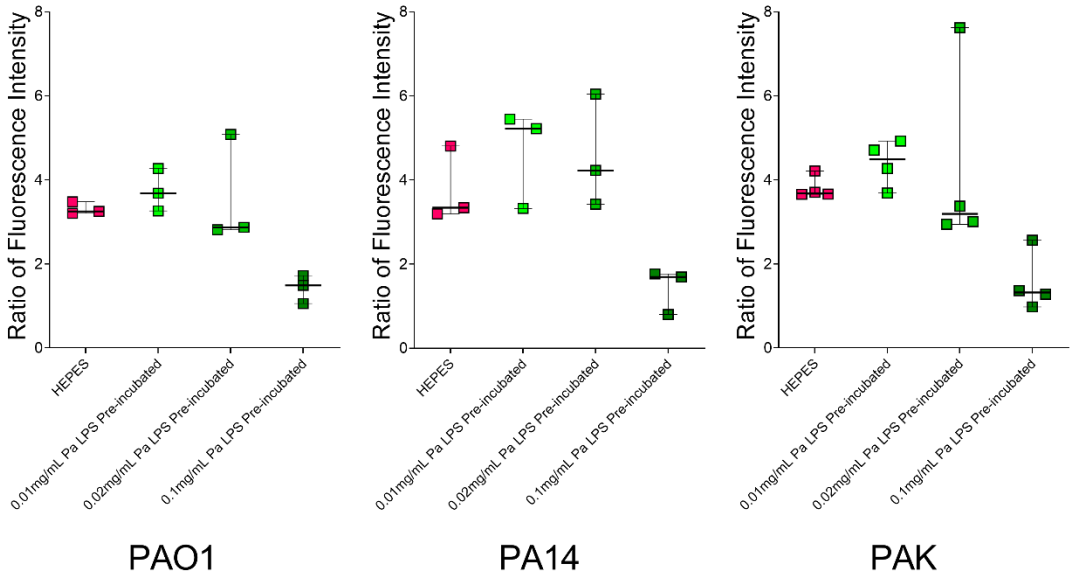


Figure 6-7. Mean ratio of DiSC$_3$(5) fluorescence over 15mins for 50mg/L GA Treatment:No Treatment suspensions of P. aeruginosa strains PAO1, PA14 and PAK after pre-incubation of GA with 0.01, 0.02 and 0.1mg/mL P. aeruginosa LPS. Pre-incubation of GA with LPS did not have a significant impact on GA activity for any of the three reference strains. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

Pre-incubation of GA with LPS at concentrations of 0.01 and 0.02mg/mL did not have a significant impact of the activity of GA for any of the three strains, indicating that GA activity was retained.

6.3.2.4 Effect of LPS on GA induced cytoplasmic membrane depolarisation: Background LPS

As with pre-incubation for $DiSC_3(5)$ assays, ratios were calculated for the effect of treatment with GA vs no treatment at each of the LPS concentrations tested, as well as for results in the absence of LPS.

The presence of *P. aeruginosa* LPS in the background of suspensions for $DiSC_3(5)$ assays did not alter the activity of GA against the cytoplasmic membranes for any of tests performed across the three reference strains and three concentrations of LPS (**Figure 6-8**).

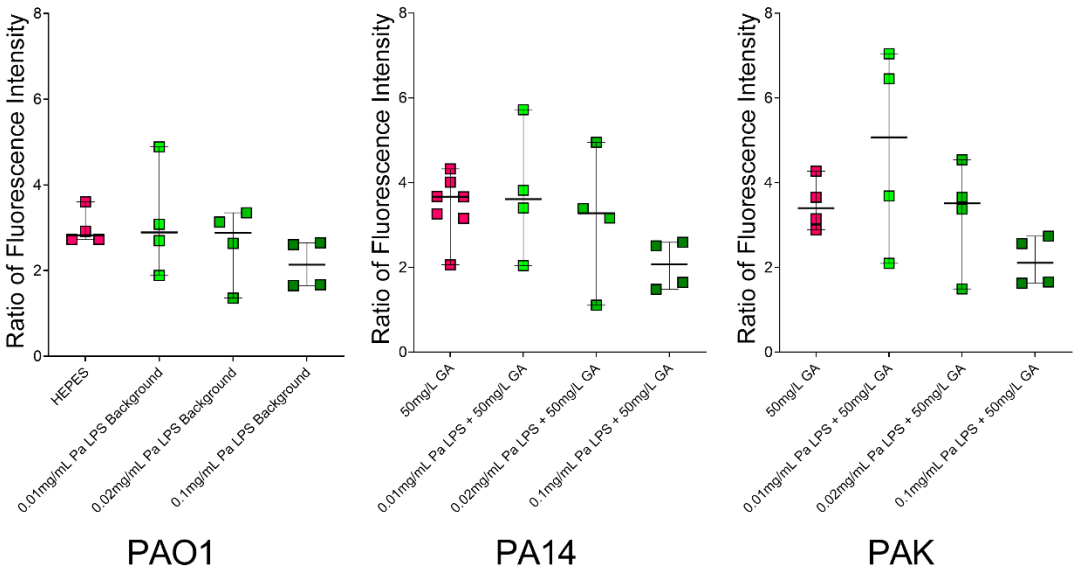


Figure 6-8. Mean ratio of $DiSC_3(5)$ fluorescence over 15mins for 50mg/L GA Treatment:No Treatment suspensions of P. aeruginosa strains PAO1, PA14 and PAK of GA in a background of 0.01, 0.02 and 0.1mg/mL P. aeruginosa LPS. Background LPS did not have a significant impact on GA activity for any of the three reference strains. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

6.3.2.5 Effect of LPS on GA induced cell wall permeabilisation: LPS-GA pre-incubation

Presence of LPS (which had been incubated at 37°C for 30mins before addition) did not have any impact on the assay. No significant changes were seen for any strain with any LPS concentration when No Treatment groups were compared using AUCs over 60mins.

Strain- and concentration-specific changes to GA's effect on cell wall permeability were seen after pre-incubation with LPS (**Figure 6-9**). PA14 was the most affected by LPS pre-incubation with significant reductions in permeabilisation by GA seen at 0.02 and 0.1mg/mL ($p<0.05$). There was also a significant reduction in the permeabilisation of PAK by GA due to LPS at 0.1mg/L ($p<0.05$). Neither strain was altered by 0.01mg/mL LPS (**Figure 6-9**).

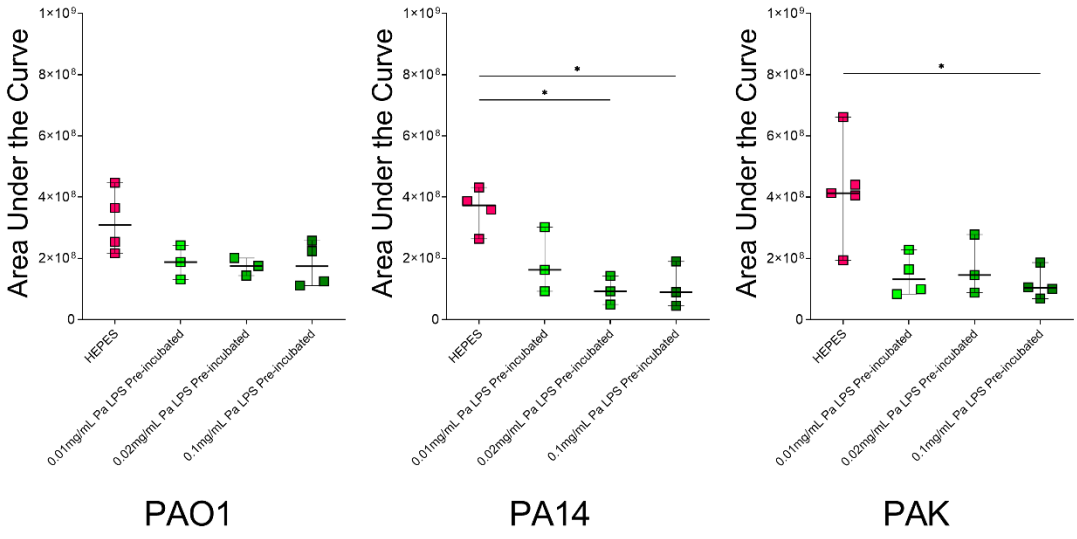


Figure 6-9. AUCs of PI fluorescence over 60mins of P. aeruginosa PAO1, PA14 and PAK during exposure to 50mg/L GA pre-incubated with 0.01mg/mL, 0.02mg/mL or 0.1mg/mL P. aeruginosa LPS. For strain PAO1, PI AUCs were not significantly reduced by pre-incubation LPS. For strain PA14, PI AUCs were significantly reduced by pre-incubations with 0.02mg/mL (p=0.04) and 0.1mg/mL LPS (p=0.04). For strain PAK, PI AUCs were significantly reduced by pre-incubations with 0.1mg/mL LPS (p=0.027). Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

Permeabilisation of PAO1 by GA was unaltered by LPS pre-incubation at any of the concentrations tested.

6.3.2.6 Effect of LPS on GA induced cell wall permeabilisation: Background LPS

As with pre-incubated suspensions, the PI assay was unaffected by the presence of LPS at any concentrations tested. Comparisons of untreated suspensions of each strain with and without LPS had no significant differences.

In contrast with pre-incubation of GA-LPS, background LPS did not significantly alter the ability of GA to permeabilise the reference strains. Using multiple comparison tests for each strain, no significant differences were seen in the results of GA treated samples without LPS compared with GA treatment in a background of any of the LPS concentrations tested (**Figure 6-10**).

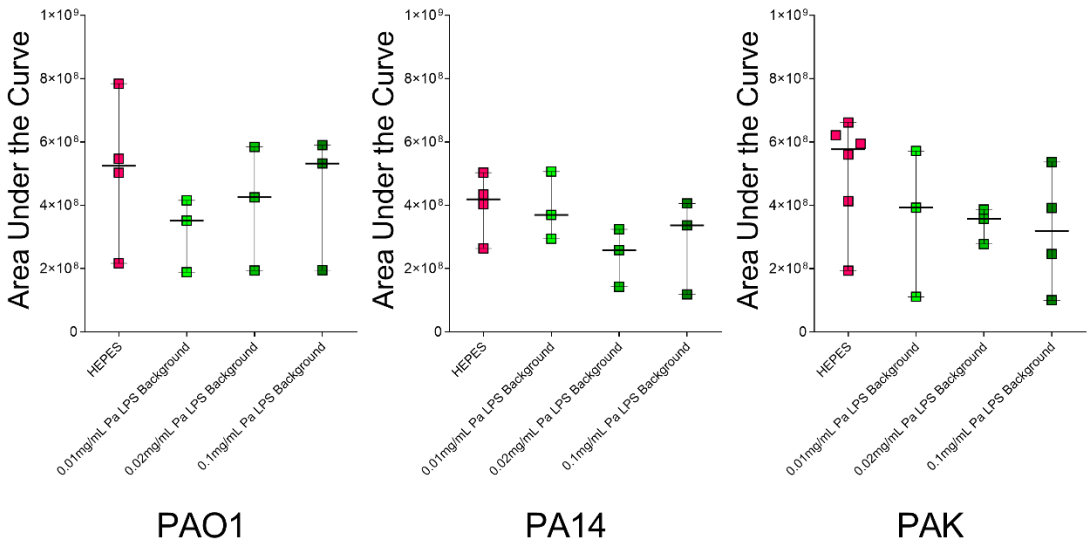


Figure 6-10. AUCs of PI fluorescence over 60mins of P. aeruginosa PAO1, PA14 and PAK during exposure to 50mg/L GA in a background of 0.01mg/mL, 0.02mg/mL or 0.1mg/mL P. aeruginosa LPS. Background LPS did not alter the PI AUCs of any of the strains at any of the LPS concentrations tested, in comparison with GA activity in the absence of LPS. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Points are biological replicates with line at median and 95% confidence intervals.

6.3.2.7 GA-LPS binding

The data above suggest that GA is binding to LPS, which is reducing efficacy of the former on bacterial cells. I therefore sought to confirm a direct interaction between GA and LPS, using a Pierce™ Chromogenic Endotoxin Quant Kit was which measures LPS concentrations in Endotoxin Units.

At all three concentrations of LPS tested (0.01, 0.02 and 0.1mg/mL), pre-incubation with 50mg/L GA at 37°C for 30mins resulted in lower levels of LPS being detected by the LAL kit than in the equivalent sample without GA. This indicates that a proportion of the LPS was bound to GA and was no longer available for detection by the kit. There was 50.7% reduction in Endotoxin Units detected for 0.01mg/mL LPS when GA was present, 56.9% reduction for 0.02mg/mL LPS and 16.9% reduction for 0.1mg/mL LPS (**Figure 6-11**). GA alone (which had also been incubated at 37°C for 30mins) was not detected by the assay.

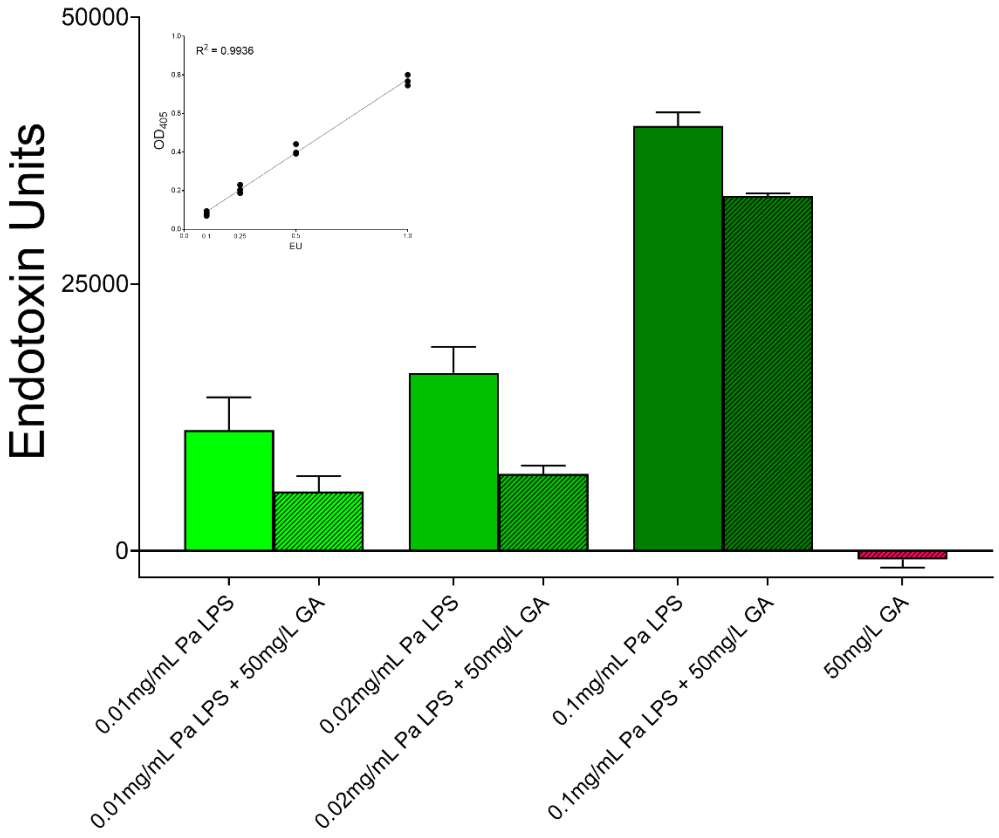


Figure 6-11. Endotoxin Units of 0.01, 0.02 and 0.1mg/mL P. aeruginosa LPS after 30mins incubation at 37°C with and without 50mg/L GA measured by Pierce™ Chromogenic Endotoxin Quant Kit. Presence of GA reduced detectable LPS from 11,296 (± 3,082) to 5,563 (± 1,443) at 0.01mg/mL LPS, from 16,666 (± 2,434) to 7,180 (± 790.5) at 0.02mg/mL LPS and from 39,791 (± 1,292) to 33,234 (± 262.3) at 0.01mg/mL LPS. GA alone was undetectable. Inset: Standard curve prepared from provided standards of E. coli (0111:B4) Endotoxin Standard. Bars are means of 3 technical replicates with standard deviation.

6.3.3 Effect of GA on the expression of TCS genes involved in AMP sensing and LPS modification genes in reference *P. aeruginosa*

Having noted the ability of GA to bind to *P. aeruginosa* LPS, the ability of *P. aeruginosa* strains to sense and react to this targeting was examined. The expression of TCS genes, which have been shown to mediate the response of *P. aeruginosa* to AMPS, and one of the known targets of this mediation, were examined by qRT-PCR. The response regulators *phoP*, *pmrA*, *cprR* and *parR* along with *arnB*, a member of the *arnBCADTEF* operon which modifies LPS

chains in response to signals from the TCS, were examined. The *P. aeruginosa* housekeeping gene *rpsL* was also measured for normalisation and analysis purposes.

The effect of GA on reference strains was compared to that elicited by AMPs colistin and LL-37, as well as No Treatment. Triplicate biological replicates were performed of each condition except LL-37 which was performed in duplicate due to limited reagent availability.

6.3.3.1 Reference *P. aeruginosa* ΔCt

The expression level of each gene under each condition tested was calculated relative to the level of *rpsL* expression under the same conditions via ΔCt. The fold changes in each gene from *rpsL* were compared for each strain. This allowed measurement of the constitutive expression levels of each of the genes and compare that to expression levels after stimulation with the AMPs tested.

For strain PAO1, of the genes tested, none was expressed at a high level, relative to the housekeeping gene and the highest ΔCt seen in untreated PAO1 cultures was 1.34 [95%CI 0.07-2.14] for *phoP*. The gene *arnB* had the lowest relative expression level with a ΔCt of 0.08 [95%CI 0.0004-0.16]. Expression of each gene under AMP stimulation showed no significant variance for any of the genes tested for PAO1, indicating that expression levels were comparable with the control cultures (**Figure 6-12**).

