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Identifying, optimising and characterising antimicrobial peptides against *Pseudomonas aeruginosa*

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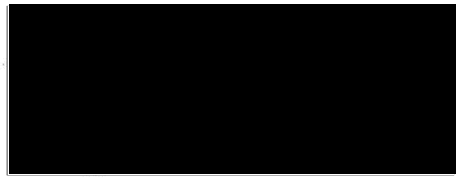
Declaration

I, Jurnorain Gani, declare that all the work presented in the thesis is my own (unless clearly stated otherwise).



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In the name of Allah, The Most Gracious, The Most Merciful

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Publications, Posters and Presentations

Publications

- Koehbach, J., **Gani, J**., Hilpert, K. and Craik, D.J. (2021) 'Comparison of a short linear antimicrobial peptide with its disulfide-cyclized and cyclotide-grafted variants against clinically relevant pathogens', *Microorganisms*, 9(6), p. 1249.
    - Partial results are reproduced in Table 3.5, p 104
- Hilpert, K., **Gani, J**., Rumancev, C., Simpson, N., Lopez-Perez, P M., Garamus, V M., von Gundlach, A R., Markov, P., Scocchi, M., Mikut, R., Rosenhahn, A. (2021) 'Rational Designed Hybrid Peptides Show up to a 6-Fold Increase in Antimicrobial Activity and Demonstrate Different Ultrastructural Changes as the Parental Peptides Measured by BioSAXS', *Frontiers in Pharmacology*, 12.
    - Partial results are reproduced in Table 3.6, p 106
- von Gundlach, A., Ashby, M P., **Gani, J**., Lopez-Perez, P M., Cookson, A R., Huws, S A., Rumancev, C., Garamus, V M., Mikut, R., Rosenhahn, A., Hilpert, K. (2019) 'BioSAXS Measurements Reveal That Two Antimicrobial Peptides Induce Similar Molecular Changes in Gram-Negative and Gram-Positive Bacteria', *Frontiers in Pharmacology*, 10.
- Ashby, M., Petkova, A., **Gani, J**., Mikut, R. and Hilpert, K. (2016) 'Use of Peptide Libraries for Identification and Optimization of Novel Antimicrobial Peptides', *Current Topics in Medicinal Chemistry*, 17(5), pp. 537–553.
- Onime, L.A., Thomas, B J., **Gani, J**., Alexander, P., Waddams, K E., Cookson, A., Fernandez-Fuentes, N., Creevey, C J., Huws, S A. (2021) 'The rumen eukaryotome is a source of novel antimicrobial peptides with therapeutic potential', *BMC Microbiology*, 21(1), p. 105.
- Yu, K., Lai, B.F., **Gani, J**., Mikut, R., Hilpert, K. and Kizhakkedathu, J.N. (2015) 'Interaction of blood components with cathelicidins and their modified versions.', *Biomaterials*, 69, pp. 201–11.

Conference Presentations

- **9th International Meeting on Antimicrobial Peptides, 2019, Utrecht, Netherlands**
    - Disentangle The Gordian Knot – Separating antimicrobial and haemolytic activity in short antimicrobial peptides

Conference Posters

- **8th International Meeting on Antimicrobial Peptides, 2018, Edinburgh, United Kingdom**
    - Naturally occurring peptides targeting *Pseudomonas aeruginosa*
- **7th International Meeting on Antimicrobial Peptides, 2017, Copenhagen, Denmark**
    - Characterisation of novel antimicrobial peptides targeting Gram-negative bacteria
- **6th International Meeting on Antimicrobial Peptides, 2016, Leipzig, Germany**
    - Development of natural and novel antimicrobial peptides targeting *Pseudomonas aeruginosa*
    - BioSAXS as a high-throughput method to classify the mode of action of antimicrobial compounds

Abstract

Over the last century, antibiotics have played a pivotal role in global health. The ever-growing threat of multidrug-resistant organisms, coupled with a declining antibiotic pipeline, has led to an urgent need for novel antimicrobial agents. One bacterial pathogen of concern is *Pseudomonas aeruginosa.* The World Health Organisation has declared it a "critical priority" pathogen for developing new antibiotics against it. It is intrinsically resistant to many antibiotics and is an opportunistic pathogen.

Antimicrobial peptides (AMPs) have been touted as potential novel antimicrobial agents. They are present in all kingdoms of life and play a vital role in the innate immunity of mammals. Two streams of antimicrobial peptides were evaluated. First, *in silico,* artificially designed short peptides (nine amino acids in length), which were predicted to be bioactive against *P. aeruginosa* and be non-haemolytic, were selected. Second, naturally occurring peptides (4 – 17 amino acids in length) from the Antimicrobial Peptide Database 3 (APD3) database, classified as antibacterial, were chosen.

Each library was synthesised using the Spot technique and screened against a luminescent *P. aeruginosa* strain and human erythrocytes. Promising candidates were identified with potent antimicrobial activity against *P. aeruginosa* (MIC 4 – 8 μg/mL) and low haemolytic toxicity (HC_{50} 200 – >512 μg/mL). To create new libraries, three peptides from the novel and one from the natural peptide stream were subjected to amino acid substitutions. The libraries were screened as above, with an additional screen in human serum and a multidrug-resistant *Escherichia coli* isolate, which revealed no promising candidate. Further engineering strategies were applied to a novel peptide which varying success.

Mode of action studies revealed that both novel and natural AMPs rapidly kill *P. aeruginosa*. Biological Small-Angle X-Ray Scattering (BioSAXS) revealed that peptides and conventional antibiotics mechanistic actions could be differentiated upon exposure to bacteria. Visualisation of bacteria treated with natural and novel peptides revealed disruption to the

membrane and intracellular changes. Furthermore, resistant studies revealed that a novel peptide, peptide 7, does not easily induce bacterial resistance.

Peptides were evaluated in the presence of simulated human physiological conditions to predict systemic *in vivo* activity. *In vivo* evaluation revealed only mild toxicity at high concentrations but unfortunately could not rescue the wax moth larvae, *Galleria mellonella,* from *P. aeruginosa* infection, although it did delay the infection.

In conclusion, this study demonstrated that using the Spot synthesis technology can identify potent peptides against *P. aeruginosa* and minimal mammalian cell toxicity *in vitro*. Moreover, bioinformatic analysis and machine learning can help predict new potent peptides. In addition, this study showed a potential of a novel application of the BioSAXS to differentiate modes of action for *P. aeruginosa* and enable faster decision making to develop novel antimicrobials more efficiently.

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Abbreviations

AA	Amino acid
ACN	Acetonitrile
Ala	Alanine
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BioSAXS	Biological Small Angle X-Ray Scattering
BOC	Tert-butyloxycarbonyl
BPB	Bromophenol Blue
$CaCl_2$	Calcium chloride
CDC	Centers for Disease Control and Prevention
CF	Cystic Fibrosis
CFU/mL	Colony-forming units per millilitre
CNS	Central Nervous System
Cys	Cysteine
DIC	N,N′-Diisopropylcarbodiimide
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ECDC	European Centre for Disease Prevention and Control
$FeCl_3$	Iron chloride

Fmoc	9-Fluorenylmethyloxycarbonyl
g	Grams
g/mol	Grams per mol
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
HC_{75}	75 % haemolytic concentration
His	Histidine
HOBt	Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
IC_{50}	Half maximal inhibitory concentration
IC_{75}	75 % bacterial inhibitory concentration
Ile	Isoleucine
IMC	Isothermal microcalorimetry
IV	Intravenous
KCl	Potassium chloride
LC-MS	Liquid Chromatography-Mass Spectrophotometry
Leu	Leucine
LMSL	Lasioglossin Multiple Substitution Library
LSSL	Lasioglossin Single Substitution Library
Lys	Lysine
M	Molar
MATC	More Active Than Control
MATP	More Active Than Parent
Mbp	Million base pairs
MDR	Multi-drug resistance
Met	Methionine

mg/kg	Milligram per kilogram
mg/mL	Milligram per millilitre
$MgCl_2$	Magnesium chloride
MH	Mueller-Hinton
MH Br	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
mL	Millilitre
mL/min	Millilitre per minute
mM	Milimolar
MOA	Mode of action
MRSA	Methicillin-resistant *Staphylococcus aureus*
N	Central nucleoid
NaCl	Sodium chloride
NH_2	Amino group
NL	Natural Library
nm	Nanometeres
NMI	N-Methylimidazole
NMP	N-methylpyrrolidone
NOL	Novel Original Library
NOMSL	Novel Original Multiple Substitution Library
NOSSL	Novel Original Single Substitution Library
O/N	Overnight
OM	Outer membrane
OMV	Outer membrane vesicles
OPM	Oscillations per minute
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline

PCA	Principal Component Analysis
Phe	Phenylalanine
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
PIVL	Pre-*in-vivo* Library
PP	Periplasm
Pro	Proline
R	Ribosomal particles
RBC	Red blood cells
RCF	Relative centrifugal force
RelIC$_{75}$	75 % relative inhibitory concentration
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Room temperature
SATC	Similar Activity to Control
SATP	Similar Activity to Parent
SEM	Standard error of mean
Ser	Serine
SPPS	Solid Phase Peptide Synthesis
S. aureus	*Staphylococcus aureus*
TB	Tuberculosis
TEM	Transmission Electron Microscopy
TFA	Trifluoroacetic acid
TG	Tris and glucose mix
TGB	Tris, glucose and bacteria mix
Thr	Threonine
TI	Therapeutic Index
TIPS	Triisopropyl saline

TP	Therapeutic Potential
Tris	Tris(hydroxymethyl)aminomethane
Trp	Tryptophan
Tyr	Tyrosine
UPLC	Ultra-Performance Liquid Chromatography
UTI	Urinary Tract Infection
v/v	Volume per volume
Val	Valine
w/v	Weight per volume
WATC	Weaker Activity Than Control
WATP	Weaker Activity Than Parent
$ZnCl_2$	Zinc chloride
α	Alpha
β	Beta
<	Less than
>	Greater than
≤	Equal to or less than
≥	Equal to or greater than
°C	Degrees Celcius

1 Introduction

1.1 History of Antibiotics and Discovery

Antibiotics are defined as an agent that inhibits or disrupts bacterial growth (Waksman, 1947). Alternatively, the term antimicrobial is also more commonly used in current times, encompassing other microbial organisms, including viruses, fungi, protozoa and bacteria (Burnett-Boothroyd and McCarthy, 2011). First discovered in the early 20th century, antibiotics have significantly reduced illness and death associated with bacterial infectious diseases. Before their deployment, bacterial infections often led to poor outcomes and high mortality (Adedeji, 2016). Antibiotics are therefore an imperative pillar in medicine and arguably one of the most significant accomplishments in the history of medicine.

The first reported antibiotic used widely was known as arsphenamine, which was discovered by Paul Ehrlich in the early 1900s and used to treat syphilis infections; caused by the bacterium *Treponema pallidum* without damaging host cells (Kaufmann, 2008). Arsphenamine and the like became the most commonly prescribed drugs in that era (Voegtlin *et al.*, 1922). It paved the way for various subsequent discoveries of novel antibiotic classes, which were efficacious and non-toxic. Some notable early discoveries were penicillin (Fleming, 1929), which prevented bacterial cell wall synthesis in the peptidoglycan layer and is classed as a beta-lactam (Fernandes *et al.*, 2013). Domagk first discovered the sulphonamide class in the 1930s (Domagk, 1935), which act as competitive inhibitors of the enzyme dihydropteroate synthase (DHPS) (Henry, 1943). The 'golden era' of antibiotics came to fruition in the 1940s – 1960s, in which a multitude of antibiotic compounds and classes were discovered (Figure 1.1). It includes streptomycin, an aminoglycoside that inhibits protein synthesis discovered by Schatz *et al*. in 1944 (Schatz *et al.*, 1944; Mingeot-Leclercq *et al.*, 1999). Chlortetracycline, a tetracycline antibiotic that binds to the 30S ribosomal subunit causing inhibition of peptide synthesis, was reported in 1948 by Duggar (Duggar, 1948;

Chopra and Roberts, 2001). Chloramphenicol, the first amphenicol antibiotic which prevents the protein chain from elongating, thereby inhibiting protein synthesis, was discovered by Rebstock *et al*. in 1949 (Rebstock *et al.*, 1949). Nalidixic acid, the first quinolone, was found by Lesher and colleagues in the 1960s (Lesher *et al.*, 1962).

Following this 'golden era,' the discovery of new antibiotic classes decreased substantially as a result of failed antibiotic leads and many large multinational pharmaceutical companies severing antibiotic research programmes, with the lack of financial incentives amongst the primary reason (Jackson *et al.*, 2018)

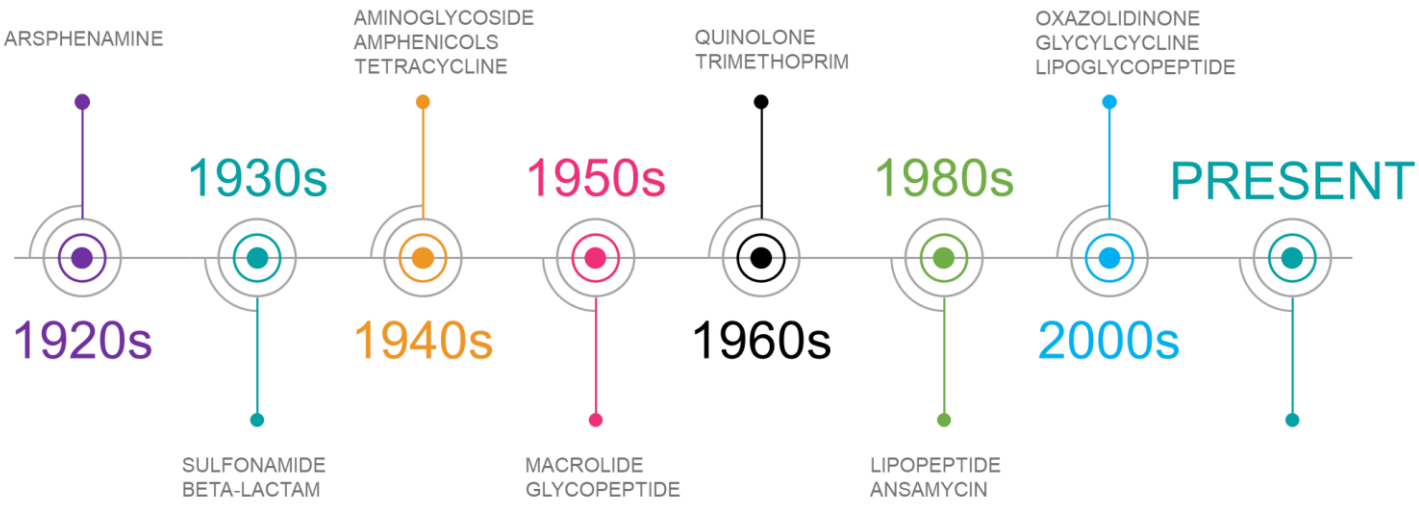


Figure 1.1 Antibiotic Discovery Timeline

The timeline of discovery of new classes of antibiotics. There have been gaps in discovery over the past 50 years, with very few compounds being discovered, notably in the 70s, 90s and the present time.

1.2 Antibiotic Resistance

Antibiotics are used to treat already present life-threatening bacterial infections such as septicaemia and pneumonia. Still, an important use for them is as prophylaxis for many different medical procedures, such as cardiac and necrotising fasciitis surgery and organ transplantations (Soave, 2001; Misiakos *et al.*, 2014). Antibiotic resistance was already reported as early as the 1940s among the sulphonamide class, one of the first classes of antibiotics to be discovered (Kirby and Rantz, 1943). Over the years, there has been a rapid emergence of antibiotic resistance. It is defined as the ability of bacteria to survive exposure to an antibiotic used to treat the infection-causing organism.

The cause and rise of antibiotic resistance can be attributed to various issues. It includes the wide availability of antibiotics, indiscriminate use and being a commodity in unregulated markets, all leading causes (Grundmann *et al.*, 2011). In many countries,' antibiotics are available to purchase without a prescription, which has led to them being used for headaches, sore throats, and the common cold, in which antibiotics have minimal to no effect. The misuse results in unnecessary antibiotic exposure and can often lead to increased antimicrobial resistance (AMR) (Jackson *et al.*, 2018). Inadequate prescribing by clinicians coupled when patients being incompliant by not finishing the course of medication or stockpiling for future use has also contributed to the rise of AMR (Tong *et al.*, 2018). Patients receiving suboptimal doses of antibiotics, which are enough to lower the infection, are an additional cause. (Carmeli *et al.*, 1999). Those factors have increased mutagenesis/gene transfer, thus increasing AMR rates (Ventola, 2015).

The use in animal husbandry is regularly cited as one of the leading causes of antibiotic resistance. Most antibiotics in total usage are used amongst livestock (He *et al.*, 2020). China is the largest consumer of antibiotics, approximately 162,000 tonnes, of which over half were administered to animals (Zhang *et al.*, 2015). Moreover, 80 % of antibiotics sold in the USA are used for livestock (Martin *et al.*, 2015). Continued usage has led to resistant strains arising in essential antibiotics. One example is colistin, a last-resort antibiotic, where AMR has been

reported on farms in China. However, it has been banned in several countries for animal use (Perez and Bonomo, 2020).

One of the most disturbing aspects of antibacterial resistance is multidrug resistance (MDR), defined as a bacteria surviving exposure to three or more antibiotic classes (Magiorakos *et al.*, 2012). MDR usually occurs in many cases in healthcare environments and mostly in situations where the patient is in a critical or vulnerable condition, such as those in intensive care, neonatal units or immunocompromised (Tsai *et al.*, 2014; Garnacho Montero *et al.*, 2015). MDR bacteria that have been of particular concern are the "ESKAPE" organisms, a group of Gram-positive and Gram-negative bacteria compromising *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species (Boucher *et al.*, 2009). Should antibacterial resistance continue at its current trajectory and without any meaningful interventions, it could lead to the death of more than 10 million people per annum by the year 2050 (Figure 1.2) and 100 trillion dollars of economic loss (O'Neill, 2016).

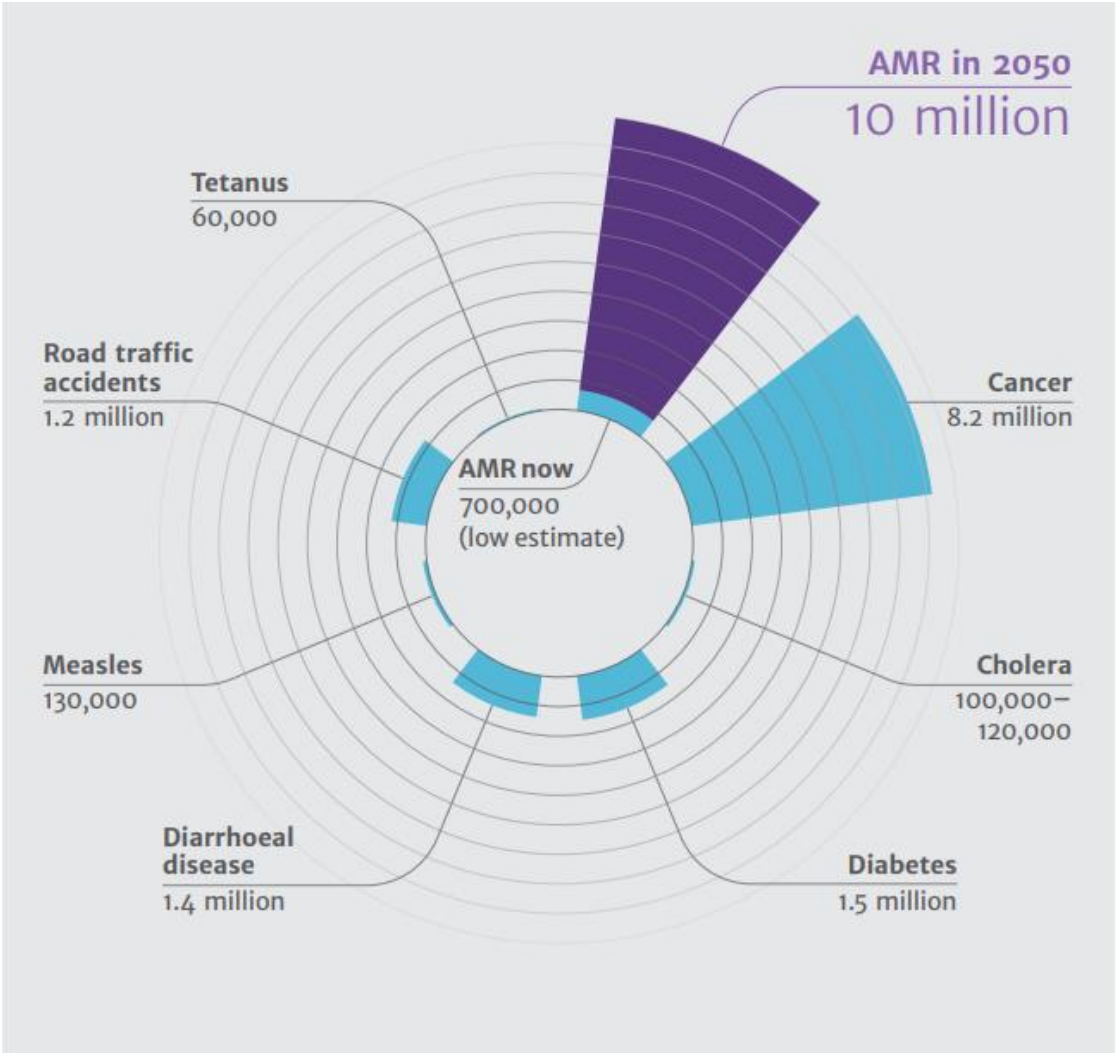


Figure 1.2 Potential major causes of death in 2050

Compared to other major causes of death in 2050, annual deaths are attributed to AMR if no intervention is taken. The image was taken from (O'Neill, 2016).

1.3 *Pseudomonas aeruginosa*

1.3.1 History and Discovery

P. aeruginosa is a rod-shaped non-fermenting Gram-negative bacterium, 1.5 – 3 micrometres (μm) in length and 0.5 – 0.7 μm in width, belonging to the *Pseudomonadaceae* family (Wu and Li, 2015; Spagnolo *et al*., 2021). It grows in aerobic conditions but can also grow anaerobically in the presence of nitrate (D'Agata, 2015). It is primarily found in the environment, soil and water and possesses a characteristic odour (Hardalo and Edberg, 1997). It is an opportunistic pathogen that can cause disease in humans, animals and plants (Cantas *et al.*, 2013). It was first discovered in 1882 by Carle Gessard, who isolated blue and green pigments from bandages on wounded soldiers (Gessard, 1984). The samples were then tested using Pasteur's cultures and found to grow between 35 – 38°C in neutralised urine and carrot extracts (Gessard, 1984). *P. aeruginosa* is one of the most common nosocomial pathogens worldwide, particularly affecting the elderly, the immunocompromised, and those in intensive care and is a major concern in those with cystic fibrosis (Davies, 2002; Paramythiotou and Lucet, 2004). One of the reasons it is so challenging to treat is because *P. aeruginosa* can adapt incredibly well to its environment (de Bentzmann and Plésiat, 2011). It has one of the largest genomes in the bacterial world, containing 6.26 million base pairs (mbp), which encode 5567 genes. Meanwhile, *Escherichia coli* has 4257 encoded genomes within 4.46 mbp (Stover *et al*., 2000). Moreover, the number of genes can be increased by acquiring genes via horizontal gene transfer. *P. aeruginosa* is ranked among the top isolated pathogens in healthcare settings, accounting for about 10 % of hospital-acquired infections (Wu and Li, 2015). It can utilise a wide range of materials for its nutrients and survive on a limited amount of nutrients (Abdelraouf *et al.*, 2011). Therefore, it can survive in harsh conditions. It has reportedly been present in many natural and artificial environments ranging from swimming pools, hot tubs, inner soles of shoes, taps, vegetation, contact lens solutions and needles among drug users (Hardalo and Edberg, 1997; Paterson, 2006; Mena and Gerba, 2009).

1.3.2 Epidemiology, Pathogenesis and Disease

P. aeruginosa is responsible for hospital-acquired infections (HAI) and is a cause of high mortality and morbidity rates (D'Agata, 2015). In Europe, in 2019, approximately 14 % of the isolates tested were classed as multidrug-resistant, i.e. resistance to three or more antibiotics was observed (European Centre for Disease Prevention and Control, 2020). Moreover, it is amongst the top five most frequently reported organisms in Europe (European Centre for Disease Prevention and Control, 2020). In the USA, it was responsible for more than an estimated 230,000 infections in the past five years, with 30,000 hospitalisations and more than 5000 deaths (Centers for Disease Control and Prevention, 2019). Furthermore, it has been classified as a "serious threat" pathogen by the Centers for Disease Control (CDC). Globally, it has been categorised as a critical priority pathogen by the World Health Organisation (WHO), for which novel antimicrobial agents are required (Tacconelli *et al.*, 2018).

The opportunistic nature of *P. aeruginosa* means it can cause many different conditions in different locations, from the central nervous system (CNS) to bones and soft tissue, such as ecthyma gangrene (Varghese *et al.*, 2011). Diseases of significant concern which *P. aeruginosa* exacerbates include cystic fibrosis (CF), which presents in the lungs (Davies, 2002) and sepsis, which shows in the bloodstream and can be fatal (Kang *et al.*, 2003). Table 1.1 further outlines some of the infections caused and where it is manifested.

Table 1.1 Diseases caused by P. aeruginosa in humans

The different diseases that *P. aeruginosa* can potentially cause demonstrating its opportunistic nature. It also shows its ability to adapt to varying environments to cause illness.

Infection	Body Part	Reference
Meningitis and brain abscesses	Central Nervous System (CNS)	(Gupta *et al.*, 1990; Huang *et al.*, 2007)
Otitis externa	Ears	(van Asperen *et al.*, 1995; D'Agata, 2015)
Bacterial keratitis and Endophthalmitis	Eye	(Eifrig *et al.*, 2003; Sy *et al.*, 2012)
Endocarditis	Heart	(Fleiszig and Evans, 2002)
Pneumonia and Cystic Fibrosis (CF)	Respiratory tract	(Davies, 2002; Tsuji *et al.*, 2014)
Diarrhoea and Enterocolitis	Gastrointestinal tract	(Kim *et al.*, 2001; Ohara and Itoh, 2003)
Urinary Tract Infection	Urinary Tract	(Strand *et al.*, 1982; Mittal *et al.*, 2009)
Osteomyelitis	Bones and joints	(Carek *et al.*, 2001)
Bacteremia	Bloodstream	(Bisbe *et al.*, 1998)
Ecthyma gangrenosum and Cellulitis	Skin	(Habif, 2005; Varghese *et al.*, 2011)

1.3.3 P. aeruginosa – Treatments and Trials

P. aeruginosa is sensitive to some antibiotic classes, including but not limited to quinolones and aminoglycosides; however, it has demonstrated intrinsic resistance against many others (Moore and Flaws, 2011). Inherent resistance has been shown in the beta-lactam class of antibiotics, the most frequently prescribed class of antibiotics (Okamoto *et al.*, 2001; Dolk *et al.*, 2018). The current treatment regimen for multidrug-resistant cases is limited to the last-resort antibiotic colistin, which was previously shelved due to its toxic side effects (Falagas and Kasiakou, 2006). However, alarmingly there have been reports of *P. aeruginosa* resistance to colistin (Goli *et al.*, 2016; Perez and Bonomo, 2020). With an urgent requirement for novel antibiotics for *P. aeruginosa,* only one antibiotic, murepavadin, an LptD inhibitor, is a new class specifically for *P. aeruginosa* in clinical trials (Martin-Loeches *et al.*, 2018). The rest of them are already existing classes (Table 1.2). It is very likely that should these antibiotics, currently in trials, be deployed clinically, resistance is very likely to occur sooner, thus reducing their effectiveness. Overall, of all antibiotics currently in trials, only a third of them have indications towards Gram-negative bacteria; therefore, it is currently an area that requires urgent attention (Breidenstein *et al.*, 2011).

Furthermore, only two companies with antibiotics in clinical trials are considered among the top 50 pharmaceutical companies, with 95 % of the others being small companies. Additionally, most small companies are pre-revenue, meaning no commercialisation from previous products. One way to potentially attract large pharmaceuticals back into developing new antimicrobials could be via incentives, reimbursement and bonuses (Rex and Outterson, 2016; Simpkin *et al.*, 2017; Morel *et al.*, 2020).

Table 1.2 Antibiotics in clinical trials

A glance at some of the antibiotics in phase II or III trials. It only shows antibiotics that have an indication towards Gram-negative bacteria. Data was collated and extrapolated (Pew Charitable Trusts, 2021)

Name	Company	Antibiotic Class	Phase
Finafloxacin	MerLion	Fluoroquinolone	II
POL7080	Polyphor	LptD inhibitor	III
Ceftobiprole	Basilea	β-lactamase inhibitor	II
S-649266	Shionogi	Cephalosporin	III
Omadacycline	Paratek	Tetracycline	III
Eravacycline	Tetraphase	Tetracycline	III
Carbavance	Rempex	β-lactamase inhibitor + Carbapenem	III
Cefepime + taniborbactam	VenatoRx Pharmaceutical	β-lactamase inhibitor + Cephalosporin	III

1.3.4 P. aeruginosa – Resistance Mechanisms

P. aeruginosa has developed resistant to virtually all classes of antibiotics (Livermore, 2002). The resistance mechanisms broadly fall under intrinsic, acquired, and adaptive categories (Blair *et al.*, 2015). Intrinsic resistance refers to the innate ability of *P. aeruginosa* to nullify the efficacy or activity of an antibiotic due to its structural or functional characteristics (Blair *et al.*, 2015). The processes which fall under this category include efflux pump systems, outer membrane permeability and inactivating enzymes (Pang *et al.*, 2019). Acquired resistance refers to *P. aeruginosa* gaining antibiotic resistance due to either acquiring resistance genes via horizontal transfer or mutational change (Poole, 2011). Adaptive resistance usually occurs in response to a stimulus (i.e. antibiotic dose) through transient alterations of genes and/or protein expression (Pang *et al.*, 2019). Adaptive resistance amongst *P. aeruginosa* is mainly seen with persister cells and biofilm formation (Pang *et al.*, 2019). An overview of the different resistance mechanisms for *P. aeruginosa* is shown in Figure 1.3.

1.3.4.1 Efflux Pumps

Efflux pumps are one of the most common ways for bacteria to eject compounds that it deems harmful. There are five different families of pumps, which include ATP-binding cassette (ABC), multidrug and toxic compound extrusion (MATE), major facilitator superfamily (MFS), small multidrug resistance (SMR) and resistance-nodulation-division (RND) family (Sun *et al.*, 2014). The proteins originating from the RND family play an essential role in resistance by *P. aeruginosa* (Nikaido and Takatsuka, 2009). The RND pumps consist of outer membrane porin channel protein, cytoplasmic membrane transporters, and periplasmic linker proteins, which combined make up 12 RND pumps expressed by *P. aeruginosa* (Adamiak *et al.*, 2021). Four RND pumps contribute to resistance, labelled Multidrug efflux (Mex) and outer membrane porin (OprM). It includes MexCD-OprJ, MexEF-OprN, MexAB-OprM and MexXY-OprM (Poole, 2004; Piddock, 2006; Lister *et al.*, 2009). MexCD-OprJ is responsible for the

expulsion of β-lactams (Gomis-Font *et al.*, 2021). MexEF-OprN is responsible for expelling aminoglycosides and quinolones (Poole, 2005a, 2005b). Moreover, MexAB-OprM expels both β-lactams and quinolones (Poole, 2005b). Some *P. aeruginosa* strains have shown overexpression of these pumps, thus broadening the resistance horizon (Pang *et al.*, 2019).

1.3.4.2 Outer Membrane Permeability

Outer membrane permeability acts as a selective barrier to prevent antibiotic penetration. It is particularly prevalent in Gram-negative bacteria due to a bilayer of phospholipids and lipopolysaccharides embedded with porins that form protein channels (Delcour, 2009). The porins can be classified into four groups responsible for allowing and restricting different substances, including specific, non-specific, gated and efflux porins (Hancock and Brinkman, 2002). In *P. aeruginosa*, the OprB, OprD, OprE, OprO and OprP are specific porins; OprF protein is a non-specific porin; and OprC and OprH are gated porins (Pang *et al.*, 2019). The class of efflux porins include OprM, OprN and OprJ (Hancock and Brinkman, 2002). The outer membrane permeability in *P. aeruginosa* is severely limited and up to 100-fold less than *E. coli* (Yoshimura and Nikaido, 1982). The absence of OprD, for example, increases resistance to carbapenems (El Amin *et al.*, 2005). Meanwhile, the overexpression in certain conditions of OprH has been associated with resistance to gentamicin, an aminoglycoside and polymyxin B, a lipopeptide (Young *et al.*, 1992; Macfarlane *et al.*, 1999).

1.3.4.3 Antibiotic Inactivating Enzymes

Antibiotic-inactivating enzymes lead to the modification or breakdown of antibiotics, diminishing their effectiveness (Pang *et al.*, 2019). *P. aeruginosa* produces β-lactamases and aminoglycoside-modifying enzymes (Poole, 2005; Wolter and Lister, 2013). The β-lactamases are produced by the *ampC* gene, which causes hydrolysis of the β-lactam ring, causing the amide bonds to break, thus leading to the inactivation of β-lactam antibiotics (Wright, 2005). Aminoglycoside resistance occurs when there is a modification to the amino and glycoside structure in the antibiotics, caused by the inactivation of aminoglycoside modifying enzymes,

namely, aminoglycoside phosphotransferases, acetyl-transferases, and nucleotidyl-transferases (Wright, 2005; Alekshun and Levy, 2007).

1.3.4.4 Acquired Resistance

Resistant genes can be carried on transposons, plasmids and integrons, and *P. aeruginosa* can acquire these genes via horizontal gene transfer (Alekshun and Levy, 2007). Integrons have shown a propensity to disseminate resistance amongst *P. aeruginosa* strains (Henrichfreise *et al.*, 2007). They are mobile genetic elements that insert a gene cassette (containing the resistant gene) into another bacteria. The main mechanisms of horizontal gene transfer involve transformation, the uptake of DNA, transduction, the transfer of DNA and conjugation, and transfer for DNA via cell-to-cell contact (Arber, 2014). There have been many reports of β-lactam and aminoglycoside resistance amongst *P. aeruginosa* (Bonomo and Szabo, 2006; Yan, 2006; Cavalcanti *et al.*, 2015).

Mutational changes can cause modification of the drug target and reduce antibiotic uptake, in addition to the mechanisms mentioned above, to enable *P. aeruginosa* (or other bacteria for that matter) to survive. Interference with antibacterial targets is a common strategy used to avoid the action of antibiotics. It can be achieved by protecting the targets and modifying the target sites (Munita and Arias, 2016). Quinolone resistance mutations in genes were observed in genes encoding DNA gyrase (gyrA and gyrB) and/or topoisomerase IV (parC and pare), which led to a decreased binding affinity with *P. aeruginosa* (Bruchmann *et al.*, 2013). Furthermore, it has been reported that strains of *P. aeruginosa* that undergo mutational changes within the ribosome have shown high levels of resistance within the aminoglycoside class (El'Garch *et al.*, 2007). Spontaneous mutations can mediate a reduction in the uptake of antibiotics to the porins (which have a size exclusion limit), whereby the overexpression of these porins reduces membrane permeability (Fernández and Hancock, 2012). The OprD protein has been attributed to resistance amongst carbapenems, especially imipenem (Ochs *et al.*, 2000; Fang *et al.*, 2014).

Biofilm mediated resistance is an adaptive mechanism of *P. aeruginosa,* whereby an aggregate of organisms adhere to each other on living or non-living surfaces (Pang *et al.*, 2019). It has been particularly problematic for patients suffering from cystic fibrosis (Ciofu *et al.*, 2015). Resistance to antibiotics generally occurs due to the prevention of antibiotic penetration; however, sensitivity can easily be restored once bacteria lose their biofilm protection (Walters *et al.*, 2003). Persister cells within *P. aeruginosa* strains are not genetically resistant to antibiotics; however, they can tolerate a higher concentration of antibiotics (Balaban *et al.*, 2013). Also, they tend to be slow-growing, metabolically inactive and comprise about 1 % biofilm cells (Wood *et al.*, 2013). Persister cells remain viable after an antibiotic attack and do not grow in the presence of antibiotics but do so after, once the antibiotic is no longer present (Van den Bergh *et al.*, 2017).

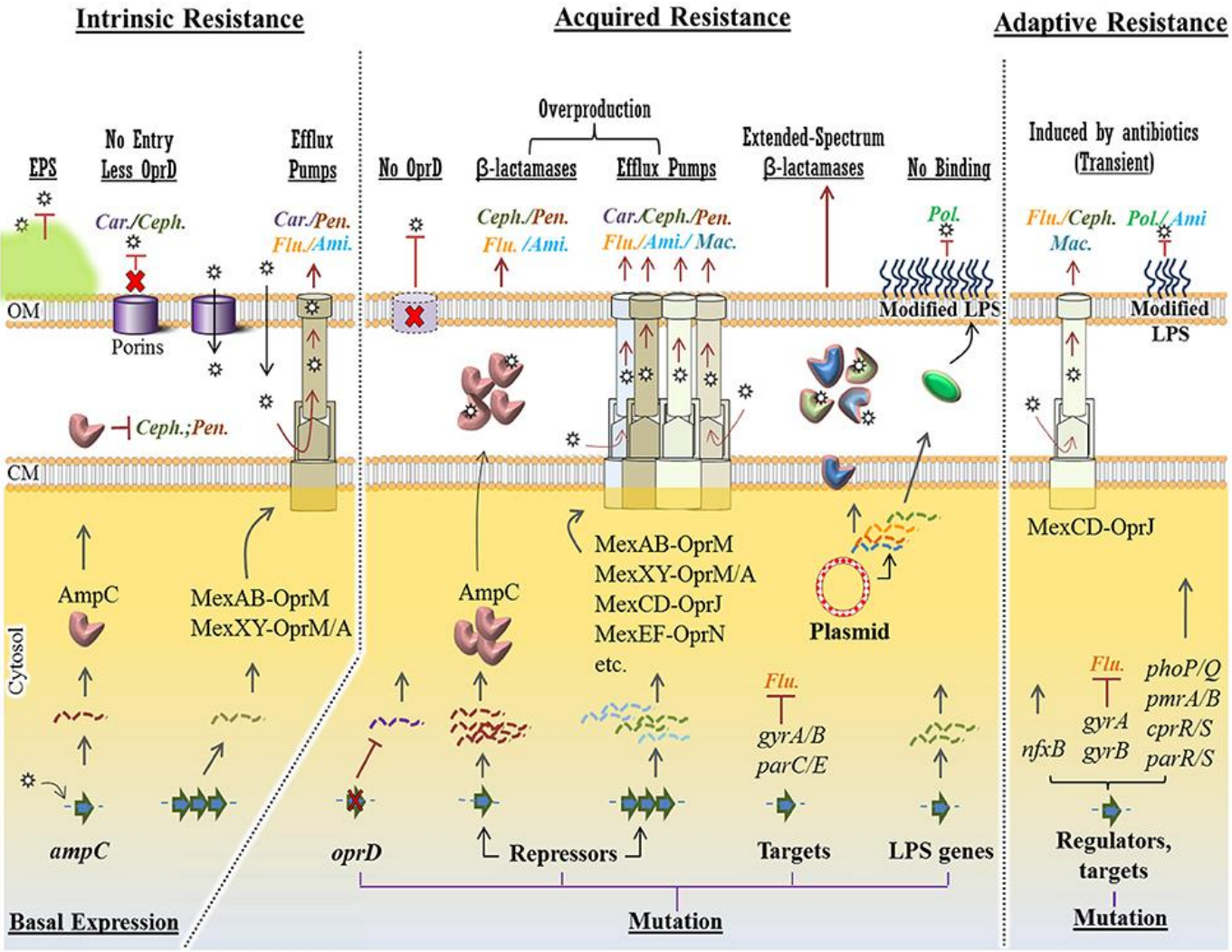


Figure 1.3 Pseudomonas aeruginosa resistant mechanisms

An overview of the different types of resistance mechanisms that can be used by *P aeruginosa* to lead to resistance to antibiotics. Car = Carbapenems, Ceph = Cephalosporins, Pen = Penicillins Ami = Aminoglycosides, Flu = Fluoroquinolones, Mac = Macrolides and Pol = Polymyxins. EPS = extracellular polymeric substances, CM = cytoplasmic membrane and OM = outer membrane. Image taken from (Moradali *et al.*, 2017)

1.4 Peptides

1.4.1 Background

Peptides are a short chain of amino acids formed together via a peptide bond. They are distinguished from proteins by their shorter length, although the cut-off is mainly arbitrary at 50 amino acids (Carton and Strohl, 2013). Peptides are found within all living organisms and are integral to virtually all manners of biological activity. In the higher kingdoms of living organisms, they are biologically synthesised through the transcription of genetic code, namely DNA. Transcription is the biological process of copying a specific DNA gene sequence into a messenger molecule, mRNA, which carries the code for a given peptide. From the mRNA, a chain of amino acids is joined together to form a peptide molecule. Twenty naturally occurring amino acids can be combined with an enormous variety to give rise to a diversified range of peptides. Moreover, this can also lead to the formation of different secondary structures of peptides, such as α-helices, β-sheets, extended and mixed (Eisenberg, 2003; Crisma *et al.*, 2018).

1.4.2 Therapeutics

Peptides have been used therapeutically for almost 100 years, with insulin being the first widely used peptide therapeutic (Banting *et al.*, 1922). Since its introduction onto the market, peptides have been used therapeutically for various indications, such as cancer, HIV and multiple sclerosis (Kavanagh *et al.*, 1989; Matthews *et al.*, 2004; Lalive *et al.*, 2011). Currently, they make up about 5 % of the global pharmaceutical market, with insulin being the first and is still the most common peptide therapy, with approximately $25 billion worth of sales (Muttenthaler *et al.*, 2021). They have been derived from various strategies such as natural sources and rational design and used for different therapies (Figure 1.4 and Table 1.3).

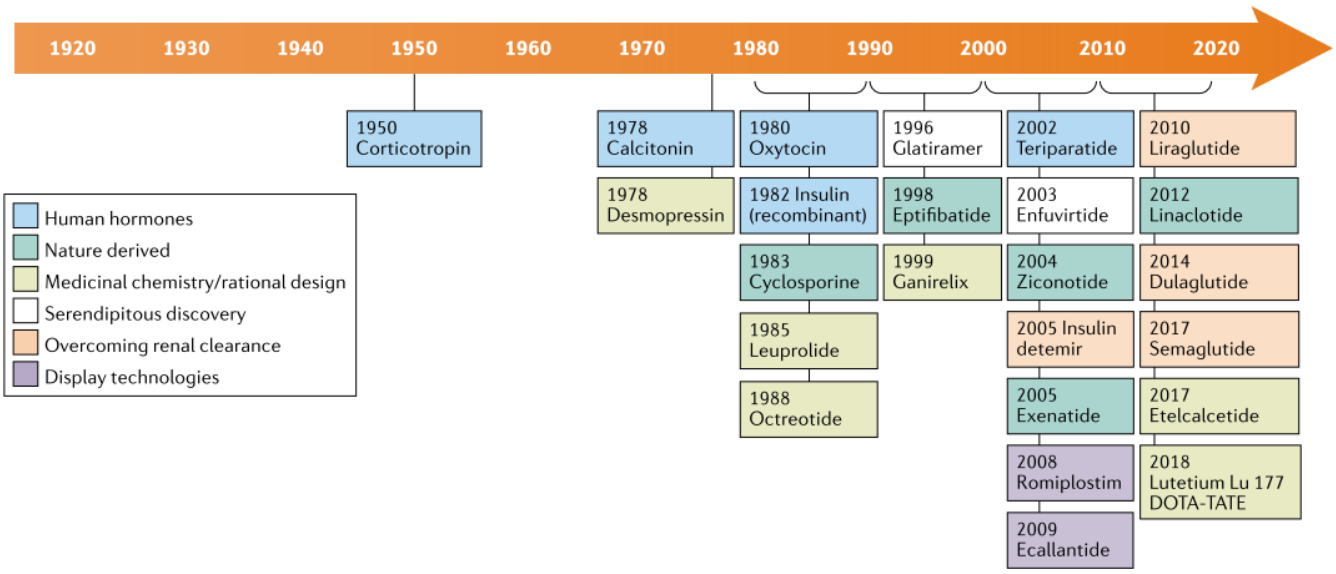


Figure 1.4 Peptide approval and origin

The regulatory approval of selected peptide therapeutics and their origin of discovery. Peptides have been isolated from different sources and this figure highlights the different technologies used to discover and develop such therapeutics. Originally isolated from human sources, it has since been greatly diversified. Figure modified from (Muttenthaler *et al.*, 2021).

Table 1.3 Current peptide therapeutics examples

The current peptide-based therapies on the market and their therapeutic indications

Peptide	Therapeutic Indication	Reference
Insulin	Diabetes	(Lau and Dunn, 2018)
Goserelin	Cancer	(Mansel *et al.*, 2004)
Etelcalcetide	Hyperparathyroidism	(Block *et al.*, 2017)
Cyclosporine	Immunosuppressant	(Tedesco and Haragsim, 2012)
Lanreotide	Acromegaly	(Giustina *et al.*, 2017)
Glatiramer	Multuple Sclerosis	(Lalive *et al.*, 2011)

1.4.3 Synthetic Peptide Synthesis

Peptide therapeutics have been possible due to great leaps in peptide isolation and discovery innovation, as initially peptides were isolated directly from animals for therapeutic use for almost 100 years (Lee *et al.*, 2019). However, in 1963, Robert Bruce Merrifield revolutionised the peptide field by inventing solid-phase peptide synthesis (SPPS), which allowed synthetic peptides to be produced (Merrifield, 1963). As a result, this led to Merrifield being awarded the Nobel Prize in Chemistry in 1984.

SPPS relies on the repeated chemical attachment of amino acids using solid support to the desired peptide and then releasing the peptide from that support (usually resin). SPPS is now employed for most peptide syntheses worldwide (Behrendt *et al.*, 2016). Within SPPS, two approaches are used primarily: 9-fluorenylmethyloxycarbonyl (Fmoc) or the *tert*-butyloxycarbonyl (BOC). Fmoc chemistry is the most widely used to synthesise peptides, as the process is mild and less complex chemically (Behrendt *et al.*, 2016). The Fmoc strategy utilises a base, usually piperidine (20 % in DMF), which removes the Fmoc group to expose the α-amino group for the incoming activated amino acid. In contrast, BOC conditions are acidic (Albericio, 2000). BOC uses hydrogen fluoride as a de-protection strategy for the amino acid side chains, which is highly corrosive and toxic. In contrast, Fmoc usest trifluoroacetic acid, which is generally considered safer.

1.5 Antimicrobial Peptides

1.5.1 History and Discovery

The first antimicrobial peptides (AMPs) to be isolated were from the soil bacterium *Bacillus brevis*, named tyrothricin in 1939 (Dubos, 1939). Tyrothricin contained a mixture of two unique peptides, gramicidin and tyrocidine. Rene Dubos demonstrated that tyrothricin had bioactivity with *in vitro* conditions against Gram-positive bacteria and infection protection with *in vivo* conditions (Dubos, 1939). The first eukaryotic derived AMP to be discovered was from the leukocytes of rabbits in 1956 by James Hirsch (Hirsch, 1956). Since then, there have been a few peptide-based antibiotics have been used clinically. Well-known ones used clinically include daptomycin and colistin (Eliopoulos *et al.*, 1986; Hachem *et al.*, 2007). Moreover, echinocandins, a lipopeptide group of peptides, have been used clinically to treat fungal infections (Mroczyńska and Brillowska-Dąbrowska, 2020).

AMPs play an integral part in the innate immune system in mammals and are present in virtually all organisms (Hancock and Sahl, 2006). Within humans, AMPs are secreted in response to infection at various sites. The most studied AMP family from humans are the human defensins (Phoenix *et al.*, 2013). In particular, human beta-defensins (HBD) are expressed in various epithelial sites alongside some immune cells, macrophages, and monocytes (Pazgier *et al.*, 2006; Diamond *et al.*, 2008). HBDs have shown extensive antimicrobial activity against various organisms, including Gram-positive and negative bacteria and protozoa, fungi, and viruses (Maisetta *et al.*, 2008; Arnett and Seveau, 2012). Another human peptide, LL-37, belonging to the cathelicidin group, expressed in epithelial and immune cells, has also shown broad-spectrum antimicrobial activity (Gordon *et al.*, 2005; Tjabringa *et al.*, 2006).

Over the years, there has been the discovery of many different AMPs from different sources. More than 3000 naturally occurring peptides have been discovered and deposited

into different databases such as APD3, DRAMP, LAMP and CAMP (Zhao *et al.*, 2013; Wang *et al.*, 2015; Waghu *et al.*, 2016; Kang *et al.*, 2019).

1.5.2 Features and Characteristics of AMPs

Despite the immense diversity within naturally occurring AMPs, there are some common features, despite millennia of evolution. They are generally cationic (positively charged) and up to 50 amino acids in length (Huan *et al.*, 2020). AMPs from naturally occurring sources have been from many organisms and consisted of different lengths and four main structures (Figure 1.5). The cationic nature of AMPs has been widely attributed to electrostatic interactions with bacterial membranes to enact inhibition of bacterial growth (Travkova *et al.*, 2017). Moreover, structural conformations of peptides also aid bioactivity. α-Helical peptides such as cecropin and mellitin are known pore-formers. Although AMPs are generally cationic, anionic AMPs also exist and have been isolated and characterised, such as dermcidin (Lai *et al.*, 2007; Dennison *et al.*, 2018)

AMPs have gained traction within the scientific community as potential novel antimicrobials due to several advantageous properties. These include the propensity to kill rapidly and broadly and not induce classical antibiotic-resistant mechanisms (Molchanova *et al.*, 2017). Furthermore, AMPs are active against MDR pathogens and can be easily engineered, such as incorporating non-natural modifications (Zasloff, 2002; Ageitos *et al.*, 2017; Torres *et al.*, 2019).

AMPs have also got some weaknesses that have to be addressed to make them more developable compounds. Weaknesses include cytotoxicity against eukaryotic cells, proteolytic stability in serum, and other physiological conditions (such as low pH and ions) hindering activity (Boto *et al.*, 2018; Mahlapuu *et al.*, 2020). Furthermore, logistical issues surrounding peptides are being addressed, such as the cost of manufacture and commercial scaling up (Seo *et al.*, 2012; Mahlapuu *et al.*, 2020).

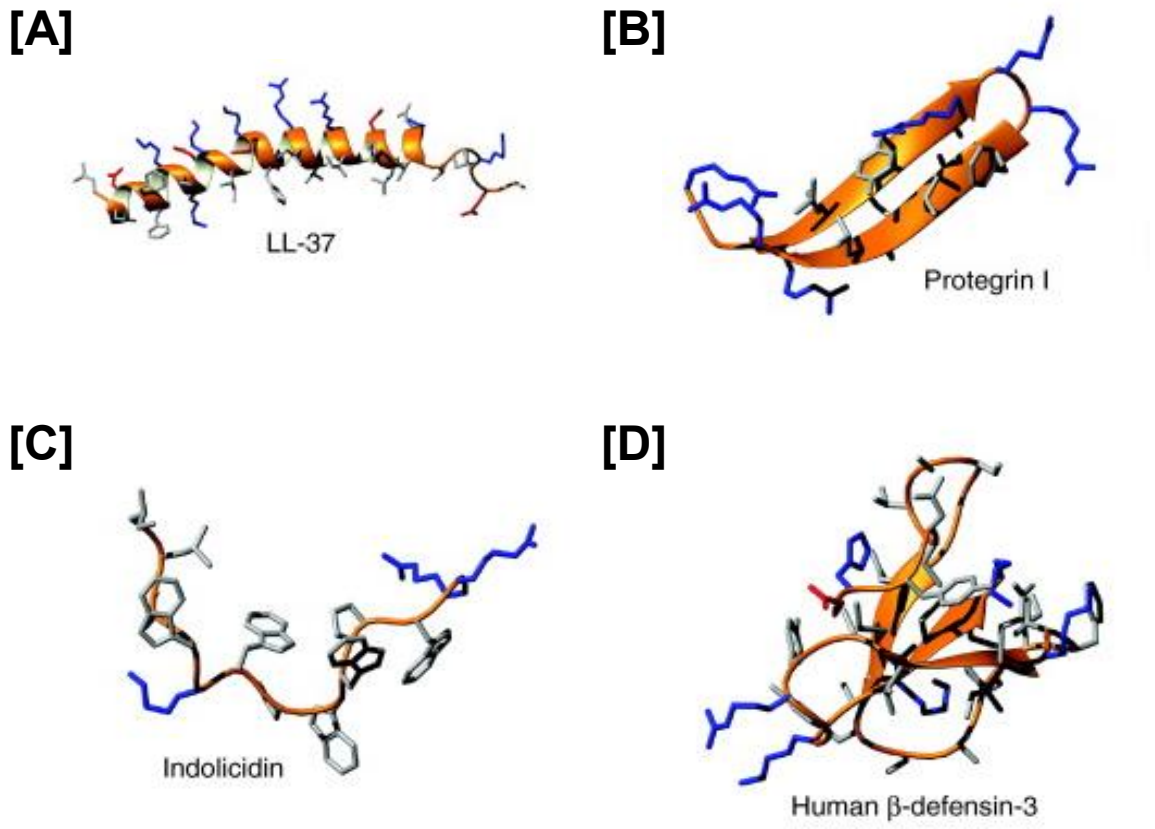


Figure 1.5 Antimicrobial peptide structures

Some well known antimicrobial peptides and their structures which they exhibit. **[A]** α-helical structure (PDB code: 2K60), **[B]** β-sheet structure (PDB code: 1PG1), **[C]** extended structure (PDB code: 1G8C) and **[D]** a mixed structure (PDB code: 1KJ6). All images sourced and adated from (Nguyen *et al.*, 2011)

1.5.3 AMPs in Clinical Trials

Despite the challenges faced with AMPs, many AMPs have entered clinical trials at various stages and indications (Table 1.4). It was evident that most of the AMPs in clinical trials are topical or mucosal use. It indicates that the systemic route of application is still posing difficulties en masse. Some other well-studied AMPs undergoing clinical evaluation are linear AMPs omiganan, pexiganan, and DPK-060, with reported activity against a wide range of antimicrobial infections (Mahlapuu *et al.*, 2020).

Table 1.4 Antimicrobial peptides in clinical trials

A snapshot of different antimicrobial peptides, their uses, application, and current clinical trials status. The clinical trial reference number stated is from ClinicalTrials.gov

Name	Company	Use	Application	Phase (Clinical Trial Reference Number)
CLS001	Cutanea Life Sciences	Papulopastular Rosacea, Acne, verruca	Topical	III (NCT02547441)
Reltecimod	Atox Bio Ltd	Necrotising Soft Tissue Infections	Intravenous	III (NCT02469857)
PAC113	Pacgen Lifesciences	Oral candidiasis	Mucosal	III (NCT00659971)
Novexatin	Novabiotics	Fungal Nail Infection	Topical	II (NCT02933879)
Brilacidin	Cellceutic	Oral mucositis	Mucosal	II (NCT02324335)
Lytixar	Lytix Biopharma	MRSA	Topical	II (NCT01223222)
C16G2	Armata Pharmaceuticals	Dental Caries	Topical	II (NCT02594254)

1.5.4 Library Creation and Spot Synthesis

Clinical trials and entering the marketplace are the latter stages of drug development. However, amongst the earliest stage of drug development, and more specifically antimicrobial peptide drug development, identification and optimisation of potential hit candidates are required. For AMPs, a common starting point could be to explore existing naturally occurring AMPs as a template or even consider synthetic rationale design. Peptides existing in nature can be identified from databases, as mentioned before (Zhao *et al.*, 2013; Wang *et al.*, 2015; Waghu *et al.*, 2016; Kang *et al.*, 2019). In contrast, synthetic rationale designed peptides can be generated from *in silico* methods and machine learning capabilities (Mikut and Hilpert, 2009; Ashby *et al.*, 2016). Furthermore, to synthesise and screen some of these large peptide libraries, a high-throughput methodology is required to whittle down the number of potential lead candidates.

In the early 1990s, Ronald Frank devised a revolutionary method to synthesise peptides onto a different solid phase, cellulose membranes, termed "spot synthesis" (Frank, 1992). The spot synthesis technique was further developed to include an automated robotic synthesiser (Figure 2.1). It allowed each spot on the membrane to contain a peptide with a sequence Figure 2.4**)** and enabled mass production of peptides at a meagre cost and very little labour compared to synthesis on resin. The peptide quality is usually similar to the resin; however, it is usually not purified (López-Pérez *et al.*, 2017). Identity, homogeneity and quality of the synthesis of the peptides can be determined using liquid chromatography-mass spectrometry (LC-MS) and high-performance liquid chromatography (HPLC). Spot synthesised peptides are sufficient to carry out high throughput antimicrobial screens reliably to identify potential hits (Hilpert *et al.*, 2005). Once hits are identified, the peptides can be scaled up using resin synthesis to increase the yield and further characterised.

Other ways to chemically generate peptide libraries and synthesise via Spot include the "multi-pin" and "tea "bag method (Geysen *et al.*, 1984; Sällberg *et al.*, 1991). An alternative

strategy is by using a biological approach. The biological approach to library creation and synthesis include phage display and ribosomal display (Xie *et al.*, 2006; Lindner *et al.*, 2011).

1.5.5 Peptide Engineering

The chemically synthesised peptides using SPPS are generally synthesised using resin. Post bioactivity characterisation, peptides may undergo modification to improve their profile and attractiveness as a therapeutic. One widely used method to engineer peptides is to switch from L-amino acid to the D-amino acid form. This instantly improves the proteolytic stability of the peptides and is an isomer of the L-form (Feng and Xu, 2016). Moreover, it has been reported by Manabe and Kawasaki that the D-form of the peptides they tested showed increased antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* (Manabe and Kawasaki, 2017). Other engineering strategies explored have been lipidation and glycosylation, particularly the optimal fatty acid length for lipidation, showing increased activity against a range of pathogens with a short AMP (Grimsey *et al.*, 2020).

The cyclisation strategy has been associated with increased stability and exertion of biological functions of particular peptides (Hayes *et al.*, 2021). Dathe *et al*. found that cyclised variants of arginine and tryptophan-rich peptides increased their potency towards *E. coli* compared to their respective linear variants (Dathe *et al.*, 2004). Moreover, Mwangi *et al*. found that a modified cyclised version of peptide cathelicidin-BF-15, ZY4, was more potent against a wide range of pathogens including *P. aeruginosa* (Mwangi *et al.*, 2019). Furthermore, they found that ZY4 exhibited minimal toxicity and demonstrated high stability *in vivo*.

One of the most straightforward engineering techniques is substituting amino acids of a peptide within a sequence. Apidaecin, a peptide isolated from the western honey bee, underwent such substitution with all amino acids and led to some variants having up to a 64 - old increase in potency against *E. coli, P. aeruginosa* or *S. aureus* (López-Pérez *et al.*, 2017). Other engineering strategies which have been employed and shown antimicrobial potency include conjugating AMPs with bacteriophages, antibodies and existing antibiotics (Mishra *et al.*, 2015; Defraine *et al.*, 2016; Touti *et al.*, 2018).

1.5.6 Mode of Action

There have been many described modes of action for AMPs (Yeaman and Yount, 2003; Brogden, 2005; Benfield and Henriques, 2020). One of the primary targets is the bacterial membrane in various ways (Epand and Vogel, 1999; Benfield and Henriques, 2020). The initial engagement of most AMPs is through electrostatic interactions, whereby the positive charge originating from the amino acid residue of the peptide binds to the negatively charged components on the cell wall (Zasloff, 2002). The negative charge is attributed to teichoic acids in Gram-positive bacteria and lipopolysaccharide (LPS) on the surface of Gram-negative bacteria (Yeaman and Yount, 2003). The membrane model proposed for α-helical structured peptides is the barrel stave model. The hydrophobic portion of the AMP faces outwards, and the hydrophilic portion binds to the membrane (Brogden, 2005). Only peptide alamethicin has been reported to employ the barrel stave mechanism as its mode of action (Qian *et al.*, 2008). The carpet model suggests that a high concentration of the peptide (regardless of structure) gathers at the target membrane surface and covers it in a carpet-like manner, leading to displacement changes in the membrane fluidity, resulting in membrane disruption (Shai, 2002). Peptides cecropin P1 and aurein 1.2 employ this mechanism and other α-helical peptides (Boman *et al.*, 1993; Oren and Shai, 1998; Fernandez *et al.*, 2012). The toroidal model for AMPs postulates the expansion of the lipid head, causing curvature of the membrane by the peptide being inserted in the membrane channel, culminating in its disruption (Yeaman and Yount, 2003). Peptides such as melittin, protegrins (a family of peptides ranging from 16-18 amino acids in length isolated from porcine white blood cells), magainin-2 and lacticin Q have all been reported to employ the toroidal mechanism (Yang *et al.*, 2001; Lazaridis *et al.*, 2013; Lee *et al.*, 2015). Three well-known models for the membrane peptide interaction are schematically represented (Figure 1.6)

Although the membrane has been touted as the primary target for AMPs, many AMPs act intracellularly without disrupting the membrane. The mechanisms include binding to DNA, inhibiting cell wall synthesis and inhibiting DNA, RNA and protein synthesis (Figure 1.7).

Peptides such as buforin II, Tachyplesins (family of peptides horseshoe crabs) and indolicidin inhibit DNA synthesis (Nakamura *et al.*, 1988; Park *et al.*, 1998; Ghosh *et al.*, 2014). RNA and protein synthesis can be inhibited by peptides HNP-1, PR39 and CP10A (Boman *et al.*, 1993; Friedrich *et al.*, 2001). Other forms of intracellular targeting involve lipid II sequestration carried out by mutacin 1140 and ribosome pathway inhibition shown by DM3 (Hasper *et al.*, 2006; Le *et al.*, 2016). Some peptides have a multimodal mechanism of action with multiple intracellular targets. These peptides include buforin II and indolicin (Le *et al.*, 2017).

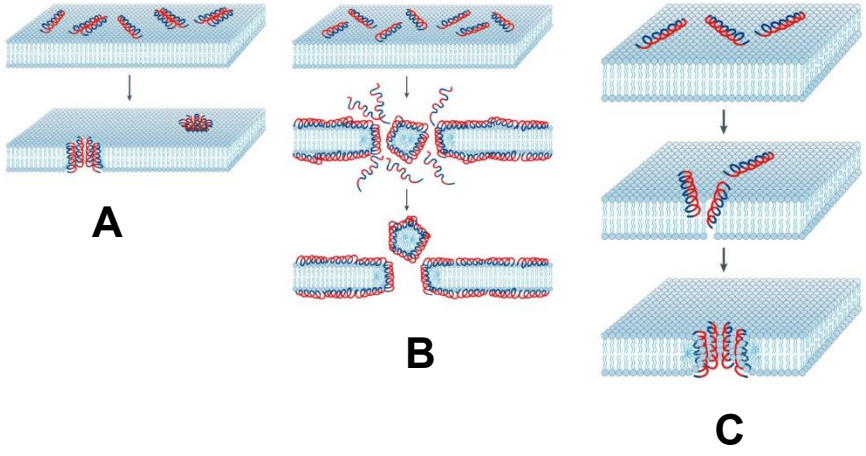


Figure 1.6 Schematic representation of membrane acting AMPs.

The three well-known proposed membrane pore forming mechanisms are: Barrel stave (A), Carpet model (B) and Toroidal model (C), which all lead to disruption of the cell membrane and eventually cell death. Modified and taken from (Brogden, 2005).

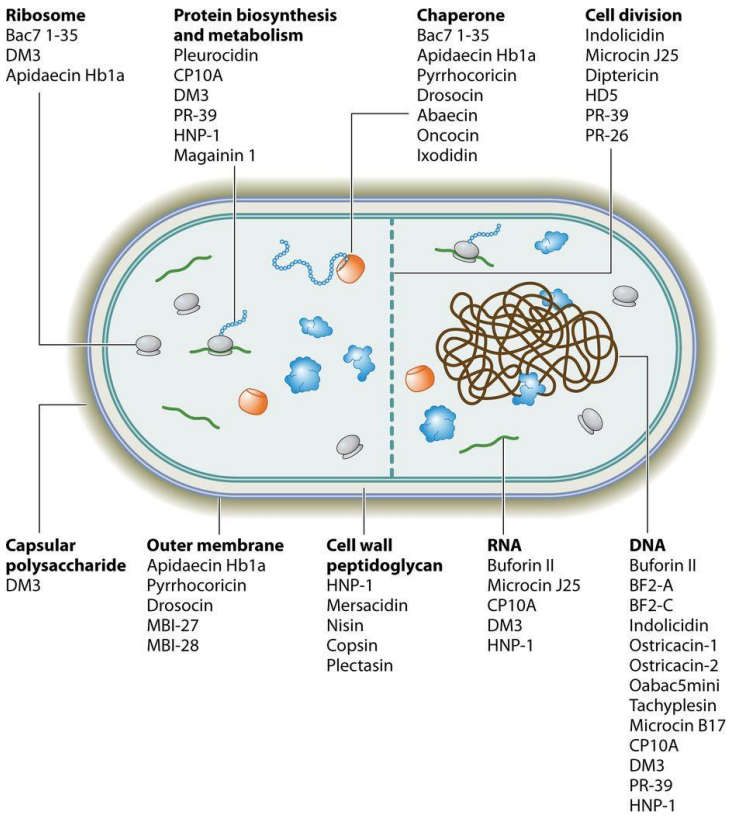


Figure 1.7 Intracellular targets for AMPs.

A schematic representation of the various proposed intracellular targets and the peptides which utilise these targets. Modified from (Le *et al*., 2017).

1.5.7 Toxicity, Translation and In Vivo

One of the main hindrances to AMP development has been the problem of toxicity. It has been evident with many peptides, particularly from nature with antimicrobial potency but with a high level of toxicity. Peptides such as melittin and tachyplesins fall into this category (Ramamoorthy *et al.*, 2006; Ko *et al.*, 2020; Koehbach *et al.*, 2021). It results in peptides with a low therapeutic value. Therefore, it is paramount to identify and exclude peptides with toxicity earlier on in the development stage. Even with rationally designed peptides with potent antimicrobial activity, it has coincided with an increase in toxicity. It was reported from the haemolytic toxicity of 3000 peptides (unpublished) from Mikut *et al*. (Mikut *et al.*, 2016). Haemolytic toxicity particularly would exclude peptides from being administered systemically. Hence, early identification of toxicity can reduce development time and cost burden.

Another issue with AMPs is predicting how their *in vitro* activity will translate into an animal model, namely murine. Often, many peptides show promising and excellent *in vitro* data, but unfortunately, are found non-efficacious in a murine model. It is suggested due to the sheer number of peptides reported and published as potent *in vitro*, but far lower number published *in vivo* in comparison. Testing many *in vitro* parameters that simulate *in vivo* physiological conditions could be worthwhile to counteract such potential issues. Conditions that can possibly be explored are serum (AMPs are known to degrade in serum) and cations. A short linear L-peptide, Peptide 7 (OptP7), demonstrated diminished antimicrobial activity in serum and fast degradation in serum (Koehbach *et al.*, 2021). In contrast, the D-version of the same peptide remained mainly intact in serum, although with reduced activity in the presence of serum (unpublished). Another peptide that has shown reduced antimicrobial activity in the presence of serum was human beta-defensin-3 (Maisetta *et al.*, 2008).

Also, interaction with ions within the blood, such as sodium, magnesium and calcium, has caused the reduction of antimicrobial activity for many peptides against a plethora of different pathogens, including *P. aeruginosa* (Moerman *et al.*, 2002; Saido-Sakanaka *et al.*, 2005; Maisetta *et al.*, 2008; Ko *et al.*, 2020). The plethora of different parameters that cause

the reduction or total inhibition of activity should be investigated as early as possible to avoid a halt in potential candidate development later.

In vivo evaluation of any novel drug usually passes through a rodent model. AMPs that pass the hindrances mentioned above regarding their *in vitro* activity may be ideal candidates to put forward into a rodent model. Peptides such as DP7, AA139, ZY4 and WLBU2 have shown efficacy against *P. aeruginosa* in murine infection models (Deslouches *et al.*, 2005; Mwangi *et al.*, 2019; Elliott *et al.*, 2020; Yin *et al.*, 2020). However, testing within rodent models attracts a high cost, ethical considerations and specialist husbandry facilities which can slow down the development and transition of AMP from *in vitro* to *in vivo*. In recent years, alternative animal models have been explored to bridge the gap between translation.

A nematode, *Caenorhabditis elegans,* has been used to evaluate many disease states, including antimicrobials. They are easy to cultivate and are highly differentiated. They have muscle cells, hypodermis, a nervous system, intestine, gonads, glands, and an excretory system (Artal-Sanz *et al.*, 2006). Uccelletti *et al*. found that AMPs extracted from frogs skin evaluated in this model using *P. aeruginosa* infection could potentially increase worm survival by up to 30 % (Uccelletti *et al.*, 2010). Also, Mohamed *et al*. found that a short D-peptide, D-RR4 protected *C elegans* from lethal doses of MDR *P. aeruginosa* and *A baumannii* (Mohamed *et al.*, 2017).

Galleria mellonella (the larvae of the greater wax moth) has recently gained traction as an alternative infectious disease model because it is cost-effective, easy to rear, and lacks extensive ethical considerations (Ramarao *et al.*, 2012; Parthuisot *et al.*, 2018). Moreover, it can be incubated at optimal bacterial growth temperatures (i.e. 37 °C) and remarkably has structural similarities to the innate immune system similarities to mammals (Tsai *et al.*, 2016). The D-enantiomer version of LL-37, a human cathelicidin, rescued 60 % of *P. aeruginosa* infected worms, compared to the control after 96 hours (Dean *et al.*, 2011). It should be noted that *P. aeruginosa* is a natural pathogen towards *G. mellonella*, with inoculums as low as 10 CFUs demonstrating 100 % death of larvae after 48 hours (Andrejko *et al.*, 2014).

Other alternative models for exploring antimicrobials are *Drosophila melanogaster* (fruit fly) and *Danio rerio* (zebrafish). Antimicrobials were evaluated using *P. aeruginosa* and MRSA, and it was found that they delayed death and increased survival of the flies (Heo *et al.*, 2009; Thomsen *et al.*, 2016). AMPs were used to evaluate their toxicity towards zebrafish (Morash *et al.*, 2011; Cebrián *et al.*, 2019). Also, AMPs were evaluated for their efficacy to protect zebrafish against aquatic bacteria infections (Ding *et al.*, 2012; Price *et al.*, 2019).

1.6 Aims and Objectives

Novel antimicrobial compounds against multidrug-resistant bacteria are needed. This study aims to identify, optimise, characterise and engineer antimicrobial peptides against *Pseudomonas aeruginosa* that also possess low toxicity against human cell lines. Additional aims include further characterisation of promising candidate AMP hits.

Specific objectives

1. Synthesise libraries of peptides obtained from naturally occurring sources and *in silico* designed sources, using Spot synthesis.
2. Screen libraries against a luminescent strain of *P. aeruginosa* and human erythrocytes.
3. Screen subset of libraries against multidrug-resistant *E. coli* in the presence of serum
4. Characterise hit AMPs regarding their antimicrobial and haemolytic toxicity.
5. Engineer the hit AMPs through amino acid substitution, isomerisation, hybridisation and cyclisation.
6. Investigate selected candidates' potential mode of action by assessing killing kinetics, biological small angle X-ray scattering (BioSAXS) and morphological changes.
7. Evaluate a subset of AMPs from different categories in simulated physiological conditions, including human serum, albumin and ions.
8. Evaluate *P. aeruginosa* pathogenesis, peptide toxicity and efficacy in a *Galleria mellonella* model.

2 Material and Methods

2.1 Peptide Synthesis – Spot technology

2.1.1 Preparation and functionalisation of membrane

A cellulose filter membrane (Whatman grade 50) was cut to 10.5 cm by 15.5 cm. Four membranes in total were created, which allowed for 640 peptides sequences to be manufactured. The four membranes were functionalised with glycine to prepare for Spot synthesis, which results in an ester. For the incubation, 5.94 grams of Fmoc-glycine (Bachem) was dissolved in 80 mL of dimethylformamide (DMF) (VWR). In addition, 3 mL of *N,N'*-diisopropylcarbodiimide (DIC) (Merck Life Science UK) and 3 mL N-methylimidazole (NMI) (Merck Life Science UK) were added. The membranes were left overnight on a Stuart SSL4 see-saw rocker (Cole-Palmer) to rock at 11 oscillations per minute (OPM) at room temperature (RT). The following day, the activation mixture was discarded, and the membranes were washed three times with 20 mL DMF per membrane per wash, with three minutes at 20 OPM for each wash. To cleave the Fmoc protection group, 25 mL of 20 % piperidine (Merck Life Science UK) in DMF (*v/v*) was added to each membrane and incubated on a shaker (15 OPM) at RT for 20 minutes. The membranes were subjected to 5 x 20 mL ethanol (VWR) and 5 x 20 mL DMF washes per membrane for 3 minutes each (24 OPM). Then 20 mL 0.01 % bromophenol blue (Merck Life Science UK) in Ethanol (500 µL bromophenol blue and 49,500 µL ethanol) were added to each membrane to confirm the presence of free amine groups. Afterwards, each membrane was washed three times in 20 mL ethanol and left to dry for 18 hours overnight in a fume hood at RT.

2.1.2 Automated Spot Peptide Synthesis

Before starting the automated peptide synthesiser, Intavis Multiprep RSi using the Spot module and standard Fmoc chemistry, sequences were input into the Multipep software. The required volume for reagents was prepared in accordance with the software and loaded into the synthesiser. Fmoc-protected amino acids obtained from Bachem were dissolved in N-methylpyrrolidone (NMP) (VWR) to achieve a 0.5 M solution from previously frozen stock at -80 ˚C. Hydroxybenzotriazole (HOBt) and DIC were prepared as a 1.1 M solution in NMP, 20 % Piperidine in DMF (VWR) and a capmix mixture of 5 % acetic anhydride *v/v* (Fluka) in DMF was prepared. A 0.1 % bromophenol blue (BPB) in ethanol (*m/v*) was prepared and stored at RT for later use in staining the spots.

The first activated amino acid solutions were automatically distributed onto the membranes by pre-activating the amino acids using HOBt, DIC and NMP. An overview of the INTAVIS Spot synthesiser with spot module is shown in Figure 2.1, with a detailed and simplified overview of the synthesis in Figure 2.2 and Figure 2.3. In most cycles before the final cycle, 0.1 % bromophenol blue was used to stain the membrane, indicating the presence of free amine groups (Figure 2.4A) and indicating each peptide's position. Each spot was marked with a mechanical pencil at the centre of the spot.