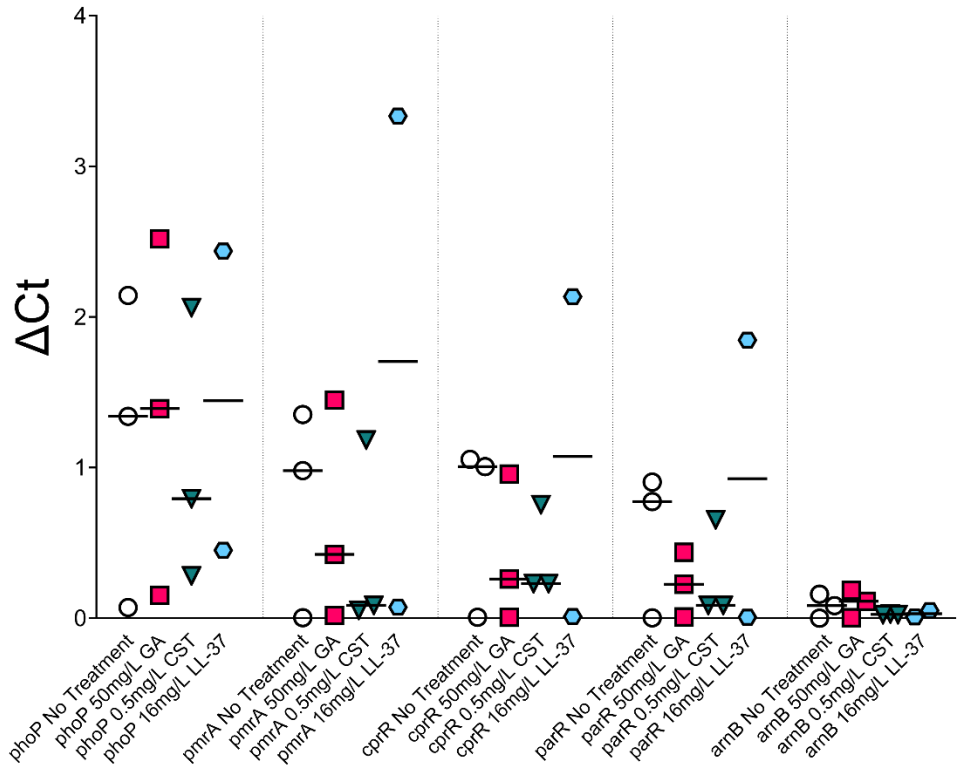


Figure 6-12. Gene expression levels relative to housekeeping gene rpsL (ΔCt) of genes phoP, pmrA, cprR, parR and arnB from P. aeruginosa strain PAO1 after exposure to AMPs GA (50mg/L), CST (0.5mg/L) and LL-37 (16mg/L). No significant variance was seen for any gene across treatment groups. Kruskal-Wallis test with Dunn's mutlipte correction. Points are biological replicates with line at median.

As with PAO1, *phoP* was the highest expressed gene of those tested for PA14 in the absence of AMP stimulation with ΔCt of 1.65 [95%CI 1.61-2.93]. Also in common with PAO1, *arnB* had the lowest expression in control cultures of PA14 at ΔCt 0.004 [95%CI 0.002-0.02]. Under AMP stimulation none of the genes showed any variance for any gene in their expressions, relative to the housekeeping gene (**Figure 6-13**).

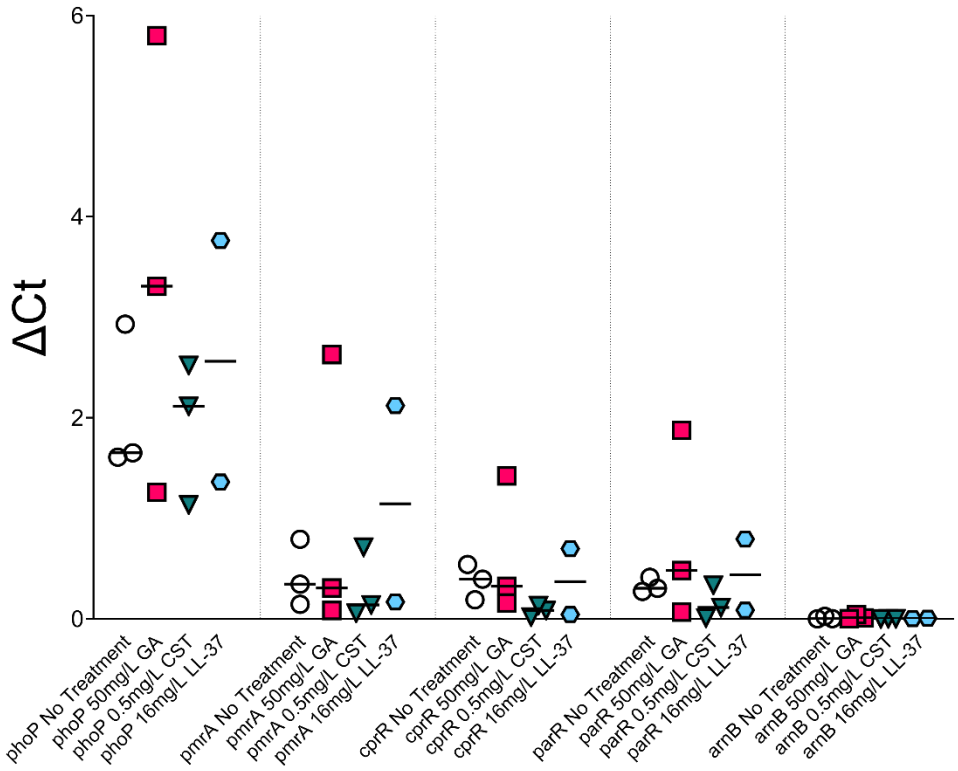


Figure 6-13. Gene expression levels relative to housekeeping gene rpsL (ΔCt) of genes phoP, pmrA, cprR, parR and arnB from P. aeruginosa strain PA14 after exposure to AMPs GA (50mg/L), CST (0.5mg/L) and LL-37 (16mg/L). No significant variance was seen for any gene across treatment groups. Kruskal-Wallis test with Dunn's mutlipte correction. Points are biological replicates with line at median.

Once again, for PAK, the gene *phoP* had the highest level of constitutive expression (and the highest level of an of the reference strains) of any of genes tested with No Treatment; ΔCt was 2.90 [95%CI 0.37-2.92]. Also as before, arnB showed the lowest expression level of the genes tested in PAK with ΔCt 0.006 [95%CI 0.0005-0.24]. PAK stimulation with AMPs did not result in any variance in the ΔCt for any of the genes tested (**Figure 6-14**).

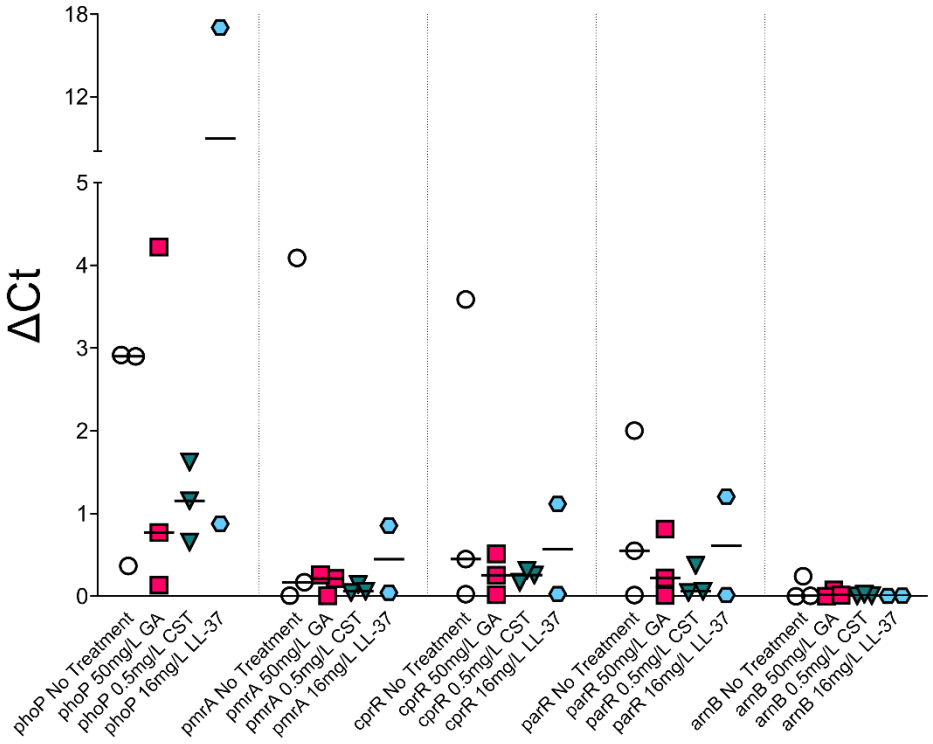


Figure 6-14. Gene expression levels relative to housekeeping gene rpsL (ΔCt) of genes phoP, pmrA, cprR, parR and arnB from P. aeruginosa strain PAK after exposure to AMPs GA (50mg/L), CST (0.5mg/L) and LL-37 (16mg/L). No significant variance was seen for any gene across treatment groups. Kruskal-Wallis test with Dunn's mutlipte correction. Points are biological replicates with line at median.

6.3.3.2 Reference *P. aeruginosa* ΔΔCt

The expression of each gene after stimulation by AMPs during growth to mid-log phase was calculated relative to the same gene without stimulation (ΔΔCt) for each biological replicate of each strain. A fold change of ≥2 resulting from AMP presence was considered a notable increase.

For strain PAO1, the greatest expression increase by AMPs was seen for *arnB* with both GA (ΔΔCt 2.21 [95%CI 0.71-7.27) and LL-37 (ΔΔCt 8.55 [95%CI 0.32-16.77]) resulting in ≥2-fold increases in expression (**Figure 6-15**). Genes *phoP* (ΔΔCt 40.6 [95%CI 1.82-6.31]) and *pmrA* (ΔΔCt 9.13 [95%CI 3.40-14.86]) expression were also increased by LL-37. None of the

remaining genes, exposed to GA, were increased in their ΔΔCt in PAO1. None of the genes tested showed a ΔΔCt increase when treated with CST under these conditions.

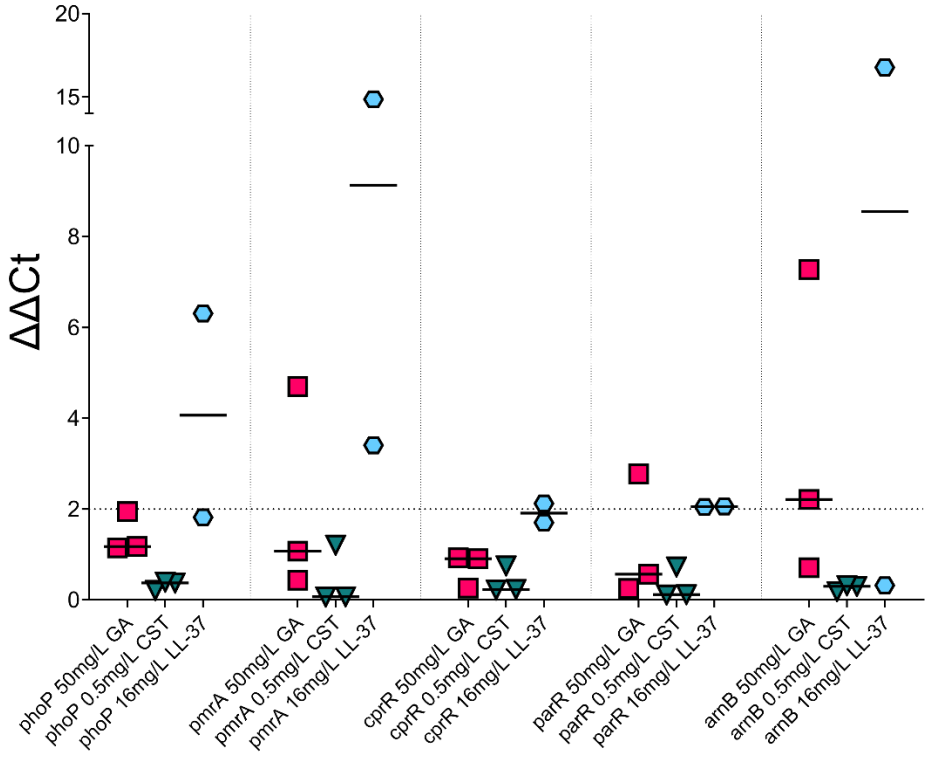


Figure 6-15. Gene expression levels relative to expression in No Treatment cultures (ΔΔCt) of genes phoP, pmrA, cprR, parR and arnB from P. aeruginosa strain PAO1 after exposure to AMPs GA (50mg/L), CST (0.5mg/L) and LL-37 (16mg/L). Points are biological replicates with line at median. A fold change ≥2 was considered notable.

GA stimulation of strain PA14 resulted in increased expression of *pmrA* with a ΔΔCt of 2.04 [95%CI 0.24-3.32]. Neither CST nor LL-37 resulted in ≥2-fold increase in *pmrA*. None of the other PA14 genes tested were stimulated to a ≥2-fold increase by GA and the only other gene-combination to reach this level for PA14 was *arnB* by LL-37 (ΔΔCt 4.29 [95%CI 1.92-6.67]) (**Figure 6-16**).

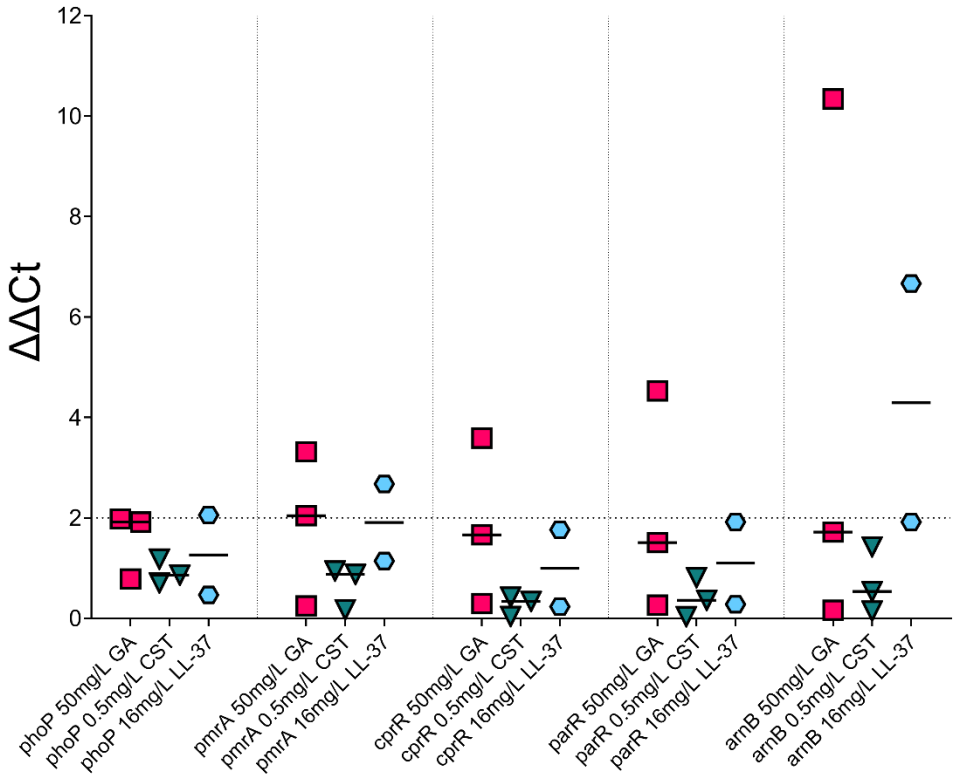


Figure 6-16. Gene expression levels relative to expression in No Treatment cultures (ΔΔCt) of genes phoP, pmrA, cprR, parR and arnB from P. aeruginosa strain PA14 after exposure to AMPs GA (50mg/L), CST (0.5mg/L) and LL-37 (16mg/L). Points are biological replicates with line at median. A fold change ≥2 was considered notable.

For strain PAK, only LL-37 was stimulatory for the ΔΔCt of the genes tested; *phoP* (ΔΔCt 4.20 [95%CI 2.53-5.87]), *pmrA* (ΔΔCt 5.08 [95%CI 5.03-5.13]) and *arnB* (ΔΔCt 11.54 [95%CI 10.30-12.78]) were increased. Neither GA nor CST resulted in a change in ΔΔCt ≥2-fold for any of the genes tested (**Figure 6-17**).