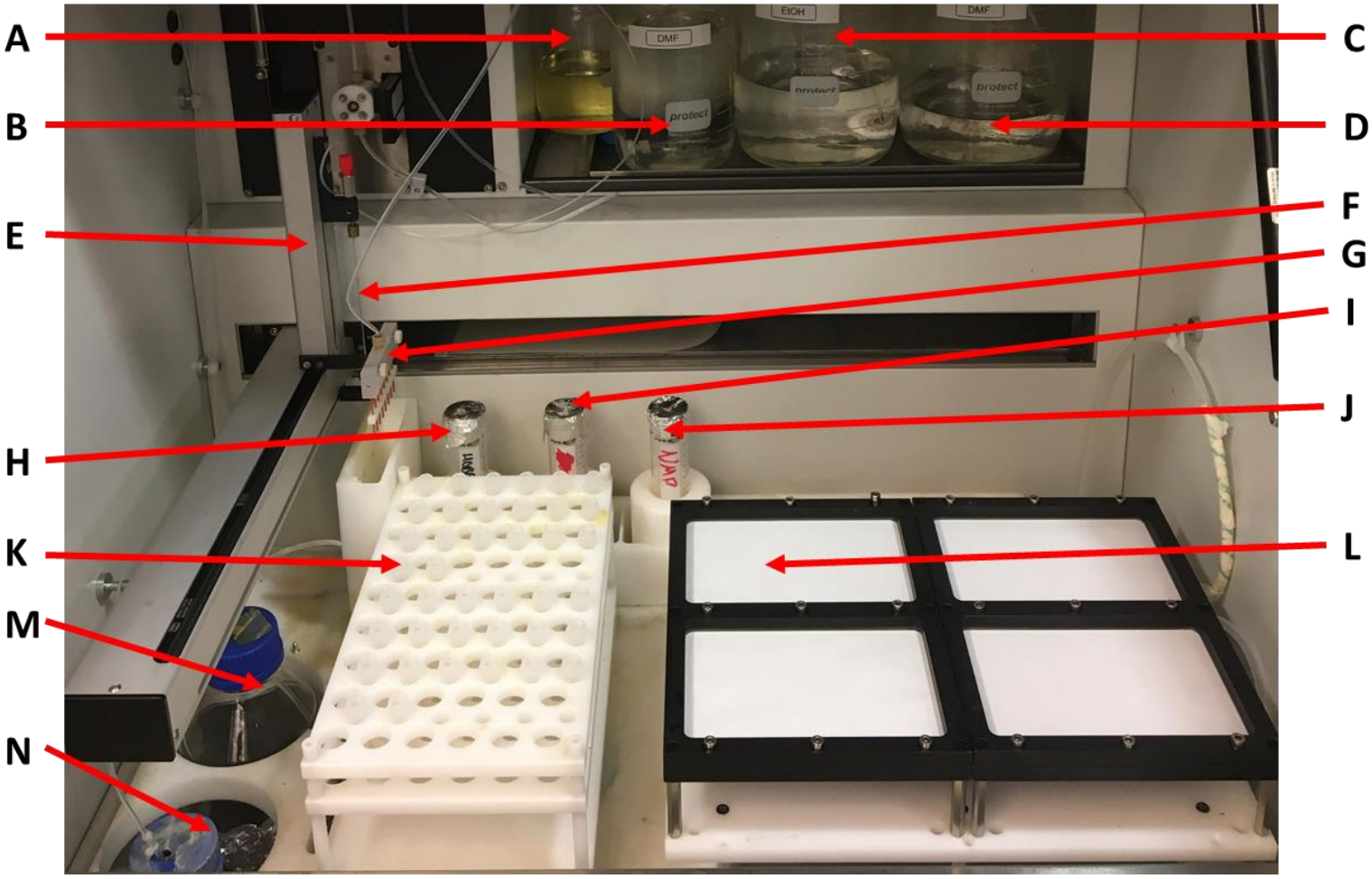


Figure 2.1 Typical Spot synthesis setup on the INTAVIS synthesiser

Intavis Multipep RSi synthesier with Spot module attachment. **A:** solvent vapour trap, **B:** 1 litre glass duran filled with DMF, for washing and rinising needle. **C:** 2 L glass duran filled with ethanol, for washing membranes. **D** is a 2 L glass duran filled with DMF, for washing membranes. **E** is the needle and manifold arm rotor. **F** is the needle, which couples the amino acids, and spots them onto the membrane. **G** is the 8 pronged manifold which washes membrane with either DMF, ethanol, capmix or piperidine. **H** is 1.1 M HOBt in NMP, **I** is 1.1 M DIC in NMP and **J** is just NMP alone. **K** is the vials (2 mL) which stores the amino acid in the rack. The lower half of the rack contained empty vials which served as a mixer vial for activating corresponding amino acids. **L** is the cellulose membrane holder on a rack, connected to an extraction pump. **M** contains 20 % piperidine in DMF in a glass duran, and **N** is a glass duran containing capmix, which is 5 % aceteic anhydride in DMF.

Cycle 1	Cycle 2 – 9	Final Cycle
Activate AA (ratio of 0.2 µl 1.1 M HOBt + 0.2 µl 1.1 M DIC + 0.01 µl NMP + 0.5 µl 0.5 M AA per spot per coupling) into mixer vial	Prime Manifold and Wash Membrane: 20% Piperidine X 2 (25 ml per wash)	Prime Manifold and Wash Membrane: 20% Piperidine X 2 (25 ml per wash)
AA Coupling Round 1 (0.9 µl aliquoted)	Wait 5 minutes after each wash	Wait 5 minutes after each wash
AA Coupling Round 2 (0.9 µl aliquoted)	Prime Manifold and Wash Membrane: DMF x 5 (25 ml per wash)	Prime Manifold and Wash Membrane: DMF x 5 (25 ml per wash)
Rinse Needle: with DMF	Prime Manifold: DMF	Prime Manifold and Wash Membrane: Ethanol x 5 (20 ml per wash)
Prime Manifold and Wash Membrane: Cap mix x 2 (6 ml per wash)	Pause (any cycle before cycle 7)	Rinse Needle: with DMF
Wait 5 minutes	Stain Membrane: BPB (10 ml 0.01% v/v BPB in Ethanol) This step only used in conjunction with pause	**End**
Prime Manifold and Wash Membrane: DMF x 5 (25 ml per wash)	Wait 5 minutes	
Prime Manifold and Wash Membrane: Ethanol x 5 (20 ml per wash)	Mark SPOT (with mechanical pencil in centre of blue spot)	
Rinse Needle: with DMF	Prime Manifold and Wash Membrane: Ethanol x 5 (20 ml per wash)	
Continue to next cycle	Activate AA	
	AA Coupling Round 1	
	AA Coupling Round 2	
	Prime Manifold and Wash Membrane: Cap mix x 2 (6 ml per wash)	
	Wait 5 minutes	
	Prime Manifold and Wash Membrane: DMF x 5 (25 ml per wash)	
	Prime Manifold and Wash Membrane: Ethanol x 5 (20 ml per wash)	
	Rinse Needle: with DMF	
	Repeat until cycle 9, then continue to final cycle	

Figure 2.2 Detailed outline of Spot synthesis

A typical detailed outline for the synthesis of a nine-mer peptide, using the Spot module of an Intavis Multipep RSi peptide sysnthesiser.

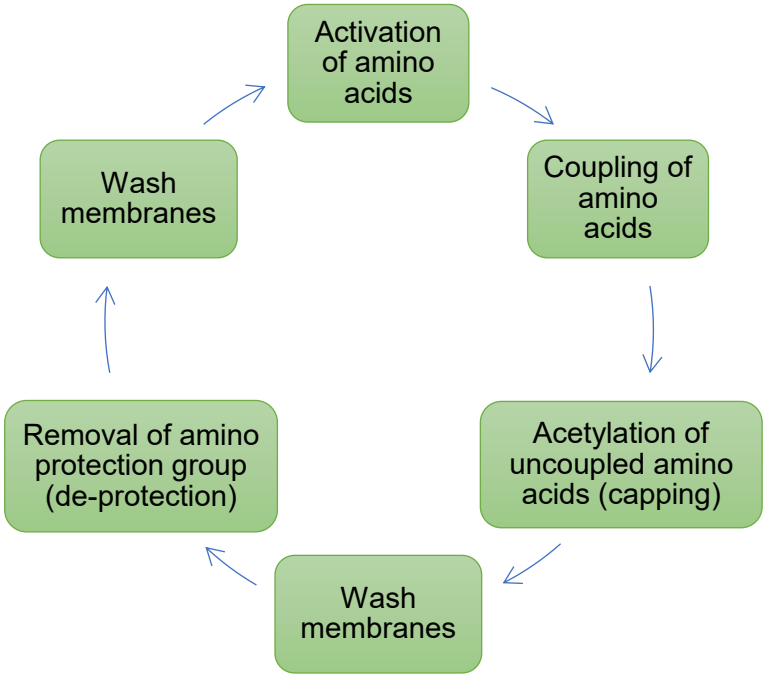


Figure 2.3 Schematic representation of solid phase peptide synthesis.

The schematic shows the simplified steps which were performed for one round of coupling of an amino acid in the automated synthesiser. This is a repeated process until the final cycle.

2.1.3 Side Chain Deprotection

The membranes were removed from the synthesiser and left to dry overnight at RT. To remove the acid-labile moieties from the peptides on the membrane, two cleavage solutions were prepared:

Solution 1: 90 mL Trifluoroacetic acid (TFA) (Merck Life Science UK), 5 mL dichloromethane (DCM) (Acros Organics), 3 mL triisopropyl saline (TIPS) (Acros Organics) and 2 mL H_2O.

Solution 2: 50 mL trifluoroacetic acid (TFA) (Merck Life Science UK), 45 mL dichloromethane (DCM) (Acros Organics), 3 mL TIPS and 2 mL H_2O.

First, 25 mL of Solution 1 was added to each membrane, left to bathe for 30 minutes, and then discarded. Next, 25 mL Solution 2 was added to each membrane, and this was left rocking (20 OPM) at room temperature for 2 hours and then discarded. Then each membrane was subject to a 3 x 20 mL DCM wash to remove TFA. Then 3 x 20 mL ethanol, 3 x 20 mL DMF, 3 x 20 mL Tris, and 3 x 20 mL H_2O washes per membrane were performed on a rocker at 25 OPM for 3 minutes per wash. The membrane was then left to dry overnight (18 hours) at room temperature.

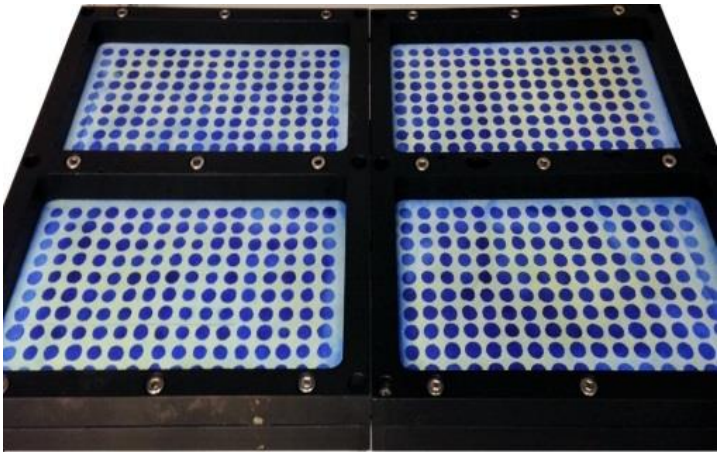
2.1.4 Ammonia gas cleavage

The dried membranes were placed in a glass desiccator (VWR), and a vacuum seal was created. Ammonia (NH_3) was used to cleave the peptides off the membranes to allow solubilisation and create a peptide amide. NH_3 (BOC Industrial Gases) was transferred to an ammonia resistant balloon (Deutsch & Neumann) from an NH_3 canister. The ammonia was transferred into the desiccator via the slow release of the vacuum seal. The membranes were left overnight and then taken out of the desiccator.

2.1.5 Preparation of spots

The peptides on the cellulose membrane were hole-punched out using a one-hole standard paper puncher (Figure 2.4B) and placed into 150 - 200 µL of autoclaved deionised water in a 96 well plate (Corning). The plate was then covered in a heat-sealing aluminium foil (Fisher Scientific) to prevent evaporation and stored in the freezer at -20 ˚C. The plates were left at RT for 30 minutes and briefly centrifuged at 1000 RCF for 5 minutes before use. The concentration of a selection of peptides ranged from 200 – 300 µg/mL, determined by using a spectrophotometer at 280 nm (Nanodrop 2000).

[A]



[B]

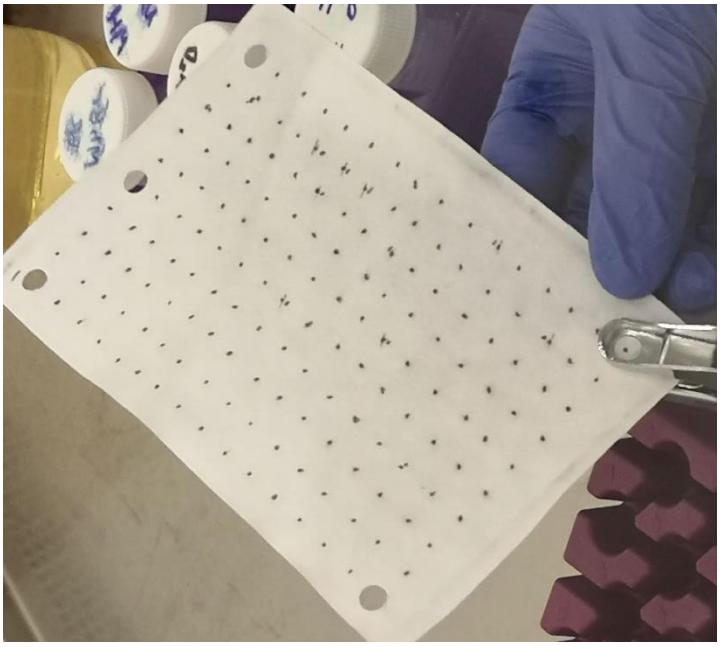


Figure 2.4 Spot staining and punch out.

Following the addition of 0.1 % BPB (*w/v*) to the Spot synthesis, the appearance of **[A]** occurs, due to the presence of free amino groups; which indicates the position of the peptide. After being marked with a mechanical pencil and post synthesis, they are punched out with a **[B]** single hole puncher and solubilised in water.

2.2 Peptide Synthesis - Resin

2.2.1 Resin Preparation

Using the Intavis Multiprep RSi column module, high Loading Resin (Rapp-Polymere) was weighed in accordance to scale requirement, which was determined by the following formula:

$$Weight\ of\ resin\ per\ peptide\ (g) = \frac{Scale\ of\ synthesis\ (mMol)}{Resin\ loading\ capacity\ (mMol)}$$

The following reagents were prepared before the synthesis:

- Amino acids with Fmoc protection (Bachem) were prepared as a 0.5 M solution in NMP
- 4 M N-methylmorpholine (NMM) (Merck Life Science UK) in NMP
- 5 M 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Carbosynth) in NMP
- 20 % Piperidine *v/v* (Acros Organics) in DMF, either freshly prepared or from stock
- 5 % Acetic anhydride *v/v* (Fluka) in DMF, referred to as Capmix

Using the Multipep software, the resin was initially swelled with DMF before commencing onto the automated synthesis.

2.2.2 Automated Peptide Synthesis

Before starting the automated peptide synthesiser, Intavis Multiprep RSi, with the column module, sequences were input into the Multipep software. The required volume for reagents was prepared in accordance with the software and loaded into the synthesiser: Fmoc protected amino acids dissolved in NMP to achieve a 0.5 M solution from previously frozen stock at -80 ˚C; 5 M HBTU dissolved in NMP and DIC prepared as a 1.1 M solution, also dissolved in NMP and neat NMP all placed into 50 mL microfuge tubes (BD Falcon). Solutions of 20 % Piperidine *v/v* in DMF (VWR) and capmix mixture of 5 % acetic anhydride *v/v* (Fluka) in DMF were placed into glass bottles.

2.2.3 Sidechain deprotection and cleavage

TFA was used to cleave the peptide from the resin, and ice-cold tertiary-butyl ether (Merck Life Sciences UK) was used to precipitate the peptide. The TFA cleavage solution consisted of 19 mL TFA, 500 µL DCM, 300 µL TIPS and 200 µL H_2O, which was required for ten peptides, and left to shake at 800 OPM for three hours. They were then precipitated into 20 mL Tert-butyl ether, vigorously shaken, spun at 3000 RCF for five minutes and left to dry overnight after removing the supernatant. The next day, the pellet was suspended in water, frozen at -80 ˚C and lyophilised using a VirTIs Benchtop Pro 3XL freeze drier with the 50 mL microfuge tube lids replaced with non-residual wipes (Kimtech Science). Complete lyophilisation was indicated upon the appearance of usually an off-white powder within the tube.

2.3 Peptide Homogeneity & Purification

2.3.1 Homogeneity

A small grain of the lyophilised sample was taken with a spatula and dissolved in 100 µL of a 20 % HPLC acetonitrile (ACN) (VWR) in HPLC water (VWR). The solution was then placed in a 150 µL insert (Fisher Scientific) and placed into a 2 mL flat glass vial (Fisher Scientific). Homogeneity was determined through high-performance liquid chromatography (HPLC) using a Shimadzu LC-2010A equipped with a Shimpack VP-ODS column. The column was isocratically equilibrated to 5 % ACN for 3 minutes, and a 50 µL of the peptide was loaded onto the column. Then, a 5 – 70 % HPLC ACN/0.1 % TFA solvent gradient was used over 32.5 minutes at a flow rate of 1 ml/min, followed by 3.5 minutes of 100 % ACN to rinse the column and a further 6 minutes at 5 % ACN to equilibrate it for the following sample. The trace was analysed, and homogeneity was determined.

2.3.2 Purification

The dried peptides were dissolved in liquid chromatography-mass spectrophotometry grade water/acetonitrile/TFA (Fluka) to a ratio of 80:20:1 (*v/v/v*). Those which were unable to be dissolved using this strategy were then subjected to the following until dissolved: first, heated up to 60 ˚C in a water bath (Pharmacia Biotech), ACN concentration was increased up to 40 %, then underwent sonication, or was re-lyophilised and dissolved in 25 – 40 % DMF in water. 1 mL of the peptide sample was aliquoted into a 2 mL glass vial (Fisher Scientific) and placed into the sample rack. The peptides were purified using reverse-phase high-performance liquid chromatography-mass spectrophotometry (HPLC/MS), linked to electrospray (Shimadzu LC-2020), equipped with a preparative Phenomenex, Jupiter C_{18} column. The column was isocratically equilibrated to 10 % ACN for 10 minutes and loaded with 1 mL of the peptide solution. 10 – 40 % solvent gradient of ACN/0.01 % TFA over a period of 20 minutes at a flow rate of 15 mL/min. The absorbance at 220 nm and the mass-to-charge ratio of the eluent was monitored, and the peak fractions were collected manually. Fractions were pooled as required the purification run was analysed to determine homogeneity (see 2.3.1). The collected fractions were frozen at -80 ˚C and lyophilised using the VirTIs Benchtop Pro 3XL freeze drier.

2.4 Storage and Preparation of Bacterial Cultures

2.4.1 Bacterial Strains

Various bacterial strains were utilised in this study for multiple experiments (Table 2.1). The strains were supplied either with the bacteria plated on an agar plate, lyophilised or frozen stock.

Table 2.1 Bacterial strains

The various bacteria, the variant (strain) of the bacteria and the different experiments in which they were used.

Bacteria	Strain	Experiments
Pseudomonas aeruginosa	PA01 – From Robert Hancocks Laboratory	Minimum Inhibitory Concentrations (MICs), Time Kill, Resistance Assay and Transmission Electron Miscopy and Biological Small-Angle X-Ray Scattering
Pseudomonas aeruginosa	H174	Luminesce Antimicrobial Screening
Escherichia coli	K12	Minimum Inhibitory Concentrations
Escherichia coli	#15 – Multi-drug resistant Clinical Isolate from St Georges University of London (SGUL)	Antimicrobial Screening in the presence of human serum
Staphylococcus aureus	E-MRSA 15	MICs
Klebsiella pneumoniae	Clinical Isolate (SGUL)	MICs
Acinetobacter baumannii	Clinical Isolate (SGUL)	MICs
Enterococcus faecalis	VRE ATCC 51299	MICs

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2.4.2 Bacterial storage and agar plate preparation

All strains of bacteria were stored in Mueller-Hinton (MH) broth (PanReac AppliChem) containing 7 % Dimethyl sulfoxide, DMSO (Merck Life Science UK) in a -80 ˚C freezer using cryogenic vials (Merck Life Science UK). Mueller-Hinton agar was used as the media to grow all strains of bacteria and any subsequent bacteria used. The agar was prepared using the following mixture to create approximately 40 plates: 16.8 g Mueller-Hinton (Merck Life Science UK) and 10.8 g Agar (Merck Life Science UK) with 800 mL demineralised water then autoclaved. Plates were poured at 20 mL per plate and stored at 4 ˚C. *P. aeruginosa* or other bacteria were streaked onto MH agar plates (Fisher Scientific) and left overnight at 37 ˚C. The following day the plates were taken out of the incubator, wrapped in cellophane and left in a class II cabinet. Fresh plates were streaked every week, as and when required.

2.4.3 Preparation of Inoculum

Using a sterile loop (VWR), three morphologically similar bacterial colonies were picked and inoculated into 3 mL Mueller-Hinton broth (MH Br). The culture was incubated overnight (O/N) in a 37 ˚C shaking incubator (225 RPM). Optical density was checked at 600 nanometres (nm) of a 1:10 dilution (100 µL overnight bacterial culture and 900 µL MH broth), compared with a blank of MH Br using an Amersham Biosciences Ultrosepc 2100 Pro spectrophotometer (General Electric). Various dilutions were plated out, and colonies were counted the following day. An absorbance of 0.3 (with 1 in 10 dilution) equated to approximately 3 x 10^9 colony-forming units per millilitre (CFU/mL) for a neat O/N culture.

2.5 Luminescence Screening of P. aeruginosa

2.5.1 Preparation of culture and reagents

Luminescent *P. aeruginosa* (H174) was obtained from a cryo stock and prepared as described (see 2.4.2 and 2.4.3). Solutions of 100 mM sterile tris(Hydroxymethyl) (Merck Life Science UK) (pH 7.3), along with 2 M filter sterilised glucose (Merck Life Science UK), were prepared. A Tris/glucose (TG) mixture was created to give a solution containing 20 mM glucose.

2.5.2 Antimicrobial screen

The O/N culture was diluted 1:50 (200 µL bacterial culture and 9800 µL MH broth) and left to incubate, shaking at 37 ˚C (225 RPM), to allow the optical density at 600 nm (OD_{600}) to reach between 0.3 and 0.35; which took approximately two hours. The following amounts were a single plate. The culture was then added to the mixture of Tris/glucose (400 µL bacterial culture, to 10000 µL of TRIS/glucose solution) to give Tris/glucose/bacteria (TGB). A 110 µL solution of TGB was added to each well on the first row (A1 – A12) of a white, opaque, flat bottom, 96 well plate (Nunc), and 70 µL of TGB was added to the rest of the wells except the last row. Before any peptide was added to the plate, it was subjected to a test luminescence reading, ensuring that *P. aeruginosa* was giving off luminescence. To the first row (A1 – A12), 40µL of peptide (which included an in-house control) was added and serially diluted two-fold down the plate seven times, with the last dilution being discarded. The last row consisted of 6 negative controls (TG only, H1 – H6), and the other 6 were growth controls (TGB solution). An overview of the plate layout is demonstrated in Figure 2.5.

The plates were incubated at 37 ˚C for 4 hours before the luminescence was measured using a Tecan INFINITE 200 PRO spectrophotometer (Tecan Group). Once the antimicrobial activity was measured, 70 µL of MH broth was added to each of the wells and left to incubate overnight at 37 ˚C for visual inspection the following day for any bacterial recovery.

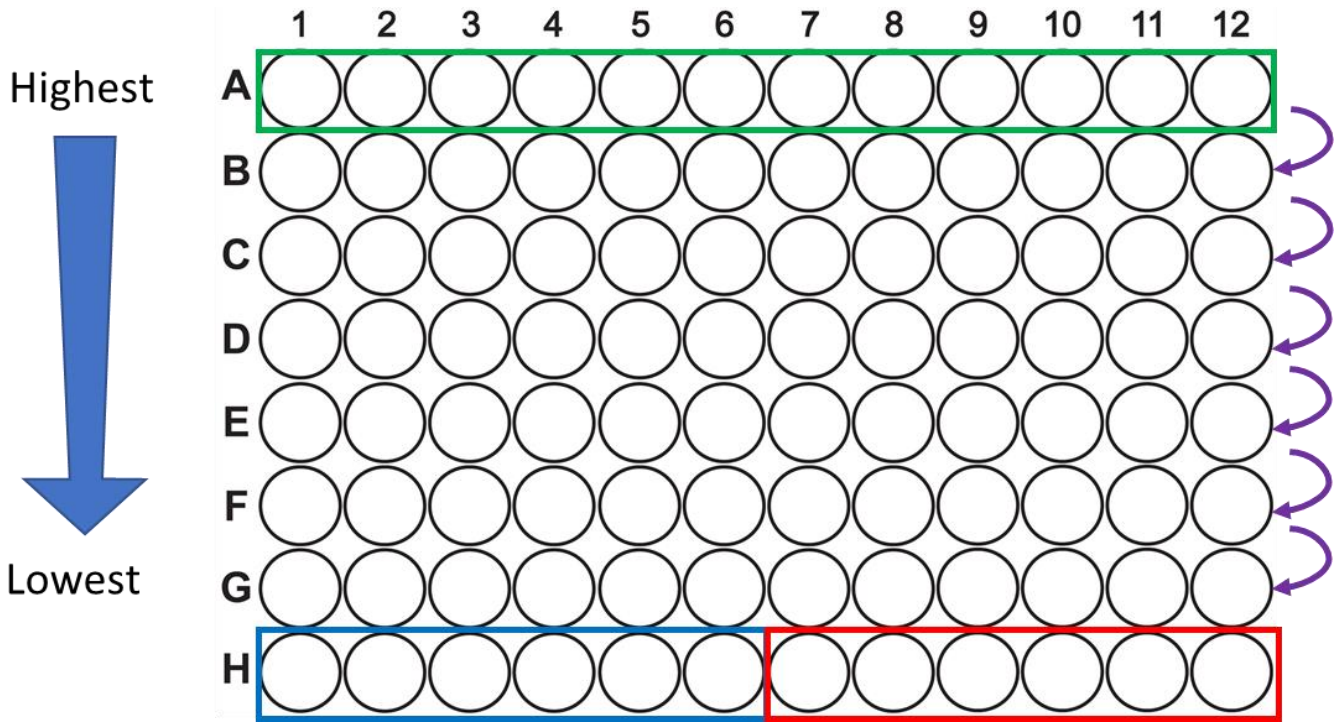

Figure 2.5 Antimicrobial screen layout plate

P. aeruginosa and peptides (highest concentration), including control are placed on the top row (green region). Subsequent twofold dilutions occur (purple arrows) until row G, which is the lowest concentration of peptide. The blue region served as a media control (i.e. only tris/glucose solution), with the red region representing the growth control (i.e. untreated bacteria). Blue arrow represents highest to lowest concentration of antimicrobial peptide.

2.5.3 Analysis

The data was analysed using SciXminer, formerly known as GaitCAD, which is an software tool running on MATLAB®. The generated excel file from the antimicrobial and haemolytic activity screens were formatted in the way required for analysis by SciXminer (Mikut, 2010). The data was analysed using a set method of parameters as described in Mikut, 2010. This gave rise to four distinct groupings of each individual peptide which was termed "more active than control/parent", "similar active to control/parent", "weaker activity than control/parent" and "inactive" in accordance with the relative inhibitory concentration$_{75}$ (comparison of 75 % of bacterial burden compared to the control peptide).

The relative IC_{75} (RelIC_{75}) values and their respective values was as follows:

1. More active than control/parent (MATC/P) = 0 – ≤ 0.5
2. Similar activity to control (SATC/P) = > 0.5 – ≤ 5
3. Weaker activity than control/parent (SATC/P) = > 5 – ≤ 20
4. Inactive = > 20

Data which was considered anomalous was labelled as an artefact, and was categorised as such, if the following conditions were met:

1. A positive correlation between peptide concentration and luminescence/fluorescence intensity whereby there was a > 30 % increase in luminescence/fluorescence when the peptide concentration was doubled or the luminescence/fluorescence increased by > 1 % of the maximal luminescence/fluorescence on the plate when the peptide concentration doubled (antimicrobial screen only).
2. Unusually high IC_{75} or low Haemolytic Concentration$_{75}$ for the control peptide/drug at the highest concentration, whether due to a pipetting error or contamination. This would result in entire plate being considered as an artefact.
3. Unusually low levels of luminescence/fluorescence in untreated wells, or very high absorbance in the untreated red blood cells. This would also result in entire plate being considered as an artefact.

2.6 Screening in the presence of Human serum

2.6.1 Preparation of culture and reagents

A multidrug-resistant *E. coli* #15 clinical isolated from blood culture was obtained from a cryo stock and prepared as described (see 2.4.1 and 2.4.2). A solution of 120 µg/mL resazurin (Merck Life Science UK) was made up in a 50 mL tube, protected from light via aluminium foil wrap around and stored in the fridge at 4 ˚C and used when required. Two solutions were made in the following way:

Solution A: 13 % Human serum (ZenBio) + 3.9 x 10^5 CFU/mL (log phase culture)

Solution B: 10 % Human serum + 3.5 x 10^5 CFU/mL (log phase culture).

2.6.2 Antimicrobial screen

The O/N culture was diluted 1 in 25 (400 µL bacterial culture and 9600 µL MH broth) and left to incubate shaking at 37 ˚C (225 RPM), to allow the optical density at 600 nm (OD_{600}) to reach between 0.2 and 0.25 and in log phase. This took approximately 2 hours. The bacterial culture was adjusted to match the aforementioned solutions of A and B. Solution A (140 µL) was added to the top rows (A1 – A12) in a flat bottom, black, opaque plate (Corning). Spot produced peptides (including control) were also added (40 µL) into the wells where solution A was added. Solution B (90 µL) was added to the rest of the wells. From the top row a twofold serial dilution was performed until row G, and discarded. The same occurred with the control peptide from H1-H6. Wells H7 – H9 served as an untreated control, and H10 – H12 contained human serum and media only . The plates were incubated at 37 ˚C for 4 hours, then 10 µL of previously prepared resazurin was added to each well and incubated for a further 2 hours. The plates were then measured for fluorescence readings (535/590 nm) using a Tecan INFINITE 200 PRO spectrophotometer (Tecan Group). The data was analysed in accordance with 2.5.3.

2.7 Haemolytic Assay

2.7.1 Blood Collection & Ethics

Fresh blood was drawn from the researcher themselves, at St Georges Hospital by a trained phlebotomist, and transported in vacutainers containing 0.109 M sodium citrate buffer (BD). No ethical approval was required, due to the blood not being stored and used on the day it was drawn.

2.7.2 Preparation & Centrifugation

Blood was centrifuged (1000 RCF for 5 minutes) in 2 mL microfuge and the supernatant, which contained plasma, was removed. The red blood cell pellet was re-suspended and mixed slowly via pipetting in phosphate-buffered saline (PBS) (pH 7.4) (Merck Life Science UK) before re-centrifugation; this process was repeated three times. Dilutions of blood and Triton X-100 (positive control) were created as follows: 4 % red blood cell (RBC) solution (4 % haematocrit) were created for 10 plates totalling 75 mL (3 mL RBC, 72 mL PBS), 5.14 % RBC was created for 10 plates totalling 20,000 µL (1028µL RBC, 18,972 µL PBS) and Triton-X (Alfa Aesar) 0.45 % was created for 10 plates totalling 890µL (4 µL Triton X-100, 886µL water).

2.7.3 Haemolytic activity screen

First, 140µL of 5.14 % RBC was added to the first row (A1-A12) of a 96 well plate (Corning). Second, 90µL 4 % RBC was subsequently added to all the other wells except the last row. Then, 40µL of peptide (including 0.1 % Triton X-100, which served as a positive control), was added to the first column of each plate (totalling 180µL and giving 4 % RBC concentration) and serially diluted two-fold across the plate, with the last dilution being discarded. The last row consisted of six negative controls (PBS only), while the other six were untreated controls (untreated 4 % RBC). The plate was incubated for 1 hour at 37˚C. Then it was centrifuged (1000 RCF for 5 minutes at 4 ˚C). Following the formation of RBC pellets, 70 µL of supernatant was removed from each well and transferred into a clear, flat, round bottom 96 well plate (Nunc), ensuring no pellets were disturbed. The plates were read under a Tecan INFINITE pro 2000 spectrophotometer at 450 nm for the absorbance.

2.7.4 Analysis

The data analysis conformed to a method similar to the one mentioned in 2.5.3; however, the groups were termed as "strongly haemolytic", "medium haemolytic", "weak haemolytic" and "non-haemolytic." In this instance, relative HC_{75} was generated, as opposed to the relative IC_{75} and absorbance (release of haemoglobin) was measured instead of luminescence/fluorescence.

The relative HC_{75} values and their respective classification was as follows:

1. Strong Haemolytic = < 0.5
2. Medium Haemolytic = ≥ 0.5 – < 1
3. Weaker Haemolytic = ≥ 1 – < 1.5
4. Non-Haemolytic = > 1.5

The haemolytic activity was determined for peptides synthesised on resin in three independent assays, with fresh blood used each time. HC_{50} was determined of the respective peptide by evaluating through non-linear regression. Log_{10} concentration was determined. Then, the absorbances for each respective compound were normalised, with 100 % cell lysis represented by 0.1 % Triton X-100. At the same time, untreated RBCs represented 0 % cell death. Non-linear regression, using the variable slope, was applied using log (peptide inhibitor) vs normalised response in Graphpad Prism, version 9.

2.8 Therapeutic Potential

To assess which peptides were viable candidates for further investigation, a therapeutic potential was determined for Spot synthesised peptides. The therapeutic potential combines both antimicrobial activity and haemolytic activity to give an output of an arbitrary value between 1 to 100. The higher the therapeutic potential, the more promising the overall candidate is and was worthy of consideration for further characterisation. In order for a peptide to achieve a higher value, the follow remits have to be met:

1. A low haemolytic activity, i.e., increased HC_{75}, which indicates weaker haemolysis
2. A high antimicrobial activity, i.e., decreased $RelIC_{75}$, which indicates strong antimicrobial activity
3. A high IC_{75} relation value, suggests a large therapeutic window, i.e. higher selectivity window between antimicrobial activity and haemolytic activity

$$IC75\ Relation = \frac{HC75}{RelIC75}$$

The therapeutic potential of each peptide was calculated via SciXMiner using the following equation:

$$\mathrm{TP} = 100 \times \exp(-\alpha\mathrm{Activity} \times (\mathrm{RelIC75})^2 \times (1 - \exp(-\alpha\mathrm{Haemolysis} \times \mathrm{HC75})^2 \times (1 - \exp(-\alpha\mathrm{Relation} \times (\mathrm{IC75Relation}))^2$$

Each condition was weighted by applying the following parameters:

αActivity = 0.2, αHaemolysis = 0.5, αRelation = 0.1

Each peptide was allocated a class based upon its therapeutic potential value, with the parameters below.

Therapeutic potential:

1. None = < 25
2. Weak = ≥ 25 – < 50
3. Moderate = ≥ 50 – < 60
4. Strong = > 60

2.9 Minimum Inhibitory Concentration

2.9.1 Preparation of Bacteria, Peptides and Antibiotics

O/N cultures of bacteria were prepared as previously described (see 2.4.3) and diluted to achieve a concentration of 6 x 10^5 CFU/mL according to the OD_{600}. The peptides were prepared by weight at a concentration of 1 mg/mL using sterilised deionised water (*w/v*). They were kept at -20 ˚C and allowed to thaw to room temperature before use.

All peptides were synthesised and purified in-house as per 2.2 except the following peptides and antibiotics, which were procured from as per below (Table 2.2)

Table 2.2 List of purchased antimicrobial compounds

List of peptides and antibiotics and their respective supplier from which they were purchased. All compounds came as solid off-white powder.

Type	Name	Supplier
Peptide	5D	Bachem
Peptide	7D	Bachem
Peptide	Cyc-7	Synpeptide
Peptide	U-HYB	Synpeptide
Peptide	Cyclotide-7	University of Queensland (Koehbach *et al.*, 2021)
Peptide	Melittin	Merck Life Science UK
Peptides	Api88, Api137, Onc72, Onc112	Leipzig University
Antibiotic	Ciprofloxacin	Merck Life Science UK
Antibiotic	Gentamicin	Carbosynth
Antibiotic	Tobramycin	Merck Life Sciences UK
Antibiotic	Meropenem	Carbosynth
Antibiotic	Cefepime	Fisher Scientific
Antibiotic	Polymixin B	Fisher Scientific

2.9.2 MIC assay – Standard and diluted media

To each well, 50 µL of the MH broth (PanReac AppliChem, prepared as per the manufacturer's instructions) was added across a round bottom, polypropylene 96 well plate (Corning). Then, 50 µL of peptide or antibiotic (which served as a control) was added into the first column (A1 – H1) and serially diluted two-fold across the plate. The excess 50 µL was discarded following the last dilution. Then, 50 µL of bacteria containing 6 x 10^5 CFU/mL was added across all wells except column 11 (negative control, media only), while row 12 acted as growth control, containing untreated bacteria. The plate was left in an incubator at 37 ˚C and evaluated the following day (18 ± 2 hours) via visual inspection. Diluted media was defined as 20 % MH broth, which was achieved by diluting standard MH broth in sterile demineralised water. All other procedures were the same as those used for the standard media assay.

To check for correct cell numbers used, as well as to observe whether any contamination occurred, a control plate was made with the bacteria (3 x 10^5 cells) diluted 10-fold three times and 5 µL of each dilution was spotted onto an MH agar plate split into a quadrant.

2.9.3 MIC assays – In the presence of serum components

Determination of antimicrobial efficacy of selected peptide candidates in the presence of human serum (HS), human serum albumin (HSA), and ions was performed. First, 50 µL of the MH broth (standard) was added across a round bottom polypropylene 96 well plate (Corning). Then, 50 µL of selected peptides and antibiotics were diluted serially as per 2.8.2. The final concentration of human serum off-the-clot (absence of anti-coagulant), filter sterilised (Zen-Bio) in a well was either 10 % or 25 %. To achieve a final serum concentration of 10 %, a 20 % serum mixture with 7.5 x 10^5 CFU/mL from an O/N culture was made before being added to the wells containing peptides or antibiotics. A final serum concentration of 25 % was achieved with a 50:50 mix of serum and 1.2 x 10^6 CFU/mL, before being added to the respective wells. Wells containing serum and media only served as a negative control, while untreated serum/bacterial mix served as a growth control.

Albumin is the most abundant protein in human serum and can be expressed in differing ways, therefore HSA expressed from different sources was evaluated. HSA direct from humans and expressed in a yeast, *Pictia pastoris* (Merck Life Science UK) were diluted in sterile water to a concentration of 500 mg/mL. The HSA stocks were then filter sterilised using 0.2 µm filter (Merk) and 10 µL of the stock was added to each well as required after the aforementioned peptide/antibiotic serial dilutions in standard media. The final concentration of HSA in each well was equivalent to 25 % human serum concentration and at a physiological concentration of 10 mg/mL (equivalent to the presence in 25 % serum presence).

To evaluate the effect of human serum components, MICs were carried out in the presence of ions either alone or all combined at their physiological concentrations. Physiologically relevant concentrations of particular ions were as follows (Merck Life Science UK): sodium chloride (NaCl - 150 mM), calcium chloride ($CaCl_2$ - 2.5 mM), iron chloride ($FeCl_3$ - 4 µM), magnesium chloride ($MgCl_2$ - 1 mM), potassium chloride (KCl – 4.5 mM) and zinc chloride ($ZnCl_2$ – 8 µM) were supplemented in either standard or minimal media.

2.9.4 MIC assay – concentrated bacteria

To determine the relevant concentrations of antimicrobial substance to be used for Electron Microscopy (2.12) and BIOSAXs (0), MICs were evaluated against 2 x 10^8 bacterial cells. O/N culture was diluted 1 in 25 and incubated at 37 ˚C for approximately two hours to achieve bacterial log phase, with an OD_{600} of 0.2 equivalent to 2 x10^8 cells. All other procedures for this assay followed 2.9.2. The next day, wells were visually inspected as per usual; however, those MICs which were unable to be determined adequately (due to cell debris), were spotted out in MH agar and checked next day to evaluate any growth. Furthermore, 20 µL of a 120 µg/mL (*w/v*) of filter sterilised resazurin was added to the entire plate and left to incubate for 2 hours. Cell viability was determined via a colorimetric change with blue indicating no cell viability, and pink indicating cells were alive.

2.9.5 MIC assay – different pathogens

In addition to *P. aeruginosa,* pathogens which formed the ESKAPE(*coli)* group were used (Boucher *et al.*, 2009). The extra bacteria used are outlined in Table 2.1. They were all assayed in the same manner as laid out 2.9.2, with the exception of VRE. O/N cultures of VRE was grown in brain heart infusion (Merck Life Science UK), with the neat culture diluted 1 in 100 to achieve a concentration between 1 – 3 x 10^5 CFU/mL. The rest of the assay for VRE mirrored 2.9.2.

2.10 Cell Toxicity

2.10.1 Cell culturing and maintenance

Human embryonic kidney cells (HEK-293, CRL-1573, ATCC, LGC Standards) were thawed from liquid nitrogen (-196 °C) tank and plated onto 75 cm^2 flasks (Corning) along with 15 mL of the following Dulbecco's Modified Eagles Medium (DMEM, Merck Life Science UK) mixture used to maintain the cells: 450 mL DMEM, 5 mL penicillin streptomycin (10,000 IU/mL, Merck Life Science UK), 5 mL L-glutamate (Merck Life Science UK) and 50 mL foetal bovine serum (FBS, Merck Life Science UK). The flask was kept at 37 °C, with 90 % humidity and 5 % CO_2 conditions, with cells being regularly passaged by adding 2 mL trypsin to detach the cells, and then suspending cells at a 1:6 ratio into 15 mL of fresh DMEM when confluence of about 70 % was observed. A further DMEM media mixture for assays was made which excluded penicillin streptomycin.

2.10.2 Cytotoxicity Assay

Upon passaging, the cell concentration was determined by haemocytometer and subsequently diluted to achieve 750,000 cells/mL in 16 mL to allow for 75,000 cells per well to be spread across the plate. After,150 µL of the cell suspension was added to each well in a black, clear bottom 96 well plate (Grenier Bio-One) and was left to incubate overnight. The following day, the wells were replaced with either 100 µL fresh assay media and 100 µL of pre-determined peptides at specific concentrations, or 0.1 % Triton X-100 (positive control), or media (untreated control), and left to incubate for 4 hours. 20 µL of 0.1 mg/mL solution of resazurin (Merck Life Science UK) was added across the plate and left to incubate overnight. The next day, fluorescence was measured at 535/590 nm using a Tecan INFINITE 200 PRO spectrophotometer.

2.10.3 Analysis

The raw fluorescence readings were inputted into Graphpad Prism, then normalised according to 0 % cell viability with 0.1 % Triton X-100, and 100 % viability with media treated wells. Further to this, the normalised data was subjected to statistical analysis in the form of non-linear regression (log concentration vs variable slope) to obtain a curve and determine IC_{50} of the peptides. Error bars indicated standard deviation from three experimental repeats.

2.11 Time Kill

A fresh O/N bacterial culture was prepared using MH broth; using methods previously described (see 2.4.3). The O/N culture was diluted 1 in 40 in 10 mL and incubated at 37 ˚C for approximately two hours to achieve bacterial log phase. The log phase inoculum was then adjusted to $1x10^6$ CFU/mL with MH broth. Within a 2 mL microfuge tube (STARLAB), the peptides were added to the inoculum at a concentration that was threefold the MIC against the wild type strain of *P. aeruginosa*. The tubes were placed in a shaking incubator (225 RPM) at 37 ˚C. At certain predetermined time points between 0 minutes and 18 hours: 10, 40, 60, 120, 240 and 1080 minutes for the peptides and for the untreated control: 0, 60, 120, 240 and 1080 minutes, 100 µL samples were taken out. Furthermore a 1:10 dilution series, five times using 10mM Tris buffer (pH 7.5) was performed using the 100 µL sample, then 5 x 5 µL were spotted for each dilution onto MH agar plates at 37 ˚C for 18 ± 2 hours. After the incubation period, the colonies were counted and the colony forming units, CFU/mL for each time point, were determined.

2.12 Electron Microscopy

2.12.1 Sample Preparation – Microbiology

O/N culture of bacteria was grown log phase to achieve 2 x 10^8 CFU/mL, peptides were added at twice the MIC value (see 2.8.3) and left to incubate for a desired time at 37 ˚C in a 2 mL microfuge tube. The samples were removed and centrifuged at 1000 RCF for 5 minutes. For washing the sample, the supernatant was removed, and the pellet was re-suspended in a 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7, Merck Life Science UK) and centrifuged again as aforementioned; this was performed a further two times. The pellet was then suspended in 2.5 % Glutaraldehyde in cacodylate buffer (Merck Life Science UK) and left shaking (200 RPM) at room temperature for one hour. The sample was centrifuged at 1000 RCF for 5 minutes and washed with PIPES buffer twice as mentioned above. Finally, the pellet was suspended in 50 µL PBS and kept refrigerated until required.

2.12.2 Sample Preparation – Electron Microscopy

The following steps were kindly performed by the Image Resource Facility at St George's, University of London. The sample containing PBS was centrifuged (1000 RCF for 5 minutes), and the supernatant was carefully removed. Step 1: 1000 µL of 0.1M cacodylate buffer (Merck Life Science UK) was added to the tube, vortexed to disrupt the pellet and placed on a rotator for 8 minutes (Fisher Scientific). Microfuge tubes were centrifuged as mentioned above with the supernatant removed, and the process was repeated with 0.1M cacodylate solution. Step 2: 2 % osmimum tetroxide (Taab) was added and placed on the rotator for a further 30 minutes. Step 1 was repeated again. Samples were then dehydrated using ascending concentrations of 70 % ethanol twice (10 minutes each), twice at 90 % (10 minutes each) and thrice in absolute ethanol (15 minutes each) were was all on rotator). Samples were centrifuged before each supernatant was removed and vortexed after the new solution was added. Resin agar was made up using agar 100 (Agar Scientific), methyl nadic anhydride (MNA - Scientific Laboratory Supplies), dimethylbenzylamine(BDMA - Scientific Laboratory

Supplies) and dodecenyl succinic anhydride (DDSA - Scientific Laboratory Supplies) and protected from moisture exposure. Then, 100 µL of propylene oxide (PO) (Scientific Laboratory Supplies) was added to the dehydrated samples thrice at 5 minutes on the rotator with centrifugation, followed by the removal of the supernatant, the addition of fresh PO and resuspension of the pellet. A 50:50 mixture of PO/resin was added to the sample, vortexed and put on a rotator for a further 45 minutes. Then the sample was centrifuged and the supernatant removed. 100 % resin was added to the sample and further rotated for 60 minutes with the microfuge lid opened. The sample was then centrifuged and replaced with fresh resin ensuring the tube was adequately labelled. The samples were then placed at 60 ˚C for 48 hours. Finally, using a razor blade and cutting guide, the microfuge tube casing was removed and the resin blocks were stored in the relevant specimen box until further use.

The following part of the protocol was performed by the researcher themselves. Semi-thin sections (1 µm) of the resin block were initially cut using a Ultramicrotome Leica EM UC7 (Leica Microsystems) and stained with toluidine blue (VWR). The section was then examined under a light microscope to determine areas of interest and potential investigation. After determination of the area of interest, ultra-thin slices (100 nm) were cut from the resin using the ultramicrotome and placed onto copper grids (Merck Life Science UK) and left to dry for 24 hours. Manual staining of the sections was performed by placing the grids on a drop of uranyl acetate (UA) for 10 minutes at RT in a glass petri dish (Fisher Scientific) layered with parafilm (VWR), where the sample was placed. The grids were then rinsed in double distilled water several times. Prior to the addition of lead citrate (LC), pellets of sodium hydroxide (NaOH) were placed around the petri dish to absorb CO_2 and prevent LC precipitation. The grids were placed on a LC drop for 10 minutes, with the petri dish covered using a lid. The grids then underwent several washes in double distilled water, left to dry and then placed in a grid box (Scientific Laboratory Supplies) and used when desired.

2.12.3 Imaging

A Hitachi H-7100 Transmission Electron Microscope (TEM) equipped with an AMT 16-megapixel high-resolution camera was used to image the samples. Initially, the chiller was turned on, followed by the creation of a vacuum. Samples were placed under a vacuum and the images from each sample were acquired at three different magnifications, ×3000, ×10000 and ×20,000, and for at least four images at each magnification were captured. The images were analysed using ImageJ to determine if there were any changes to the size of the different samples, while visual analysis was employed to interpret the morphological changes to the cells.

2.13 Small Angle X-Ray Scattering For Biological Samples

O/N cultures of either *P. aeruginosa* were diluted 1 in 50 in 40 mL of MH broth and placed into a shaking incubator at 37 ˚C until an OD_{600} of about 0.2 was reached; which equated to 2 x 10^8 CFU/mL of log phase cells. A total of 1.5 mL of this log phase culture was added to 2 mL microfuge tubes (STARLAB) with the desired antibiotic/peptide concentration. The concentration was 2 x MIC from the selected peptides/antibiotics tested at 2 x 10^8 CFU/mL determined from 2.9.4. All the samples, including untreated bacteria, were placed in a shaking incubator set to 37 ºC and 225 RPM. All samples, peptides, antibiotics and untreated bacteria were incubated for 40 minutes,

After removing the samples at the desired incubation time, the samples were centrifuged at 10,000 RCF for 5 minutes using a SciSpin Mini Microfuge, with the supernatant being removed and the pellet being suspended in a 0.1 M PIPES buffer. This was performed twice. The pellet samples were then resuspended in a 2.5 % Glutaraldehyde PIPES buffer (*v/v*) and left to shake at RT on a platform shaker. Afterwards, the samples were washed thrice with 1 mL PBS. Following the final washing step, the pellet was suspended in 50 µL of PBS and left refrigerated at 4 ˚C until required. The samples were then shipped to Germany for the small angle scattering experiments, which were performed

at the biological small-angle X-ray scattering (BioSAXS) P12 beamLine, at the European Molecular Biology Laboratory (PETRA III, Deutsches Elektronen-Synchrotron DESY) in Hamburg. The samples were centrifuged with a sigma microfuge and resuspended in PBS (from 100 – 150 µL) to achieve turbidity with no debris in the sample. 20 µL of the samples were transferred to a strip of 100 µL PCR tube strips (Eppendorf).

The following procedure was kindly carried out by Dr Christoph Rumanacev, from The Ruhr-University Bochum, Germany. A photon flux of 5 x 10^{12} s^{-1} was focused to a spot measuring 0.2 mm x 0.1 mm (horizontal x vertical) and the resulting diffraction pattern was recorded with a Pilatus 2M detector (Dectris, Switzerland). The sample (20 µL) was delivered into a cooled glass capillary (20 °C) by an automated sample robot. Each sample had an exposure time of 0.05 seconds with 20 diffraction patterns being recorded. Before and after every sample, the background was measured. After angular integration to obtain one-dimensional scattering curves, the background subtraction was performed. To avoid the introduction of artefacts by radiation damage, curves collected in subsequent illuminations were compared by a standard F-test. Only curves collected before the occurrence of radiation damage were further processed. These primary data processing steps were performed using the automated data pipeline SASFLOW.

Scattering data was analysed using the open source data mining MATLAB® toolbox SciXMiner (Formerly known as Gait-CAD). To compensate for the experimental variation of the cell density, the data was normalised to the initial region (0.04 nm^{-1} to 0.05 nm^{-1}) assuming that the overall shape of the bacterial cell remained unaffected. For the principal component analysis, the log of the scattering data was used and the range had to be restricted (0.055 nm^{-1} to 0.2869 nm^{-1}) due to the low intensity of some scattering curves. The principal component analysis was an easy visualisation that preserved the differences of the investigated scattering curves. The sample points were projected to a lower dimensional parameter space, built by so-called principal components. These principal components were orthogonal to each other and removed the redundancies caused by correlations of the sample points. They were computed by finding the eigenvalues of the covariance matrix of the 94

sample points. The first two principal components were found to describe the variations due to antibiotic treatments.

For reasons of better visualisation, a centred PCA starting from the mean of all scattering curves $I_m(q)$, $I(q) = I_m(q) + A \times PC1(q) + B \times PC2(q)$. Consequently, each scattering curve could be reproduced by two linear coefficients (A, B). To provide evidence of reproducibility between two measurements, the duplicates of a subset of samples were measured.

2.14 Isothermal microcalorimetry

To determine metabolic changes to a bacterium upon interaction with peptides/antibiotics, IMC was used. CalScreener by Symcel was used to set up the assays. O/N cultures of desired bacteria were grown in MH broth and adjusted to 3 x 10^5 CFU/mL prior the addition of peptides. The experimental procedure followed the same protocol as an MIC (see 2.9.2). The conditions evaluated via the calScreener were Peptides 7L and ciprofloxacin activity against *P. aeruginosa.*

For all experiments, the calScreener, a 48-channel (calPlate) isothermal microcalorimeter with its corresponding pre-sterilised plastic inserted into calWells was used (Figure 2.6). The calPlate, containing the calWells was placed in a chamber set at 37℃ and rested upon a heat-flux detecting sensor, the thermopile. The sensor was attached to a heat-sink with a large mass compared with the cell culture cups. All the heat produced was transferred to the heat-sink, giving rise to a signal in the thermopile sensor proportional to the heat flow, which was the measured output. All experiments were done in triplicate, with untreated bacteria serving as a negative control, and blank media and/or blank media serving as contamination quality control.

The data was expressed as heat-flow over time, using microwatts (μW), and measured continuously using the software calView software (Symcel). The initial 45 minutes of incubation was used as a baseline to correct any potential sample variation, with measurements taking place at 10 minutes intervals for a period of up to 20 hours.

Figure 2.6 Isothermal microcalorimetry setup

Metabolic activity was evaluated when peptides and bacteria interacted. It was measured using **A:** the calScreener, and monitored via **B:** calView software, which measured the activity in real time. **C** is the calWells in which the bacteria and peptide mixture or sample conditions were placed in. the calWells containing the sample are then placed into **D:** the calPlate, which provided high heat sensitivity and was inserted into the calScreener. Image taken and modified from symcel.com

2.15 Resistance Assay

A 50 µL aliquot from an O/N culture of *P. aeruginosa* was subcultured in 4950 µL MH Br in a 15 mL microfuge tube, which was subsequently left to incubate at 37 °C at 225 RPM. The next day, 12 1.5 mL microfuge tubes were prepared with the addition of different concentrations (0.25, 0.5, 1, 2 and 4 X the MIC) of peptide 7 and gentamicin into 990 µL of MHBr. Into each microfuge tube, 10 µL of the subcultured solution was added. Furthermore, the final two microfuge tubes had MH Br only and untreated bacteria. All microfuge tubes were incubated at 37 °C at 225 RPM. The next day (18 ± 2 hours), each microfuge tube was inspected. The tube at the lowest concentration with clear visibility was classified as the interim MIC.

Ten 1.5 mL microfuge tubes were prepared with the following concentrations for either peptide or antibiotic: ½, 1, 2 and 4 X the interim MIC in 990 µL of MHBr. Furthermore, the inoculated with 10 µL of bacteria from the previous days ½ X interim MIC and two tubes contained either MHBr only or ½ X interim MIC culture from the previous day. All tubes were incubated at 37 °C at 225 RPM.

This procedure was repeated for 25 days, with three days, 50 µL ½ X the interim MIC culture streaked out into MH agar plate to check for contamination. Furthermore, every five days, the ½ X interim MIC culture was immersed in 7 % DMSO in case of contamination and potential future use. After 25 days, the daily interim MICs were used to determine the resistance of *P. aeruginosa* towards either peptide or antibiotic and the point at which resistance occurred for either compound.

2.16 Stability

2.16.1 Human Serum Stability

The following was kindly performed out by Dr Johannes Koehbach from The University of Queensland, Queensland, Australia. Human serum from a male with AB plasma (Merck Life Science UK) was centrifuged (13000 RCF) for 10 minutes to remove lipids. Then, 100 µM of peptides 7, 7D, Cyc-7 and Cyclotide-7 were incubated in 100 % serum at 37 °C for 24 hours. At time points 0, 30, 60, 180, 360 and 1440 minutes, 40 µL of the samples were removed. Then, 3 µL TFA was added to the removed peptide-serum sample to give a final TFA concentration of 7 % and precipitate the serum proteins. Samples were centrifuged (13000 RCF) for 10 minutes and the supernatant was diluted 50:50 with H_2O/ACN. The amount of peptide remaining was measured using a Shimadzu UPLC-MS using a C18 column at 214 nm using a solvent gradient for ACN and H_2O.

2.16.2 pH Stability

The following was also kindly performed by Dr Johannes Koehbach from The University of Queensland, Queensland, Australia. Water was adjusted to pH 1.2 and 7.5 using hydrochloric acid and sodium hydroxide (Merck Life Science UK), respectively. Then, 20 µM of peptides were incubated in the buffers at 37 °C for 24 hours, and then stability was measured using a Shimadzu UPLC using a C18 column at 214 nm using a solvent gradient for ACN and H_2O.

2.17 Galleria mellonella

2.17.1 Maintenance

Galleria mellonella (larvae of the great wax moth) were purchased from Live Foods UK Limited. They were in the final instar larval stage and stored in the packaging in which they arrived, which consisted of wood shavings, until used for experimentation. They were kept at 4 °C, in the dark and used within 10 days of shipment.

2.17.2 Assessing pathogenicity of *P. aeruginosa* in *G. mellonella* larvae

First, 2 mL of O/N culture of *P. aeruginosa* PA01 in MH Br was centrifuged at 5000 RCF for 5 minutes in a SciSpin Mini Microfuge. The supernatant was discarded and the pellet was suspended in 2 mL PBS. OD_{600} measurements were taken to determine the approximate CFU/mL of the PBS suspension. The suspension was then diluted to obtain a series of different bacteria concentrations ranging from 1 x 10^2 to 1 x 10^6 CFU/mL.

G. mellonella larvae were split into three groups: injection with bacteria, injection with vehicle (i.e. PBS) and no injection at all. They weighed between 200 – 300 mg and each group consisted of 12 larvae.

The larvae which were subjected to injections were placed between the thumb and index finger with the legs exposed. Then, 20 µL of solution containing either bacteria or PBS was injected into the last left proleg using a U-100 insulin syringe (Terumo Corporation). They were then placed into a petri dish containing some wood shavings and incubated at 37 °C and observed daily for survival for five days. Larvae were considered dead if they fully melanised or did not respond to any physical stimuli.

To determine if correct inoculum concentrations were used and no contamination occurred, all bacterial concentrations were serially diluted 10-fold and 5 x 5 µL were spotted for each dilution onto MH agar plates and incubated 37 °C and visually analysed the next day.

2.17.3 *Evaluating toxicity of peptides in G. mellonella larvae*

Peptides/antibiotics were dissolved in PBS up to 5 mg/mL. Depending on the concentration required, 10 to 20 µL of peptides and antibiotic gentamicin were injected into the last left proleg of the larvae using a U-100 insulin syringe. The dosage of peptides equated to 250 to 500 mg/kg. The antibiotic dosage injected was 250 and 500 mg/kg. Each group consisted of eight worms. Larvae were then placed into a petri dish containing some wood shavings and incubated at 37 ˚C and observed daily for survival for five days. Larvae were considered dead if they did not respond to any physical stimuli.

2.17.4 Efficacy of peptides in G. mellonella using a P. aeruginosa systemic infection model

A 1 mL of O/N culture of *P. aeruginosa* PA01 in MHBr was centrifuged at 5000 RCF for 5 minutes in a SciSpin Mini Microfuge. The supernatant was discarded and the pellet was suspended in 2 mL PBS. OD_{600} measurements were taken to determine the approximate CFU/mL of the PBS suspension, based on previous findings. The suspension was then diluted to obtain bacteria concentration 1 x 10^3 CFU/mL. Larvae weighed between 200 – 300 mg and each group consisted of 12 larvae. They were then infected with 20 µL *P. aeruginosa* solution or PBS into the last left proleg using a U-100 insulin syringe, and left to incubate at 37 ˚C for 30 minutes in a petri dish, containing wood shavings. To determine if correct inoculum concentrations were used and no contamination occurred, all bacterial concentrations were serially diluted 10-fold and 5 x 5 µL were spotted for each dilution onto MH agar plates and incubated 37 ˚C and visually analysed the next day.

The larvae were split into five groups for treatment purposes:

1. Vehicle (i.e. PBS) only
2. *P. aeruginosa* - 10^3 CFU/mL only i.e. untreated
3. 400 mg/kg of L-peptide
4. 400 mg/kg of D-peptide
5. 250 mg/kg of Gentamicin

Post 30-minute incubation, 10 to 20 µL of peptides/antibiotic (depending on concentration) were injected into the last right proleg of each larvae – i.e. the opposite side of where bacteria was injected into, and left to incubate at 37 ˚C. After six hours of incubation, a same second dose of antimicrobial agent or PBS was administered. They were returned to incubate for a period five days. Survival of the larvae was observed daily, with melanised larvae and those that did not respond to stimuli considered dead.

3 The Development of Novel Antimicrobial Peptides Targeting *Pseudomonas aeruginosa*

3.1 Introduction

Mikut *et al*. developed a machine learning algorithm that has been refined over many iterations from various antimicrobial and haemolytic screening data, which gave rise to promising predictive peptides with strong antimicrobial activity from 3000 candidates (Mikut *et al.*, 2016). In order to quantify the antibacterial effects, a screening procedure was performed using a luminescent reporter strain of *P. aeruginosa* (Hilpert and Hancock, 2007). This luminescence is dependent on 1,5-dihydroriboflavin 5'-(dihydrogen phosphate) ($FMNH_2$), which is a reduced form of flavin mononucleotide (Winson *et al.*, 1998). When treated with antimicrobial peptides, it has been shown that a decrease or loss of luminescence correlated with a reduction or loss of CFU counts (Hilpert and Hancock, 2007). Furthermore, Mikut *et al*. evaluated the toxicity of 3000 peptides against human erythrocytes (unpublished). Such methods allow high throughput identification of the most promising candidates, which can later be scaled up, characterised and developed further. The haemolytic screening procedure is a well-established method. The use of fresh human blood allows for robust reporting, as blood acquired from blood banks is of unknown age and adds uncontrolled variability. Uncontrolled elements include the processing of the blood, transportation and storage of the blood, and the potential for the blood even to be haemolysed (Choudhury and Mathur, 2011; van der Meer and Pietersz, 2015). Furthermore, using sheep or horses' blood is not an ideal comparison to human blood due to different organisms and blood components (McCutcheon *et al.*, 2011; Molchanova *et al.*, 2017).

Therefore, it is paramount to discover novel antimicrobial agents that are safe for human use by eliminating toxic peptides. Furthermore, it is also essential to establish a large therapeutic window between antimicrobial and haemolytic activity. For the purposes of this study, it was decided it would only be worth considering peptides that have a minimum 50-fold difference between the antimicrobial activity and haemolytic toxicity, i.e. a selectivity index of at least 50.

This chapter explores the peptides generated from previous work by Mikut *et al*. (Mikut *et al.*, 2016). High throughput screening enabled the downstream of candidates, which were further optimised. Substitution analysis is a valuable tool to determine if any particular amino acid or combination of amino acids can lead to a stronger or weaker candidate. Further rationale engineering strategies of the most promising candidates were further explored to yield potentially a better candidate.

3.2 Study Aims & Objectives

The antimicrobial peptides used in this chapter were designed from the analysis and optimising prediction based on a semi-random library of 3000 peptides (Mikut *et al.*, 2016). The further iteration of these peptides resulted in 560 peptides being predicted with low haemolytic activity but strong antimicrobial activity against *Pseudomonas aeruginosa*. Furthermore, optimisation strategies were employed with the ancillary aim to improve bioactivity and/or reduce toxicity for selected candidates.

Specific Objectives

1. Synthesis of 560 short synthetic peptides library, termed Novel Original Library (NOL)
2. An antimicrobial screen of the NOL using a luminescent strain of *P. aeruginosa* and haemolytic activity against human erythrocytes.
3. Computational assessment of the NOL by SciXMiner (a toolbox of MATLAB®) was used to identify the candidates with the promising therapeutic potential
4. A systematic substitution analysis of the three most promising candidates to identify optimised variants, termed Novel Original Single Substitution Library (NOSSL), was screened as objective 2.
5. Favourable substitutions were combined to generate a new library, termed Novel Original Multiple Substitution Library (NOMSL), and screened as per objective 2
6. The NOMSL was also screened against a multi-drug resistant *E. coli* isolate in the presence of human serum.
7. Synthesis of the best performing peptides from the screens was scaled up on resin, purified, and further assessed by minimum inhibitory concentration (MIC) against *P. aeruginosa*, broad-spectrum activity against other pathogens, haemolytic activity and toxicity towards HEK-293 cells.
8. Further engineering strategies of selected peptides was performed through cyclisation, peptide conjugation and scaffold insertions, which were also characterised using various assays.

3.3 Novel Original Library Screen

3.3.1 Design of Library

The original library originated from Mikut *et al*. in 2016 when they performed a series of screens against a luminescent strain of *P. aeruginosa* (H1001). The 3000 peptides that were tested were analysed for their performance using a machine learning approach (Mikut *et al.*, 2016), alongside human erythrocyte toxicity data (unpublished), to give rise to 560 peptides, termed the NOL. They were generated to maximise therapeutic potential, i.e. high antimicrobial activity and low haemolytic toxicity.

The 560 peptides featured a biased inclusion of specific amino acids, namely arginine (Arg), isoleucine (Ile), lysine (Lys), tryptophan (Trp) and valine (Val). This resulted from them appearing the most frequently in highly active peptides in the larger library study (Mikut *et al.*, 2016). Arg represented 30 % of the total amino acids used, with Lys, Trp, Ile and Val present in 20 %,16 %, 13 % and 10 % of the total amino acid outlay, respectively. The rest of the 11 % amino acids used comprised the other 10 of 15 amino acids, shown in Figure 3.1.

There tended to be an increased proportion of Arg at the C-terminus of the peptides, which was most likely due to Arg previously showing an increased therapeutic potential when positioned at the terminal. Finally, there was a restriction to the number of Trp in a sequence, capped at two, due to an excess of Trp causing toxicity, confirmed in the toxicity models (unpublished). The library generated aimed to strike a balance between hydrophobicity and charge, which was also shown to increase its therapeutic potential (unpublished).

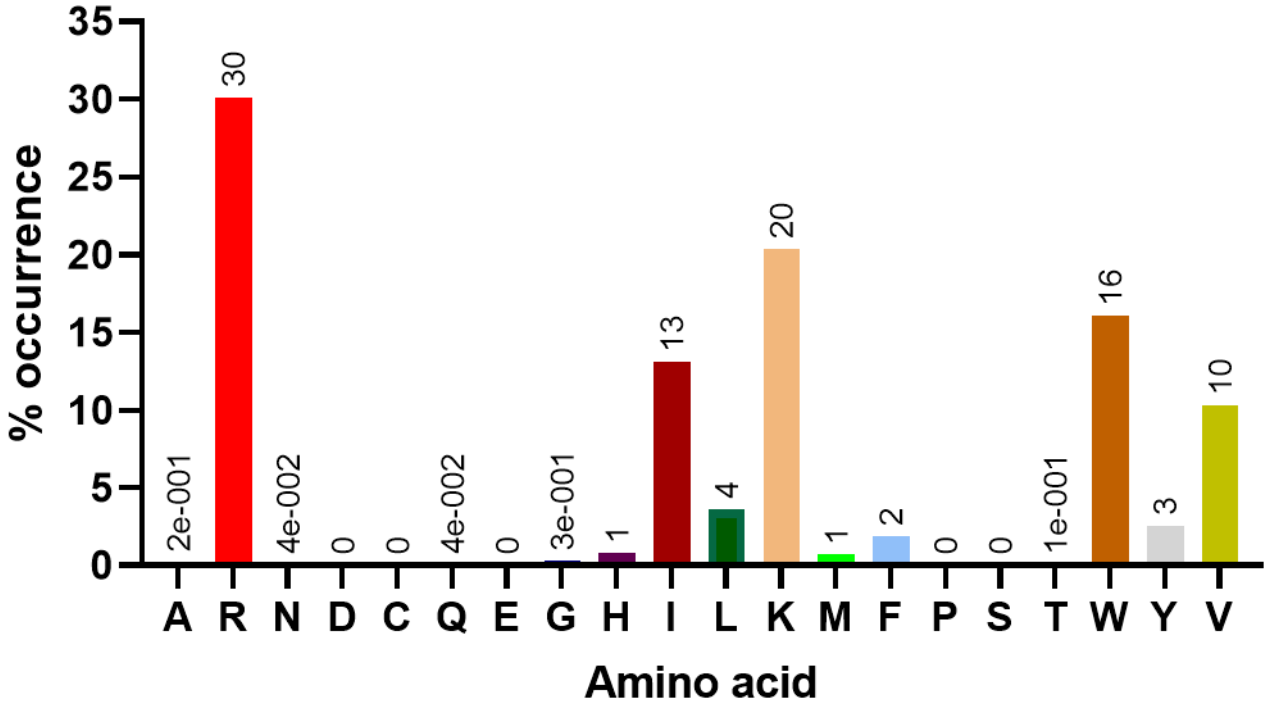

Figure 3.1 Amino acid composition of the NOL

The amino acid frequency in the NOL from 15 amino acids was used as part of their sequence. Asparagine (Asp), glutamine (Gln) and threonine (Thr) had the lowest occurrence, with Arg and Lys most frequently occurring. The remaining five amino acids were excluded because, upon evaluation, the therapeutic potential of peptides was deemed weak with their inclusion.

3.3.2 Antimicrobial Screen

The peptides were synthesised on a cellulose membrane and screened against a luminescent strain of *P. aeruginosa* (H174), which had a *lux* gene inserted into its plasmid, which gave rise to luminesce (Hilpert and Hancock, 2007). The antimicrobial data was inserted into SciXminer (formerly known as Gait-CAD), which calculates the relative IC_{75} (75 % bacterial inhibition relative to a control peptide). The in-house peptide control (KRRWRIWLV) produced a MIC of 16 µg/mL. The in-house control was one of the best AMP hits from the previous 3000 peptide library generated by Mikut *et al*.; thus, it was an excellent candidate to serve as a control (Mikut *et al.*, 2016).

The activity of each peptide was determined by using the method laid out by Mikut (Mikut, 2010) and defined in 2.5.3. Of the 560 peptides, 2 % (12 peptides) were more active than the in-house control, and 9 % (48 peptides) had similar activity to the control (SATC). The overwhelming majority, 86 % (483 peptides) and 3 % (17 peptides) were categorised as weak or inactive, respectively (Figure 3.2).

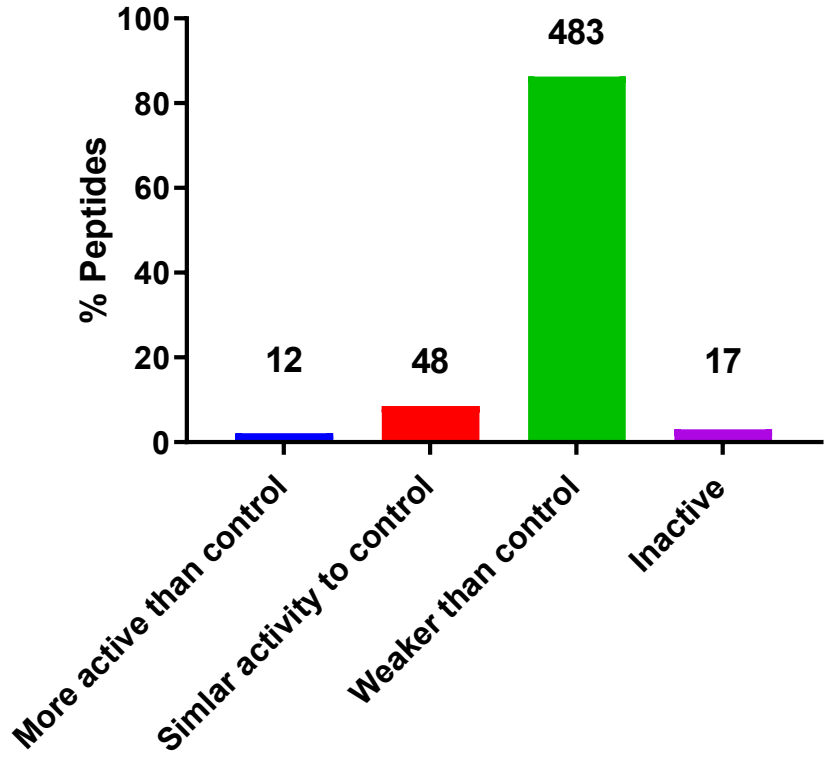

Figure 3.2 Antimicrobial activity of NOL

Using the relIC$_{75}$ value, each peptide from the library was categorised into one of four groups indicating its level of antimicrobial activity. Above each respective bar is the number of peptides that fall within each category.

3.4 Haemolytic Screen

Fresh human erythrocytes were used in this assay to determine the toxicity of the short antimicrobial peptides. The haemolytic data was inserted into SciXminer (formerly known as Gait-CAD), which calculated the relative HC_{75} (75 % haemolytic concentration). Each peptide was assigned one of four activity groups (Figure 3.3). The control used for the haemolytic screen was 0.1 % Triton X-100, which represented 100 % haemolysis. In this assay, the four groups were labelled "strong haemolytic", "medium haemolytic", "weak haemolytic", and "non-haemolytic." "Strong haemolytic" was equivalent to the in-house control used, while "non-haemolytic" was equivalent to the untreated RBCs.

A combined 2 % (11 peptides) showed medium and weak haemolytic activity, with 2 and 9 peptides in each category, respectively. The overwhelming majority, 98 % (549 peptides), showed no detectable haemolysis, and no peptide was classified as "strong haemolytic."

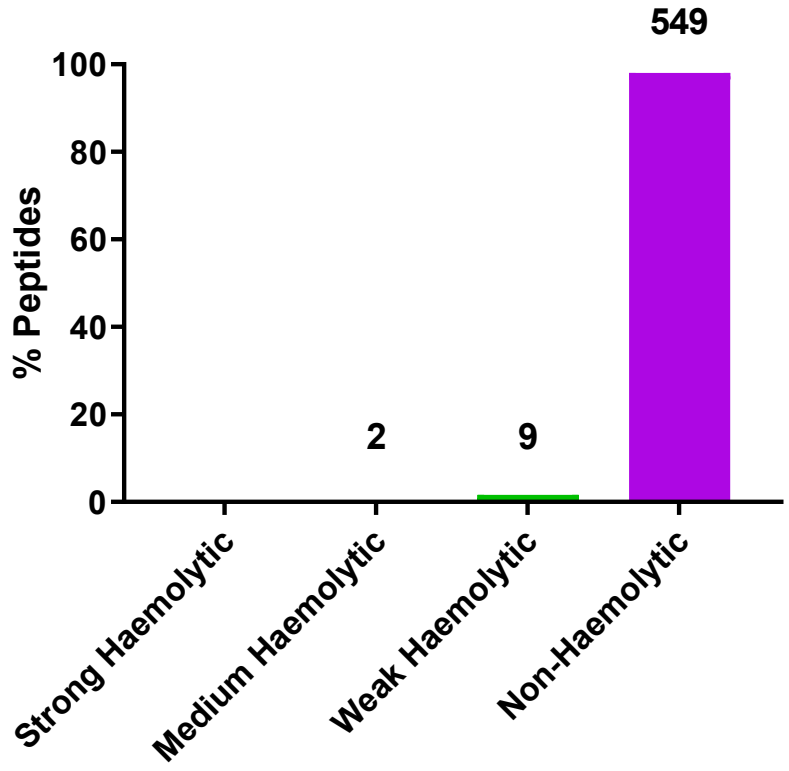

Figure 3.3 Haemolytic activity screen of the NOL

Each peptide was then assigned a class amongst four groups, based upon its HC_{75} value to signify its level of haemolytic activity. Above each respective bar is the number of peptides that fall within each category.