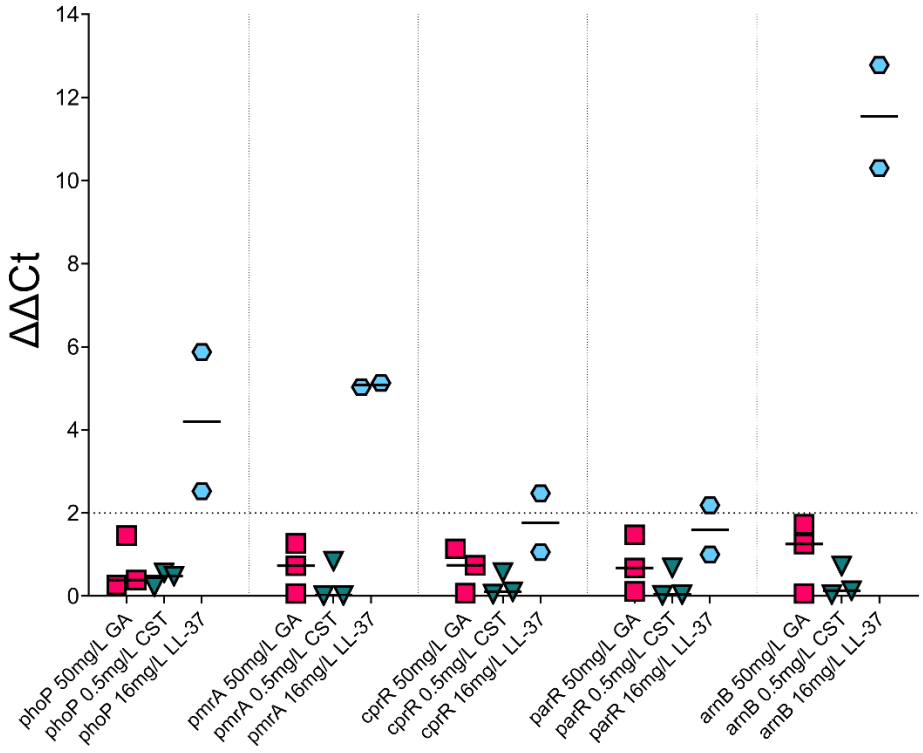


Figure 6-17. Gene expression levels relative to expression in No Treatment cultures (ΔΔCt) of genes phoP, pmrA, cprR, parR and arnB from P. aeruginosa strain PAK after exposure to AMPs GA (50mg/L), CST (0.5mg/L) and LL-37 (16mg/L). Points are biological replicates with line at median. A fold change ≥2 was considered notable.

6.3.4 Two component systems of clinical *P. aeruginosa* from cystic fibrosis involved in AMP sensing and the effect of GA on the expression of these TCS and LPS modification genes

6.3.4.1 Sensor Gene Sequences in clinical *P. aeruginosa*

Mutations in the genes of the sensor protein of TCSs have previously been strongly associated with antibiotic resistance, particularly to AMPS. The sensory protein genes of TCSs PhoPQ, PmrAB, CprRS and ParRS were extracted from the genomes of 11 clinical *P. aeruginosa* from cystic fibrosis and aligned in MEGA-X. Translated protein sequences of the aligned DNA sequences were examined for non-synonymous changes resulting in amino acid differences in the sequences of each gene.

6.3.4.1.1 PhoQ

Three amino acid changes were found in the PhoQ protein across the clinical strains examined. All three changes were found in different clinical strains; two in the periplasmic sensor domain of the protein (RBH294 and RBH065) and one in the HATPase_c domain (RBH422) (**Figure 6-18**, **Table 6-5**).

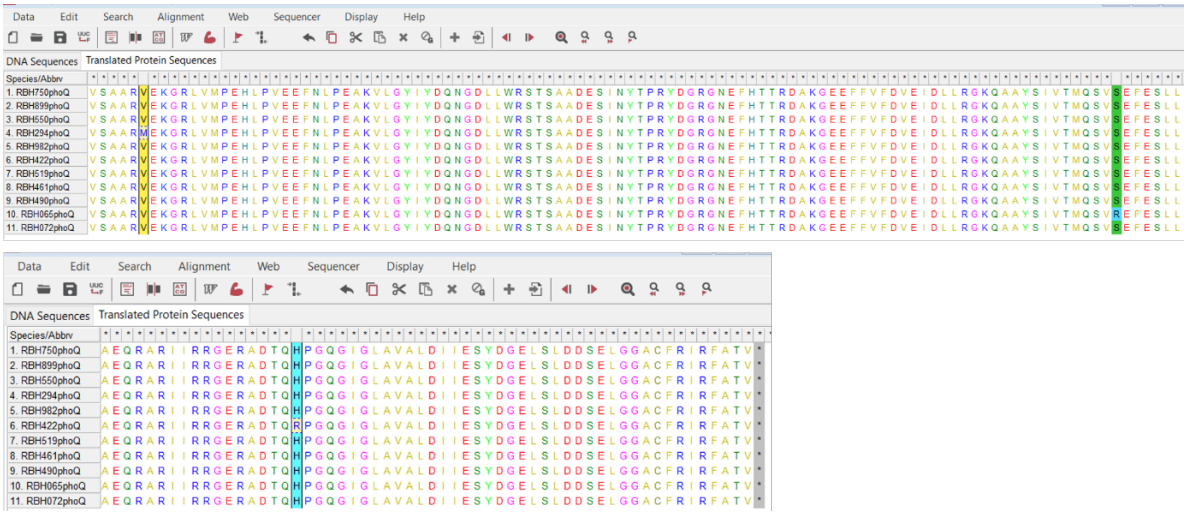


Figure 6-18. MEGA alignment of translated sequences of PhoQ from 11 clinical P. aeruginosa from cystic fibrosis. Amino acid changes were found in strains RBH294 (V57M), RBH065 (S153R) and RBH422 (H408R).

6.3.4.1.2 PmrB

Six amino acid changes were found in the PmrB protein across the clinical strains examined. Four of these changes were found in a single strain, RBH899; two outside any known domains/motifs (S2P and A4T), one in a transmembrane domain (V15I) and one in the sensor portion (G68S). Another amino acid change was found in the sensor domain in RBH490 (A83P) and the last change found was in the HAMP domain of RBH065 (E218D) (**Figure 6-19**, **Table 6-5**).

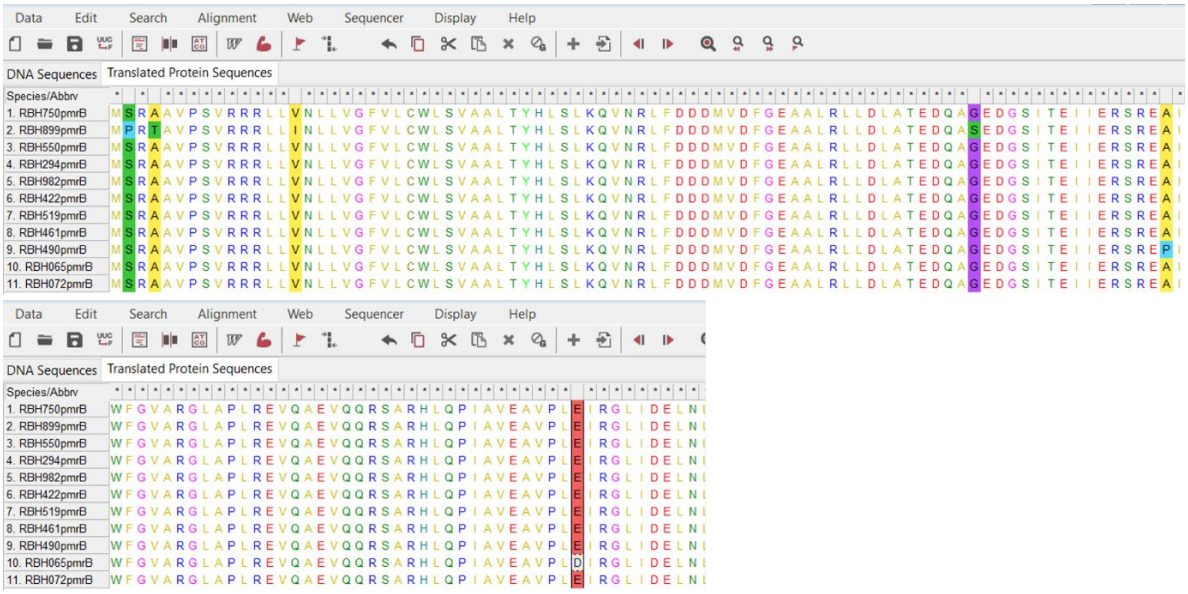


Figure 6-19. MEGA alignment of translated sequences of PmrB from 11 clinical P. aeruginosa from cystic fibrosis. Amino acid changes were found in strains RBH3899 (S2P, A4T, V15I and G68S), RBH490 (A83P) and RBH065 (E218D).

6.3.4.1.3 CprS

As with PmrB, six amino acid changes were found among the 11 sequences of CprS and, also in common with PmrB, four of these were found in the strain RBH899. Changes in this strain were found across the CprS gene; in a transmembrane domain (T16S), in the sensor domain (D32N), in the histidine kinase (G282A) and at E386D. The two remaining changes were found; in the transmembrane domain of strain RBH519, with an identical change to one of those found in RBH899, (T16S) and in the second transmembrane domain of RBH422 (S173L) (**Figure 6-20**, **Table 6-5**).

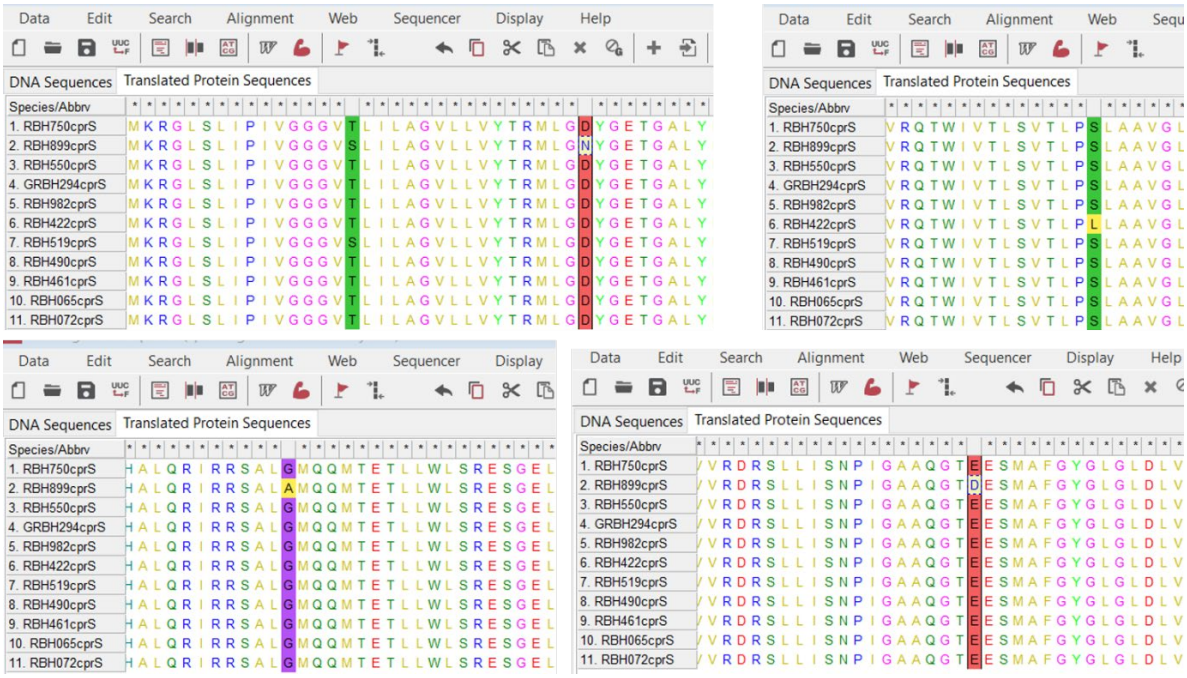


Figure 6-20. MEGA alignment of translated sequences of CprS from 11 clinical P. aeruginosa from cystic fibrosis. Amino acid changes were found in strains RBH3899 (T16S, D32N, G282A and E386D), RBH519 (T16S) and RBH422 (S173L).

6.3.4.1.4 ParS

Two amino acid changes were found in the translated protein sequences of ParS across the 11 clinical strains. In strain RBH519 a change was found in the periplasmic domain (A55P) and in strain RBH072 an amino acid change was found in the histidine kinase domain (A215T) (**Figure 6-21**, **Table 6-5**).

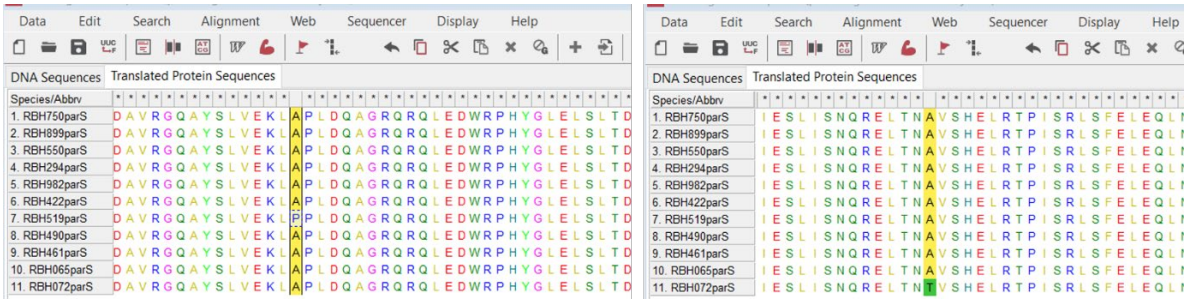


Figure 6-21. MEGA alignment of translated sequences of ParS from 11 clinical P. aeruginosa from cystic fibrosis. Amino acid changes were found in strains RBH519 (A55P) and RBH072 (A215T).

Of the 16 amino acid changes found across these four sensor genes in the 11 clinical strains examined, half were found in a single strain RBH899, and all of these were found in two genes, PmrB and CprS. Three strains had two amino acid changes each; RBH065 (in PhoQ and PmrB), RBH422 (in PmrB and CprR) and RBH519 (in CprS and ParS).

Five of the clinical strains examined here had no amino acid changes in any of the four sensor genes examined.

Table 6-1. Non-synonymous changes found in the gene sequences of sensory components of Two Component Systems of 11 clinical P. aeruginosa strains from cystic fibrosis.

Sensor	Domain	Amino Acid Change	Strain
PhoQ	Periplasmic Sensor	V57M	RBH294
		S153R	RBH065
	HATPase_c	H408R	RBH422
PmrB		S2P	RBH899
		A4T	RBH899
	Transmembrane 1	V15I	RBH899
	Periplasmic Sensor	G68S	RBH899
		A83P	RBH490
	HAMP	E218D	RBH065
CprS	Transmembrane 1	T16S	RBH899
			RBH519
	Periplasmic Sensor	D32N	RBH899
	Transmembrane 2	S173L	RBH422
	Histidine Kinase	G282A	RBH899
		E386D	RBH899
ParS	Periplasmic Sensor	A55P	RBH519
	Histidine Kinase	A215T	RBH072

HATPase_c, histidine kinase-like ATPases; HAMP, histidine kinases, adenylyl cyclases, methyl-binding proteins, and phosphatases

6.3.4.2 Effect of GA on TCS and LPS modification gene expression in Clinical *P. aeruginosa*

Clinical strains were tested in the same manner for as reference *P. aeruginosa* for gene expression with and without GA.

6.3.4.2.1 Clinical *P. aeruginosa* ΔCt

Treatment of clinical *P. aeruginosa* strains with GA resulted in a significant increase in the ΔCt of genes *pmrA* (p=0.042) and arnB (p=0.0068), compared with untreated cultures, indicating that these genes were stimulated by GA exposure (**Figure 6-22**).

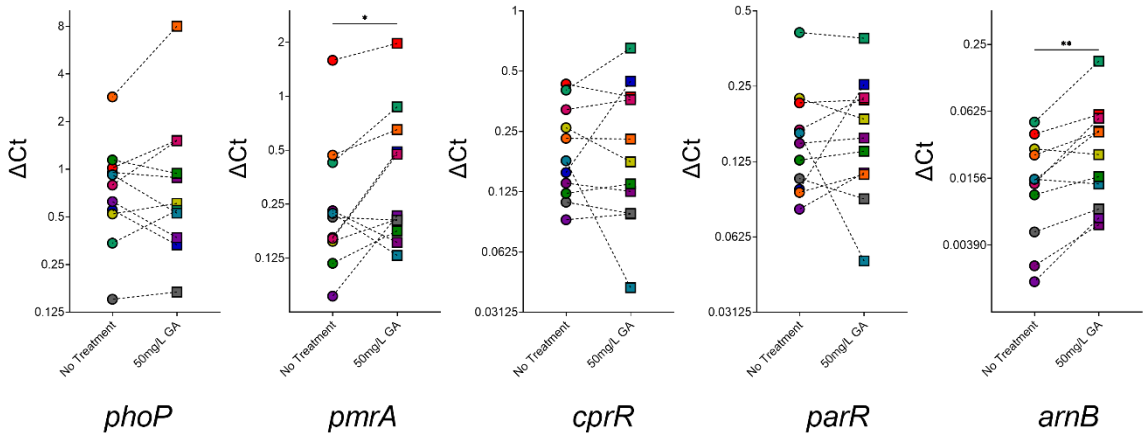


Figure 6-22. Gene expression levels relative to housekeeping gene rpsL (ΔCt) of genes phoP, pmrA, cprR, parR and arnB from 11 clinical P. aeruginosa strains from CF after exposure to 50mg/L GA. Genes pmrA (p=0.042) and arnB (p=0.0068) had significant increases in their ΔCt after treatment with GA. None of the other genes tested were significantly changed. Wilcoxon tests. Colour-matched points are biological medians of clinical strains without and with GA-treatment.

There was no significant differences in the ΔCt expression levels of *phoP* between No Treatment and GA-treated cultures across the 11 clinical strains tested (**Figure 6-22**). As with reference strains, *phoP* had the highest constitutive expression among the genes tested in clinical strains; ΔCt of 0.79 [95%CI 0.34-1.15] in untreated cultures. One clinical strain had high constitutive ΔCt expression of *phoP*, comparable with the highest *phoP* expressing reference strain, PAK. Strain RBH490 had a ΔCt of 2.81 [95%CI 1.76-8.42] untreated and 8.03 [95%CI 6.47-9.39] GA-treated.