3.5 Therapeutic Potential

Therapeutic potential (TP) was the assessment of all the NOL peptides synthesised on Spots. It was assessed by combining both antimicrobial and haemolytic data to determine the best candidates to characterise further (Figure 3.4). To determine a TP, it must meet at least the criteria laid out in 2.8.

If none of the conditions was met, the TP was automatically calculated as 0, thus "none."

A strong TP was found in 19 % of the peptides (103), with 10 % (58 peptides), 41 % (231 peptides), 30 % (168 peptides) was observed as showing medium, weak and none, respectively. The higher the TP value,

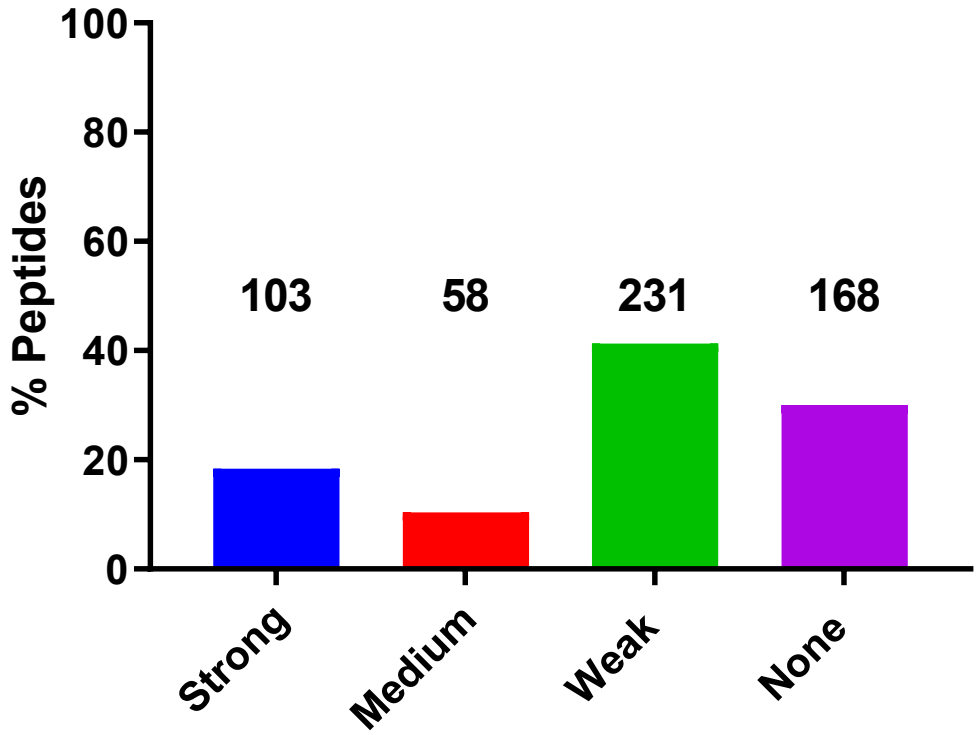

Figure 3.4 Therapeutic Potential of NOL screens

Antimicrobial and haemolytic activity data were combined to assess TP. They were assigned into one of four groups in accordance with the analysis performed by SciXMiner. Above each respective bar is the number of peptides that fall within each category.

3.5.1 Features That Maximise Therapeutic Potential

The peptide library was generated on the hypothesis that the peptides in this study would have antimicrobial activity towards *P. aeruginosa* while having minimal toxicity towards human erythrocytes. Therefore a postulation that they would exhibit the most promising therapeutic potential. Overall, the bias of the peptide sequence was proportioned towards mostly charged and hydrophobic amino acids. However, specific rules were inherent to the design within a peptide sequence. The rules indicated that overall, no more than 1/3 of peptides are charged or hydrophobic. According to the amino acid histogram with the classification of peptides (Figure 3.5A), the most active peptides had a noticeably higher proportion of Trp and a high probability of Ile in their sequence. The inactive peptides possessed the highest amounts of Lys, glutamic acid (Glu), methionine (Met) and threonine (Thr) proportionally. A few patterns were observed from the positional analysis of the sequences (Figure 3.5B). The "more active than control (MATC)" group tended to have Arg at the beginning of the sequence, i.e. C-terminus, with the middle of the sequence containing more Ile and Trp towards the end of the sequence. However, the "similar to control group" had W spread out similarly across the sequence. The "inactive" peptides showed a wide distribution of L across most positions of the sequence.

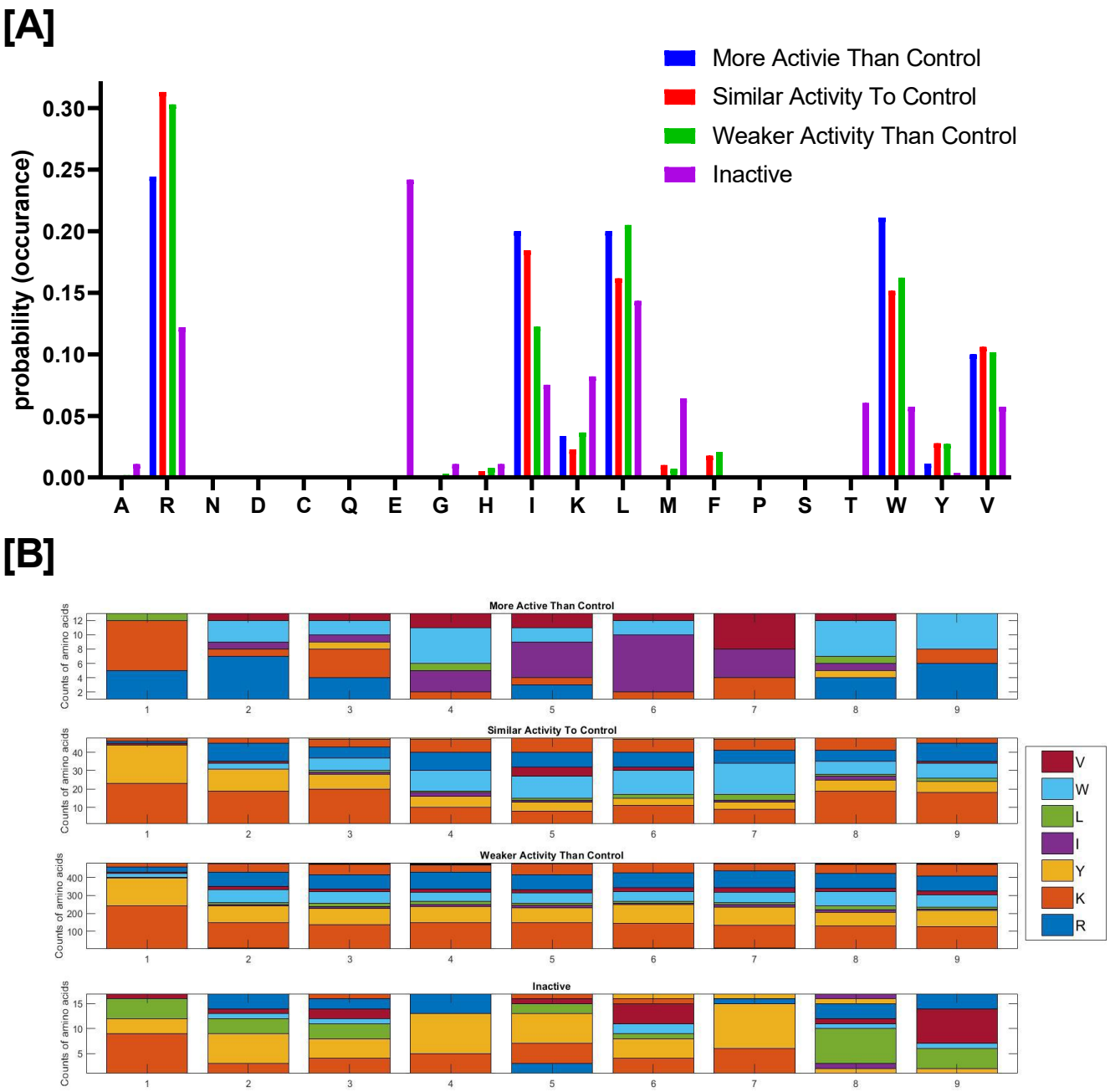

Figure 3.5 Amino acid profile and positional analysis for NOL.

[A], the proportion of amino acids in respect to their categorised antimicrobial activity, with the hydrophobic occurrences showing the most potent antimicrobial activity, and **[B]**, the positional contribution of specific amino acids in relation to the antimicrobial activity.

3.6 Validation of Novel Original Library Screen

Synthesis of 12 peptides from the original screen, with different activities, was scaled up on resin at 25 μM scale, followed by reverse-phase HPLC-MS purification (Table 3.1). Peptide 7 performed the best against wild type *P. aeruginosa* with a MIC of 4 μg/mL, followed by 5 and 9, both of which showed a MIC of 8 μg/mL, with all three peptides falling into the MATC. Peptide 2 and 8 had MICs of 32 μg/mL, despite being in the SATC) class. However, peptides 1, 4 and 6 were the same MIC and matched the in-house control. Peptides 3, 10 and 11 were in the "weaker activity than control (WATC)" category and showed MICs of 32 or 128 μg/mL, with the "inactive" peptide 12 showing a MIC of >256 μg/mL.

The general trend for the HC_{50} of the peptides revealed that the less antimicrobial activity the peptide displayed, the less haemolytic the peptide was. Peptides from the SATC category had HC_{50} ranging from 622 – 1448 μg/mL, while MATC grouped peptides ranged from 849 – 1490 μg/mL. The WATC and inactive peptides demonstrated HC_{50} range from 1481 – 12431 μg/mL.

The selectivity index (SI) was calculated as a ratio between 50 % population inhibition concentration at which haemolytic effects were observed and the concentration at which the minimal antimicrobial effects were observed (TI = min [HC_{50}]/MIC). The SI for peptides in the MATC category ranged from 78 – 308, with peptides 5 and 7 showing the highest with 202 and 308, respectively. The SATC peptides had SI between 28 and 53, while the weak or inactive peptides had SI between 9 and >49.

The amphipathicity of the peptides indicated that bola-amphiphilic and detergent-like peptides contributed to the potency of antimicrobial activity the most.

Table 3.1 Resin peptides from the NOL

A selection of peptides, including the most promising peptides from the NOL screen, were scaled up onto resin their MIC was determined. At least three independent experiments were performed, with their modal value stated on the table. The HC_{50} was determined using fresh human RBCs HC_{50} is reported as the average of three independent experiments. The Selectivity Index was calculated using the ratio between the MIC of the peptide and HC_{50}. All experiments were subjected to at least three independent biological repeats, with the modal value reported for the MICs. Amphipathicity was determined on the basis of the sequence configuration.

Key: MATC = More Active Than Control, SATC = Similar Activity To Control and WATC = Weaker Activity Than Control.

ah = amphipathic helix, b = Bola-amphiphile, d = detergent-like, m = mixed

Peptide	Sequence	Antimicrobial Classification	*P. aeruginosa* (µg/mL)	HC_{50} (µg/mL)	SI	Amphipathicity
1	KVIIVWRRK	SATC	16	849	53	b
2	VRRWRIIVR	SATC	32	1490	46	m
3	RWRWRRVRL	WATC	128	1481	12	ah
4	RIRIKWRFV	SATC	16	1560	98	m
5	RVKWWIRVR	MATC	8	1614	202	b
6	KIFWRLRVK	SATC	16	1448	90	ah
7	KRRVRWIIW	MATC	4	1230	308	d
8	RKWKIWIKR	SATC	32	1214	38	m
9	KRRIWIVWR	MATC	8	622	78	b
10	RKWLRRRIW	WATC	32	922	28	m
11	RWWIRRIRR	WATC	128	2108	9	b
12	WIVRRRRFV	Inactive	>256	12431	>49	m
Control	KRRWRIWLV	Control	16	425	27	d

3.7 Systematic Substitution Analysis

3.7.1 Antimicrobial Screen

Peptides 5 (RVKWWIRVR), 7 (KRRVRWIIW) and 9 (KRRIWIVWR) were taken further for optimisation, based on them displaying superior potency as shown from both the screens and validation. Although peptides 4 and 6 had a greater TI than peptide 9, the consistently two-fold greater antimicrobial activity of 9 resulted in it being included in the next investigative stage.

The three peptides were subjected to a single systematic substitution analysis in each position of the peptide sequence, which gave rise to a library of 513 (171 peptides per parent) peptides. The library herein was termed "Novel Original Single Substitution Library (NOSSL)." The three parental peptides were the respective controls for antimicrobial activity screening. Substituted peptides derived from peptide 5 (Figure 3.6A) showed that 9 % (16 peptides) performed better than the parent, i.e. more active than parent (MATP). 61 % (105 peptides), 26 % (47 peptides), and only 3 % (7 peptides) were similar, weaker, and completely inactive, respectively. The library from peptide 7 (Figure 3.6B) did not show any peptide which performed better; however, 31 % (53 peptides) and 52 % (89 peptides) were classified as having similar and weaker activity than the parent. Furthermore, 17 % (29 peptides) showed no activity. Derivatives of peptide 9 (Figure 3.6C) also showed no improvement; however, 46 % (79 peptides) and 49 % (83 peptides) were classed as "similar activity to parent (SATP)" and "weaker than the parent (WATP)", respectively. Only 5 % (9 peptides) showed no antimicrobial activity in comparison.

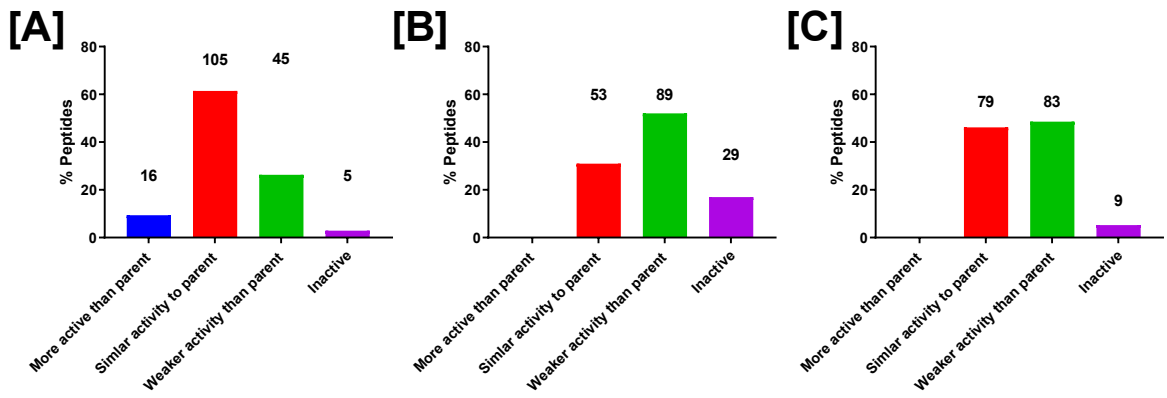

Figure 3.6 Antimicrobial activity of NOSSL.

Three peptides from the NOL underwent systematic amino acid substitution. They were classified into four groups based on screening with *P. aeruginosa*. **[A]** Peptide 5 substitution, **[B]** Peptide 7 and **[C]** Peptide 9. *n* = 171 peptides from each parent peptide. Above each respective bar is the number of peptides that fall within each category.

3.7.1.1 Positional Analysis of Antimicrobial Activity

A heatmap was created to visually analyse the alterations within a specific location of the sequence in order to determine which mutations resulted in a change in antibacterial activity. Peptide 5 (Figure 3.7A) had the most promising substitutions overall, with Ile, Lys and tyrosine (Tyr) substitutions amongst those which increased, showing favourable activity. Positions 4 and 6 showed the least flexibility. The substitution of Trp or Ile mainly led to a decrease or complete inactivity in those positions. Furthermore, it was observed that substitutions with amino acid Pro through the peptide sequence mainly led to a decrease in activity. However, no clear pattern of any particular amino acid led to increased potency, albeit Ile and Lys showed the greatest proportion of increased antimicrobial potency than the remaining amino acids. Moreover, the increased antimicrobial potency coincided with the peptide exhibiting a more detergent-like and bola-amphiphilic structure.

Peptide 7 variants (Figure 3.7B) did not show any peptide which performed better and was the most rigid to change. Many substitutions between positions 4 and 9 showed decreasing levels or complete inactivity when substituted for a single amino acid, particularly position 6. It was notably shown with the substitution of proline (Pro), which led to complete inactivity irrespective of the positions that were replaced. Positions 1, 2 and 3 of peptide 7 showed broadly similar activity across the spectrum, suggesting that it was more flexible and open to changes within those three positions while still maintaining activity.

Peptide 9 (Figure 3.7C) variants also demonstrated low flexibility to change. There was no increase in potency amongst the substitutions. Similar antimicrobial activity to the parent peptide was mainly observed in positions 2 and 9. Most substitutions in positions 4, 6, 7 and 8 led to either a decrease or complete inactivity. The substitutions within positions 2, 3 and 9 of peptide 9 overall retained similar activity for most substitutions.

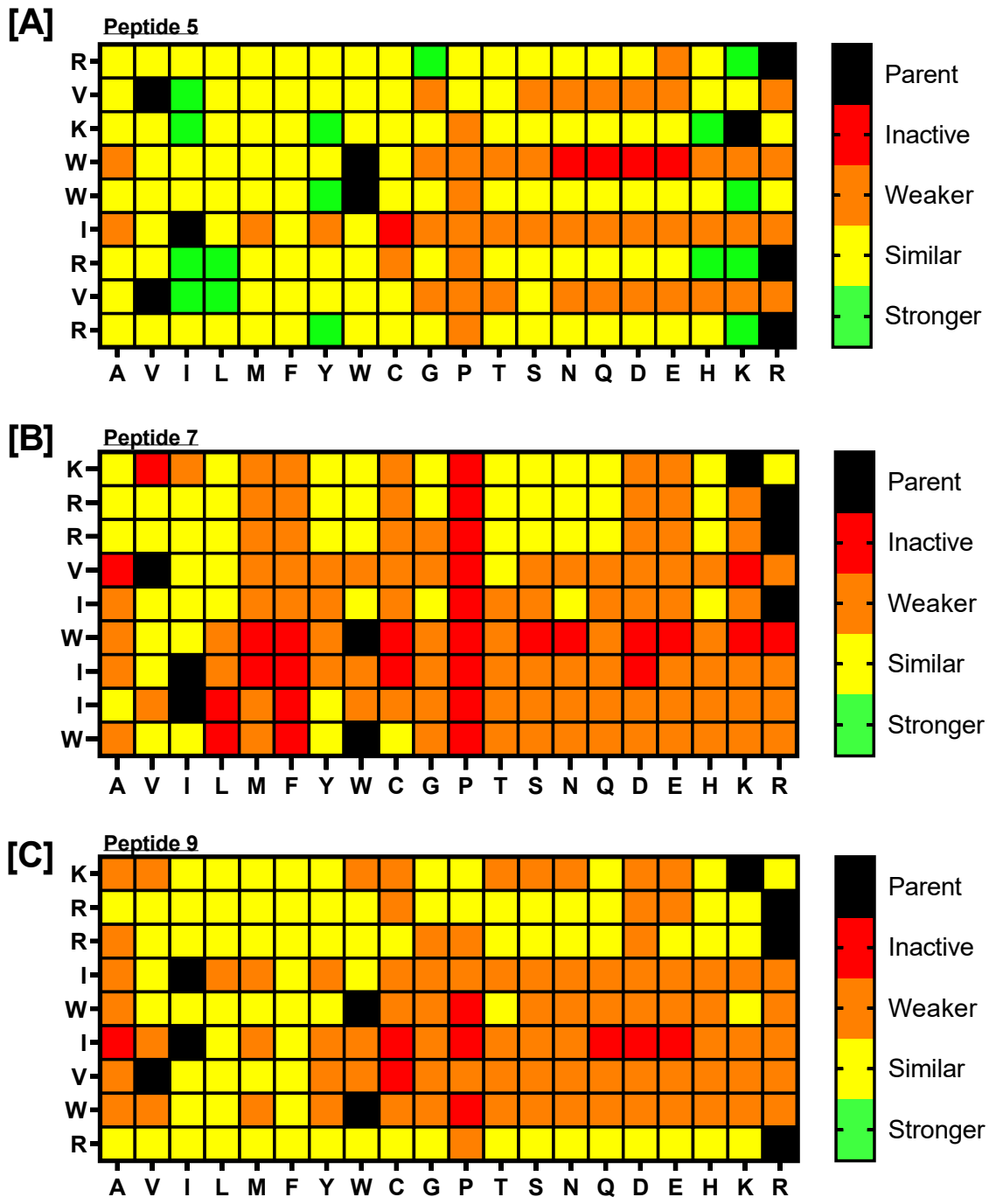

Figure 3.7 Heatmap of single substitution of three novel AMPs.

A group 171 peptides was generated for each parent. The antimicrobial activity classification in relation to the parent peptides of single systematic amino acid substitution of three peptides **[A]** Peptide 5, **[B]** Peptide 7 and **[C]** Peptide 9. The parental sequence is shown vertically with the effect of a single amino acid substitution, of all 20 amino acid shown in each position.The $relIC_{75}$ of Stronger peptides = 0 – ≤ 0.5, similar = > 0.5 – ≤ 5, Weaker = > 5 – ≤ 20, Inactive = > 20

3.7.2 Haemolytic Screen

To determine if any peptides from the NOSSL were worthwhile taking forward for further development, only peptides from the MATP and SATP were screened for their haemolytic activity. In total, 31 peptides were tested (Figure 3.8), consisting of derivates of best performers from peptide 5 analogues and the better performers (those with the lowest relIC$_{75}$ values) amongst the "similar activity to parent" category from peptides 7 and 9. The haemolytic screen was performed as previously mentioned (3.4). 16 % (5 peptides) were classified as being strong haemolytic, with 23 % (7 peptides) being categorised as having medium haemolytic. 42 % (13 peptides) demonstrated weak haemolytic activity, with only 19 % (6 peptides) being classified as non-haemolytic.

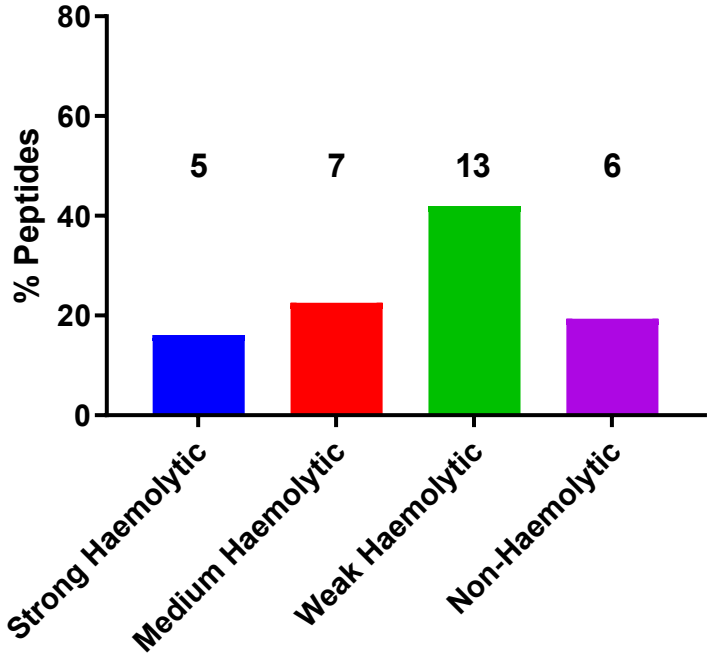

Figure 3.8 Haemolytic assay screen for the best performing peptides

The best performing peptides from the NOSSL antimicrobial screen (*n* = 31), were screened for their haemolytic activity. The peptides were subjected to same screening as before in NOL haemolytic screen, and classified accordingly after a relative comparison with 0.1 % Triton X-100 (positive control) and untreated RBCs served as the negative control. Above each respective bar is the number of peptides that fall within each category.

3.7.3 Validation of NOSSL Screens

A selection of 18 peptides from all three parents consisting of different classes were synthesised on resin at a 25 μM scale and purified using reverse-phase HPLC-MS. From the derivates of parent 5, the five peptides chosen and classified as MATP did not show a lower MIC than the parent (Table 3.2). They all either demonstrated the same MIC (peptides 5.1, 5.3 and 5.5) as the parent at 8 μg/mL or were two-fold greater at 16 μg/mL (peptides 5.2 and 5.4). However, except for 5.3 and 5.5, all variants from the SATP showed less haemolytic toxicity at the highest concentration tested compared to the parent. Peptides 5.6 and 5.7, derived from peptide 5, categorised as WATP in the antimicrobial screen, showed MICs of 32 μg/mL. Finally, peptide 5.8 had a MIC >64 μg/mL, which correlated with the antimicrobial screen.

From the derivates of peptide 7, none showed superior activity in the antimicrobial screen, and only WATP and inactive classified peptides were able to be synthesised and tested. Unfortunately, two peptides were lost before characterisation and were in SATP class. From the WATP class, peptides 7.1 and 7.2 had MICs of 32 μg/mL and 16 μg/mL, which correlated with the antimicrobial screen. Peptide 7.3 was inactive, evident with an MIC of > 64 μg/mL. All peptides from the progeny of 7 had either similar or remarkably less haemolytic toxicity at the highest concentration.

None of the derivates of peptide 9 was classified as MATP and non-haemolytic in the screen. Unfortunately, the peptide 9.1 had a MIC of 16 μg/mL and displayed more potent haemolytic activity. Furthermore, peptides 9.1 and 9.3 demonstrated a MIC of 32 and 16 μg/mL, despite being in the SATP category. Peptide 9.2 showed an MIC of 8 μg/mL and similar haemolytic toxicity as its parent, which correlated with the screen. Finally, peptide 9.4, which was inactive, displayed an >64 μg/mL, which correlated with the designated classification within the antimicrobial screen. There were no observed patterns regarding the amphipathicity of the peptides.

Table 3.2 NOSSL resin peptides.

Several peptides from different activity classes had their MIC determined against *P. aeruginosa* PA01, and their % haemolysis was determined relative to the positive control, 0.1 %Triton X-100. The respective sequences, homogeneity and status within the antimicrobial screen are also displayed. The MIC is the modal value of three independent experiments, with % haemolysis the average of three independent experiments.

Key: MATP = more active than parent, SATP = similar activity to parent, WATP = weaker activity than parent, b = Bola-amphiphile, d = detergent-like, m = mixed

Peptide	Sequence	Purity (%)	Class in substitution screen	MIC (μg/mL)	Haemolysis (%) at 512 μg/mL	Amphipathicity
5	RVKWWIRVR	>95	Parent	8	30	b
7	KRRVRWIIW	>95	Parent	4	35	d
9	KRRIWIVWR	>95	Parent	8	41	b
5.1	KVKWWIRVR	>95	MATP	8	15	b
5.2	RVKWWIKVR	>95	MATP	16	22	b
5.3	RVYWWIRVR	>95	MATP	8	45	b
5.4	RVKWYIRVR	>95	MATP	16	5	b
5.5	RVKWWIRVY	>95	MATP	8	50	m
9.1	KRRIWIVWG	>95	SATP	16	70	d
9.2	KRRIWIVLR	>95	SATP	8	62	b
9.3	KRMIWIVWR	>95	SATP	32	52	b
9.4	KRRIWIMWR	>95	SATP	16	50	b
5.6	TVKWWIRVR	>95	WATP	32	12	m
7.1	LRRVRWIIW	>95	WATP	32	40	m
7.2	KRRTRWIIW	>95	WATP	16	21	d
9.5	KRRIDIVWR	>95	Inactive	>64	1	m
5.7	RVKWWIRVP	>95	WATP	32	1	m
5.8	RVKDWIRVR	>95	Inactive	>64	0	b
7.3	KRRPRWIIW	>95	Inactive	>64	0	d

3.7.4 The Spectrum of Antimicrobial Activity

Antimicrobial peptides can exhibit both specific and broad-spectrum activity. The peptides from the NOSSL (MATP and SATP) and the original parents were tested against a modified panel of Gram-positive and negative bacteria, known as the ESKAP(*E. coli*) pathogens which are a burden hospital settings (Table *3.3*). The peptides all performed well against *Enterococcus faecalis* (VRE)*, Acinetobacter baumanii, Klebsiella pneumoniae* and *E. coli* with a MIC range of 1 – 8 µg/mL. Overall the best performance was against *A baumanii* with all MICs either 1 or 2 µg/mL. The peptides tested against VRE were similarly low in MIC, ranging from 1 – 4 µg/mL. Against MRSA, the MICs were demonstrably higher with a varying range of 8 – >64 µg/mL. Some peptides such as 5.2, 9.1 and 9.2 showed MICs of the same value against *P. aeruginosa* and MRSA at 16 µg/mL.

Table 3.3 NOSSL peptides against ESKAP(E. coli) pathogens

The peptides from NOSSL were subjected to testing against various pathogenic bacteria. The bacteria tested were *Enterococcus faecalis* (VRE ATCC 51299), EMRSA-15, *Klebsiella pneumonia* and *Acinetobacter baumanii* clinical isolates, *P aerguniosa* PA01, and *E. coli* K12. Experiments were conducted at least three independent times, and the most frequently occurring value, i.e. mode, was reported. The results from section 3.6 were also transcribed in this table for comparison purposes.

Peptide	MIC (μg/mL)					
	VRE	**MRSA**	***Klebsiella pneumonae***	***Acinotobacter baumanii***	***P. aeruginosa***	***Escherichia coli***
5	2	8	4	2	8	2
5.1	2	16	8	2	8	2
5.2	2	16	8	2	16	2
5.3	4	16	2	2	8	4
5.4	2	32	4	2	16	4
5.5	4	16	4	2	8	4
7	1	8	2	2	4	1
9	4	8	4	2	8	1
9.1	4	16	2	1	16	1
9.2	4	16	8	2	8	2
9.3	4	>64	8	2	32	8
9.4	4	16	2	1	16	4

3.8 Multiple Substitutions Antimicrobial Screen

The most favourable substitutions from the NOSSL screen for the three-parent peptides 5, 7 and 9 were combined with either two or three amino acid substitutions to create more variants. Although peptides 7 and 9 had no better performing antimicrobial activity peptide, using previous knowledge of amino acid properties as well as previous data (SATP groups), it was hypothesised that the addition of specific amino acids should do either or a combination of the following:

- Decrease toxicity
- Improve antimicrobial activity
- Demonstrate bioactivity in the presence of human serum

The original parental peptides remained as th controls and served as a reference for classifying the four respective groups. The substitutions were inserted into the positions where favourable activity was shown in 1.7. The library generated from these three substitutions was termed Novel Original Multiple Substitution Library (NOMSL). In addition to the antimicrobial screen with luminescent *P. aeruginosa*, the three peptide libraries underwent another antimicrobial screen with a multi-drug resistant *E. coli* isolate in the presence of 10 % human serum. The *E. coli* with human serum assay was measured after both six- and 24-hours incubation.

3.8.1 Antimicrobial Screen – Peptide 5 Multiple Substitutions

The double or triple substitutions of amino acids in each position gave rise to 157 new variants for peptide 5. The substitutions consisted of the following amino acids: phenylalanine (Phe), histidine (His), Ile, leucine (Leu), Lys, Arg, Trp and Tyr, and were spread across the sequence, depending on the activity from the NOSSL.

Against *P. aeruginosa*, only 2 % (3 peptides) showed improved activity compared to the original parent. Of those three peptides, only double substituted peptides yielded better

performance against *P. aeruginosa*. Furthermore, these particular peptides tended to retain their bola-amphiphilic tendency. For peptides with similar activity to the parent, 36 % (58 peptides) were observed within this class. From this classification, most had a triple substitution. However, most of the peptides were weaker, with 58 % (94 peptides) being classified as such, with again most peptides from triple substitutions with 28 peptides, compared to 66 peptides with double substitutions. Only two peptides were classed as inactive, and they were both triple amino acid substitutions (Figure 3.9A and Figure 3.9B). The weaker acting peptides tended to shift towards a detergent-like structural confirmation; a shift from the original bola-amphiphile structure.

Using an *E. coli* MDR isolate in the presence of human serum allowed for more stringent discrimination of antimicrobial activity and revealed candidates that maintain activity in a human serum environment, which partially represents physiological conditions. After a six-hour incubation, 38 % (60 peptides) showed greater activity than its parent in the presence of serum, of which 19 peptides had double, and 41 peptides had triple substitutions. Post 24-hour incubation under the same classification; eight other peptides were increased amongst the triple substitution, bringing the total to 49. The SATP classification after 6-hour incubation revealed 46 % (70 peptides), of which 19 peptides were double substitutions, and 51 peptides were triple. Furthermore, after 24 hours of incubation, there were two extra peptides in the dual substitution group (21 peptides), followed by an increase of a single peptide to the triple substitution bringing the total to 52 peptides within that subgroup. 15 % of peptides were classified into the WATP group with six peptides from double substitutions. At the same time, there were 17 peptides for triple substitution post-6-hour incubation.

Interestingly, after 24 hours in the WATP class, there was an overall reduction of peptides, with four peptides with a double substitution and eight peptides from the triple substitution. Only two peptides were classified as inactive and from double and triple substitution apiece (Figure 3.9C – Figure 3.9F). They both remained inactive after 24 hours as well.

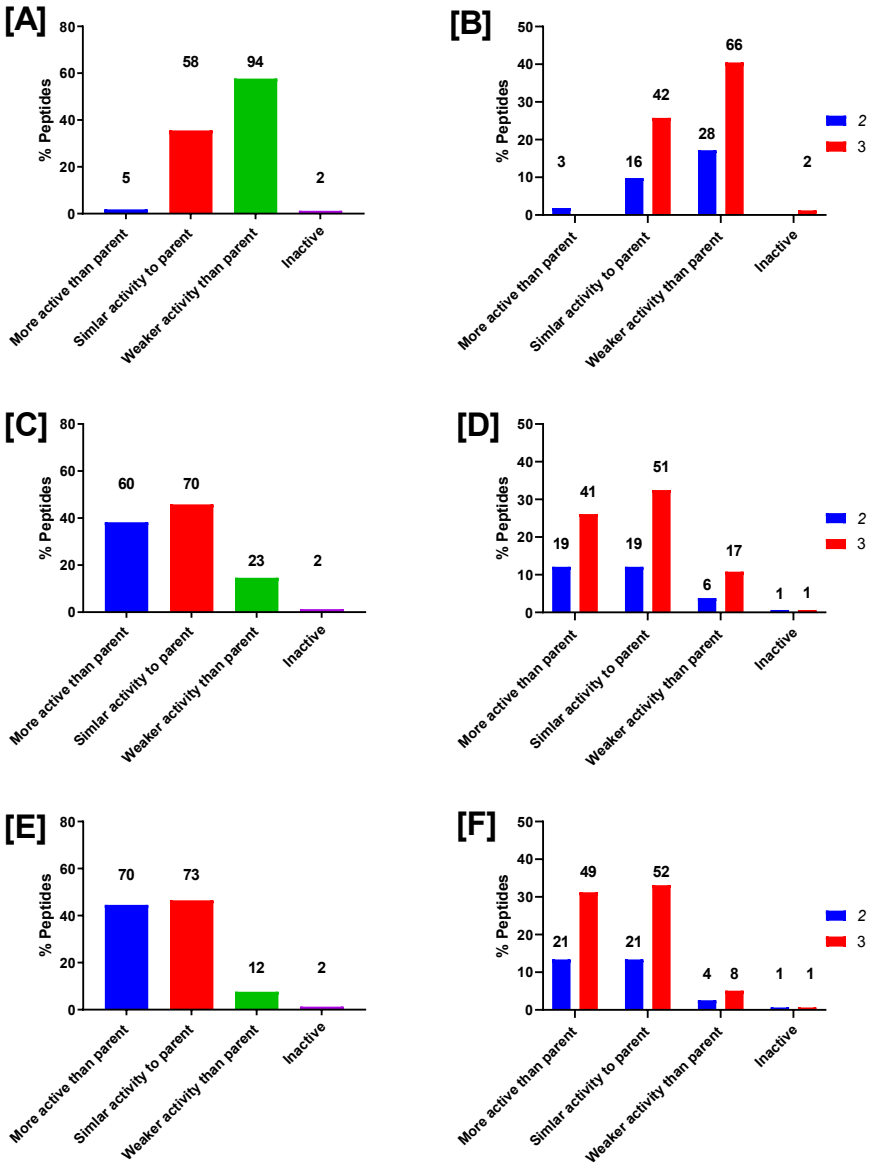

Figure 3.9 Peptide 5 – Multiple Substitutions

A total of 157 peptide derivates from peptide 5 were screened for their antimicrobial activity. **[A]** shows the classification *of P. aeruginosa* screen, with **[B]** representing the individual substitution breakdown. An *E. coli* MDR isolate was tested in the presence of human serum and incubated for 6 hours, with the **[C]** showing the classification and **[D]** representing the individual substitution breakdowns. **[E]** is the classification post 24 hours and **[F]** gives the breakdown of individual substitutions with the MDR isolate. Individual breakdown, 2 = double substitution and 3 = triple substitution. Above each respective bar is the number of peptides that fall within each category.

3.8.2 Antimicrobial Screen – Peptide 7 Multiple Substitutions

From the numerous substitutions of peptide 7, 76 peptides arose, and only double or triple substitutions were performed. The amino acids substituted include glycine (Gly), His, Ile, Phe, Lys, Val and Tyr. They were placed within various positions of the sequence depending on the outcome from the NOSSL screens. It should be noted, that none of the peptides outperformed the parent peptide 7, in the NOSSL screens. Instead, it was proposed that existing knowledge of amino acid characteristics likely to induce more strong antibacterial activity to be used. The luminescent antimicrobial screen (Figure 3.10A and Figure 3.10B) against *P. aeruginosa* did not identify a peptide with greater antimicrobial activity that the parent peptide. The activity of 32 % (24 peptides) of the peptides was similar to that of the original peptide 7 sequence, of which 14 peptides were double substitutions, while ten peptides were from triple substitutions. The activity of 59 % (45 peptides) was weaker than the parent and consisted of 14 peptides featuring double substitutions and 31 peptides with triple substitutions. Complete inactivity was seen in 9 % (7 peptides) of the peptides and were only in the triple substituted peptide subgroup.

From the screen with MDR *E. coli*, none of the peptides yielded better antimicrobial performance after 6- or 24-hours incubation (Figure 3.10C – Figure 3.10F). However, 12 % (9 peptides) did show similar activity to the parent after 6 hours, with seven peptides subjected to double substitutions and two peptides containing triple substitutions. It increased to 21 % (16 peptides) after 24-hour incubation, with 12 and 4 peptides from double and triple substitutions. Around 50 % of peptides demonstrated weaker activity for both the 6- and 24-hour incubation period. Furthermore, 21 peptides were from the double substitution after 6 hours, reducing to 16 after 24 hours. On the other hand, triple substituted peptides saw an increase of 6 peptides after 24 hours from 17 to 23 peptides. 38 % of peptides after 6 hours (29 peptides, of which all were triple substitutions) showed no activity, reducing it to 27 % (21 peptides) after 24 hours.

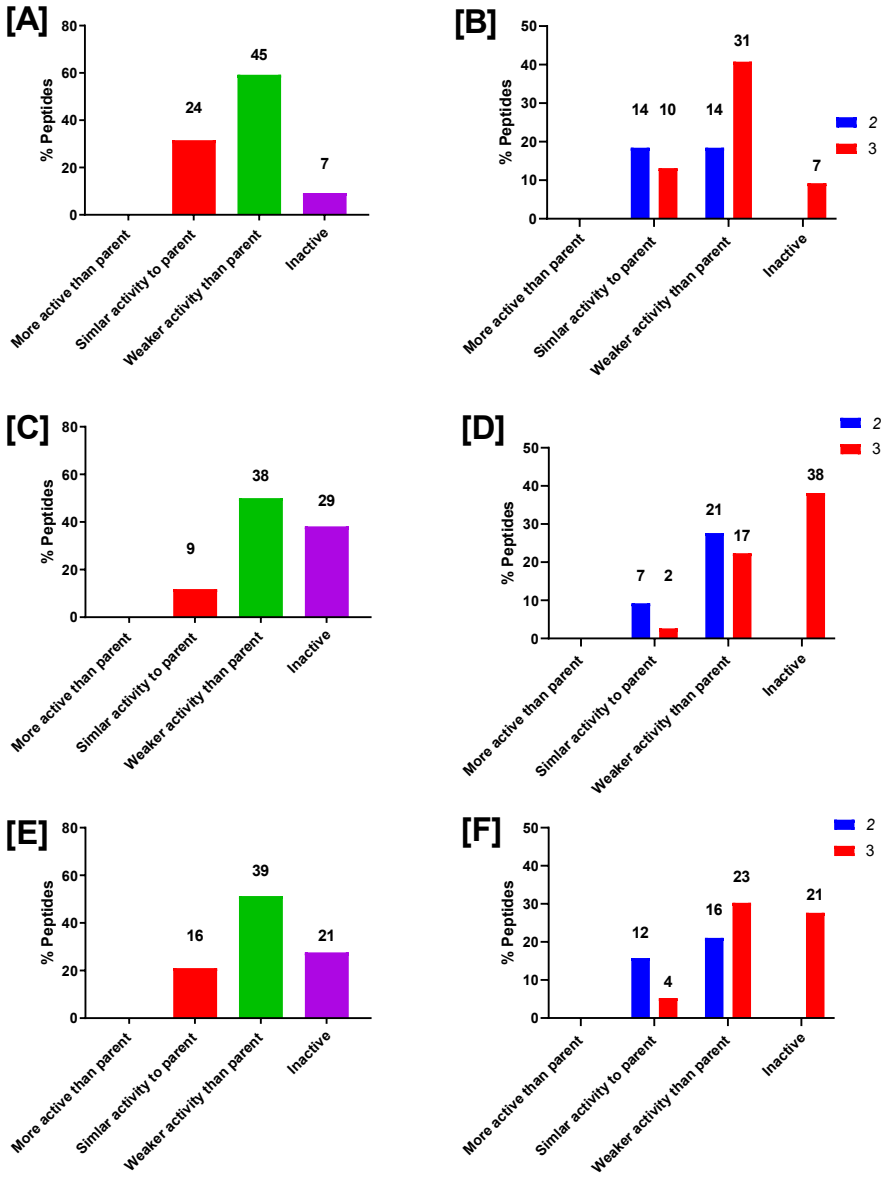

Figure 3.10 Peptide 7 – Multiple Substitutions

A total of 76 peptide derivates were generated from either a double or triple substitution. They were tested against a luminescent strain of *P. aeruginosa* and classified into four groups, relative to parent peptide 7. **[A]** shows the classification of *P. aeruginosa* screen, with **[B]** representing the individual substitution breakdown. An *E. coli* MDR isolate was tested in the presence of 10 % human serum and incubated for 6 hours and fluorescence measured, with **[C]** showing the classification and **[D]** representing the individual substitution breakdowns. **[E]** is the classification post 24 hours fluorescent reading and **[F]** gives the breakdown of individual substitutions with the MDR isolate. Individual breakdown, 2 = double substitution and 3 = triple substitution. Above each respective bar is the number of peptides that fall within each category.

3.8.3 Antimicrobial Screen – Peptide 9 Multiple Substitutions

Multiple double and triple substitutions of peptide 9 gave rise to 111 peptides, with the following amino acids substituted within: Pro, Arg, Trp, Phe, Tyr, Val, Lys, Ile and Leu. Again, like peptide 7 previously, the substitutions were based on previous and existing knowledge of amino acid properties.

There was unfortunately, no better performers detected with the luminescent *P. aeruginosa* screen (Figure 1.11A and Figure 1.11B). Similar activity to the parent peptide was shown in 20 % (22 peptides), consisting of 14 and 8 peptides from double and triple substitutions, respectively. Most peptides were classified as "weaker than the parent" at 72 % (80 peptides). The WATP class was subdivided into 22 peptides for double substitution and 58 peptides for the triple substitute variants. Only triple substituted variants were classed as inactive, 8 % (9 peptides).

Against MDR *E. coli* isolates (Figure 3.11C – Figure 3.11F), six peptides (5 %) showed greater antimicrobial activity after the 6-hour incubation period, reducing to 5 peptides after 24 hours. It included four peptides from the double substitution being reduced to three peptides after 24 hours. 23 % of the peptides showed similar activity to the parent (26 peptides), with 13 peptides each from double and triple substitution after 6 hours. Moreover, this was increased to 20 (double substitution) and 47 (triple substitution) peptides after 24 hours, taking the total proportion to 32 %. Also, 44 % of peptides (49 peptides) showed weaker activity after 6 hours, increasing to 59 peptides after 24 hours. It consisted of double substitute variants being reduced to 12 from 15 peptides; however, there was an increase from 34 to 47 peptides for the triple substituted peptides. The inactive class had a proportion of 27 % for peptide 9, with 4 and 26 peptides from the double and triple substitution variants, respectively, after 6 hours. However, that was reduced to 12 peptides, of which 11 peptides were triple substitution variants after 24 hours.

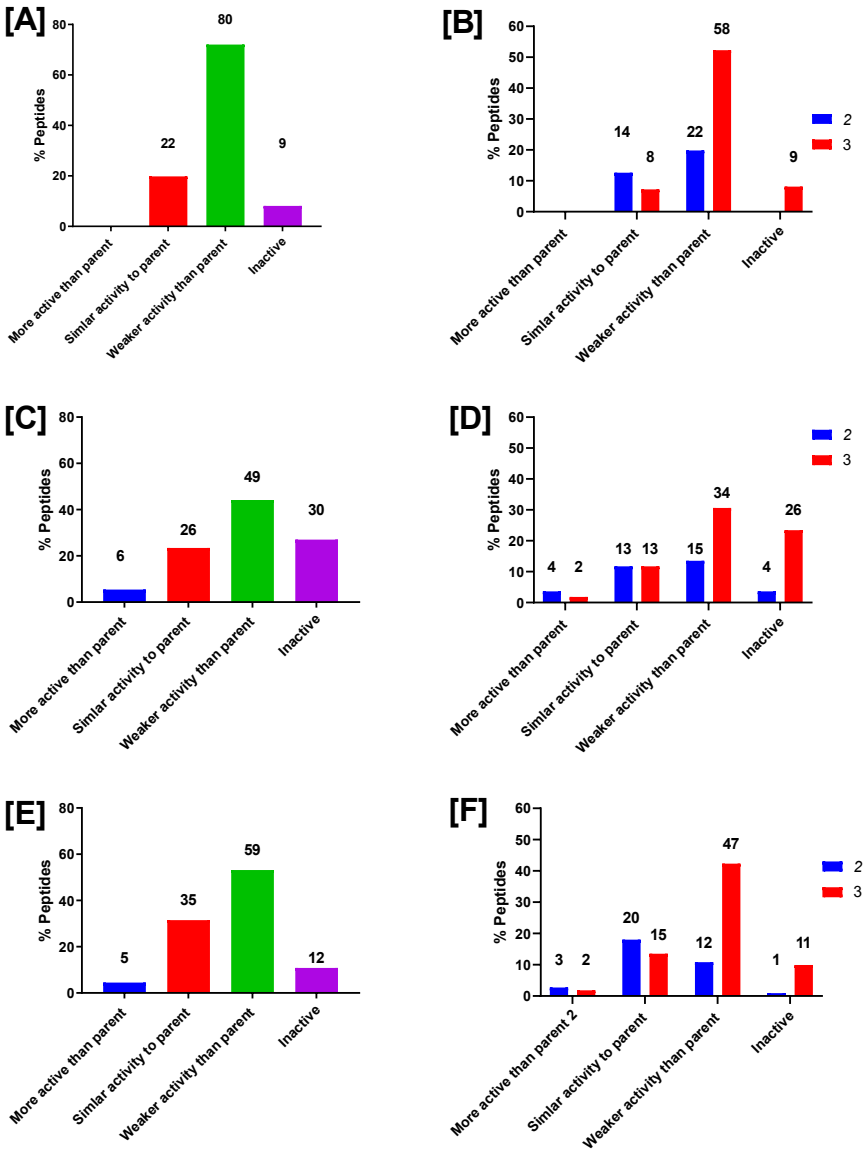

Figure 3.11 Peptide 9 – Multiple Substitutions

A total of 113 peptide derivatives were generated from either a double or triple substitution. They were tested against a luminescent strain of *P. aeruginosa* and classified into four groups, relative to parent peptide 9. **[A]** shows the classification of *P. aeruginosa* screen, with **[B]** representing the individual double or triple substitution breakdown. An *E. coli* MDR isolate was tested in the presence of 10 % human serum and incubated for 6 hours and fluorescence measured, with **[C]** showing the classification and **[D]** representing the individual substitution breakdown amongst the classifications. **[E]** is the classification post 24 hours fluorescence reading and **[F]** gives the breakdown of individual substitutions with the MDR isolate. Above each respective bar is the number of peptides that fall within each category.

3.8.4 Haemolytic Screen

MATP and SATP performers in the antimicrobial screens were further assessed for haemolytic activity. Haemolytic activity was analysed and classed with the same parameters as previously (NOSSL screen) using fresh human blood (Figure 3.12).

From peptide 5 NOMSL variants, there were 82 peptides (all from the MATP classification). Peptide 7 had 36 variants, and peptide 9 had 24 variants tested. Out of a total of 145 peptides (including the three parental controls), the vast majority of them displayed strong haemolytic activity (79 % or 115 peptides). It was followed by the remaining variants showing medium haemolytic activity at 19 % or 27 peptides. Only the parental peptides were non-haemolytic, which made up the remaining 2 %. At this point, it was decided that due to the increased toxicity, it did not warrant further investigation and scale-up, based on previous screening and validation of Spot peptides.

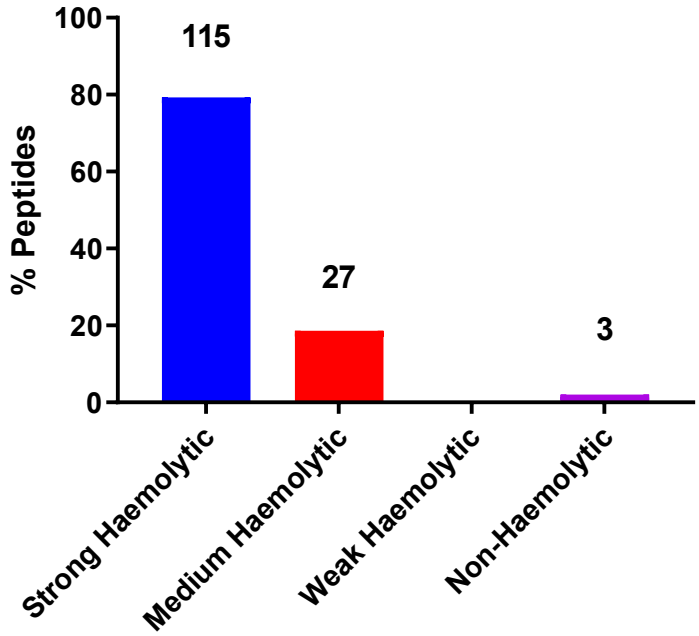

Figure 3.12 Multiple substitution haemolytic activity

Haemolytic activity screen using fresh human blood, with the multiple substitution variants of three parental peptides identified from the NOL screen. The proportion of total peptides (including controls) were assessed, and classified into their respective groups in comparison with the positive control, 0.1 % Triton X-100. Above each respective bar is the number of peptides that fall within each category.

3.9 Peptide Engineering

It was decided that the most promising peptides which had the most potent antimicrobial activity, coupled with minimal toxicity, were peptides 5 and 7 from the NOL, as the further iterations assessed did not yield peptides with improved therapeutic potential. They underwent engineering via isomerisation, cyclisation and hybridisation. They were subjected to the same biological experiments as this chapter, including MIC, haemolytic activity and HEK-293 activity.

3.9.1 Isomerisation

The peptides tested in this chapter, whether by Spot or resin, have so far consisted only of L-amino acids. To determine the activity of its respective enantiomer, peptides 5 and 7 had their full D variant synthesised.

The MIC against *P. aeruginosa* remained the same regardless of the peptide variation, with peptides 5L and 5D showing 8 µg/mL. Peptide 7L, synthesised in-house and 7D (purchased from Bachem), showed a MIC of 4 µg/mL. The HC_{50} for both D-variants of the peptides proved to be more toxic at more than a twofold increase compared to the L-variant. 5L demonstrated an HC_{50} of 1614 µg/mL, while 5D showed 696 µg/mL. Meanwhile, peptide 7L showed an HC_{50} of 1230 µg/mL, while 7D showed 532 µg/mL (Table 3.4).

Table 3.4 Isomerisation of peptides 5 and 7

They were both synthesised using D-amino acids. The bioactivity assays were carried out under the same conditions simultaneously. MICs reported were a modal value of at least three independent experiments. HC_{50} was performed with fresh human blood each time and was reported as the average of at least three independent experiments.

Peptide	Sequence	MIC (µg/mL)	HC_{50} (µg/mL)
5L	RVKWWIRVR	8	1614
5D	rvkwwirvr	8	696
7L	KRRVRWIIW	4	1230
7D	krrvrwiiw	4	532

3.9.2 Cyclisation and Scaffold

Peptides 5 and 7 underwent head-to-tail cyclisation via a double beta-alanine and cystine on either end of the termini connected through a peptide bond (Table 3.5). Further, peptide 7 was inserted into a scaffold, the MCoTI-II cyclotide, a very stable (in various conditions) peptide originating from the *Momordica cochinchinensis* plant, known as Gac (Hernandez *et al.*, 2000). Both of these strategies were employed as it was hypothesised that they would be bioactive, stable, and non-toxic. Cyclised peptides were bought from SynPeptide. The cyclotide peptide was kindly synthesised and purified by Dr Johannes Koehbach from the University of Queensland, Australia. They were each subjected to MICs under standard and diluted media conditions, as well as haemolytic activity. Data here was presented as µM due to the vast differences in molecular weight between the different peptides.

Peptide 5 MICs were reduced to 1.5 µM in diluted conditions, which resulted in a 4-fold improvement compared to standard MH broth. Peptide Cyc-5 was observed to have a MIC of 197 µM using MH broth. The MIC was reduced to 25 µg/mL when in the presence of 20 % MH broth, representing an almost eightfold improvement in potency. Both peptide 5 and Cyc-5 had an HC_{50} greater than the highest concentrations tested.

A fourfold increase in potency was also observed with peptide 7, with a reported MIC of 0.75 μM in diluted media conditions compared to standard media conditions. The cyclised variant of peptide 7, Cyc-7, had a MIC of 142 μM in standard conditions with an improvement of almost 16-fold shown in minimal media at 9 μM. Cyclotide-7 also had weak activity against *P. aeruginosa* in standard conditions ranging from a MIC of 31 μM and showing significant improvement in diluted conditions to a MIC 4 μM, which represented an almost 8-fold increase in its activity. The haemolytic activity for peptide 7 and its two modified variants had an HC_{50} greater than the highest concentrations tested.

Table 3.5 Cyclised and Scaffold Peptides

MICs of peptide 5 and 7 variants were performed in standard and diluted conditions (20 % MH Br). MICs were reported as the modal value of at least three independent experiments. HC_{50} is reported as the average of three independent experiments. Part of this table is reproduced from (Koehbach *et al.*, 2021)

Name	**Sequence**	**Class**	**MIC against *P. aeruginosa* (μM)**		**HC_{50} (μM)**
			MH Br	**20 % MH Br**	
5	RVKWWIRVR	Parent	6	1.5	>395
Cyc-5	(CAA-RVKWWIRVR-AAC: Head to Tail)	Cyclised	197	25	>286
7	KRRVRWIIW	Parent	3	0.75	>390
Cyc-7	CAA-KRRVRWIIW-AAC: Head to Tail	Cyclised	142	9	>284
Cyclotide-7	Cyclo-CICRGNGYCKRRVRWIIW CPKILKKCRRDSDCPGA	Cyclotide Scaffold	31	4	>123

3.9.3 Hybridisation/Conjugation

Hybridisation involves combining two or more peptides. It was done on the basis that a combination of two or more bioactive or promising peptides could lead to a potent candidate. In this section, novel peptides were attached with a known consensus sequence via a linker to improve their antimicrobial activity. The consensus sequences used were RKPPYLPRPRPRPL (C1) and RWRRPIRRRPIRPPFWR (C2). These two sequences are derived from work done by the Schochi group from The University of Trieste, Italy. They found the C1 and C2 to be active against Gram-negative bacteria and exhibit a potentially different mode of action compared to peptides 5 and 7. Moreover, C2 has shown improved bioactivity compared to its parental analogue (Mardirossian *et al.*, 2019).

Peptide 7 was conjugated with standard Fmoc chemistry with a triple glycine linker before or after the consensus sequences (see Table 3.6). Peptide 7 was also used as a scaffold for cyclotide as well and assessed. Different conditions of media were used to examine if any notable activity changes occurred because of the modifications.

The consensus sequence, C1, on its own did not display any antimicrobial activity under standard MH broth, however, an MIC of 35 μM was observed in diluted media conditions. Peptides H1 and H2 had a MIC of 4.8 and 2.4 μM, respectively, under standard conditions. The only difference in their sequence was the position of the parent and consensus being swapped at the C and N-termini. Both performed the same under diluted media conditions, displaying a MIC of 1.2 μM. Furthermore, H1 and H2 had the same haemolytic activity, with HC_{50} above the highest concentration tested.

Peptide H3, H4 and H5 had the same sequence, with the combination C2 and peptide 7 being distinguished by their respective linkers in the middle of the sequence. H3 contained a triple glycine linker between C1 and peptide 7. H4 contained a triple lysine linker, while H5 contained a triple lysine-glycine linker. All three reported similar antimicrobial activity under all the conditions tested. MICs of 1.8 – 2 μM were reported in standard conditions, with 0.9 - 1 μM being reported in diluted conditions. C2 and H3 demonstrated similar haemolytic activty.

H4 presented a lower HC_{50} at 82 μM, and H5 showed greater HC_{50} than the highest concentration tested. Ultra-hybrid (U-HYB) also demonstrated similar antimicrobial activity to H3, H4 and H5 and equivalent toxicity to H3.

Table 3.6 Peptide 7 hybridisation

Peptide 7 was conjugated with two known consensus sequences. Peptide 7 was also inserted into a cyclotide scaffold at three different positions. MICs were reported as the modal value of at least three independent experiments. The blue highlighted portion of the sequence represents the linker between the two sequences, with underlined sequence representing the consensus 1 sequence and bold representing the consensus 2 sequence. This table is partially reproduced from (Hilpert *et al.*, 2021).

Name	**Sequence**	**Class**	**MIC *P. aeruginosa* (μM)**		**HC_{50} (μM)**
			MH Br	**20 % MH Br**	
7	KRRVRWIIW	Novel	3	0.75	>390
C1	VRKPPYLPRPRPRPL	Consensus 1	>278	35	>278
H1	VRKPPYLPRPRPRPLGGGKRRVRWIIW	Hybrid	4.8	1.2	>102
H2	KRRVRWIIWGGGVRKPPYLPRPRPRPL	Hybrid	2.4	1.2	>102
C2	**RWRRPIRRRPIRPPFWR**	Consensus 2	53	1.7	106
H3	**RWRRPIRRRPIRPPFWR**GGGKRRVRWIIW	Hybrid	2	1	102
H4	**RWRRPIRRRPIRPPFWR**KKKKRRVRWIIW	Hybrid	2	1	82
H5	**RWRRPIRRRPIRPPFWR**KGKGKGKRRVRWIIW	Hybrid	1.8	0.9	>125
U-HYB	**RWRRPIRRRPIRPPFWR**KGKC-S-S-CKGKRRVRWIIW	Hybrid with Disulphide Bridge	2	1	102

3.10 Cytotoxicity

HEK-293 cells are a widely used mammalian cell line. It was essential to garnering information about how novel peptides would behave with human cells. Selected peptides from the NOL, NOSSL and modified variants of peptides 5 and 7 were evaluated (Figure 3.13). The fluorescence values were normalised to a positive control (0.1 % Triton X-100) and negative control (peptide diluent, i.e. water treated HEK-293), representing 0 % and 100 % cell viability. Non-linear regression analysis was used to extrapolate the IC_{50} values of the peptides.

Peptides 5 and 7 from the NOL screen showed IC_{50} of ≥256 µg/mL, while peptide 9 demonstrated an IC_{50} of 160 µg/mL. The peptides from NOSSL (and subsequently derivatives of peptide 5) showed IC_{50} of ≥256 µg/mL (5.1), 240 µg/mL (5.3) and 300 µg/mL (5.5). Furthermore, melittin, a well-known antimicrobial peptide, had an IC_{50} of 12 µg/mL (Figure 3.13A).

Modified variants of peptide 5 all showed an IC_{50} ≥256 µg/mL, regardless of which modification was used. Meanwhile, peptide 7 variants equally showed IC_{50} ≥256 µg/mL, except for peptide 7D, which showed an approximate IC_{50} of 216 µg/mL (Figure 3.13B).

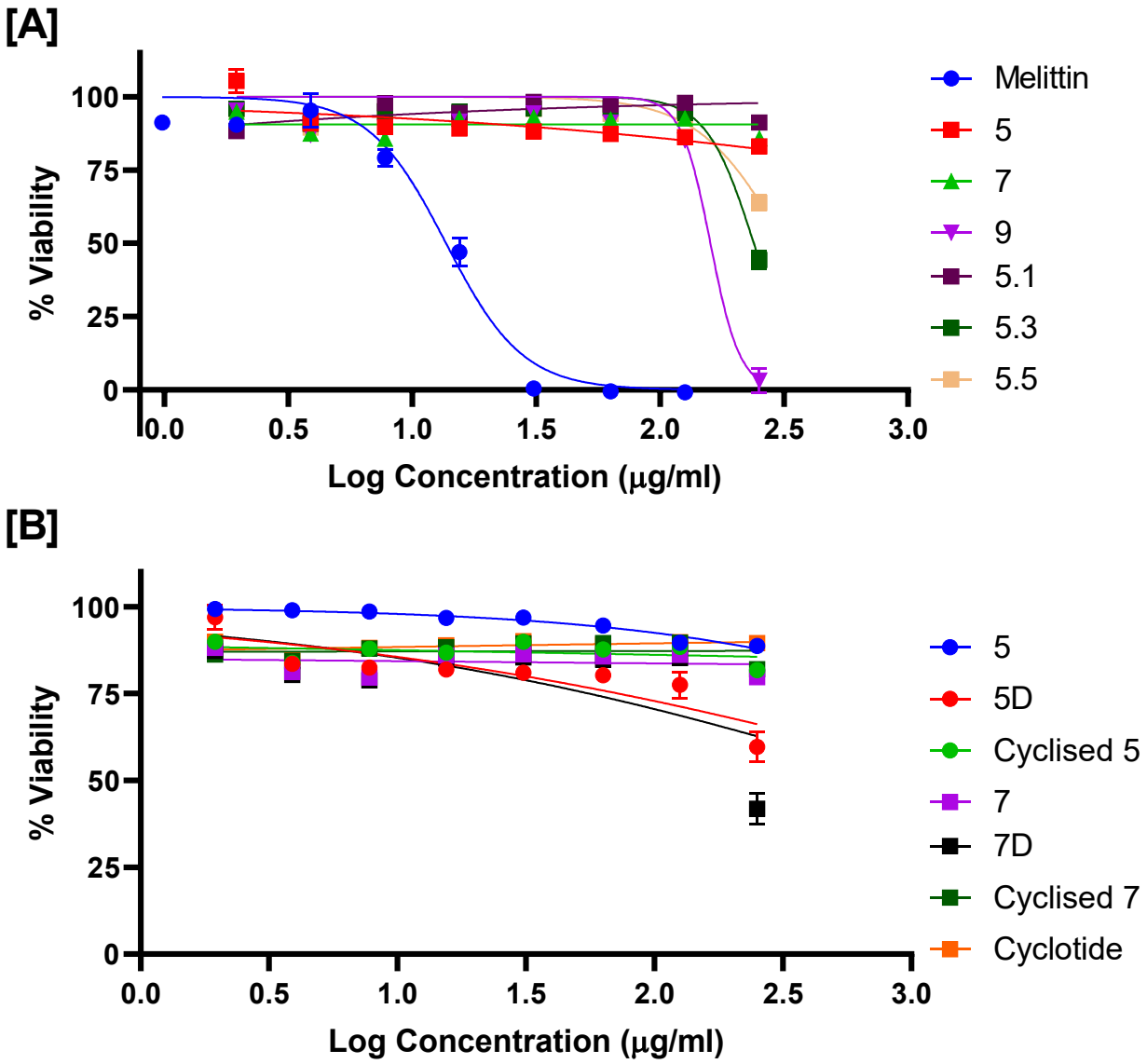

Figure 3.13 Cytotoxicity of Novel Peptides towards HEK-293

Peptides from **[A]** OLS, NOSSL and **[B]** modified variants of peptides 5 and 7 were tested against HEK-293 cells. The data was normalised to H_2O treated cells (negative control, no death) and 0.1 % Triton X-100 treated cells (positive control, 100 % death). Each peptide was tested in duplicate with at least three biological repeats. Error bars represent the standard deviation, and melittin was added further as another positive control.

3.11 Discussion

Original Library Antimicrobial and Haemolytic screen

This chapter examined whether short peptides designed from predictions utilising the iterations of 3000 peptides led to peptides with antimicrobial activity towards *P. aeruginosa* and were non-toxic towards human erythrocytes (Mikut *et al.*, 2016). From the NOL screen, it could be seen that several peptides did show increased antimicrobial activity compared to the in-house control in the screening with luminescent *P. aeruginosa*. The peptides were screened against a luminescent variant of *P. aeruginosa* as it is more sensitive than the fluorescence approach to detecting inhibition in bacterial growth (Hilpert and Hancock, 2007). It was more surprising that the haemolytic effect observed was also very minimal. Many studies performed before using short synthetic peptides produced promising antimicrobial data. However, there was difficulty in reducing toxicity; therefore, haemolytic effects have been one of the major hindrances in advancing antimicrobial peptide development (Lee and Hodges, 2003; Rathinakumar *et al.*, 2009; Starr *et al.*, 2018). The use of fresh human blood served as an advantage as it removed variability connected to the age of the donor and storage length of blood.

Furthermore, the use of human blood is a better representative of human physiological conditions than sheep or horse blood, which is commonly used in antimicrobial development (Dennison and Phoenix, 2014). Due to the large numbers of peptides from the NOL presented as non-haemolytic, the therapeutic potential was primarily driven by antimicrobial activity. However, overall antimicrobial activity served as a good indicator in identifying potential candidates.

The features that tended to promote antimicrobial activity were based on a prediction from Mikut *et al*. using different descriptors and modelling by taking amino acid occurrence, distribution, hydrophobicity, charge, and other descriptors into account (Mikut *et al.*, 2016). The same set of peptides from Mikut *et al*. was also assessed for their toxicity and showed 97 % of the peptides showed weak to strong toxicity towards human erythrocytes (unpublished

data). The predictions made by Mikut's software and analysis via SciXminer (formerly Gait-CAD) show that the outcome of such predictions was successful (Mikut, 2010). The peptides which showed the most potent antimicrobial activity had a high proportion of tryptophan. This pattern was also observed with peptides with potent antimicrobial activity against *Mycobacterium tuberculosis* (Ramón-García *et al.*, 2013). Overall, harmonising antimicrobial activity and toxicity levels (an optimum level), balancing charge, and hydrophobicity was important.

Maintaining both high antimicrobial activity and low toxicity was paramount in transitioning towards progressing antimicrobial development for systemic infections. Otherwise, it would not pass through the required regulatory approvals. Finally, a limitation of this method, i.e. synthesis of peptides on Spots, was that the concentrations of all the peptides could not be determined due to the vast numbers. Also, the peptides were not purified. However, the quantity produced was sufficient to perform assays to allow whittling down thousands to the tens of candidates (Hilpert *et al.*, 2007).

Validation of Novel Original Library Screening

The validation of the novel original library screen gave rise to three promising candidates, which were further characterised and optimised. The synthesis scale-up onto resin allowed for more accurate and robust bioactivity to be determined as appropriate antimicrobial and toxicity levels could be determined. From the overall sequences that were the most promising from the screens, it was clear that peptides with a greater hydrophobicity contributed heavily to the antimicrobial activity against *P. aeruginosa*. It was evident in many studies and is usually due to the membrane permeating ability of hydrophobic residues amino acids (Kim *et al.*, 2014; Lei *et al.*, 2019).

Usually, increased levels of hydrophobicity lead to increasing toxicity (Chen *et al.*, 2007). However, increasing it beyond a particular threshold also reduces antimicrobial activity (Chen *et al.*, 2007). Therefore, keeping the hydrophobicity levels balanced is paramount, based on the toxicity models (unpublished). Interestingly, a vast majority of the NOL peptides

overall contained a higher proportion of charged amino acids in relation to hydrophobic residues. However, the more charged peptides tended to show weaker or limited activity against *P. aeruginosa*, thus indicating that hydrophobicity played a more critical role in determining the antimicrobial activity for these short peptides.

The MICs for these peptides against *P. aeruginosa* ranged from 4 – 32 μg/mL for strong active to moderately active peptides. It was indicative of an optimal sequence configuration with very similar sequences that posed the most promising antimicrobial activity. The haemolytic activity of the peptides from the NOL screen showed weak haemolytic activity, despite being classed as non-haemolytic in the screen. They appeared to be weakly haemolytic at higher concentrations due to the vastly increased concentrations of peptides used. The main reason for such observation was primarily due to the low concentrations of peptide synthesised on a Spot; thus, the discrimination was primarily within lower concentrations ranges. The haemolytic toxicity of peptides has been one of the main hindrances to advancing it towards clinical development. Thus, rapid screening from many peptides synthesised on membranes was helpful. However, the haemolytic activity in the scale-up was acceptable. It demonstrated the importance of establishing peptides' toxicity early in the development phase. The therapeutic index indicated how selective such peptides were within the concentration ranges tested. The higher the therapeutic index, the greater the cell selectivity (Zhu *et al.*, 2007). Peptide 7 has been tested and demonstrated a MIC of 8 μg/mL (Grimsey *et al.*, 2020). However, 4 μg/mL is the MIC observed more consistently in this study. Peptides 5 and 7 showed up to 4-fold greater SI than peptides from the same peptide class with similar antimicrobial activity, as per Table 3.1. Therefore, along with peptide 9, they were the three peptides chosen to be further characterised.

Engineering Strategies (Substitutions & Modification)

Several different optimisation strategies were employed for select peptides to explore the possibility of improving antimicrobial activity while maintaining low toxicity. The peptides 5, 7 and 9 were chosen because they had the most potential from the NOL screen and subsequent validation. The first strategy was to perform single systemic amino acid substitution, which showed only peptide 5 substituted peptides had more activity than the parent. Peptide 7 and 9 did not yield a single better performing candidate. The substitution of a single amino acid into each position of the three most promising compounds showed that overall, there was an almost 50 % chance that activity would be reduced instead of increasing. This was demonstrated with 47 % of the total number of peptides showing reduced or devoid activity, compared to only 6 % improvement overall, mainly attributed to peptide 5. However, it should be noted that a similar proportion (47 % of total peptides) of the single substitute variants had activity equivalent to the parent. Therefore, this suggested that there was scope for minor changes within the sequence.No single position within the sequence was responsible for the bacterial inhibition. A similar observation was also demonstrated with a peptide derived from bovine, bactenecin, which underwent a single systematic single amino acid substitution, with 23 % of the total variants showing similar activity to the parent (Hilpert *et al.*, 2005). Where there was a noticeable improvement of antimicrobial activity, it coincided mainly with the incorporation of either hydrophobic (Phe and Trp), charged (Lys and Arg) or uncharged polar or non-polar amino acids (Gly and Gln). However, the inclusion of negatively charged amino acids, namely Asp and Glu, led to dramatically weaker activity, consistent with earlier findings (Hilpert *et al.*, 2005). Many studies have shown that incorporating charged (such as Arg and Lys) or hydrophobic amino acids (such as Leu) led to an increase in antimicrobial activity (Jiang *et al.*, 2008; Tan *et al.*, 2014). Also, it has been shown that specific substitutions of amino acids can lead to more reduced toxicity (Irazazabal *et al.*, 2016). A good amphipathic balance is crucial for the activity of these peptides, with a pattern that the c-termini being polar and charged, the n-termini being either hydrophobic or charged, and the core remaining hydrophobic.

Despite showing better overall activity in the antimicrobial screen, the peptides scaled up onto resin proved the contrary. The peptides derived from peptide 5, which performed better, were either the same MIC or higher and had more haemolytic potency. For the haemolytic potency, it appears that the prediction-based algorithm developed by Professor Ralf Mikut using SciXminer achieved the optimal balance in deciphering non-haemolytic peptides. For the antimicrobial activity, this could have been due to the minute discrepancies in the screen, which led to the peptide being classified as more active than being similar to the control. Furthermore, it could also be a concentration deficiency as it was not possible to measure the concentration of every single spot synthesised peptide. It could also be due to the sensitivity of the luminescence emitted from this strain, leading to the category of activity shift (Hilpert *et al.*, 2005). It appears that the addition of non-polar amino acids, such as Gly, caused either a reduction in the antimicrobial (Sani *et al.*, 2017) or caused the peptide to be selective towards particular bacteria (Ilić *et al.*, 2013). It was further observed within the antimicrobial screen that the addition of negatively charged amino acids aspartic acid and glutamic acid tended to reduce or nullify activity altogether. It further strengthened the need to balance the sequence and that losing even a single charge could have detrimental effects on antimicrobial activity.

Incorporating particular amino acids for the NOMSL was mainly predicated on them providing bioactivity in human serum, being less toxic and/or more active (Hilpert and Hancock, 2007; Li *et al.*, 2013; Zhu *et al.*, 2014). The secondary – tertiary substitutions involved swapping out more hydrophobic residues for less hydrophobic, such as Trp for Phe, Leu and Ile. Also, Arg was frequently replaced with Val to achieve a similar goal regarding the charge. Furthermore, these were initially screened against *E. coli* in the presence of 10 % human serum to allow for more robust discrimination. Moreover, it allows for the emergence of particular peptides, a condition (i.e. human serum) that has been one of the major pitfalls of antimicrobial peptides (Knappe *et al.*, 2010). Unfortunately, the best performing substituted peptides from all three screens proved to be mainly toxic towards human erythrocytes. It was

not surprising due to the increased inclusion of Arg, Trp and Lys. Overall, the more substitutions, the weaker the antimicrobial activity, which was shown by the vast majority of the peptides with triple substitutions being classified as weaker than the parent. The rationale of choice for specific amino acids chosen was primarily based on their properties, in particular charge and hydrophobicity. For example, replacing an Arg with a Lys was predicated on Arg's associated toxicity, mainly due to its membrane permeabilisation protentional (Li *et al.*, 2013; Q. Li *et al.*, 2017). However, in other instances, the substitution of K was more toxic than R (Yang *et al.*, 2018). This indicated that there are no set rules concerning peptides as a whole, but rather each has its own features and different levels of accommodation towards substitutions.

Hydrophobic substitution of Trp appeared to weaken the antimicrobial activity overall for these peptides. This indicated that having two tryptophan residues in the sequence was optimal for allowing peptide bioactivity. Even though the combination of amino acids did not result in a strong candidate in this study, the combination of a favourable amino acid approach allowed some groups to design highly potent peptides antimicrobial activity. Knappe *et al*. found that combining two favourable substitutions for the peptide oncocin resulted in unmasking an analogue that was 10-fold more potent against *P. aeruginosa* and 100-fold more potent against *S. aureus* (Knappe *et al.*, 2016). Furthermore, Hilpert *et al*. found that multiple favourable substitutions of peptide Bac2A led to increased potency across many pathogens, particularly a 25-fold increase in antimicrobial activity against *P. aeruginosa* and a 34-fold increase in antimicrobial activity against *E. coli* (Hilpert *et al.*, 2005). Screening a multi-drug resistant *E. coli* in the presence of human serum was used to discriminate peptides due to many peptides producing inferior activity and being proteolytically degraded in human serum (Knappe *et al.*, 2010). The case of using *E. coli* over *P. aeruginosa* in this instance was due to L-peptides requiring high levels of concentration to produce an inhibitory effect and to elicit any discrimination between peptide activities. From all the substitutions performed, alongside the validation, there were no better-performing candidates overall in comparison to the three

original parents. Therefore, it was decided to proceed with peptides 5 and 7 from the NOL from this stage for further optimisation strategies.