Genes *cprR* and *parR* were unchanged in their ΔCt by GA-treatment and there was no indication of high levels of constitutive expression of either gene in untreated cultures (**Figure 6-22**).

6.3.4.2.2 Clinical *P. aeruginosa* ΔΔCt

As with ΔCt, the greatest impact on ΔΔCt resulting from GA treatment was seen for gene *arnB*. The ΔΔCt of arnB was the highest of the genes tested at 1.55 [95%CI 1.16-2.08]

over the 11 strains tested and was the gene with the greatest proportion of strains with ≥2-fold increase in ΔΔCt (RBH750, RBH550, RBH294 and RBH519) (**Figure 6-23**).

Also in common with ΔCt comparisons, the next most affected gene in its ΔΔCt by GA treatment was *pmrA* with a ΔΔCt over the 11 clinical strains of 1.39 [95%CI 0.82-2.52]. Two of the clinical strains tested had ≥2-fold increase in their ΔΔCt results after stimulation with GA (RBH750 and RBH294) (**Figure 6-23**).

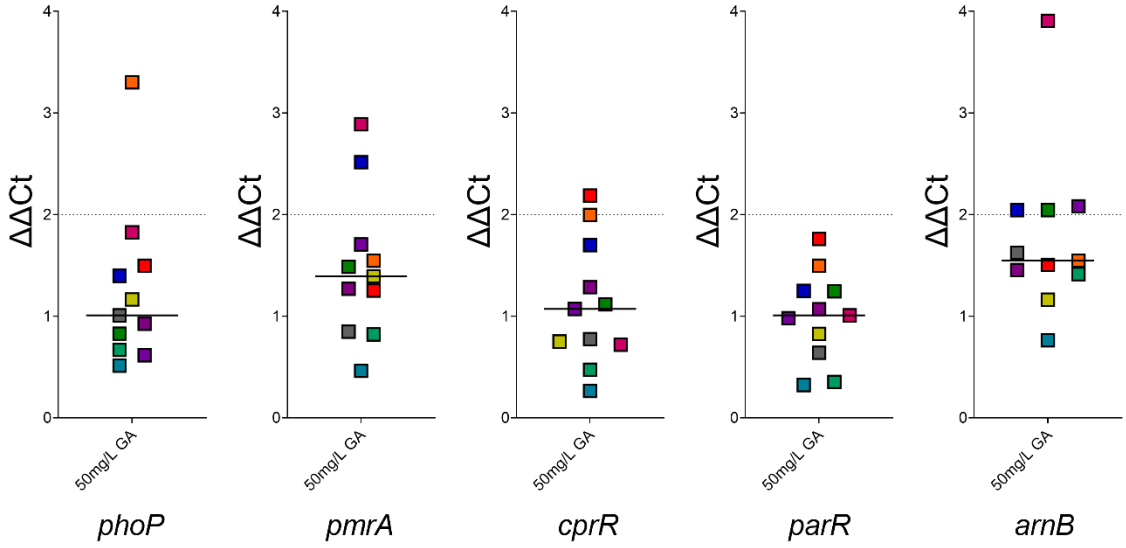


Figure 6-23. Gene expression levels relative to expression in No Treatment cultures (ΔΔCt) of genes phoP, pmrA, cprR, parR and arnB from 11 clinical P. aeruginosa strains from CF after exposure to 50mg/L GA. Points are medians of biological replicates for each strain with line at median. A fold change ≥2 was considered notable.

For the remaining genes tested, GA treatment did not stimulate expression as the ΔΔCt for each across the clinical strains indicated no change in expression between No Treatment and GA treatment; *phoP* ΔΔCt was 1.01 [955CI 0.62-1.83], *cprR* was 1.07 [95%CI 0.47-1.99] and *parR* was 1.01 [95%CI 0.36-1.49].

One clinical strain had a *phoP* ΔΔCt with a ≥2-fold increase and this was the same strain with a high *phoP* ΔCt indicating that this strain, RBH490, was both constitutively expressing *phoP* and was further stimulated in its *phoP* expression by GA. An additional strain, RBH065, was stimulated by GA in the expression of *cprR* which had a ΔΔCt with a ≥2-fold increase. No strain had a ≥2-fold increase in ΔΔCt for gene *parR* (**Figure 6-23**).

6.3.4.3 Correlations of changes in gene expression after GA treatment in clinical *P. aeruginosa*

6.3.4.3.1 TCS genes with *arnB*

Having measured the effect of GA treatment on TCS expression and a gene which these TCSs regulate (*arnB*) in clinical *P. aeruginosa*, correlation between TCS expression and *arnB* expression was examined to determine a potential pathway by which GA is sensed by *P. aeruginosa* clinical strains and whether or not that signal is transmitted to LPS-modification machinery of *arnBCADTEF*.

Correlation of the ΔΔCt results of *phoP*, *pmrA*, *cprR* and *parR* with *arnB* revealed that expression of only one TCS gene was correlated with *arnB* expression; *pmrA* (**Figure 6-24**). With a Spearman r of 0.80 [95%CI 0.37-0.95] *pmrA* and *arnB* were positively correlated (p=0.0047) (**Table 6-2**).

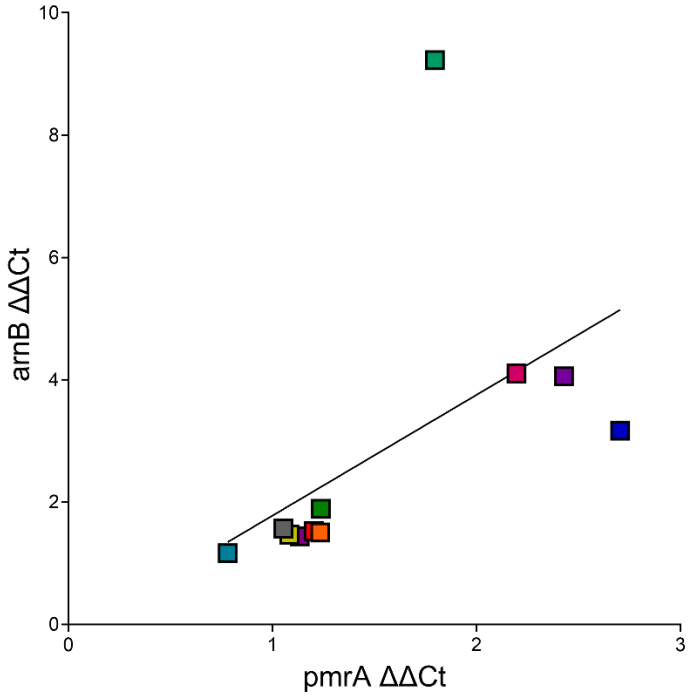


Figure 6-24. Correlation of ΔΔCt results of clinical P. aeruginosa from cystic fibrosis after treatment with 50mg/L GA for expression of genes pmrA and arnB. Expression of pmrA and arnB were positively (Spearman r 0.8 [95%CI 0.37-0.95]) and significantly (p=0.0047) correlated. Spearman Correlation of biological replicates.

Table 6-2. Correlation of arnB ΔΔCt with the ΔΔCt results of each of the TCS genes tested for clinical P. aeruginosa after exposure to 50mg/L GA. Only pmrA expression was positively correlated with arnB expression. Spearman Correlation of biological replicates.

	Spearman r	p value
phoP	0.16 [-0.49-0.71]	0.63
pmrA	**0.80 [0.37-0.95]**	**0.0047 (**)**
cprR	0.18 [-0.49-0.72]	0.59
parR	0.23 [-0.44-0.74]	0.5

6.3.4.3.2 TCS genes with each other

Cross-talk between different TCS has been reported in response to AMPs and therefore correlation between TCS genes in response to GA treatment was examined.

Expression of *cprR* and *parR* in response to GA treatment were positively correlated in clinical *P. aeruginosa* (Spearman r 0.94 [95%CI 0.76-0.98], p<0.0001) (**Figure 6-25**).

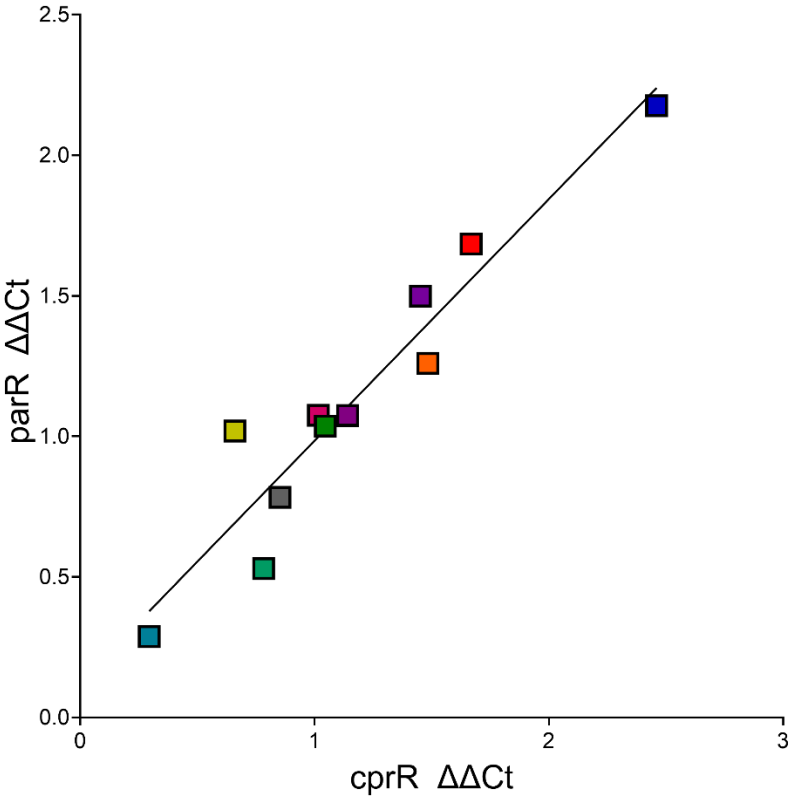


Figure 6-25. Correlation of ΔΔCt results of clinical P. aeruginosa from cystic fibrosis after treatment with 50mg/L GA for expression of genes cprR and parR. Expression of cprR and parR were positively (Spearman r 0.94 [95%CI 0.76-0.98]) and significantly (p<0.0001) correlated. Spearman Correlation of biological replicates.

Correlation, to a lesser extent, was also noted for the genes *pmrA* and *parR* after exposure to GA with a Spearman r of 0.64 [95%CI 0.04-0.89] (p=0.0402) (**Figure 6-26**). No correlation was seen between any of the TCS genes examined (**Table 6-3**).

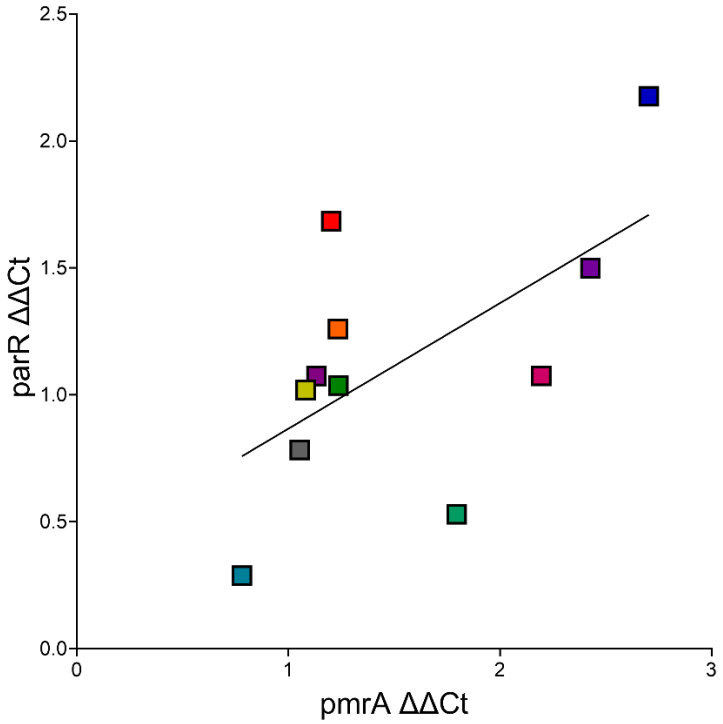


Figure 6-26. Correlation of ΔΔCt results of clinical P. aeruginosa from cystic fibrosis after treatment with 50mg/L GA for expression of genes pmrA and parR. Expression of pmrA and parR were positively (Spearman r 0.64 [95%CI 0.04-0.89]) and significantly (p=0.0402) correlated. Spearman Correlation of biological replicates.

Table 6-3. Correlation of ΔΔCt results of each of the TCS genes tested with each other for clinical P. aeruginosa strains after exposure to 50mg/L GA. Expression of pmrA was positively correlated with parR expression. Expression of cprR was positively correlated with parR expression. Spearman Correlation of biological replicates.

	pmrA	***cprR***	***parR***
phoP	r=0.17 [-0.49-0.71] p=0.61	r=0.31 [-0.38-0.78] p=0.36	r=0.42 [-0.26-0.82] p=0.20
pmrA	-	r=0.57 [-0.06-0.88] p=0.071	**r=0.64 [0.04-0.89]** **p=0.04 (*)**
cprR	-	-	**r=0.94 [0.76-0.98]** **p<0.0001 (****)**

6.3.5 LPS modification by L-Ara4N

6.3.5.1 Reference *P. aeruginosa*

Modification levels of LPS were compared using the Polymyxin Resistance Ratio (PRR), that is the Abundance of Unmodified Lipid A:Abundance of Modified Lipid A[178].None of the treatments tested resulted in a significant change in the PRR of any of the reference *P. aeruginosa* (**Figure 6-27** and **6-28**, **Table 6-4**).

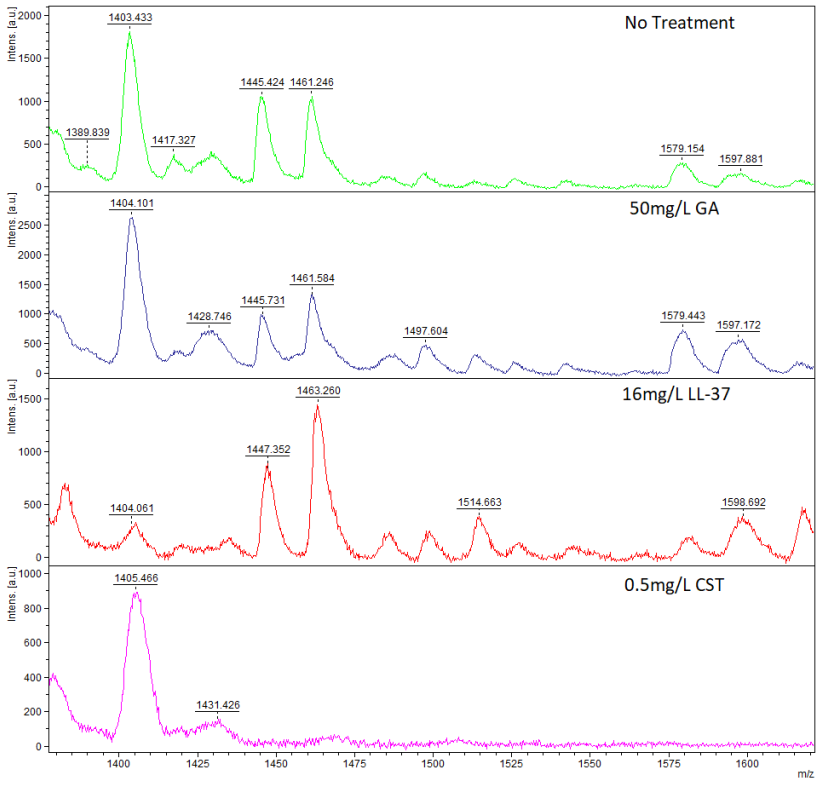


Figure 6-27. Example MALDI-TOF spectra of P. aeruginosa PAO1 Lipid A after treatment with GA (50mg/L), LL-37 (16mg/L) and CST (0.5mg/L). Native Lipid A peaks are m/z ~1404, 1442 and 1462. Lipid A modified with L-Ara4N are at m/z ~1577 and 1597.

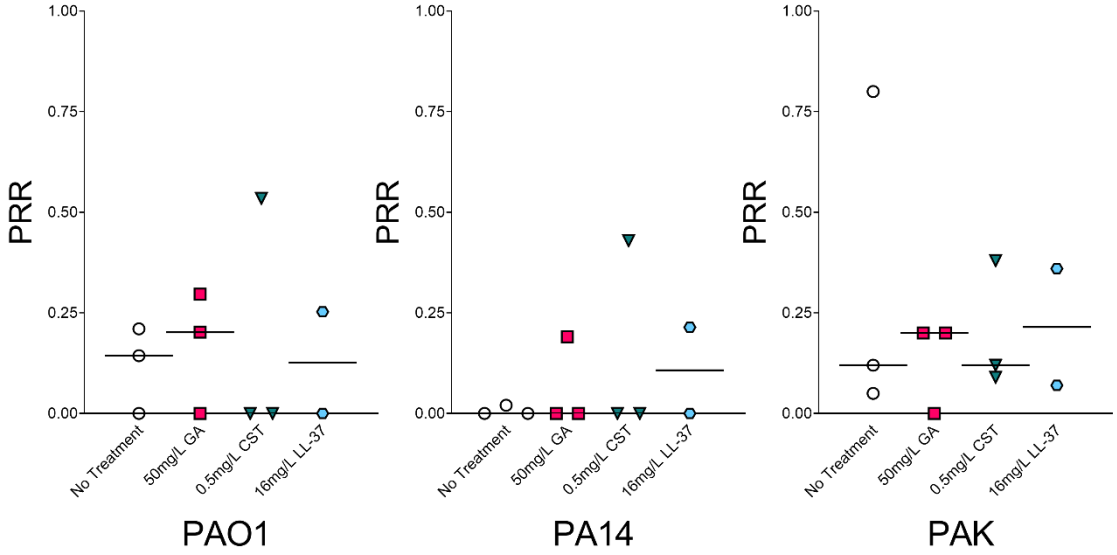


Figure 6-28. Polymyxin Resistance Ratios of P. aeruginosa reference strains PAO1, PA14 and PAK after stimulation with GA (50mg/L), CST (0.5mg/L) or LL-37 (16mg/L). No significant change was seen in the level of L-Ara4N addition to Lipid A for any of the conditions tested (Kruskal-Wallis test with Dunn's mutlipte correction). Biological replicates with lines at medians.