Isomerisation of peptides has been shown to retain or improve antimicrobial activity in some instances (Carmona *et al.*, 2013). Isomers of the naturally L-amino acids also protect against proteolytic degradation (Carmona *et al.*, 2013; Lu *et al.*, 2020). The isomerised peptides showed a lower therapeutic window than their L-amino acid counterpart, indicating an increased toxicity level, as antimicrobial activity remained unchanged (Feng and Xu, 2016). This was also evident in the cytotoxicity assay with HEK-293. The D-peptides showed the most cell viability reduction compared to the other modifications tested at the higher concentrations.

Peptides 5 and 7 were cyclised via cysteine in a head-to-tail manner with two β-alanines attached to each side of the termini before the cysteine. Cyclisation is a common method used extensively to improve the stability of any peptide; however, they have other notable features such as low toxicity to make them a viable peptide drug proposition (Falanga *et al.*, 2017). Furthermore, antibiotics such as daptomycin and colistin (currently on the market) are peptides of a cyclic nature (Zorzi *et al.*, 2017). The cyclisation of the short novel peptides resulted in a dramatic decrease in antimicrobial activity of up to almost 50-fold in MH broth and a 16-fold loss of activity in diluted media. It suggests that head-to-tail cyclisation was not ideal for advancing these particular peptides. Cyclotides are extremely stable peptides resistant at differing pH, high temperatures and enzymatic degradation (Gould *et al.*, 2011; Craik and Du, 2017). As a standalone compound, there have been only a few instances where antimicrobial activity has been observed (Pränting *et al.*, 2010; Strömstedt *et al.*, 2017). Peptide 7 was inserted into such a scaffold, MCoTI-II. Unfortunately, it led to weaker activity with a MIC increase of 32-fold compared to peptide 7 alone, suggesting this particular scaffold may not suit this sequence.

The hybridisation of peptides involved conjugating two or more peptides with antimicrobial activity, with the postulation that it could have a combinatorial effect. Peptide 7 was attached to two consensus sequences, C1 and C2. Both were from the Sccochi group,

as they have shown proline-rich peptides to be bioactive (Mardirossian *et al.*, 2019). Moreover, Peptide C2, known as Bac5 V291, demonstrated the lowest MIC and toxicity and was able to inhibit protein synthesis and show membrane permeabilisation (Mardirossian *et al.*, 2019). All peptides demonstrated more potent antimicrobial activity in standard MH broth than peptide 7, except the consensus sequences C1 and C2 and peptide H1. Under standard conditions, peptides H1 and H2 showed a consistent 2-fold difference in activity with one another, even though the only difference was swapping the respective sequences on either side of the linker. It suggests that it is slightly more advantageous to have C2 attached to the N-terminus and peptide 7 to C-terminus. The role of termini has played an important role in other peptides such as pleurocidin (Crusca *et al.*, 2011; Cho *et al.*, 2012). However, there was no difference between the two peptides in diluted media conditions. It indicated that specific peptides with certain properties might not all behave as expected in a previously proposed way of performing MICs with AMPs (Wiegand *et al.*, 2008). Also, concerning the haemolytic activity, it was clear that the addition of the C2 sequence increased toxicity. It was not entirely unexpected, and this is related to the amount of arginine in the sequence overall, linked with increased toxicity (Q. Li *et al.*, 2017). The triple lysine linker in H4 increases the charge; therefore, it was not surprising to observe an even lower HC_{50} compared to either the glycine linker or mixed glycine-lysine linker. Peptide U-HYB had a similar sequence to H5 but with the addition of a disulphide bridge. The addition of the disulphide bridge was in anticipation of the bacteria cleaving the disulphide bridge within its cytosol and allowing both peptides to act independently (Hilpert *et al.*, 2021). Furthermore, almost all the hybrid peptides also demonstrated antimicrobial activity against *E. coli* and MRSA (Hilpert *et al.*, 2021). The amphipathic balance of the hybrid peptides was also disrupted by increasing the charge, and hydrophobicity could have led to the reduced antimicrobial activity and also increased toxicity in some instances (Edwards *et al.*, 2016; Elliott *et al.*, 2020)

Other forms of optimisation which could have potentially been explored was the process of lipidation and glycosylation. Current antibiotic peptides daptomycin and vancomycin fall under the lipopeptide and glycopeptide, respectively, although they are only

therapeutically indicated for MRSA. Peptide 7 did, however, undergo lipidation. However, the effects were limited, and in some instances, an increase in antimicrobial activity led to an increase in toxicity (Grimsey *et al.*, 2020). Cyclisation performed here was using the head-to-tail methodology; however, side-chain-to-side-chain, head-to-side-chain, and side-chain-to-tail avenues could have potentially been explored and characterised to evaluate if any significant changes to activity could have been elucidated (Botti *et al.*, 1996; Kofod-Hansen *et al.*, 2002; Lundquist IV and Pelletier, 2002).

Broad-spectrum activity and cytotoxicity

Although these short AMPs were initially designed to be active against *P. aeruginosa*, it was intriguing to see that they were active against other problematic Gram-positive and negative bacteria, namely ESKPA(*E. coli*) (Boucher *et al.*, 2009). It was not entirely surprising given the nature of these sequences (the inclusion of amino acids such as Lys, Arg and Trp), which gave it the potential to be broad-spectrum in its antimicrobial activity profile. Some of the most potent peptides have generally displayed broad-spectrum antimicrobial activity, such as magainin, melittin and indolicidin (Subbalakshmi and Sitaram, 1998; Dathe *et al.*, 2001; Ahmad *et al.*, 2009). Furthermore, despite various activity levels against the pathogens, it was clear that these peptides do not have any major discrimination towards any specific bacterial organism. However, they still maintain relatively minimal toxicity. These findings further enable the characterisation of these peptides against other pathogens of interest. One issue that may arise due to the broad-spectrum activity could be the increased likelihood that these peptides would kill beneficial bacteria (Bhaskaran *et al.*, 2018). It is a common occurrence amongst many antibiotics, unfortunately, and some have shown extensive disruption to the microbiome of the gut with antibiotics taken orally (Stone and Xu, 2017). However, the microbiome's composition tends to recover close to pre-treatment levels after antibiotic therapy (Palleja *et al.*, 2018). Furthermore, broad-spectrum activity can increase cross-resistance amongst different bacterial species (Melander *et al.*, 2018).

Toxicity against HEK-293 showed the most promising peptide candidates to be toxic at levels at least 50 times more than the MICs reported, demonstrating a large therapeutic window. The larger the therapeutic window, the more promising the AMP for further development (Saint Jean *et al.*, 2018). The cytotoxicity assay also demonstrated that some peptides could discriminate between bacterial and mammalian cell lines, thus indicating some level of selectivity and preferential binding to bacteria. The modifications did not yield any significant reduction in cell viability except 7D. However, there was no tangible increase in antimicrobial activity. 7D showing increased cytotoxicity is likely explained by the properties of D-amino acids exhibiting a longer half-life in media, allowing it to be more toxic. Also, it has been noted that some substitutions of D-amino acids can increase cytotoxicity (Lu *et al.*, 2020).

In summary, this chapter set out to examine the use of prediction-based algorithms to design short 9-mer peptides that were active against *P. aeruginosa* and low in toxicity. It was empiracally demonstrated in the NOL screens and was successful in the haemolytic activity screen. To date, there is nothing published that uses machine learning to predict peptides of a non-haemolytic nature while still being antimicrobial.

Further iterations from the most promising candidates in the NOL were unable to produce any more viable antimicrobial potency and non-toxic peptides. Engineering strategies did not yield a substantially more active hit peptide. However, engineering did provide valuable insights into possibilities and potential avenues that can be further explored. Overall, the peptides from OLS were the only ones that warranted further investigation and characterisation.

4 Identifying and Optimising Naturally Occurring Antimicrobial Peptides

4.1 Introduction

Antimicrobial peptides are found extensively within nature from all kingdoms of life and form an important part of higher organisms' innate immune systems (Brown and Hancock, 2006). Many have been deposited into databases such as DRAMP, CAMP, and APD3, isolated from nature (Wang *et al.*, 2015; Waghu *et al.*, 2016; Kang *et al.*, 2019). There has been potential that peptides isolated in nature may act as a template to produce further antimicrobial agents. The rationale for potentially deriving AMPs for therapeutic use is that they have retained their bioactivity within species, despite the multitude of exposure to pathogens. Although these large databases provide a wealth of information pertaining to the peptides discovered, a limitation lies within the approach of antimicrobial reporting. The primary limitation is that deposited peptides have their data primarily taken from the original literature source, which invariably contain differing methodologies, making it challenging to compare peptides amongst one another. A possible way to overcome this problem is to test the peptides within the database by creating a library and using a single method against a single target organism. Menousek *et al*. and Wang *et al*. took a similar approach and screened database derived peptides against MRSA and HIV (Wang *et al.*, 2010; Menousek *et al.*, 2012). Although this serves as a good template, the use of solid-phase peptide synthesis on resin is often at a high cost and labour, which ultimately restricts the number of peptides that can be tested. An alternative method is the Spot technique, which allows for thousands of peptides to be synthesised simultaneously on cellulose support (Hilpert 2007). The yield is lower than resin; however, sufficient quantity can be produced to perform antimicrobial and haemolytic assays, which can provide valuable insight into potential lead compounds for novel antimicrobials (for *P. aeruginosa*) (Hilpert and Hancock, 2007).

4.2 Study Design

The antimicrobial peptides used in this chapter were taken from the APD3 database (Wang *et al.*, 2015). They were categorised under the "antibacterial peptides" and were between 4-17 amino acids in length. They were subjected to the same screening and further characterisation as the previous chapter. A substitution analysis optimisation strategy was employed with the ancillary aim to improve bioactivity and/or reduce toxicity for a select candidate.

Specific Objectives

1. Synthesis of 400 natural peptide library, termed Natural Library (NL)
2. An antimicrobial screen of the NL using a luminescent strain of P aeruginosa and haemolytic activity against human erythrocytes.
3. Computational assessment of the NL by SciXMiner (a toolbox of MATLAB ®) to identify the candidates with promising therapeutic potential
4. The best-performing peptides from the NL screen were scaled up, purified, and further assessed for minimum inhibitory concentration (MIC) against *P. aeruginosa*, haemolytic activity and toxicity towards HEK-293 cells
5. Systematic substitution analysis of the most promising candidate to identify optimised variants termed Lasioglossin Single Substitution Library (LSSL). Favourable substitutions were combined to generate a new library, termed Lasioglossin Multiple Substitution Library (LMSL)
6. The libraries were screened for antimicrobial activity using a luminescent strain of *P. aeruginosa* and a multidrug-resistant *E. coli* isolate in the presence of human serum, and haemolytic activity against assessed using human erythrocytes

4.3 Natural Library

4.3.1 Origin & Features of Library

The peptides chosen originated from a broad spectrum of differing species. The majority of peptides (Figure 4.1A) were from the amphibian class (51 %), insects (15 %), bacteria (12 %) and arachnids (6 %). An additional small selection (4 %) was synthetic. The library consisted of peptides between 4 and 17 amino acids.

The amino acid occurrence across all the peptides was examined (Figure 4.1B) and showed the highest frequency for Leu with an average of just under 20 %. It was followed by Ile, Lys, and Gly, each just under 10 % of the total proportion. The least common amino acids present were asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), and histidine (His), methionine (Met), threonine (Thr), and tyrosine (Tyr).

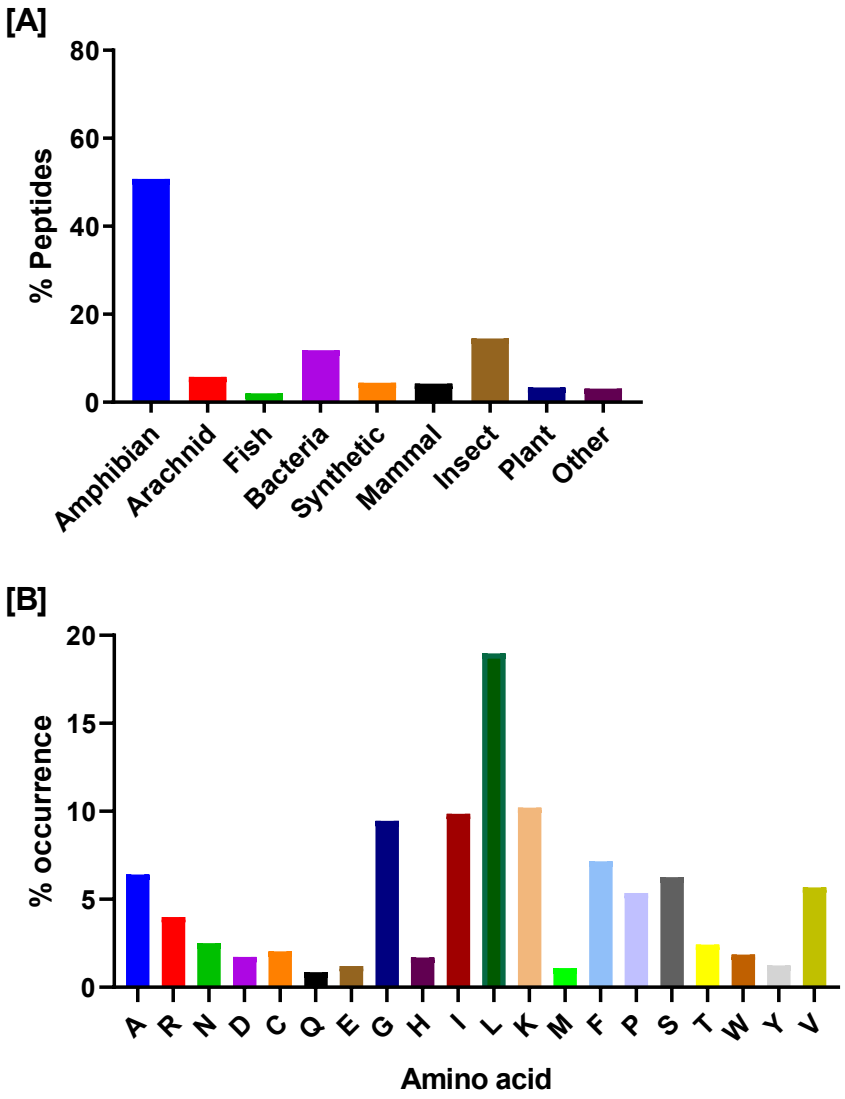

Figure 4.1 Origin and Amino Acid Composition of Natural Library

A group of 400 peptides were chosen from the APD3 database, between 4-17 amino acids in length. The peptides were classified as “antibacterial” in the database. They were synthesised using Spot technology and eventually solubilised in water, and later used for screening. **[A]** denotes the origin of the peptides and the proportional representation of each in the library, while **[B]** denoting the amino acid distribution of the entire natural library.

4.3.2 Antimicrobial Screen

The peptides were synthesised as mentioned previously (refer to section 2.1). The screening and analysis procedure against *P. aeruginosa* was similar to that of the NOL antimicrobial screen (see 2.5). Finally, in this case, the control was also the same control (KRRWRIWLV) used for the NOL antimicrobial screen.

From the 400 peptides (Figure 4.2), 5 % (20 peptides) were more active (i.e. more potent antimicrobial activity) than the in-house control. 15 % (61 peptides) had similar activity to the control. 43 % (172 peptides) and 37 % (147 peptides) were categorised as weaker than control and inactive.

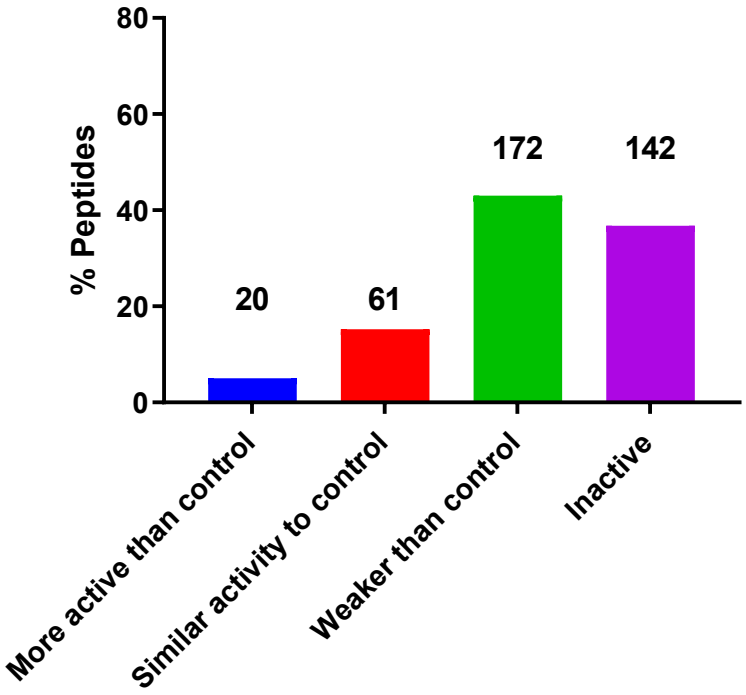

Figure 4.2 Natural Library antimicrobial screen

The 400 peptides were synthesised using Spot methodology. Using the relIC$_{75}$ value each peptide from the library was categorised into one of four groups in accordance to its antimicrobial activity. Above each respective bar is the number of peptides that fall within each category.

4.3.3 Haemolytic Activity Screen

In total, 111 peptides classified as "more active than control", "similar activity to control", including 30 peptides from "weaker activity than control" were taken forward to assess haemolytic activity (Figure 4.3). They were classified in their groups (same classification as the NOL), according to the controls of 0.1 % Triton X-100 and untreated blood.

The control 0.1 % Triton X-100, was considered to cause 100 % erythrocyte lysis and was equivalent to "strong haemolytic". In contrast, untreated blood was considered "non-haemolytic." Most of the peptides, 54 % (60 peptides) were classified as "strong haemolytic", and 36 % (40 peptides) in the medium haemolytic category. Only 9 % (10 peptides) and 1 % (1 peptide) of the peptides tested were weak haemolytic and non-haemolytic categories.

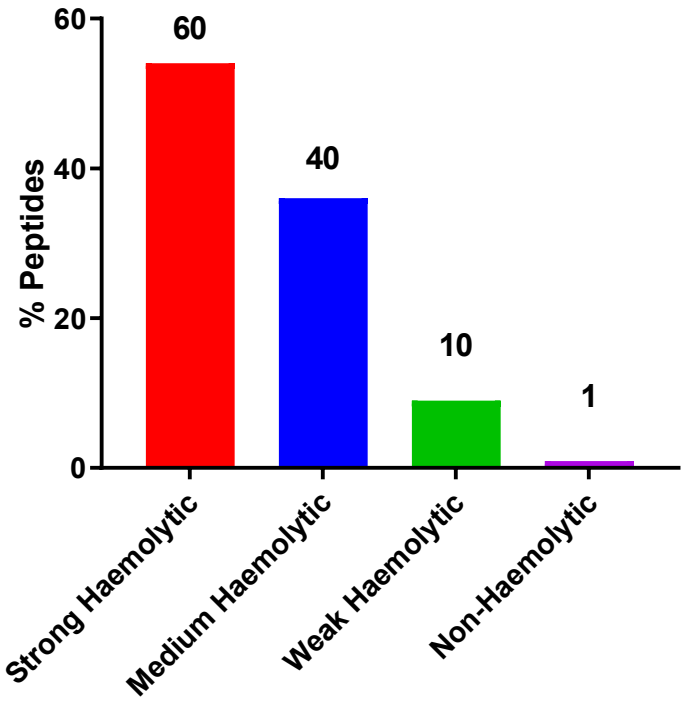

Figure 4.3 Natural Library haemolytic activity

Peptides were synthesised using Spot synthesis. The haemolytic activity of 111 peptides was determined by incubating serial dilutions of the peptide with fresh human erythrocytes. Each peptide was then assigned a class amongst four groups, based upon its HC_{75} value to signify its level of haemolytic activity. Above each respective bar is the number of peptides that fall within each category.

4.3.4 Features Promoting Antimicrobial Activity

The limited number of peptides screened for their haemolytic activity would not allow therapeutic potential to be calculated (as only a selected number was chosen). The majority of the peptides were classed as haemolytic; hence only features for antimicrobial activity were explored (Figure 4.4). Interestingly, no clear defining amino acid could suggestively contribute to antimicrobial activity. Peptides containing Asp, Glu and Gly mainly contributed to the total lack of activity in the peptides using the methodology of this chapter. There was no clear trend for the rest of the activity classes, demonstrating the vast diversity in nature.

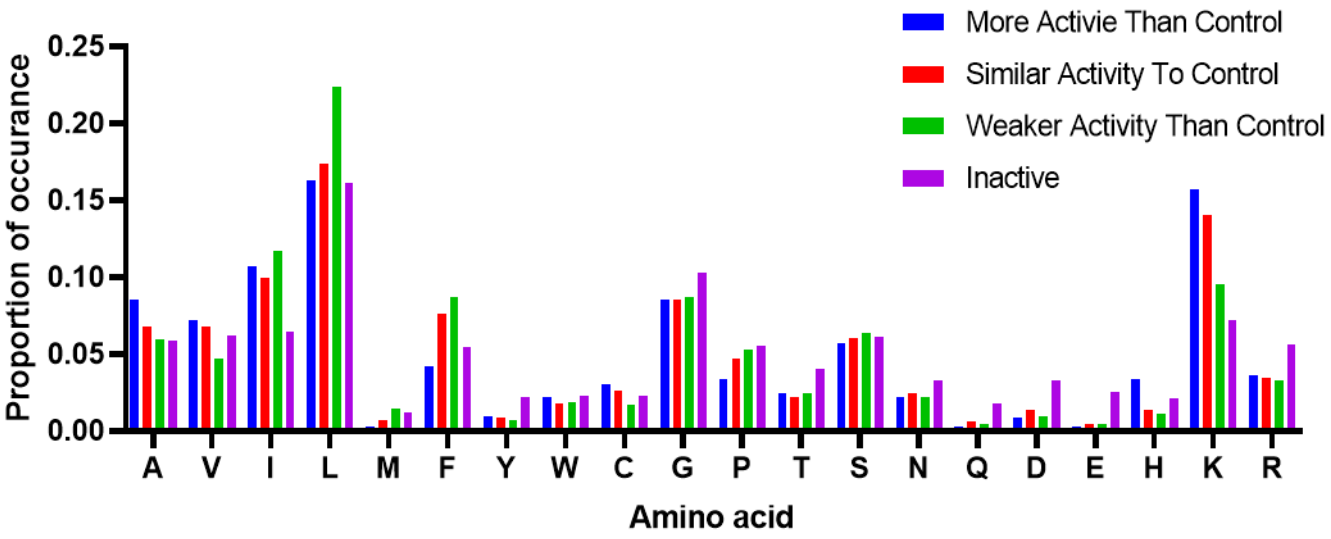

Figure 4.4 Natural Library amino acid distribution (antimicrobial screen)

An amino acid occurrence of 400 peptides which were screened for their antimicrobial activity against *P. aeruginosa*. The distribution considers the four groups in which each particular peptide was classified into. Strong = 25, Moderately active = 86, Weakly active = 161 and Inactive = 158 peptides

4.3.5 Validation of Natural Library Screening

From strong to weakly active peptides from the screens, a selection of candidates was synthesised on resin and purified as previously described to the homogeneity of at least 88 % purity (Table 4.1). The most promising peptides originated from insects (bee and wasp). Peptides classed as MATC in the antimicrobial screen, MICs were observed between 4 – 8 µg/mL. All peptides classified as SATC and WATC correlated well with their respective MICs. Moreover, the haemolytic activity correlated well between the HC_{50} and their respective classes in the screening.

The peptide with the lowest therapeutic potential was unsurprisingly CM-15 at 0.25, with the largest displayed by lasioglossin III at 50. Uperin 3.6 had a selectivity index (SI) of only 1.8, mainly attributed to its low $HC_{50,}$ in contrast with CPF-PG1, which had a TI of >4 despite its low antimicrobial activity. The larger the TI, the more scope for further development of the peptides. The control peptide MIC served as a base for the resin antimicrobial activity comparison. HC_{50} was determined from normalisation against 0.1 % Triton X-100 (100 % lysis) and untreated human erythrocytes (0 % lysis).

Table 4.1 Validation of Natural Library screens

A selection of a combination of peptides from the NL screen scaled up onto resin. All purified peptides were subjected to at least three independent experiments with the modal value reported. HC_{50} was determined using fresh human RBCs and is reported as the average of three independent experiments. The SI was calculated using the ratio between the MIC of the peptide and HC_{50}.

MICs. Key: MATC = More Active Than Control, SATC = Similar Activity To Control and WATC = Weaker Activity Than Control. NT = not tested.

Peptide	Origin	Purity (%)	Class in Screen	MIC (µg/mL)	Class in Haemolytic Screen	HC_{50} (µg/mL)	SI
Lasioglossin III	Bee Venom	>95	MATC	4	Medium	200	50
Bmkn2	Scorpion Venom	>95	MATC	8	Strong	48	6
CM-15	Cecropin/ Melittin Hybrid	>95	MATC	8	Strong	2	0.25
Mastoparan	Wasp Venom	>95	SATC	16	Strong	52	3.25
Bombolitin III	American Bee Venom	>95	SATC	16	Strong	63	3.9
Bombolitin IV	American Bee Venom	88	SATC	16	Strong	92	5.8
Dominulin B	European Paper Wasp	>95	SATC	16	Medium	129	8.1
Dominulin A	European Paper Wasp	>95	SATC	16	Medium	275	17.2
Uperin 3.6	Australian Toad	>95	WATC	256	Weak	470	1.8
CPF-PG1	Frog Skin	>95	WATC	128	Non-Haemolytic	>512	>4
Control	Peptide	>95	Control	16	Weak	425	27
Gentamicin	Conventional Antibiotic	N/A	NT	0.25	NT	NT	NT

4.4 Optimisation of Lasioglossin III

From the Natural library screen and the subsequent validation, it was clear that the best performing candidate was lasioglossin III, which warranted further scope for optimisation. For the first optimisation strategy, the lasioglossin III sequence was subjected to a single systematic substitution analysis in each position of the peptide sequence. Subsequently, the most favourable substitutions from the single substitution were also screened.

4.4.1 Single Substitution Screen – *P. aeruginosa* Screen

The single systemic substitution gave rise to a library of 285 peptides. The library herein was termed "Lasioglossin Single Substitution Library (LSSL)." An additional antimicrobial screen with an MDR *E. coli* and 10 % human serum combination was performed in addition to *P. aeruginosa*. The analysis methodology was the same as before with the novel peptides in Chapter 3, with the control being lasioglossin III. No peptide performed better than the parent (Figure 4.5A). 73 % (207 peptides) showed similar activity to the parent, while 26 % (74 peptides) showed reduced activity. Only 1 % (4 peptides) demonstrated total loss of activity.

From the heatmap (Figure 4.5B), most substitutions with Asp, Asn and Glu in most positions largely contributed to a decline in activity or inactivity. Position 3, 7 and 10 of Lasioglossin III were the most susceptible to alterations in bioactivity, with up to 70 % (14 of 20 amino acids) of the substitutions in that position causing a reduction in activity. In contrast, positions 2, 4 and 5 of Lasioglossin III had the least alternations. Only 5 % (1 of 20 amino acids) caused activity to be reduced in those positions. The rest remained similar to the parental peptide.

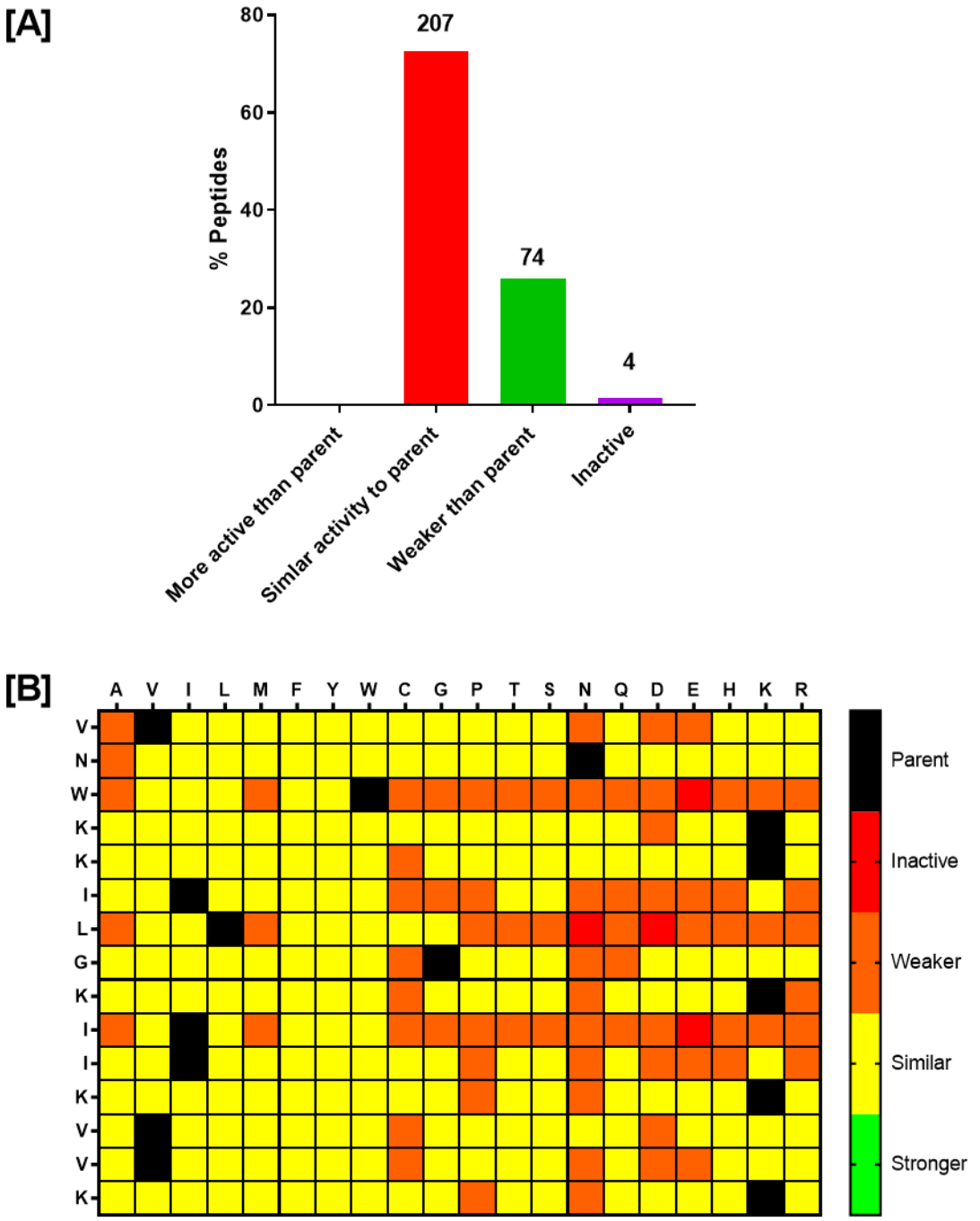

Figure 4.5 Lassioglossin Single Substitution antimicrobial screen – P. aeruginosa

The systemic single amino acid substitution of Lassioglossin led to 285 peptides being created.. **[A]** They were classified into four groups according to the activity they displayed in the antimicrobial screen. **[B]** Positional analysis of each amino acid is represented in a heatmap, showing how replacement each amino acid affected the activity ofl lassioglossin III. Above each respective bar is the number of peptides that fall within each category.

Green = stronger than parent, Yellow = Similar activity to parent, Orange = weaker than parent, Red = inactive and Black = Parental amino acid in that specific position.

4.4.2 Single Substitution Screen – E. coli + 10 % Human Serum

To ascertain the activity of the peptides in the presence of human serum, a multidrug-resistant *E. coli* in the presence of 10 % human serum was incubated alongside the peptides as mentioned previously (2.6.2). The plates were measured after 6- and 24-hours incubation.

It was observed that a majority of peptides after 6 hours (Figure 4.6A) were either weaker or completely inactive, with 53 % (152 peptides) and 4 % (11 peptides), respectively. A minority, 41 % (117 peptides), had similar activity to the parent lasioglossin III. In comparison, only 2 % (5 peptides) showed more potent activity than the parent. The peptides with weaker activity tended to contain primarily negatively charged amino acids (Asp and Glu) or neutral amino acids (Ala, Asn, Gln, Gly, Pro, Met). After 6 hours, the only peptides which showed greater activity were with charged amino acids Lys and Arg (Figure 4.6B).

After 24 hours (Figure 4.6C), the activity classes remained similar to the 6-hour incubation for the SATP class, at 2 % (5 peptides). The similar to parent group had 53 % (151 peptides), an increase from 117 peptides from the previous day under the same classification. Furthermore, the weaker than parent group had 41 % (125 peptides), reducing from 151 peptides after the 6-hour incubation. A reduction of the number of peptides in the inactive group from the 6-hour incubation was also seen, with 1 % (4 peptides) after 24-hour incubation. Observation from the heatmap (Figure 4.6D) revealed a similar trend to the 6-hour incubated peptides for peptides with negatively charged amino acids (Asp and Glu) remaining weaker than the parent. The most noticeable shift was with the peptides from the stronger than parent class. Four peptides in the more active than parent class were replaced with either Ile or His.

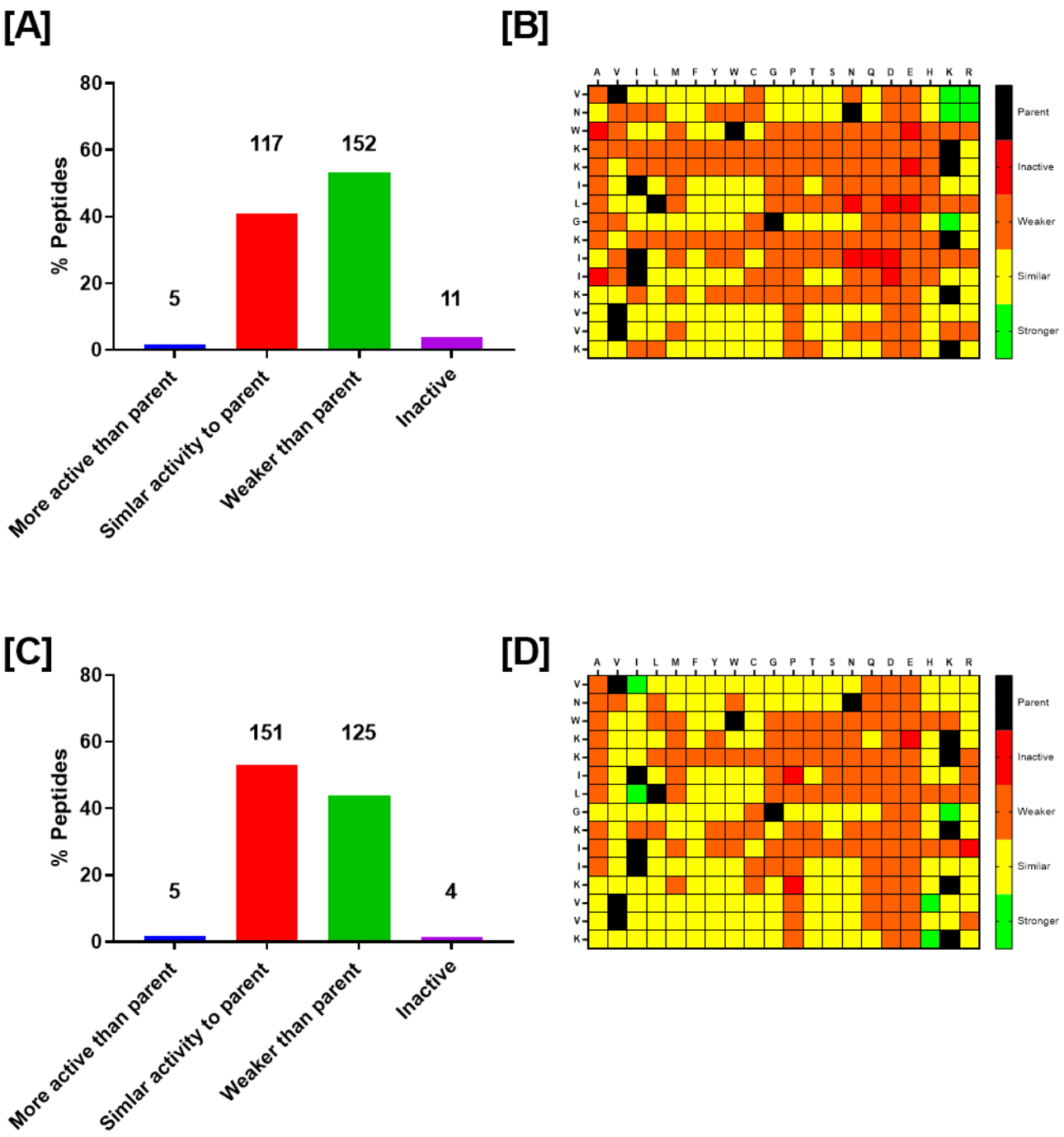

Figure 4.6 LSSL screen – MDR E. coli + 10% human serum

All peptides from the LSSL were synthesised using Spot technology. lassioglossin III served as the positive control. **[A]** shows the classification after 6 hours, with **[B]** representing the positional analysis of each amino acid and the classification that each amino substituted was classified into. **[C]** shows the classification after 24 hours, with **[D]** representing the positional analysis of each amino acid. Above each respective bar is the number of peptides that fall within each category.

4.4.3 Multiple Substitution Screen

The most favourable amino acid substitutions from the LSSL screen were inserted into lasioglossin and a total of 125 new candidates, with two – seven amino substitutions in the sequence. The substitutions were chosen in an attempt to promote greater antimicrobial potency or lower toxicity levels. This library was termed lasioglossin multiple substitution library (LMSL). The LMSL consisted of peptides under each amino acid substitution (Table 4.2).