Table 6-4. Polymyxin Resistance Ratios of P. aeruginosa reference strains PAO1, PA14 and PAK. Biological medians [95% confidence intervals].

	No Treatment	50mg/L GA	0.5mg/L CST	16mg/L LL-37
PAO1	**0.14** [0.0 – 0.21]	**0.20** [0.0 – 0.30]	**0.0** [0.0 – 0.53]	**0.13** [0.0 – 0.25]
PA14	**0.0** [0.0 – 0.02]	**0.0** [0.0 – 0.19]	**0.0** [0.0 – 0.43]	**0.11** [0.0 – 0.21]
PAK	**0.12** [0.05 – 0.80]	**0.20** [0.0 – 0.20]	**0.12** [0.09 – 0.38]	**0.22** [0.07 – 0.36]

6.3.5.2 Clinical *P. aeruginosa* from CF

There was no significant change in the PRR of clinical *P. aeruginosa* after treatment with 50mg/L GA (**Figure 6-29, Table 6-5**).

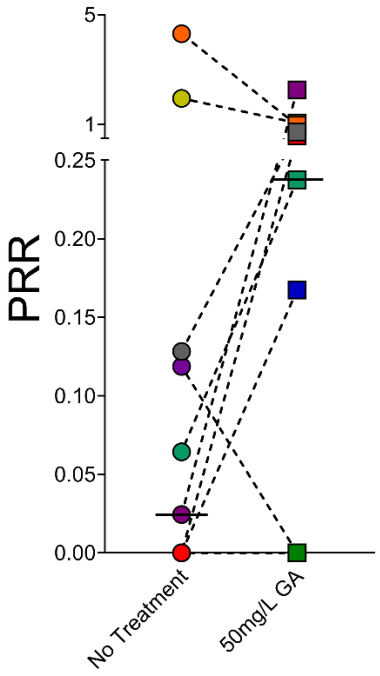


Figure 6-29. Polymyxin Resistance Ratios of clinical P. aeruginosa from people with CF after GA treatment. GA did not significantly change the level of L-Ara4N addition to Lipid A of clinical strains (Wilcoxon test). Colour-matched points are biological medians of clinical strains with lines at median.

Table 6-5. Polymyxin Resistance Ratios of clinical P. aeruginosa from people with CF after GA treatment. Biological medians [95% confidence intervals].

	No Treatment	50mg/L GA
RBH750	**0.0** [0.0 – 0.0]	**0.0** [0.0 – 0.0]
RBH899	**0.24** [0.0 – 0.05]	**2.3** [0.56 – 3.9]
RBH550	**0.12** [0.0 – 0.32]	**0.0** [0.0 – 0.0]
RBH294	**0.0** [0.0 – 0.15]	**0.17** [0.0 – 0.29]
RBH982	**0.0** [0.0 – 0.0]	**0.0** [0.0 – 0.1]
RBH422	**0.06** [0.0 – 0.07]	**0.24** [0.0 – 0.25]
RBH519	**0.0** [0.0 – 0.02]	**0.0** [0.0 – 0.08]
RBH461	**1.95** [0.0 – 2.79]	**1.05** [0.0 – 10.78]
RBH490	**4.3** [1.9 – 6.8]	**0.96** [0.0 – 4.0]
RBH065	**0.0** [0.0 – 2.2]	**0.57** [0.08 – 0.65]
RBH072	**0.13** [0.06 – 0.46]	**0.72** [0.48 – 2.6]

6.4 Discussion

In this Chapter, I have shown that in common with other cationic AMPs, GA can bind to and target *P. aeruginosa* LPS. I have shown that divalent cations of Mg^{++} were protective against some aspects of GA's activity, particularly cell permeabilisation. I have shown that this protection was dose-dependent and specific to Mg^{++} and not the result of presence of a salt. I have shown that there is a dose-dependent effect on GA mechanisms of action for LPS presence; the higher the concentration of LPS present, the lesser the effectiveness of GA. I have shown that there is a different effect on GA activity depending on if LPS is present in bacterial suspensions compared to LPS which has been allowed to bind to GA first. I have shown that GA treatment does not strongly stimulate the genes responsible for AMP sensing and LPS-modification in reference *P. aeruginosa*. I have shown that there was a strain specific stimulation of these same genes by GA in clinical *P. aeruginosa*. I have shown that neither reference nor clinical strains increased their modification with L-Ara4N in response to GA.

Lipopolysaccharides (LPS) are crucial components of the bacterial cell and one of the most important interfaces in bacterial host-pathogen interactions. LPS provides defence against cell wall attack on the membranes via modification with available divalent cations or in reaction to stimuli[335]. However, being the outermost aspect of the bacterial cell also makes LPS a target for binding particularly by AMPs which can exploit the anionic nature of unmodified LPS to bind and disrupt the outer membrane. AMPs can also competitively displace the divalent cations employed as LPS bridges to disrupt the membrane and/or facilitate their own uptake. Indeed, the targeting of LPS by AMPs does not necessarily stop at the outside of the cell[224].

LPS is also highly immunogenic, known as endotoxin, and is a component of CF sputum[36–38]. Colistin has been shown to sequester LPS[351] which, while potentially detrimental to the availability and activity of the antibiotic, may have the benefit of reducing the immunogenicity of LPS and modulating the immune response.

6.4.1 GA-LPS interactions

Divalent cations were protective against outer membrane disruption by GA for all reference strains and against cell wall permeabilisation of PA14 and PAK by GA, at 1mM $MgSO_4$ (**Figures 6-2** and **6-4**). PAK was also protected against permeabilisation by GA at 0.5mM $MgSO_4$. In the conventional model of divalent cations stabilising LPS, this result indicates an interaction between GA and *P. aeruginosa* LPS, the effects of which, Mg^{++} is partially protective against. The protective effect of divalent cations towards the outer membrane and the cell wall was in complete contrast with the effect towards the cytoplasmic membrane for GA. Cytoplasmic membrane depolarisation by GA occurred in a comparable manner for all strains, whether salts were present or absent (**Figure 6-5**). Both 0.5 and 1mM are higher than the Mg^{++} level reportedly found in CF sputum[352].

Next experiments with *P. aeruginosa* LPS present sought to explore interactions with GA in more detail and test whether extracellular LPS would overwhelm the activity of GA, which had been previously established. LPS was most protective of GA activity against outer membranes, as disruption was significantly reduced by LPS at 0.1mg/mL, whether pre-incubated or in the assay milieu (**Figure 6-6**). This suggests that GA has been bound to and sequestered by LPS at these concentrations and is no longer available to act on the outer membrane. The effect was LPS-dose dependent as sufficient GA was apparently still available to cause disruption in spite of 0.01 and 0.02mg/L LPS.

The ability of GA to depolarise cytoplasmic membranes was unaffected by LPS presence with no loss of GA activity seen for any stain or at any LPS concentration, whether pre-incubated in the background (**Figure 6-7**). These results potentially further point to the activity of GA against outer and cytoplasmic membranes as being distinct and/or related to different conformations and secondary structures of GA.

There was some contrast between the permeabilisation of *P. aeruginosa* cells by GA, whether there was pre-incubation with LPS or LPS in the background of the experiment. Pre-incubation was effective at preventing GA permeabilisation of the reference strains PA14 and PAK, with permeabilisation by GA significantly reduced at 0.02 and 0.1mg/mL LPS for PA14 and at 0.1mg/mL for PAK (**Figure 6-8**). Permeabilisation of PAO1, the strain which had previously shown the least increase in permeability by GA, was unaltered by LPS incubation. Background LPS was entirely ineffective at preventing GA activity and permeabilisation proceeded as in the absence of LPS for all strains tested.

In pre-incubated samples, there appeared to be some crossover between LPS protection against GA activity on outer membranes and permeabilisation: both effects were significantly reduced by 0.1mg/L LPS (except PAO1 permeability) (**Table 6-6**). In contrast, pre-incubation at the same concentrations did not affect GA's ability to depolarise any strain's membrane. Again, this may indicate the importance of GA conformations and secondary structures[277,278] on the areas of the bacteria which it targets, with the cytoplasmic membrane results appearing to be distinct from outer membrane disruption and permeabilisation.

Table 6-6. Comparison of results of membrane integrity assays with GA pre-incubated with or in a Background of P. aeruginosa LPS. Significance of the change resulting from treatment with 50mg/L GA on the outer membrane (OM), cytoplasmic membrane (CM) and cell wall permeability (CWP) in multiple comparison tests performed in ***Section 6.4.2.****. * p<0.05, ** p<0.01.*

		Pre-incubated				**Background**		
		0.01mg/mL	0.02mg/mL	0.1mg/mL		0.01mg/mL	0.02mg/mL	0.1mg/mL
OM	PAO1	ns	ns	**		ns	ns	*
	PA14	ns	ns	**		ns	ns	**
	PAK	ns	ns	**		ns	ns	**
CM	PAO1	ns	ns	ns		ns	ns	ns
	PA14	ns	ns	ns		ns	ns	ns
	PAK	ns	ns	ns		ns	ns	ns
CWP	PAO1	ns	ns	ns		ns	ns	ns
	PA14	ns	*	*		ns	ns	ns
	PAK	ns	ns	**		ns	ns	ns

Direct binding of GA and LPS was further confirmed by Pierce™ Chromogenic Endotoxin Quant Kit which showed that there was less LPS available for detection when LPS was present, suggesting that the LPS was bound up by GA (**Figure 6-11**).

These results show direct interaction of GA with *P. aeruginosa* LPS and this may be, at least a partial, mechanism of action of GA. Targeting of LPS structures is a common feature of cationic AMPs either as a method of self-promoted uptake or to cause membrane disruption and cell death[138,188,198,224,317,351].

6.4.2 Gene expression stimulation: reference *P. aeruginosa*

With direct interaction of GA-LPS demonstrated, the ability of *P. aeruginosa* cells to modify their LPS structures in response to GA-attack was examined. Firstly, there was little evidence of constitutive expression of TCS genes or LPS modification genes in the ΔCt of reference strains (**Figures 6-12, 6-13 and 6-14**). In common with this, colistin was not

stimulatory of any of the strains, from the ΔΔCt results with a subinhibitory concentration no resulting in a >2-fold increase.

Treatment with 50mg/L GA was also not strongly stimulatory of expression of the response regulator genes of any of the TCS tested. None of the TCS genes measured crossed the 2-fold ΔΔCt increase in PAO1 and PAK (**Figures 6-15** and **6-17**). In PA14, there was a >2-fold increase in pmrA expression, after GA treatment, indicating detection of GA via the PA14 PmrAB TCS (**Figures 6-16**). There was also some indication of increased expression of the PhoPQ TCS in PA14 by GA, although not >2-fold ΔΔCt (1.92 [95%CI 0.78-1.99]). LL-37 stimulated upregulation of PhoPQ and PmrAB in both PAO1 and PAK.

In PAO1, GA stimulated the expression of the LPS modification system gene *arnB* but this was not seen for PA14 or PAK (**Figures 6-15**, **6-16** and **6-17**). Neither was colistin an effective stimulator of *arnB*. In contrast, LL-37 treatment resulted in >2-fold ΔΔCt of *arnB* for all reference strains (with the caveat that this was for 2 biological replicates rather than 3).

While there are some suggestions in these results that the TCSs involved in AMP sensing and response are capable of detecting GA in the reference *P. aeruginosa*, the results were comparable to those of colistin – an agent to which these strains are susceptible, administered here at a sub-inhibitory concentration. Therefore, it appears that for these strains, GA at the concentration tested may not provoke the strains to mount a strong response, which could potentially negate the antibiotic potentiation shown previously (**Chapter 3**).

6.4.3 Gene expression stimulation: clinical *P. aeruginosa*

The gene sequences of the sensor genes of TCSs of clinical *P. aeruginosa* strains did not reveal any specific SNPs leading to amino acid changes which have been previously

associated with AMP resistance in *P. aeruginosa*. This was to be expected with 10 of the 11 strains analysed here being colistin-sensitive according to clinical lab characterisation. An amino acid change was detected in the sequence of CprS (S173L) of the colistin-resistant strain, RBH422 (**Table 6-1**).

Notably, there was an accumulation of amino-acid changes in a single strain (RBH899), which accounted for half of all the amino acid changes seen in all the clinical strains examined. All of these changes in RBH899 were found in two genes – *pmrB* and *cprS* – and the changes seen were dispersed across the amino acid sequences. Interestingly, the amino acid changes found in the PmrB sequence of this strain (S2P, A4T, V15I and G68S) were also present in a *P. aeruginosa* clinical strains in South Korea which were colistin non-susceptible (in the terminology of that report) with colistin MICs up to 8mg/L[159]. Those South Korean strains also had additional amino acid substitutions in PmrB, not present in RBH899, and none of the substitutions in RBH899 were predicted to change protein function by Lee and Ko, 2014[159]. Therefore, their significance in the configuration in which they feature in RBH899 is not clear. This strain was not designated colistin resistant by clinical lab testing but further testing using broth microdilution would be of interest to test for the specific colistin MIC of this strain. This was also one of the strains to show the lowest levels of potentiation by GA for tobramycin and was not significantly disrupted in its outer membrane integrity or significantly permeabilised by GA.

Treatment with GA resulted in significant upregulation of TCS gene *pmrA* and LPS-modification gene *arnB* in clinical *P. aeruginosa*. While AMPs have been shown to stimulate a variety of TCS and provide different modification outcomes, the connection between the

PmrAB system and LPS modification by ArnBCADTEF by addition of aminoarabinose to Lipid A, is a well-established pathway in the literature[33,158,180,190,195,196].

The ΔΔCt results for clinical strains did not reveal a strong overall response by the strains to GA stimulation. Summary data for the strains showed that no gene tested had a median ΔΔCt >2-fold, including the two genes which were significantly upregulated in ΔCt analyses. In fact, median results for three of the genes measured – *phoP*, *cprR* and *parR* – had ΔΔCt of ~1, meaning no change had occurred in expression levels from untreated to GA-treated across the group.

There were individual exceptions to this grouped summary. As with other results, *pmrA* was the most stimulated by GA on a strain-by-strain basis, as it had the greatest number of clinical strains with ΔΔCt >2-fold increased. However, this was still only 2 strains (RBH750 and RBH294). One strain each was stimulated by GA for *phoP* (RBH490) and *cprR* (RBH065) expression. This could mean that detection of GA by clinical strains is diffuse in nature, spread across the TCSs in each strain, or it may be that different clinical strains are reliant on different TCS to different extents for environmental sensing of AMPs.

Absent from these results is colistin-resistant strain RBH422 which did not show significant constitutive expression or a significant response to GA for any of the gene expression results here. This indicates that colistin resistance in this strain is mediated by another mechanism, not identified in the expression and genomic data analysed here and/or that GA detection and response is via a different mechanism to its colistin resistance.

An alternative explanation is that GA at 50mg/L is not exerting enough pressure on the bacteria to warrant the cells mounting a response to it. As has been shown, the killing effect of GA against *P. aeruginosa* is limited at best; no absolute MIC against reference strains

(**Figure 3-1**), short-term but limited cell death (**Figures 5-2 and 5-15**), variable levels of outer membrane disruption (**Figure 5-16**). This would be to the advantage of GA as a resistance breaker in the short term, with little defence mounted by treated cells, and in the long term, with GA exerting little evolutionary pressure to drive resistance development.

6.4.4 *arnB* expression

In clinical *P. aeruginosa* strains, expression of the LPS-modification cassette gene, *arnB*, showed the greatest stimulation by GA of any of the genes tested here. This was the case both in terms of ΔCt (**Figure 6-22**), where GA resulted in a significant increase in expression across the 11 strains, and in terms of ΔΔCt (**Figure 6-23**), where 4 individual strains had values >2-fold higher after GA treatment.

Of the 4 strains with increased ΔΔCt, 2 also showed increased ΔΔCt for expression of *pmrA* under GA stimulation (RBH750 and RBH294) while the other 2 (RBH550 and RBH519) did not have a corresponding gene in TCSs with ΔΔCt >2. This may indicate that cross-talk or signal accumulation across the TCSs results in *arnB* upregulation (as hinted at by correlation analyses [(**Table 6-3**]) or that GA being sensed by an alternative mechanism from those tested here, such as CoIRS[193,194].

Of the remaining strains which had shown TCS gene upregulation in response to GA (RBH490 for *phoP* and RBH065 for *cprR*) neither had a comparable increase in *arnB* expression. RBH490 had also shown constitutive *phoP* expression. However, there is no indication in this expression data of where these signals are transmitted.

6.4.5 *pmrA* and *arnB*

The data for clinical *P. aeruginosa* gives some indications of a link between the expression of these genes under GA stimulation. Both genes were the only ones tested to be

significantly upregulated by GA treatment via ΔCt, they were the two genes with the most individual strains to have ΔΔCt >2-fold (albeit small in number) and the two strains with >2 ΔΔCt for *pmrA* also had ΔΔCt >2 for *arnB*. Analysis of the ΔΔCt of the expression of the two genes showed they were positively correlated together (Spearman r of 0.80 [95%CI 0.37-0.95], p=0.005) (**Figure 6-24**).

6.4.6 LPS modification

GA treatment did not result in significant changes to the Lipid A portion of LPS by addition of L-Ara4N. An increase was seen in the PRR of the clinical strains – from 0.024 [95%CI 0.0-1.9] to 0.24 [95%CI 0.0-1.0] – after treatment with GA, however, individual clinical strains had a decrease in PRR after GA stimulation. This analysis only examined modification by L-Ara4N and, therefore, GA stimulation may result in different types of modification not examined here.

6.4.7 Limitations of approach

Testing the effects of divalent cations and LPS in isolation from each other will not give an indication of their effect in a complex environment such as the CF lung, where they will be present with each other as well as with many other components. For this reason, a similar approach to that suggested previously with use of CF sputum or a CF sputum proxy media, would be beneficial.

For gene expression and LPS modification studies, testing the effects of GA treatment on a strains which had confirmed resistance to other AMPs via these pathways would be of interest.

6.4.8 Future directions

The results presented here show that GA was not strongly stimulatory of the commonly reported AMP resistance mechanism of LPS modification with L-Ara4N via TCS sensing pathways. The implications of GA exposure over longer periods of exposure would be valuable to study. While this may difficult for GA alone – as it does not have a conventional MIC – it would be of particular interest to see the effect of combination GA/TOB and compare that with TOB on the development of resistance over passage in an experimental evolution study.

6.4.9 Discussion of hypothesis

The results presented here show that GA and *P. aeruginosa* LPS do interact. There was bilateral sequestration of the GA-LPS; GA was less active after contact with LPS and LPS was less detectable after contact with GA. There were moderate, gene specific levels of gene expression stimulation of TCS in response to GA treatment in both reference and clinical strains. As with gene expression, moderate (but not significant) changes were seen in LPS modification by L-Ara4N were seen after GA stimulation.

7. Interactions of glatiramer acetate with elements of the cystic fibrosis lung

7.1 Introduction

The sputum of people with cystic fibrosis is a complex, heterogenous environment and this complexity has implications for both the infectious bacteria within and for treatment strategies to eradicate them. One of the most important markers of the switch of *P. aeruginosa* from acute to chronic infections of the CF lung is the mucoid phenotype; an increase in the production of exopolysaccharides PSL, PEL and alginate[353]. These polysaccharides are key components of the *P. aeruginosa* biofilm providing the matrix in which the biofilm forms and the barrier function of biofilm[120,331,354].

P. aeruginosa in biofilms can have orders of magnitude greater resistance to antibiotic treatments than free-living, planktonic cells as a function of both the physical barrier the biofilm provides and the slowed metabolism of biofilm embedded cells. Cells aggregated together in exopolysaccharides in the CF lung are more resistant to treatments[126,355].

Extracellular DNA (eDNA) is important in establishment and maintenance of *P. aeruginosa* biofilms[127,128,130,356]. eDNA has also been implicated in *P. aeruginosa* resistance to AMPs[129]. Presence of DNA within CF sputum can be of utility to *P. aeruginosa* and may be present as a result of *P. aeruginosa*: due the increased bacterial load, due to DNA release by *P. aeruginosa* as part of 'community goods'[357] and as a component of neutrophil extracellular traps (NETs) stimulated by *P. aeruginosa*[328,358]. In CF sputum eDNA has been reported to be present at concentrations up to 9.5mg/mL[307].

Unsurprisingly in an environment characterised by acute and chronic bacterial infection, inflammation plays a large role in CF lung disease with the aggressive inflammatory response to infection contributing to progressive bronchiectasis[60–62,359,360]. It is debated whether inflammation is pathological to the CFTR defect or comes about as a result of

infection but what is clear is that CF airways are chronically inflamed. Neutrophils are characteristic of inflammation in the CF airway and are central to the excessive inflammatory response, both causing damage and being pro-inflammatory themselves[359]. Neutrophils produces extracellular traps (NETs) which involves extrusion by the cells of DNA, AMPs, proteases and more[358]. NETs are stimulated by the presence of *P. aeruginosa* in CF, facilitating a cycle of infection and inflammation – NETs stimulated in response to *P. aeruginosa* infection release goods which are beneficial to *P. aeruginosa* survival which trigger NETs[328].

Pro-inflammatory cytokines interleukins -6 and -8 (IL-6 and IL-8) are both found in the CF lung and both have been shown to be produced by epithelial cells, as well as by neutrophils and macrophages[361]. Both cytokines have been implicated in the production and chemotaxis of neutrophils, the driver of inflammation in CF[359–361].

While it is not possible to capture the full complexity of the CF sputum environment as *P. aeruginosa* encounter it in vitro, it is important to examine aspects of it with an eye towards the utility of GA in the clinic.

7.2 Hypothesis

I hypothesise that:

Elements of the CF airway will interact with GA either interfering with its antimicrobial activity or leading to a host inflammatory response

7.2.1 Aims

To investigate this hypothesis, I will:

- Assess the impact of GA on *P. aeruginosa* biofilms
- Test the effect of eDNA on the activity of GA
- Assess the impact of GA on paired epithelial cell lines

7.3 Results

7.3.1 Biofilm

The ability of GA to prevent and disrupt biofilms of *P. aeruginosa* PAO1, PA14 and PAK was examined using crystal violet assays. To test whether GA could prevent biofilm formation, strains were primed with 50mg/L and GA removed before the static assay or incubated with 50mg/L GA statically for 24hrs. To test for disruption of mature biofilm, 24 or 48hr biofilms were treated with 50mg/L GA.

7.3.1.1 24 hour aerobic biofilms

Presence of 50mg/L GA for the duration of the static incubation period resulted in significantly less biofilm being formed by PAO1, in a multiple comparison, compared with 24hr biofilms with No Treatment (p=0.0279) (**Figure 7-1**). Neither priming with GA nor administration of GA against mature biofilms for 1hr resulted in significantly changed PAO1 biofilms (**Figure 7-1**).

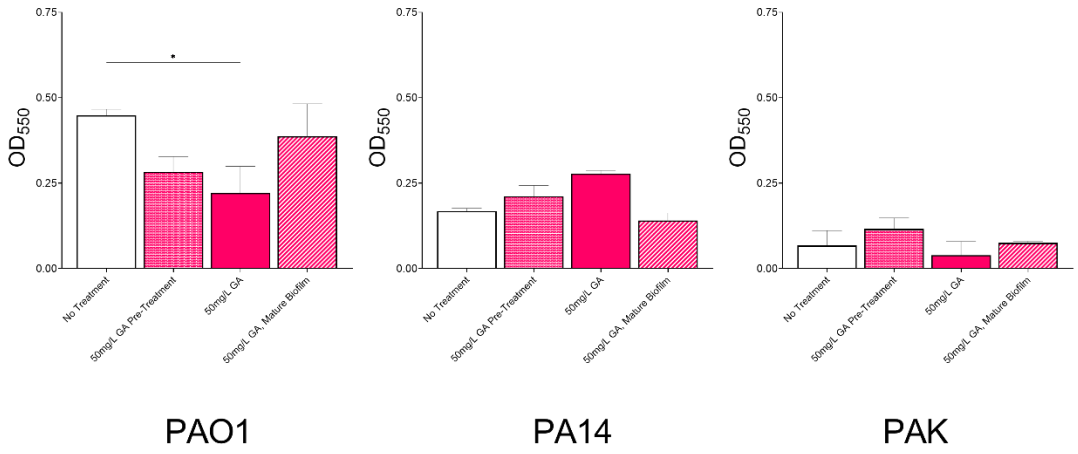


Figure 7-1. Biofilm production measured by crystal violet of P. aeruginosa PAO1, PA14 and PAK after 24hrs in aerobic conditions. Strains were either pre-treated with 50mg/L GA followed by incubation, incubated with 50mg/L GA for the duration or the experiment or had 50mg/L GA administered to mature 24hr biofilms. PAO1 incubated with 50mg/L GA for the duration of the experiment formed significantly less biofilm than No Treatment biofilm over 24hrs (p=0.0279). No other strain-condition combination tested significantly altered biofilm production. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Bar are medians of biological replicates with 95% confidence intervals.

Strains PA14 and PAK were not impacted in their ability to form biofilm, with none of the conditions tested resulting in a significant change from No Treatment biofilms (**Figure 7-1**).

7.3.1.2 24 hour anaerobic biofilms

Under anaerobic conditions, treatment with 50mg/L GA for the duration of the experiment resulted in significantly less biofilm by strain PAK, from No Treatment anaerobic PAK biofilms (p=0.0484) (**Figure 7-2**). The remaining conditions tested for PAK did not alter biofilm formation during anaerobic incubation and no differences were seen in either PAO1 or PA14.

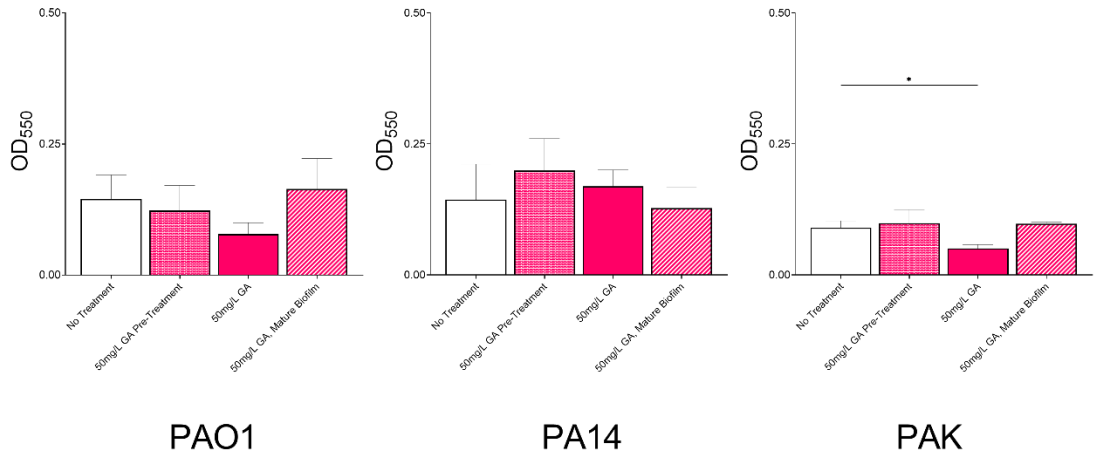


Figure 7-2. Biofilm production measured by crystal violet of P. aeruginosa PAO1, PA14 and PAK after 24hrs in anaerobic conditions. Strains were either pre-treated with 50mg/L GA followed by incubation, incubated with 50mg/L GA for the duration or the experiment or had 50mg/L GA administered to mature 24hr biofilms. PAK incubated with 50mg/L GA for the duration of the experiment formed significantly less biofilm than No Treatment biofilm over 24hrs (p=0.0484). No other strain-condition combination tested significantly altered biofilm production. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Bar are medians of biological replicates with 95% confidence intervals.

7.3.1.3 48 hour biofilms

Strains PAO1 and PA14 were also tested for GA's preventative and disruptive effects over 48hrs and no significant differences were seen for any variation of GA tested, when compared to untreated biofilms of each strain (**Figure 7-3**).

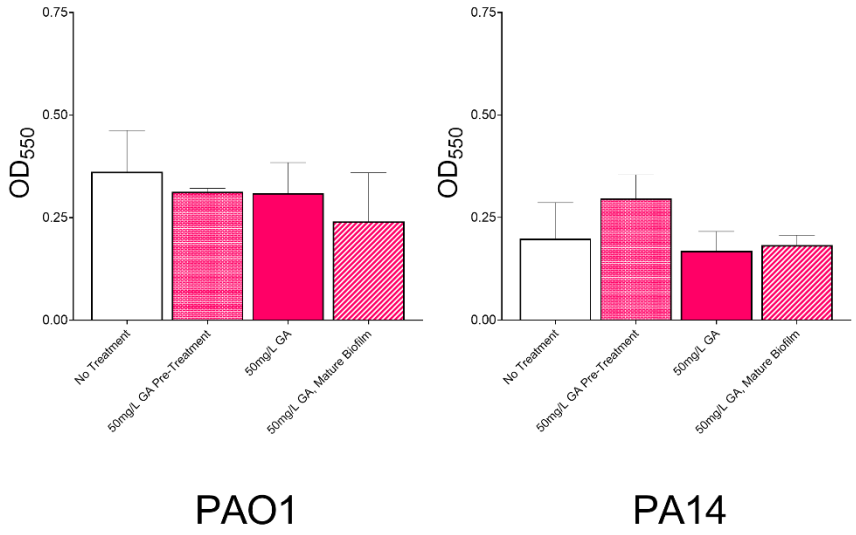


Figure 7-3. Biofilm production measured by crystal violet of P. aeruginosa PAO1, PA14 and PAK after 48hrs in aerobic conditions. Strains were either pre-treated with 50mg/L GA followed by incubation, incubated with 50mg/L GA for the duration or the experiment or had 50mg/L GA administered to mature 48hr biofilms. No strain-condition combination tested significantly altered biofilm production. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Bar are medians of biological replicates with 95% confidence intervals.

7.3.2 eDNA

7.3.2.1 Outer bacterial membrane

No differences were seen between uptake of NPN by strains in No Treatment suspensions, whether or not 1mg/mL eDNA was present indicating no effect on the strains or on the assay.

For the 3 reference strains, there was no significant difference in the GA-induced NPN Uptake Factor for GA which had been incubated with 1mg/mL eDNA compared to GA-only (**Figure 7-4**).

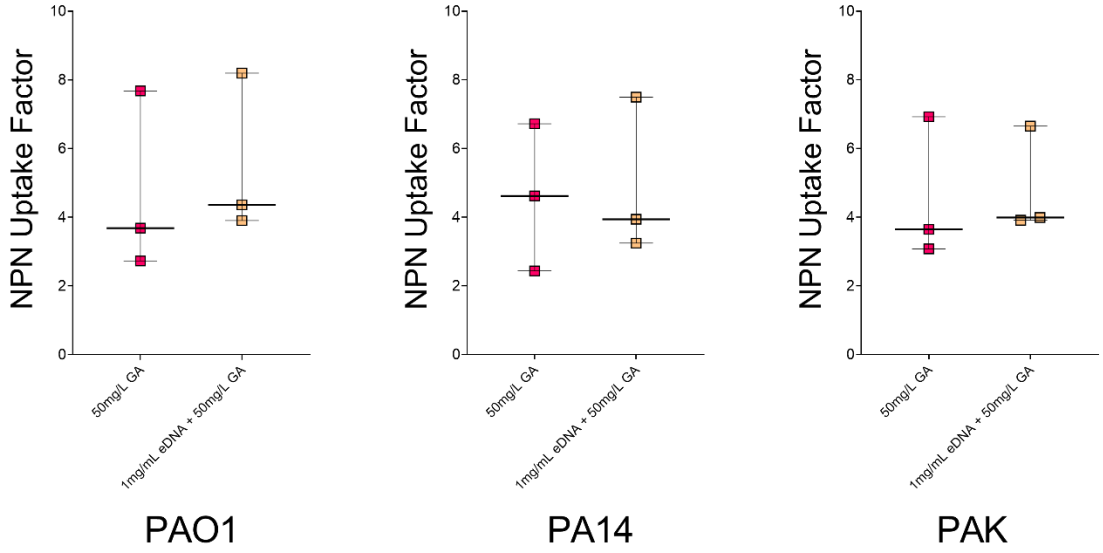


Figure 7-4. Mean NPN Uptake Factor of P. aeruginosa PAO1, PA14 and PAK over 15min exposure to 50mg/L GA pre-incubated with 1mg/mL eDNA. No significant impact on GA activity for any of the three reference strains was seen. Mann-Whitney U test. Points are biological replicates with line at median and 95% confidence intervals.

7.3.2.2 Bacterial cytoplasmic membrane

Pre-incubation with eDNA resulted in no significant difference between GA-treated suspensions, with vs without eDNA, for any of the reference strains comparing ratios of $DiSC_3(5)$ in GA Treatment:No Treatment for biological replicates (**Figure 7-5**). This indicates that pre-incubation with 1mg/mL eDNA did not significantly alter GA's activity against *P. aeruginosa* cytoplasmic membranes.

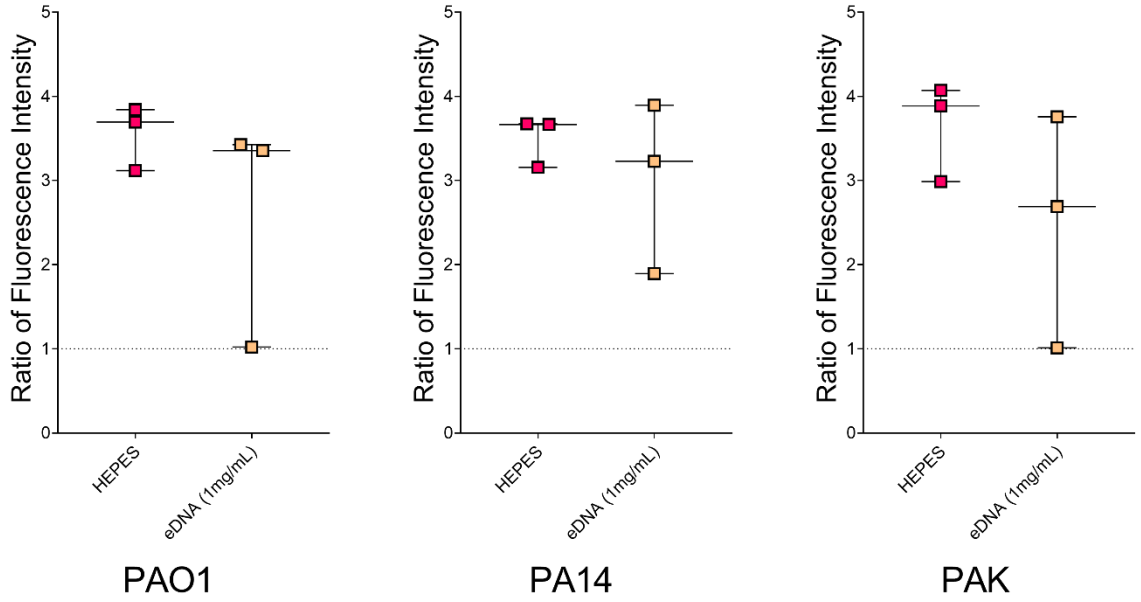


Figure 7-5. Mean ratio of $DiSC_3(5)$ fluorescence over 15mins for 50mg/L GA Treatment:No Treatment suspensions of P. aeruginosa strains PAO1, PA14 and PAK after pre-incubation of GA with 1mg/L eDNA. No significant impact on GA activity for any of the three reference strains was seen. Mann-Whitney U test. Points are biological replicates with line at median and 95% confidence intervals.

7.3.2.3 Gel Retardation Assay

At 1mg/mL DNA, DNA had not migrated along the gel at any concentration of GA tested indicting that all these GA concentrations had bound the available DNA (**Figure 7-6, Table 7-3**). At 10mg/mL DNA, DNA that had been incubated with the lowest concentration (6.25mg/L) GA was capable of migrating down the gel indicating that GA at this concentration had not bound all the available DNA. Between 12.5 and 100mg/L GA, DNA migration was not apparent for 10mg/mL DNA, indicating that GA at these concentrations had bound the available DNA. Finally, at the supra-physiological concentration of 50mg/mL DNA, migration was apparent on the gel at 6.25 and 12.5mg/L GA and partially visible at 25mg/L indicating that GA has been entirely or almost entirely bound up by DNA at these concentration

combinations. Fifty milligrams per millilitre DNA was prevented from migrating in the gel by both 50 and 100mg/L GA (**Figure 7-6, Table 7-1**).

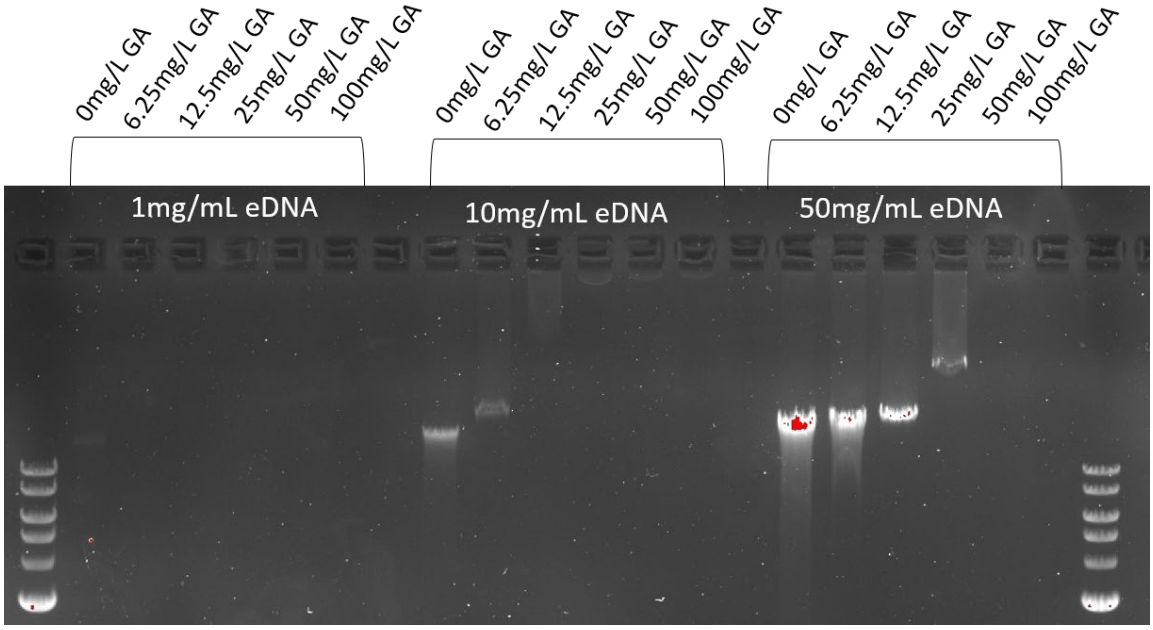


Figure 7-6. Gel retardation assay for binding of GA to DNA. DNA at concentrations of 1, 10 and 50mg/mL were incubated with GA in the range 6.25-100mg/L and migrated along a 1% agarose gel. DNA at 1mg/mL was prevented from migrating by all concentrations of GA tested. DNA at 10mg/mL, was prevented from migrating by GA in the range 12.5-100mg/L GA but not by 6.25mg/L GA. DNA at 50mg/mL migrated along the gel in the presence of 6.25 and 12.5mg/L GA. Partial migration was also seen at 25mg/L GA. With 50 and 100mg/L GA prevented 50mg/mL DNA from migrating along the gel.

Table 7-1. Results of gel retardation assay for chequerboard for GA in the range 6.25-100mg/L and eDNA in the range 1-50mg/mL. X indicates GA bound and aggregated the eDNA, preventing migration down the gel. There was partial migration at 25mg/L GA/50mg/mL eDNA.

	1mg/mL eDNA	10mg/mL eDNA	50mg/mL eDNA
0mg/L GA			
6.25mg/L GA	X		
12.5mg/L GA	X	X	
25mg/L GA	X	X	x
50mg/L GA	X	X	X
100mg/L GA	X	X	X

7.3.3 Effect of GA on the production of cytokines by epithelial cells

As a drug whose function is immunomodulation but is incompletely understood, GA was examined at increasing concentrations for the ability to stimulate cytokine production by paired human bronchial epithelial cell lines (CFBE41o-), expressing either wild type (WT) or Phe508del CFTR.

7.3.3.1 IL-6 production

In WT CFTR expressing cells, only stimulation with positive control TNFα resulted in a significant increase in IL-6 production, compared to untreated cells (p=0.0081). None of the concentrations of GA tested (6.25-100mg/L) nor CST (2mg/L) resulted in a change in IL-6 levels in these cells (**Figure 7-7**).

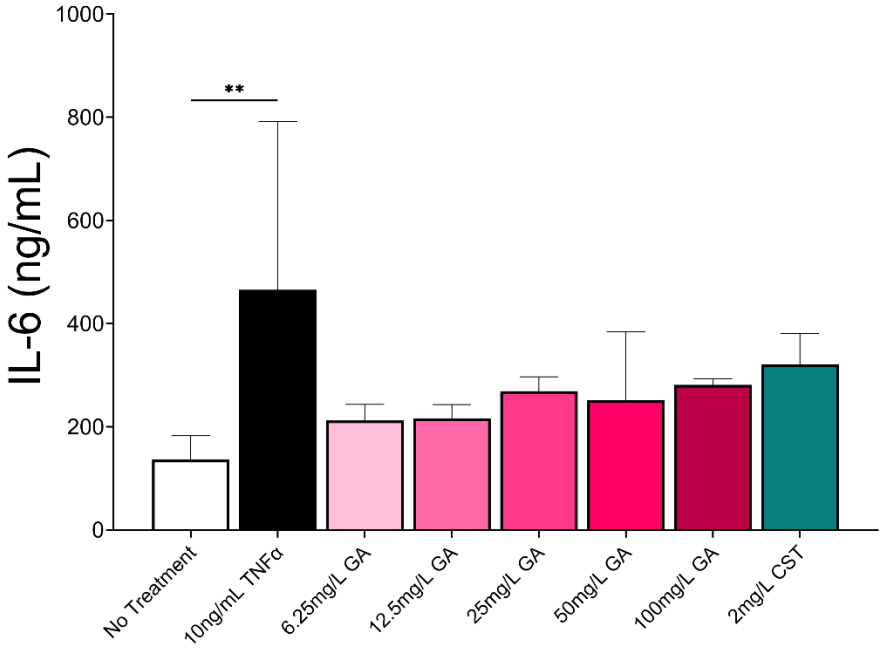


Figure 7-7. Production of IL-6 by CFBE41o- epithelial cells expressing Wild Type CFTR after exposure to GA in the range 6.25-100mg/L along with the AMP CST (2mg/L) and TNFα (10ng/mL) used as a positive control for cell stimulation. Positive control TNFα was the only condition tested to significantly increase IL-6 production by CFBE41o- cells compared with unstimulated cells (p=0.0081). Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Bars are medians of biological replicates with 95% confidence intervals.

Production of IL-6 by cells expressing Phe508del CFTR was lower in untreated cells (57.11 [95%CI 16.61-58.97] ng/mL) than in WT expressing cells under the same conditions (136.4 [95%CI 121.4-182.9] ng/mL). None of the treatments tested resulted in a change in IL-6 production by Phe508del expressing cells; the TNFα positive control response did not reach statistical significance (**Figure 7-8**).

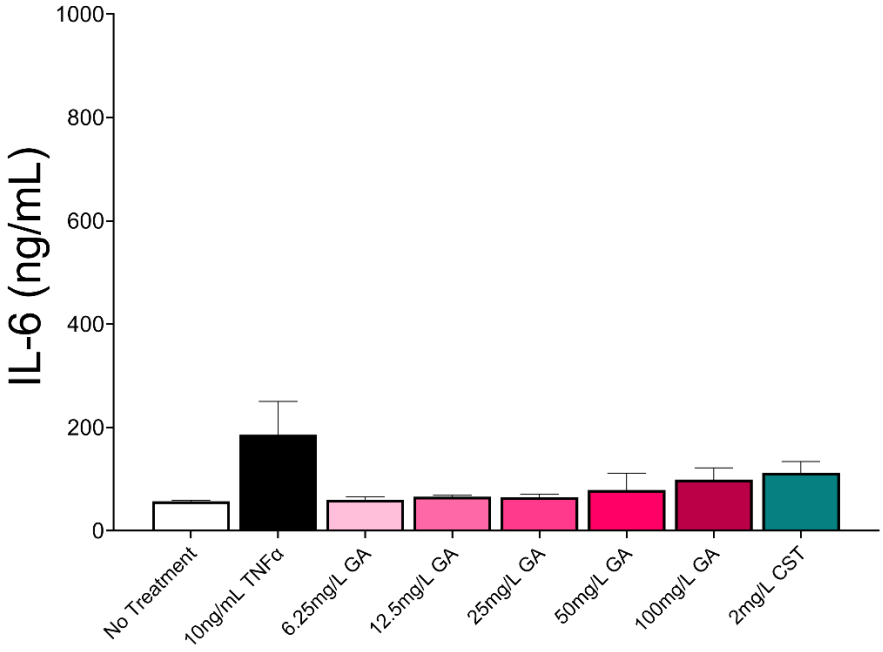


Figure 7-8. Production of IL-6 by CFBE41o- epithelial cells expressing Phe508del CFTR after exposure to GA in the range 6.25-100mg/L along with the AMP CST (2mg/L) and TNFα (10ng/mL) used as a positive control for cell stimulation. None if the conditions tested significantly increased IL-6 production by CFBE41o- cells compared with unstimulated cells. Kruskal-Wallis test with Dunn's multiple correction (adjuted p values). Bars are medians of biological replicates with 95% confidence intervals.

7.3.3.2 IL-8 production

In WT expressing cells, IL-8 production in untreated cells was below the lower limit of detection (31.25ng/mL) for all three biological replicates (**Figure 7-9**). The highest IL-8

concentration was produced during treatment with positive control TNFα (88.12 [95%CI 48.46-150.5] ng/mL) and was measurable in all biological replicates. The only other conditions which produced measurable IL-8 in three biological replicates were GA at 25mg/L (30.11 [95%CI 121.88-54.15] ng/mL), 50mg/L (52.84 [95%CI 47.69-107.0] ng/mL) and 100mg/L (47.99 [95%CI 30.54-179.6] ng/mL).

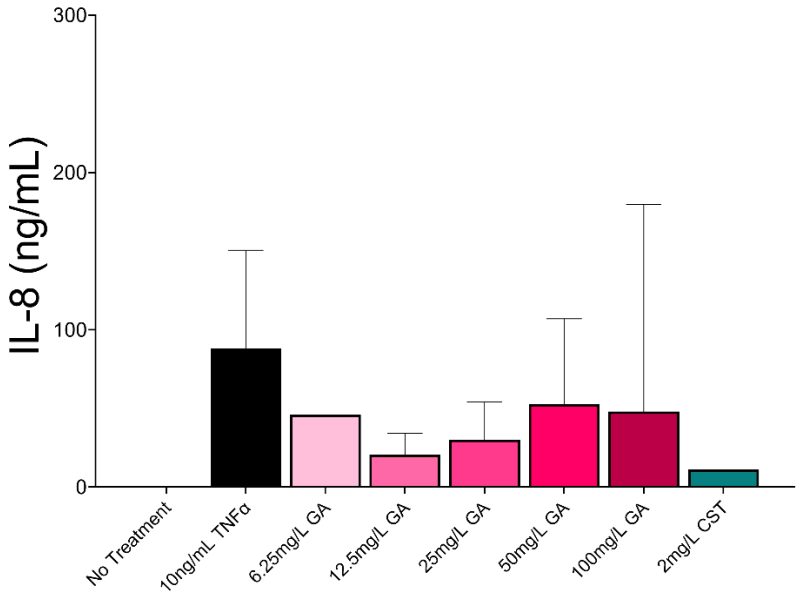


Figure 7-9. Production of IL-8 by CFBE41o- epithelial cells expressing Wild Type CFTR after exposure to GA in the range 6.25-100mg/L along with the AMP CST (2mg/L) and TNFα (10ng/mL) used as a positive control for cell stimulation. IL-8 production by cells under No Treatment was below the lower limit of detection of the assay. No variance was seen across the remaining conditions tested. Kruskal-Wallis test with Dunn's mutlipte correction (adjuted p values). Bars are medians of biological replicates with 95% confidence intervals.

In Phe508del expressing epithelial cells, IL-8 was below the lower limit of detection, regardless of stimulation (**Figure 7-9**).

7.4 Discussion

In this Chapter I have shown that GA is not an effective biofilm disruptor against reference strains of *P. aeruginosa* using a variety of experimental conditions. I have shown that eDNA does not prevent GA from acting on the membranes of *P. aeruginosa*, at a concentration of eDNA physiologically relevant to the CF lung. I have shown that GA aggregates eDNA in a dose-dependent fashion. I have shown that GA does not stimulate cytokine production by epithelial cells, expressing either Wild Type or Phe508del CFTR.

The CF lung is a complex and heterogenous environment and one of the reasons for chronic bacterial infections is the protections afforded bacteria by the lung itself, as well as the abilities of bacteria to exploit that environment. Cycles of host-pathogen interactions/behaviors are involved in driving chronicity. For example, NETs are part of the lung immune response to infection and DNA is a component of NETs[358]. Release of NETs in the lung increases the DNA content of the sputum. *P. aeruginosa* in the CF lung stimulate the release of NETs[328]. Extracellular DNA is a crucial part of the formation and maintenance of *P. aeruginosa* biofilms[127]. Biofilms allow *P. aeruginosa* to evade eradication immune systems and antibiotics. Persistence of *P. aeruginosa* induces immune responses including NETs.

These kinds of complex interactions and cycles made it important to assess some of the impacts of GA on the conditions found in CF lung. While it must recognised that studying some of these elements in isolation and in vitro only gives a glimpse into potential impacts in the CF lung, testing these conditions may give greater insights into both mechanisms of GA activity and potential confounders of that activity.

7.4.1 Biofilm

GA was not an effective against *P. aeruginosa* biofilms. GA did not prevent biofilm formation or disrupt mature biofilms, under anerobic and anerobic conditions, after 24 and 48 hours. The only exception was a reduction in biofilm by strain PAO1 formed over 24hrs when GA was present during biofilm formation (**Figure 7-1**).

These results were not unexpected given the previous results showing that GA is not inhibitory to *P. aeruginosa* when the bacteria are actively growing over a prolonged incubation period (**Chapter 3**). In addition to this, the medium used for the crystal violet biofilm assay is a defined medium containing 1mM $MgSO_4$, which was shown in **Chapter 5** to be protective against some aspects of GA's mechanisms of action. While growth in the supplemented M9 medium is likely to be slower than that in MHB, as used in earlier experiments when growth parameters during GA-treatment were measured (**Figure 3-2**), active cell division is still taking place.

These experiments were carried out using a single concentration of GA (50mg/L) and it would be of interest to see whether or not there is in a Minimum Biofilm Inhibitory Concentration (MBIC) of GA against *P. aeruginosa*, in contrast with the lack of MIC (**Chapter 3**). Antibiotic concentrations required to be effective against biofilms are higher than those for planktonically grown *P. aeruginosa* and investigating the ability of GA to synergise with tobramycin against *P. aeruginosa* biofilm, as it does with planktonic bacteria (**Chapter 3**) would be highly relevant to the CF lung environment.

The ability to aggregate, communicate and organise into biofilms is an important aspect of *P. aeruginosa* colonising the CF lung environment, avoid eradication and survive antibiotic treatments.

7.4.2 eDNA

Extracellular DNA is an important constituent of the CF lung, both human and bacterial, with implications for biofilm formation[127,130], AMP resistance[129], divalent cation availability[362], among others. Binding to DNA has been shown to be the mechanism of action of some AMPs[225] and there were indications from previous work that GA can aggregate the intracellular contents of *P. aeruginosa* cells[287]. Here, the prevention of DNA migration across an agarose gel conclusively shows that GA is capable of binding and aggregating DNA (**Figure 7-6**). This was in a dose-dependent manner; all GA concentrations tested, down to 6.25mg/L, were capable of aggregating all DNA at 1mg/mL, at 10mg/mL this only occurred with ≥12.5mg/L GA and at 50mg/mL DNA at ≥25mg/L GA.

That 10mg/mL DNA can still migrate at 6.25mg/L GA but not 12.5mg/L indicates that GA is sequestered at the lower concentration but may still be, at least partially, available at 12.5mg/L and higher. Since 10mg/mL is the upper biologically relevant eDNA concentration of the CF lung[307], the implication is that the best studied GA dose here (50mg/L) would not be rendered unavailable by eDNA in CF lung conditions. Optimistically, it might also be suggested that sequestration in the other direction (of DNA by GA) may be of some benefit, reducing DNA availability and some of its more detrimental properties.

At a lower, but still physiologically relevant CF lung concentration of 1mg/mL eDNA[307], membrane integrity assays showed that any binding of 50mg/L GA was not detrimental to the activity of GA. GA was as effective an outer membrane disruptor and cytoplasmic membrane depolariser after incubation with this concentration of DNA as it was without (**Figures 7-4** and **7-5**). It would also be of interest to test whether this is also the case for permeabilisation by

GA, but this results show that GA activity would not be confounded by the eDNA content of the CF lung.

These results may also go to explain the relative lack of impact of GA during co-treatment experiments with the fluoroquinolone ciprofloxacin (**Chapter 3**). Ciprofloxacin targets processes involved in DNA replication by *P. aeruginosa*, preventing uncoiling of DNA and therefore it's replication and division. Binding of GA to intracellular DNA would potentially have a similar impact and negating any synergistic effects, if the two agents were to target separate cellular processes.

7.4.3 Cytokines

It has been shown previously in an animal model that nebulised GA does not result in any adverse effects for the animal including no increase in inflammation[290]. With inflammation playing such an important part in the progress of CF lung disease[359,360], it was important to test whether this observation was applicable the CF lung, in vitro, testing the likelihood of GA adding to the inflammatory burden already present.

GA is not a significant stimulator of pro-inflammatory cytokines in epithelial cells. Up to the highest concentration of GA tested (100mg/L), there was no increase in IL-6 or IL-8 production by epithelial cells expressing Wild Type or Phe508del CFTR. The activity levels of CFTR in these paired cells was not confirmed in this study. Also confirmation of the viability of the cells post-treatment (with resazurin or XTT for example) would have been of interest.

GA is a known immunomodulator with most of the research into its mechanisms of immunomodulation indicating that it reduces the immune response[282]. The previous report from nebulised GA in the pig lung and the absence of a pro-inflammatory response here are encouraging indicators that administration of GA as an inhaled therapy against *P. aeruginosa*

in CF would not be detrimental. Indeed, in the case of the CF lung, where inflammation plays a large role in the progress of disease, the anti-inflammatory properties described for GA to-date may be additionally beneficial, particularly the observation that macrophages produce more anti-inflammatory cytokines after GA treatment[280].

7.4.4 Limitations of approach

Testing of both a greater concentration range of GA as well as combination effects of GA with antibiotics for biofilm eradication, rather than a single concentration would be of interest, given the knowledge of lack of efficacy of 50mg/L GA against planktonic growth.

As with previous observations about the testing of sputum components in isolation, eDNA in combination with other sputum components should be performed.

Cytokine measurement experiments would benefit from the confirmation and/normalisation of the cytokine signals to the level of epithelial cells in the wells, post-treatment. Density of cells could be measured using crystal violet or viability of cells via resazurin.

7.4.5 Future directions

The encouraging results here, that there was little stimulation of pro-inflammatory response in epithelial cells by GA, could be followed by transferring GA/TOB treatment of *P. aeruginosa* into an animal model such as a mouse model. This would allow determination of whether the combination retains the observed synergy in an *in vivo* context while testing for the immunogenicity of GA in that model.

7.4.6 Discussion of hypothesis

Reactivity of GA with elements of the CF lung were minimal. GA did not have a strong impact on *P. aeruginosa* biofilms. While GA does have the ability to bind to eDNA and aggregate it, indications are that the combination of a physiologically relevant DNA concentration with the effective concentration of GA (both in activity and synergy) against *P. aeruginosa* was not detrimental to GA activity. Finally GA was not pro-inflammatory, *in vitro*, for epithelial cells including those with a CF phenotype.

8. Conclusions

The desperate need for new antimicrobial treatments for infectious bacteria is one of the most pressing needs of our time[113,252,253,363–365]. The lack of investment in traditional antibiotic development paths due to the reduction in numbers of large pharmaceutical companies, and resultant reduction in Research and Development, as well as due to the shift in focus towards 'blockbuster' drugs, has meant an overreliance on antibiotic drugs which have been in use for many decades.

Glatiramer acetate fulfills many of the desired qualities for a new treatment strategy to combat the antibiotic resistance crisis: a drug already approved and in use and with a long safety profile[289], a structure which resembles antimicrobial peptides[277,278] and antibacterial activity[287]. It is a cationic, amphipathic peptide, both common features of natural and synthetic AMPs[277]. In contrast with many AMPs, GA is not cytotoxic but rather has a long safety history with few side-effects[289]. Its AMP-like properties translated into a moderate level of antimicrobial activity against *P. aeruginosa* in previous work[287].

The work carried out here shows that GA is an antibiotic resistance breaker against reference *P. aeruginosa* and clinical *P. aeruginosa* from people with CF. The concentrations of the aminoglycoside tobramycin required to be effective in the treatment of *P. aeruginosa* were reduced by GA. GA and tobramycin also synergise together in their activity against *P. aeruginosa* strains. This was the case for all reference strains tested and the majority of clinical strains tested. There was evidence of improved efficacy of tobramycin when given in combination with GA for all experiment types performed: growth curves, colony counts, MIC calculations and synergy analysis. Crucially, in the clinical strains where synergy was not seen, no antagonism was seen.

There was some indication of synergy between GA and ceftazidime but only in a single reference strain and further work would be required to clarify this combination effect – more strains and a more balanced approach to replicates. There was little evidence of improvement of the actions of ciprofloxacin or colistin by GA (synergy was indicated for a single PAO1 concentration but at a relatively ineffective concentration of the antibiotic) and evidence here would suggest some cross-over in the mechanisms of action of GA with each, negating synergy through facilitation of each other's activity. In common with colistin[224], GA is able to bind *P. aeruginosa* LPS and disrupt outer bacterial membranes and, in common with ciprofloxacin[366], GA can aggregate DNA/prevent DNA uncoiling. However, antagonism in overnight colony counts was not seen despite these common properties.

Calculating precise MICs for strains via colony counts is not a common method of generating MIC data, compared with broth microdilution for example, but it has allowed a more granular approach to testing the combination therapies. It also allowed for generation of two metrics of MIC (MIC_{50} and MIC_{90}) for each strain giving an intra-strain comparator. While this methodology is much more labourious than others, and therefore limited the number of clinical strains that could reasonably be tested, it allowed a greater appreciation of the changes brought about by GA in combination with tobramycin, some of which may have been missed in other assays.

This approach also has the advantage of showing that the tobramycin MIC changes resulting from GA co-treatment were not limited to dramatic shifts, for strains with already high MICs, but were the case for sensitive strains or narrower changes too. Strains which are already sensitive to an antibiotic are often neglected in these types of studies in favour multidrug resistant examples or in favour of resistant strains crossing EUCAST breakpoint

thresholds. That the advantages seen for GA co-treatment were applicable across the susceptibly spectrum, and applicable to a broad swathe of clinical *P. aeruginosa*, is of interest. The results here would be complemented by a broader but shallower look at GA-tobramycin effect on susceptibility in clinical *P. aeruginosa* – using a much larger panel of strains with a broth microdilution assay, for example.

The activity of combination treatments against sensitive, as well as resistant, strains is also of interest in potentially reducing antibiotic doses or duration of treatments regardless of strain phenotype. In people with CF, tobramycin has been associated with both ototoxicity[100] and nephrotoxicity[98] and, therefore, that the resistance breaking properties of GA being applicable across the tobramycin susceptibility spectrum is of great interest.

Given that this a project which seeks to investigate an agent as a potential solution to the antibiotic resistance crisis, future work on the development of resistance to the actions of GA on *P. aeruginosa* are required. AMPs are not thought to be strongly mutagenic for bacteria due to their rapid activity and work has shown that AMPs do not increase recombination rates in bacteria[367]. AMPs can alter non-heritable resistance[368] however but given that GA is non-cidal over long exposures it would be interesting to test for resistance development. Similarly, it would be of interest to test whether GA given in combination with tobramycin slows or stops the evolution of tobramycin resistance.

It is apparent from all the results presented here that GA is, in common with many AMPs[203,205,369–371], an outer membrane disrupting agent and takes advantage of it cationic and amphipathic properties to bind to the outer membrane of *P. aeruginosa* (possibly via Lipid A of LPS), insert itself and fatally disrupt the membrane, resulting in uncapturably rapid cell death. As Boman said in 1995[340], AMPs;

"lyse bacteria, sometimes so quickly that it is technically difficult to assess whether there are steps preceding cell lysis"

GA certainly lives up to this description. The specificity of killing by GA at 50mg/L, which is absent at both higher (100mg/L) and lower (25mg/L), suggests that cell death may require the accumulation of a specific level of charge on the cell surface, rather than a concentration of the peptide. This has been reported as a property of other cationic AMPs[372]. It may also be a factor of the ability of GA to oligomerise and to have different conformations in solution[277,278].

GA was potent in its ability to significantly increase damage to both Gram-negative membranes and to permeabilise the cells (with the exception of PAO1 permeability) in reference strains. It was the only antimicrobial tested here which significantly altered all three metrics across the reference strains, including the potent membrane active AMPs colistin and LL-37 at MIC concentrations.

The cell wall is the major barrier that must be overcome for most antibiotics to reach their targets and kill bacteria or prevent replication. The ability of GA to interact with and perturb the bacterial membranes, combined with the observed potentiation of antibiotics in combination with GA (particularly tobramycin) would strongly suggest that GA is overcoming the impermeability of *P. aeruginosa* cells (with its various mechanism of exclusion and extrusion). However, since all membranes were disturbed, this is a non-specific observation

and it is not apparent from these results on the reference strains if any one or any combination of membrane disturbances is required for potentiation.

The evidence of PAO1 would suggest that permeabilisation of the cell wall, allowing molecules external to the cell entry into the cytoplasm, is the least important of these properties of GA for antibiotic potentiation. PAO1 showed some synergy with three different antibiotics, from different classes, despite not showing as high a level of permeabilisation by GA as the other reference strains. So, while GA can permeabilise *P. aeruginosa*, this may not be necessary for antibiotic potentiation.

On a strain-by-strain basis there was less evidence of a connection between GA disrupting the outer membrane of *P. aeruginosa* clinical strains and strains which were potentiated by GA and synergised with tobramycin. The cytoplasmic membrane of clinical *P. aeruginosa* strains was the most consistently impacted by GA. In fact only two strains tested were not significantly impacted in their cytoplasmic membrane, and neither of these showed synergy with tobramycin. It was also noted that, while not statistically different, the signal for cytoplasmic depolarisation was the only one increased by GA-treatment of *S. aureus*, B*. cepacia* and *A. xylosoxidans* (*S. aureus* does not have an outer membrane).

Depolarisation of the cytoplasmic membrane was also the most consistently observed mechanism of GA action across experimental conditions and, unlike outer membrane disruption and permeabilisation, was never confounded by combination with anything else; divalent cations, pre-incubated or background LPS, eDNA. In totality, these results point to GA having two distinct mechanism of action: (i) a detergent-like outer membrane interaction which results in sloughing away of the outer membrane and an increase in cellular particles[287] resulting in outer membrane thinning and weakening followed by rapid cell death (ii) a

cytoplasmic membrane depolarising mechanism, which given the association with synergy in clinical strains, drives antibiotic potentiation. It cannot be deciphered from the assays and results here whether depolarisation of the cytoplasmic membrane by GA is due to pore formation or due to an increase in the fluidity of the liquid membrane and more sophisticated methods of measuring cytoplasmic polarisation would be required, such as high powered microscopy.

GA is a heterogenous collection of peptides which vary in composition, polymerisation order and polymer length. Previous studies have shown the ability of GA to oligomerise and adopt at least two conformations, string-like fibres and spherical particles[276–278]. It may be that different populations of GA conformations drive the different mechanisms of GA seen here. Some AMPs only take on their 'active' conformation once they come into contact with membranes[206,373].

GA binds to *P. aeruginosa* LPS, is sequestered by it and sequesters it. Results in **Chapter 6** show that after an incubation period (30mins) GA was no longer available to disrupt membranes and LPS was less detectable in solution showing bilateral sequestration. Encouragingly, the effect on GA activity was LPS dose-dependent and physiologically relevant LPS concentrations showed less impact on GA activity, allowing for the possibility that GA would still be available, or available in the appropriate conformations to be active in vivo.

As GA interacts with *P. aeruginosa* LPS, *P. aeruginosa* reacts by activating systems involved in the modification of LPS and significant increases in the expression of genes *pmrA* and *arnB* were seen in clinical strains after incubation with GA. This indicated that GA is sensed by and activates the PmrAB two component regulatory system which in turn activates LPS modification by L-Ara4N via the *arnBCADTEF* operon. This is well recognised and well-

studied mechanism of *P. aeruginosa* protection against AMP attack[33,158,180,190,195,196,198]. Despite this upregulation compared to untreated samples of the clinical strains, the overall results did not indicate strong induction of their systems (or any system tested) with median ΔΔCt results >2 for all genes tested.

Modification of LPS by L-Ara4N in response to GA was not significantly increased for either reference or clinical strains of *P. aeruginosa*. There are other methods by which *P. aeruginosa* can modify LPS (PetN, decanoic acids, palmitic acid) and testing for these modifications (and there genetics) after GA stimulation would give a broader picture of how the cells are reacting to GA.

It was encouraging to see that interactions with components of CF sputum were not strongly detrimental to GA activity; divalent cations, *P. aeruginosa* LPS, eDNA. GA did interact with and was affected by these molecules but not at physiologically relevant concentrations, GA was still active against *P. aeruginosa* cells. These elements were tested in isolation, however, and their interactions with each other and bacterial cells and other CF sputum components are likely to be much more complex than was captured here. And, while concentrations of each of these elements have been published previously, it is unclear whether these concentrations represent 'free' concentrations as, for example, divalent cations in CF sputum are equally as likely to interact with LPS from dead bacteria as live bacteria in ASL.

It may be disadvantageous to GA antimicrobial/potentiation activity for it to be able to bind and be sequestered by different elements of CF sputum. The effective concentration predominantly used here (50mg/L) works as a potentiator in these in vitro assays but getting an appropriate concentration to *P. aeruginosa* in the CF lung, while being confounded by

eDNA or LPS, will require further work. On the other hand, GA binding does not serve to only sequester the peptide but also the element to which it is bound. As has been proposed for colistin[351], this may be advantageous in the case of the extremely pro-inflammatory *P. aeruginosa* LPS (endotoxin), presenting less to the immune system by aggregation with GA[374]. Similarly, eDNA is a major component of biofilm matrix and important in biofilm formation[127] and eDNA can aid in antibiotic resistance[129] and *P. aeruginosa* persistence and therefore a reduction in eDNA availability in the CF lung may be of benefit.

This study is very much limited in scope to in vitro methods and observations. While these are necessary to show the utility of GA as an antibiotic resistance breaker, it must be recognised that many of the conditions tested here are not representative of sites of host infection, particularly the CF airway. Efforts have been made to test physiologically relevant characteristics (cations, LPS, eDNA) but these were tested in isolation of one another for the most part and it remains to be seen if a cumulative effect of these components would be detrimental to GA activity. Testing of GA activity and synergy in a medium like artificial sputum medium, or indeed sputum from people with CF, would be an interesting future direction.

One of the major factors of the CF lung which did not feature in this study is the decreased pH of CF ASL[69,70]. Acidification of the ASL has been shown to be detrimental to host immunity to bacterial infection[69,70] and acidic pHs can reduce the efficacy of some AMPs[206] and of tobramycin against *P. aeruginosa*[375]. Testing GA activity and synergy with tobramycin in media which is pH-matched to CF ASL would be a necessary next step.

People with CF are already under a huge treatment burden with a median number of 10 current treatments and 2hours per day devoted to those treatments recently reported[106]. GA is a promising drug for repurposing to CF infection with work already begun testing its

applicability as nebulised treatment[290] but it must be borne in mind that an additional treatment to the 10 already currently used may not be feasible or desired by people with CF. Indeed, in that same study of the treatment burden, people with CF already ranked "Long term nebulised antibiotics" as the 5th most important treatment they take and in the top 2 of the most burdensome[106]. While the pharmaco-properties of GA are not fully studied and understood (its extreme heterogeneity being a barrier to tracking the drug *in vivo*), the small amount of research available in humans indicates that doses of 50mg/L would be physiologically achievable[279]. A co-formulation (with tobramycin) approach to nebulisation may be a necessary for clinical deployment.

Due to our earlier work, GA was already an interesting prospect as a drug for repurposing as an antimicrobial at the beginning of this project. Much of the work reported here confirms earlier indications that GA is not a potent enough anti-pseudomonal to be considered as a treatment itself but the results here point towards a different kind of clinical application for GA. However, the restoration of efficacy of existing antibiotics through potentiation is of as much interest as new antimicrobials as it would allow current therapies to continue or be revived. The evidence presented here, in concert with previously published studies, point to GA being a strong prospect for repurposing as an antibiotic adjuvant.

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