Table 4.2 LMSL peptides break down

A breakdown of all the different amino acid substitutions was inserted into lasioglossin III. Also, the number of peptides generated from such insertion gave rise to the entire LMSL.

Amino acid substitution	Number of peptides
2	15
3	22
4	11
5	13
6	34
7	29

All 125 peptides were screened and analysed similarly to previous screens. LMSL peptides were screened against both *P. aeruginosa,* and an MDR *E. coli* isolate (in addition to 10 % human serum). The control peptide in this scenario was still the parent Lasioglossin III.

From the screen against *P. aeruginosa* (Figure 4.7A), 5 % (6 peptides) showed activity greater than the parent. The vast majority, 78% (98 peptides), showed similar activity to the parent. 17 % (21 peptides) demonstrated weaker activity than the parent. Expectedly, not a single peptide was classified as inactive against *P. aeruginosa*. Upon further analysis, the breakdown in the number of substitutes and its effect on the antimicrobial activity (Figure 4.7B). The six peptides, which were more active than the parent, had either 5 or 6 substitutions in the sequence. The similarities in both 5 or 6 substitutions were that in positions 2 and 15, they both had Lys and Val substituted with Gln and Lys, respectively. In addition, the most potent substitutions were at either at the C- or N- termini. Expectedly, all six different substitution variants were present in the similar activity to the parent class, with 6 substitutions representing over 25 % of the total number of peptides. Furthermore, none of the peptides with 6 substitutions showed any weaker activity, while the rest did.

Against *E. coli* MDR isolate with the addition of 10 % human serum, no peptide demonstrated greater activity than Lasioglossin III itself. As with the *P. aeruginosa* screen, the vast majority of peptides (82 % - 103 peptides) showed similar activity to the parent after 6-hour incubation (Figure 4.7C). The remainder of peptides showed weaker or complete inactivity at 10 % (12 peptides) and 8 % (10 peptides), respectively. From the specific amino acid substitutions (Figure 4.7D), the inactive peptides contained either 5 or 7 amino acids substitution into the sequence. Post 24-hour incubation (Figure 4.7E), the more than active and inactive class remained the same as the previous day. However, there was a shift from the similar to parent class to weaker than parent class with a 10 % reduction (82 % to 72 % or 103 peptides to 90 peptides). The breakdown of the different substitutions (Figure 4.7F) showed all amino acid substitutions numbers to be present (i.e. 2 – 7 variants).

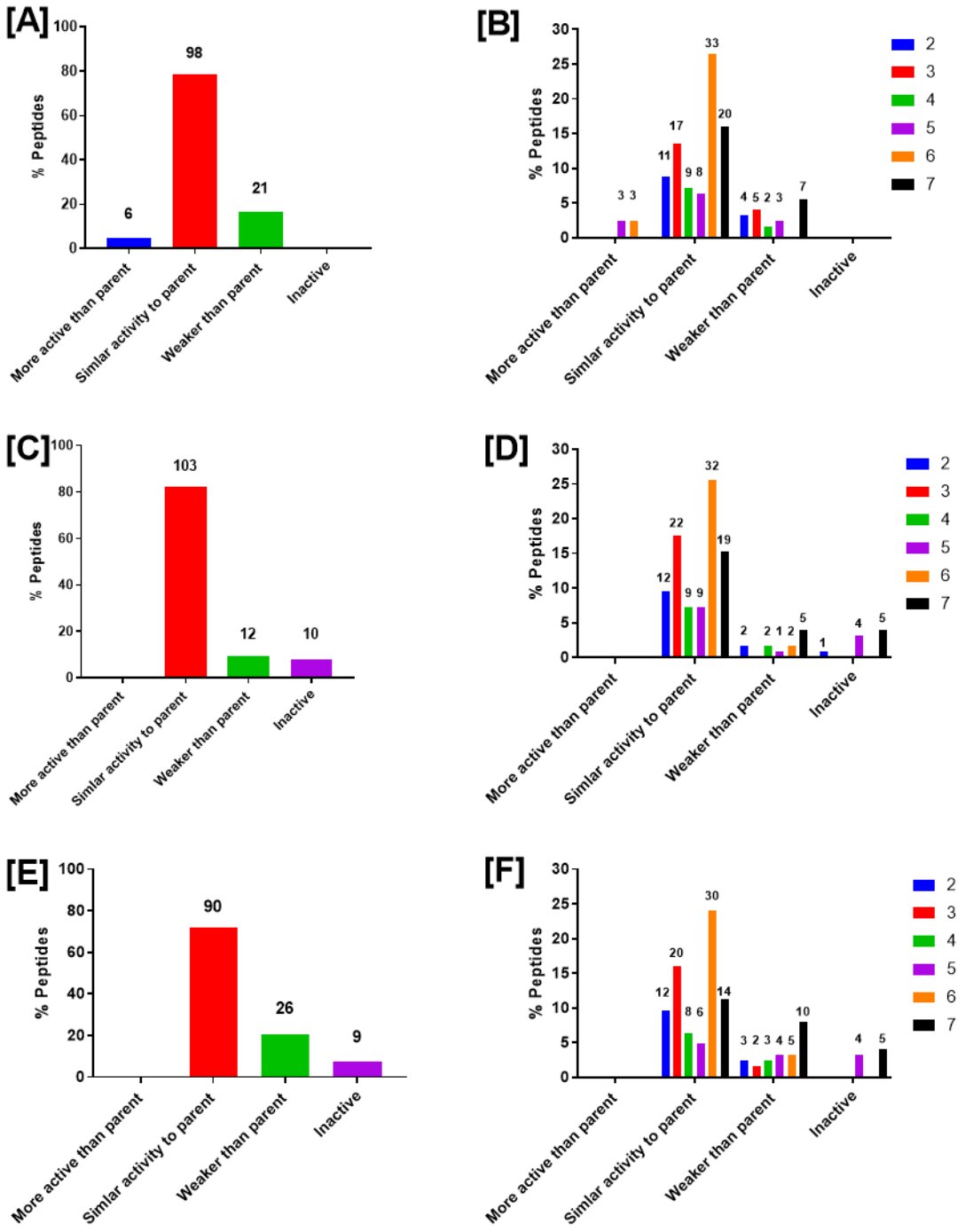

Figure 4.7 Lassioglossin Multiple Substitution Library – Antimicrobial screens

lassioglossin III derivates resulted in 125 peptides III. **[A]** Shows the breakdown as per the four activity groups, with **[B]** showing the breakdown of each amino acid substitution group. Furthermore, the peptides were screen against an MDR strain of *E. coli* in the presence of human serum and classified into their respective groups. **[C]** is the classification of peptides after 6-hour measurement, with **[D]** showing the breakdown of specific substitutions. The measure after 24-hours shows **[E]** their classification and **[F]**, the breakdown of specific substitution. Above each respective bar is the number of peptides that fall within each category.

4.4.4 Haemolytic Screen

All peptides amongst the better performers, i.e. "more active than parent" and top 10 from "similar to parent" classification, were tested in a haemolytic assay from both single and multiple substitution screens. In total, there were 44 peptides tested. All the peptides, 100 % (44 peptides), demonstrated strong haemolytic activity. All of them were similar to the positive control (0.1 % Triton X-100). No further peptide verification and scale up to resin was performed.

4.5 Discussion

Natural Library Screen

Delving into nature to aid in novel antibiotic development has long been the most explored route and led to the discovery of some of the earliest and most well-known antibiotics. Antibiotics such as penicillin and streptomycin have been derived from natural sources, fungi and bacteria, respectively (Fleming, 1929; Schatz *et al.*, 1944). Generally, the trend of identifying novel compounds has led to small and limited numbers of compounds identified. Large scale synthesis and antimicrobial studies also attracted a high-cost burden and increased staffing hours, which has resulted in limited advancement of novel antimicrobials (Jackson *et al.*, 2018). Naturally occurring antimicrobial peptides such as magainin, buforin and LL-37 have been isolated from sources such as amphibians and humans and have demonstrated activity against various pathogens (Zasloff, 1987; Park *et al.*, 2000; Gordon *et al.*, 2005). One major issue with naturally occurring peptides deposited within peptide databases is that they have been presented with a plethora of different methodologies employed. Different techniques, bacterial strains, media, and conditions within different research groups may all give different results for the same compound. This was evident with peptide cecropin P1, where the same compound produced differing results (Pillai *et al.*, 2005; Ebbensgaard *et al.*, 2015). High throughput Spot screening eradicates these differences by

employing a single systematic method for all peptides with a specific pathogen of interest (Hilpert and Mikut, 2010). However, this does not consider that specific peptides may require additional supplementation or conditions to be active. For example, peptide lebocin 4 is affected by salt (Hara and Yamakawa, 1995). It served as a good balance to consider as many peptides in a short amount of time as possible.

Furthermore, using Spot methodology and high throughput screening does not require the peptide to be of a high purity or post-synthesis modifications to be evaluated. The screening results from the NL indicate that it was possible to find potent antimicrobial peptides using a harmonised method. Furthermore, haemolytic screening reduced the number of candidates from hundreds to just a few.

The database used, APD3, contained the amphibian class of peptides primarily, mainly because they have been the most abundantly available, extensively studied and explored (Patocka *et al.*, 2018). Insects were the next most prevalent in the chosen section. Interestingly, almost all of them were from their respective venom, such as bees, wasps and scorpion venom. The secretion of venom has been characterised by many groups and could potentially serve as a template for antimicrobial development (Čeřovský *et al.*, 2009; Slaninová *et al.*, 2012; Harrison *et al.*, 2014). Despite an increased chance of a peptide containing leucine, the amino acid composition's overall features were mainly scrambled.

The peptides that demonstrated the most promising activity from the antimicrobial screen were from the venom of either insects or arachnids. Despite many of the peptides in the library originating from the amphibian class, they were mostly either similar in antimicrobial potency to the control or weaker. It should be noted here that peptides within the natural library were chosen purely based on being classed as "antibacterial" according to the APD3. No other parameter except the length of the peptide was used to select these peptides. Unfortunately, and expectedly, the peptides have a positive correlation between antimicrobial activity and toxicity against human erythrocytes. This screen shows the importance of differentiating peptides that act in a non-discriminatory manner between prokaryotes and eukaryotes (including erythrocytes). Assessment of haemolytic activity at an early stage was a very useful

tool in developing novel non-toxic antimicrobial peptides (Molchanova *et al.*, 2017). However, it should be noted that although some peptides may exhibit non-haemolytic activity, they may not be necessarily non-toxic towards other human cell lines (Greco *et al.*, 2020). Furthermore, there have been inconsistencies in haemolytic testing, with different blood sources from different animals providing differing results (Molchanova *et al.*, 2017). Applying the same method across both antimicrobial screen and haemolytic screening removes such inconsistencies that exist, as the bacteria and blood have been maintained from the same sources throughout the entire screening procedure.

Validation of Natural Library

When selecting the peptides to scale up, they mainly were classified as moderately active from an antimicrobial perspective, with weak haemolytic activity. Peptide CM-15, which fell into both strong antimicrobial and strong haemolytic activity, was synthesised as part of the validation process. Peptides uperin 3.6 and CPF-PG1 were chosen as representatives from the weaker class of peptides. Some peptides which were moderately active and had low haemolysis were excluded from further scale-up due to the complexity of synthesis and potential difficulty in dissolving these peptides in water. Peptides that contain a single cysteine are prone to oxidation. In contrast, two or more cysteine residues were likely to lead to disulphide bridge formation, including peptides from tachyplesin and protegrin families (Nakamura *et al.*, 1988; Wiedemann *et al.*, 2020). Peptides, which had a high hydrophobicity, were likely to lead to difficulties in solubilising, which was excluded (Mueller *et al.*, 2020). Peptides lasioglossin, mastoparan, bombolitin and dominulin all originate from a similar species, bees and wasps. Lasioglossin peptides have previously demonstrated wide-ranging activity against a variety of pathogens, including lasioglossin III reported having a MIC of 18.7 μM against *P. aeruginosa*, which is equivalent to 32 μg/mL (Čeřovský *et al.*, 2009). Meanwhile, in in-house laboratory testing, 4 μg/mL was obtained. However, the methodology used to obtain the MIC was different. Furthermore, Čeřovský *et al*. also demonstrated 50 % haemolysis at > 220 μM (or 388 μg/mL) in the presence of rat erythrocytes (Čeřovský *et al.*,

2009). Lasioglossin III overall demonstrated the largest therapeutic window amongst the Lasioglossin family (Čeřovský *et al.*, 2009).

Mastoparan was reported to have a MIC of 50 μM, equivalent to a MIC of 74 μg/mL against *P. aeruginosa* (Moerman *et al.*, 2002). More than a 4-fold greater MIC was observed here at 16 μg/mL. However, the methodology involved using brain heart infusion broth rather than MHBr. Furthermore, Irazazabal *et al*. reported 40 % haemolysis of mastoparan to be at 10 μM (approximately 15 μg/mL); however, it was in the presence of rat erythrocytes (Irazazabal *et al.*, 2016). In contrast, all the haemolytic experiments performed within this thesis was on fresh human blood. Bombolitin peptides have demonstrated activity against both Gram-negative and Gram-positive bacteria. In addition, it was also reported to be highly haemolytic towards porcine erythrocytes (Argiolas and Pisano, 1985; Qiu *et al.*, 2012). Peptide Bmkn2 and CM-15 showed potent antimicrobial activity but proved to be the most toxic peptides, which was not surprising, given that both are from scorpion venom and a hybrid of melittin and cecropin, which are well known toxic peptides (Zeng *et al.*, 2004; Moghaddam *et al.*, 2012). Overall, the validation process for antimicrobial and haemolytic activity correlated well with the screening and demonstrated the advatanges of initially using a high-throughput methodology to whittle down candidates.

Optimisation

The optimisation strategies followed the same trajectory as the previous chapter. Lasioglossin was subjected to optimisation as the peptide with the highest therapeutic potential. The other peptides from the natural peptide selection were deemed too toxic for further development. The optimisation process for the naturally occurring peptide was predicated on improving antimicrobial activity and reducing haemolytic toxicity. Using single substitutions, there was no greater activity against *P. aeruginosa*, thus indicating that lasioglossin III was already at its optimum regarding activity against *P. aeruginosa*. Another peptide, apidaecin (a peptide from the Western Honey Bee), did show improved activity against *P. aeruginosa* when subjected to the same single substitution methodology (Hoffmann

et al., 2012). Furthermore, peptide oncocin (a peptide from a large milkweed bug) demonstrated improved activity against *P. aeruginosa* in diluted media conditions upon applying the single substitution methodology (Knappe *et al.*, 2016).

Surprisingly from the six lassioglossin III variants candidates in the five and six amino acid substitutions, there was a noticeable improvement in antimicrobial activity against *P. aeruginosa*. The amino acids from the lasioglossin III variants, which lent to the more favourable activity, included the substitution at their respective termini. In particular, the substitution of "VN" in the parental sequence at the N-terminus with either Lys, Arg or Pro and the substitution of "VK" at the C-terminus was primarily Trp, Phe and Val (instead of Lys). Interestingly, the substitutions tended to be more positively charged residues at the N-terminus, while the C-terminus had more hydrophobic residue substitutions. The overwhelming number of peptides retained similar activity to the parent (lasioglossin III) against *P. aeruginosa*, regardless of the number of substitutions. This was not the case with the novel peptides, which tended to perform worse with the increasing number of substitutions to which they were subjected to. The small number of peptides that were either weaker or inactive in all substitutions showed that lasioglossin III is widely open to substitutions in many positions within its sequence. It does not display the same level of rigidity seen with other peptides, particularly the novel ones in the previous chapter. Other peptides which have shown more remarkable improvement in antimicrobial activity upon multiple substitutions with other pathogens was found by Knappe *et al.,* who demonstrated that double and triple substitutions produced up to a 100-fold reduction in MIC against *S. aureus* with oncocin variants (Knappe *et al.*, 2016).

Upon interaction with *E. coli* in the presence of serum, the single substituted peptide strategy produced 5 more potent peptides than Lasioglossin III when tested for 24 hours. It is interesting to note that at 6 hours, peptides with Arg and Lys substitution were more potent. However, after 24 hours, the activity shift was seen amongst His and Ile being more potent. The influence of Ile was expected, given that the hydrophobic residues could have contributed to its activity, yet His was unexpected. However, peptides rich in His have shown increased

stability in the presence of human serum and salts against *E. coli* (Lai *et al.*, 2019). From the multiple substituted peptides, there was no observable improvement in activity in the presence of serum. The overwhelming majority remained similar in activity to Lasioglossin III, regardless of the number of substitutions, thus indicating its flexibility. Due to the large numbers of similar acting variants, increasing the serum concentration between 20 – 25 % may have been better, leading to a more discriminatory parameter to distinguish activity. It has been demonstrated that the higher the serum content, the weaker the antimicrobial activity tends to be (Dong *et al.*, 2018). Also, lasioglossin variants created resulted in reduced antimicrobial activity against *P. aeruginosa*, reduced toxicity, or increased toxicity depending on the substitutions and modifications performed (Čeřovský *et al.*, 2009; Slaninová *et al.*, 2012)

Unfortunately, but not unexpectedly, all of the most promising antimicrobial active variants demonstrated strong haemolysis. This suggested that any positively acting substitution, whilst increasing antimicrobial activity, coincided with an increase in toxicity. It could primarily be explained by increasing the charge of the overall sequence due to replacing existing amino acids with charged amino acids, such as Arg and Lys. Increased charge to the overall sequence, notably the addition of Arg, has been shown increased haemolytic activity in peptides (Q. Li *et al.*, 2017).

In summary, this chapter explored screening naturally occurring antimicrobial peptides using a harmonised screening methodology. The high throughput screening revealed that most naturally occurring peptides were toxic towards human erythrocytes. Lasioglossin III was assessed to have the most promising therapeutic potential. Substitution of amino acids in varying numbers showed increased antimicrobial activity in a few select cases. However, ultimately, they demonstrated increased toxicity towards human erythrocytes. Thus no further investigation was warranted or carried out. Lasioglossin III as its original sequence was further investigated and characterised.

5 Elucidating the mode of action of naturally occurring and novel designed antimicrobial peptides

5.1 Introduction

There is a large, diverse population of AMPs with differing properties (sequence composition, structure and length of peptide), which inevitably leads to different modes of action (MOA). As previously stated, it is accepted that cationic antimicrobial peptides (AMPs) have an initial interaction with the bacterial membrane via electrostatic interactions (Benfield and Henriques, 2020). However, other MOA of peptides have been posited, which have broadly fallen into two categories: those which act extracellularly (membrane disruption, pore formation) and those which act intracellularly (RNA stoppage, DNA replication disruption) (J. Li *et al.*, 2017).

Additionally, drawing several conclusions from a single peptide and its MOA has been problematic as various factors can influence how the peptides perform. Factors such as type of bacteria, pH, and concentration levels of peptide present can alter the antimicrobial activity performance (Deslouches *et al.*, 2013; Dong *et al.*, 2018).

Historically, one of the main ways to elucidate the mode of action (MOA) was via artificial membranes to observe peptide interactions (Roversi *et al.*, 2014; Hollmann *et al.*, 2018). This methodology, although helpful, has its limitations; it is a simple model for a very complex cascade of possible interactions (Lei *et al.*, 2019). Furthermore, performing MOA studies is generally time-consuming and expensive. A novel high throughput methodology, such as biological small-angle X-ray scattering (BioSAXS), can be used to counter this drawback. It can help differentiate different MOA of known and unknown antimicrobial

compounds (von Gundlach *et al.*, 2016). Using such techniques can enable faster decision making when choosing novel compounds to develop further.

P. aeruginosa is intrinsically resistant to many antibiotics currently used clinically (Li *et al.*, 2003). The situation has been exacerbated further with last-resort antibiotics such as colistin, commonly used to treat multi-drug resistant Gram-negative bacteria, reported to develop resistance also (Goli *et al.*, 2016). Therefore, it is paramount that novel antimicrobial agents are available to treat multi-drug resistant variations of *P. aeruginosa*. Although it is commonly cited that AMPs are unlikely to infer resistance, they have been reported in laboratory conditions (Gooderham *et al.*, 2009).

This chapter explores a select number of natural and novel peptides taken from previous chapters 3 and 4 and aims to gain further insight into their respective MOA. The novel peptides especially are of interest, as there is currently limited understanding of their MOA. This chapter investigates whether the peptides selected for further characterisation are bacteriostatic or bactericidal. A novel high throughput method, BioSAXS, used to classify the MOA, was employed to seek out potentially different MOA amongst the same and different classes of AMPs. In order to gain an insight into morphological changes that occur once interaction between peptide and bacteria takes place, transmission electron microscopy was employed. In addition, the metabolic activity of bacteria upon peptide interaction was measured by isothermal microcalorimetry. Furthermore, stability in human serum and pH stability of peptide 7 and its respective variants were assessed. Finally, a multistep resistance assay was used to assess the resistance propensity towards *P. aeruginosa*.

5.2 This Study

This chapter aims to gain insight into the MOA of peptides from different sources against *P. aeruginosa*.

Specific Objectives

1. Assessment of bactericidal/bacteriostatic activity towards *P. aeruginosa*
2. Use of BioSAXS to differentiate ultrastructural changes
3. Visualisation of morphology via transmission electron microscopy (TEM) following antimicrobial treatment of *P. aeruginosa*
4. Assessment of metabolic activity with antimicrobial treated *P. aeruginosa* via isothermal microcalorimetry (IMC)
5. Determine human serum and pH stability of peptide 7 and its variants
6. Assess resistance propensity of *P. aeruginosa* to the antibiotic gentamicin and peptide 7

5.3 MICs in concentrated P. aeruginosa

MIC values were obtained under different conditions before conducting any MOA studies to ascertain the concentrations used for further studies. Firstly, a standard MIC (as previously stated) was performed using 10^5 CFU/mL. Secondly, a MIC in the presence of 10^8 CFU/mL was performed to determine the concentrations to be used for BioSAXS and TEM experiments (Table 5.1). The method for 10^8 bacterial cells differed slightly, with the bacteria being grown to 2 x 10^8 CFU/mL to achieve logarithmic growth phase and the addition of 500 µM resazurin post overnight incubation to determine cell viability visually if the unaided visual assessment was difficult.

In most cases, the MIC in the presence of 10^8 cells was increased twofold – 16-fold in comparison to the standard MIC, requiring a greater concentration of peptide to inhibit the growth of *P. aeruginosa*. A twofold increase in MICs was observed amongst all the antibiotics in concentrated conditions, except tobramycin and cefepime, where a fourfold and eightfold

increase was observed, respectively. Peptide T14 showed a twofold increase in MIC from standard to concentrated bacterial concentrations. Peptide HHC10 showed the greatest increase in MIC from 2 to 16 μg/mL, representing an eightfold increase. All other peptides showed a fourfold increase in MIC when bacterial concentration was increased 1000-fold.

Table 5.1 MICs evaluation of bacteria at 10^5 and 10^8 CFU/mL

MICs for peptides and antibiotics were determined against *P. aeruginosa* under standard (10^5 CFU/mL) and concentrated (10^8 CFU/mL) conditions. MICs were assessed by visualising the lowest concentration that a well is clear at 18 ± 2 hours and are the mode of three independent experiments is reported.

Peptide/Antibiotic	**Sequence/Class**	**MIC [10^5] (μg/mL)**	**MIC [10^8] (μg/mL)**
5	RVKWWIRVR	8	32
7	KRRVRWIIW	4	16
HHC10	KRWWKWIRW	2	32
T14	WKIVFWWRR	8	16
T69	RRWRIVVIRVRR	16	32
CM15	KWKLFKKIGAVLKVL	4	16
Lasioglossin III	VNWKKILGKIIKVVK	4	16
BmKn2	FIGAIARLLSKIF	4	32
Cefepime	β-lactam	2	16
Ciprofloxacin	Flouriquinolone	0.06	0.125
Gentamicin	Aminoglycodside	0.25	0.5
Meropenem	β-lactam	0.25	0.5
Tobramycin	Aminoglycodside	0.125	0.25
Polymixin B	Polymixin	2	4

5.4 Time-Kill Kinetics Assay

A time-kill study was performed compared to a conventional antibiotic to assess the bacteriostatic or bacteriocidal activity of novel and naturally occurring peptides. A conventional antibiotic served as a positive control in both instances, with untreated bacteria serving as the negative (growth) control. All antimicrobial agents were tested at threefold their MICs reported previously.

The novel AMPs time kill study (Figure 5.1A) was performed with peptide 5 substituted novel peptides 5.1,5.3 and 5.5 (see 3.7.3). They were chosen based on their low toxicity against human erythrocytes and strong antimicrobial activity against *P. aeruginosa*. In addition, novel peptides 7 and 9 were included. All peptides except 5.3 demonstrated a faster reduction in bacterial burden than ciprofloxacin after 240 minutes. Furthermore, all peptides except 5.3 demonstrated no regrowth after 24 hours of incubation, demonstrating bactericidal activity. Peptide 5.3 initially produced a twofold log reduction in bacterial numbers after 240 minutes, but these grew back to the initial concentration at 10^6 CFU/mL; hence a transient effect was observed between inhibition and killing.

The natural peptides (Figure 5.1B) showed a similar trend to the novel peptides. Peptide mastoparan showed no detectable growth after 10 minutes, which was the first time point post-treatment. Furthermore, peptide BmKn2 and bombolottin IV showed slight bacterial load reduction after 10 minutes but were undetectable 20 minutes post-incubation. Lasioglossin III showed no detectable growth after 60 minutes before initially showing a log reduction.

By 240 minutes of incubation, all peptide-treated viable bacteria were undetectable, with gentamicin showing a 2-log reduction in the same period. No natural peptide or antibiotic showed any regrowth except bombolottin IV, thus exhibiting a bactericidal activity. Bombolottin IV treated bacteria grew back to 10^6 CFU/mL post 24 hours, which was the initial starting concentration of the bacteria, displaying a transient killing and inhibitory tendency.

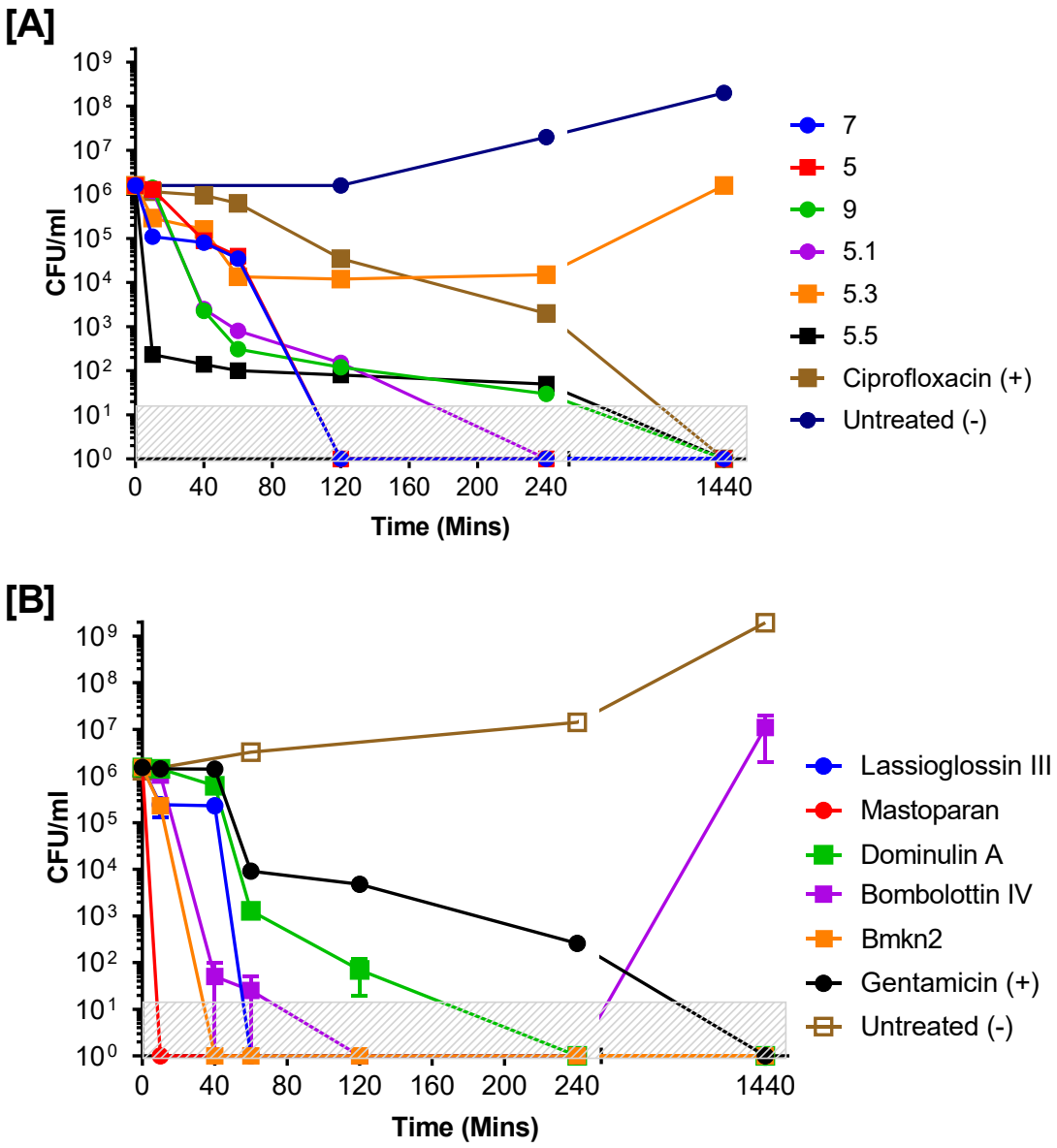

Figure 5.1 Killing kinetics of novel and natural peptides

Logarithmic *P. aeruginosa* was grown to evaluate the killing kinetics of **[A]** novel peptides and **[B]** naturally occurring peptides over 24 hours. The antimicrobial agent concentrations were thrice the MIC (Table 5.1). The shaded region represents the limits of visual detection. The results were reported as the average of three independent biological repeats, with error bars representing the standard error of the mean (SEM). (+) = positive control, (-) = negative control.

5.5 Biological Small Angle X-Ray Scattering

Biological small angle X-ray scattering (BioSAXS) was used to evaluate the differences in the MOA of antimicrobial compounds. A wide selection of peptides, in addition to antibiotics with known MOA, was included as controls. A higher density of cells was required for this experiment, and therefore required the use of 10^8 CFU/mL, with 2 x MIC at 10^8 CFU/mL (Table 5.1) was used for BioSAXS. The measurements were performed at the BioSAXS P12 at PETRA III in Hamburg, Germany, alongside colleagues from Bochum University. The data output was performed via principal component analysis (PCA) by Christoph Rumancev from the University of Bochum, who quantified the scattering curve variations upon antimicrobial treatments. PCA 1 and PCA 2 were plotted (Figure 5.2). All antibiotics and peptides are well separated within the PCA. All antibiotics fell within values between 2 to 4 and peptides between -2 to 1 for PCA 2.

Antibiotics tobramycin and gentamicin, both aminoglycosides that disrupt protein synthesis within bacteria, were close to one another within the graph. Likewise, a similar observation was seen with cefepime and meropenem, both from the beta-lactam class of antibiotics, which inhibit the peptidoglycan layer in bacterial cell walls. A similar observation was also found pertaining to short antimicrobial peptides 5, 7, HHC-10, T14 and T69, and their close proximities on the PCA plot. In contrast, peptides lasioglossin III, CM-15 and BmKn2 are known to permeabilise cell membrane and nearby on the PCA plot.

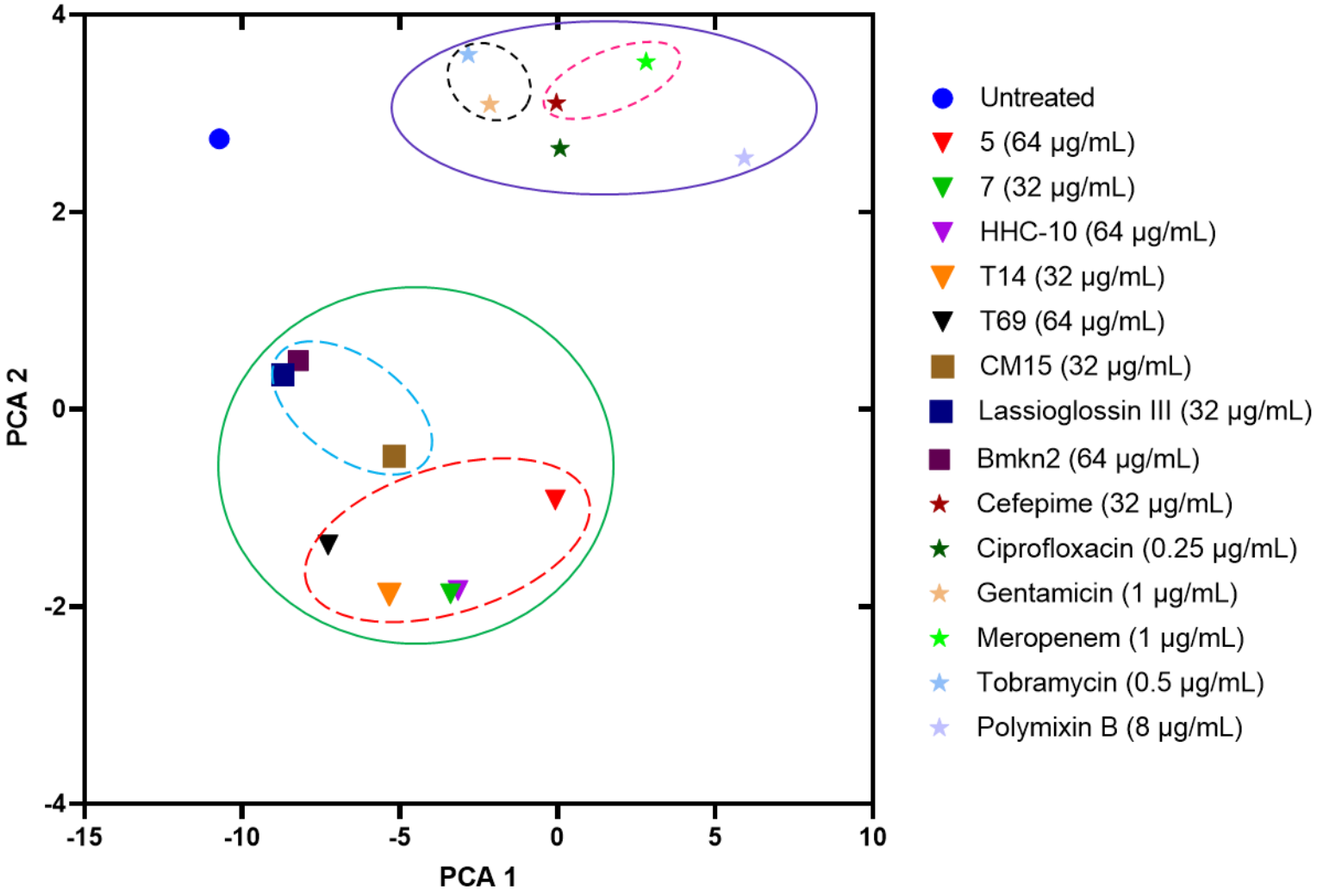

Figure 5.2 BioSAXS of AMPs and Antibiotics

Data for BioSAXS was analysed by Christoph Rumancev from Bochum, Germany. The scattering data which emerged from each sample was subjected to a PCA, of which only the first two PCAs were used (PCA 1 and PCA 2). Each data point on the plot represents a marker, which can be attributed to ultrastructural changes that occur upon antimicrobial treatment. The solid purple line denotes all antibiotic footprint, while the dashed pink and green lines within represent the same class of antibiotics. The solid green line denotes antimicrobial peptide treatment, with the dashed blue line within indicating natural peptides, and the dashed red indicating short synthetic peptides.

5.6 Transmission Electron Microscopy

In order to gain insight into the morphological changes of a variety of different types of antimicrobial compounds, five compounds in total were chosen from the novel, natural and conventional antibiotic range. Transmission electron microscopy (TEM) was employed to visually observe morphological changes in *P. aeruginosa* post antimicrobial peptide treatment (Figure 5.3). Four AMPs, peptide 5, peptide 7, lasioglossin III and BmKn2, and the antibiotic ciprofloxacin treated *P. aeruginosa* at 2 x MIC at 10^8 CFU/mL. Moreover, untreated *P. aeruginosa* served as a control and used a comparison.

Untreated *P. aeruginosa* showed well-dispersed nucleoid and undisturbed membranes. At X 20,000 magnification, the outer membrane, periplasm and plasma membrane were all visible with a homogenous cytoplasm (Figure 5.3A). After 40 minutes of treatment, peptide 5 (Figure 5.3B) showed a strongly enlarged nucleoid separated from the cytoplasm, indicating a potential intracellular mode of action. The cytoplasm was non-homogenous, demonstrated by the strong electron density. Also, there was likely elongation of the lipopolysaccharides (LPS) on the outer membrane. Peptide 7 (Figure 5.3C) showed a more mild nucleoid separation from the cytoplasm with mild electron density contrast within a non-homogenous cytoplasm than the untreated control, suggesting a potential intracellular mode of action. Moreover, LPS elongation was also visible on the outer membrane, alongside the peptidoglycan layer, but largely the membrane is in tact therefore could indicate a non-lytic mechanism.

After 40 minutes post-treatment, BmKn2 (Figure 5.3D) caused complete membrane disruption with extensive OMV release and non-homogenous cytoplasm. The structural integrity of *P. aeruginosa* was compromised upon BmKn2 treatment. In contrast, lasioglossin III (Figure 5.3E) caused a milder disruption to the bacterial membrane, and the release of outer membrane vesicles (OMV) was visible. Furthermore, a non-homogenous cytoplasm was also visible. Ciprofloxacin treated *P. aeruginosa* (Figure 5.3F) was still structurally intact, and the appearance was closer to that of the untreated bacteria.

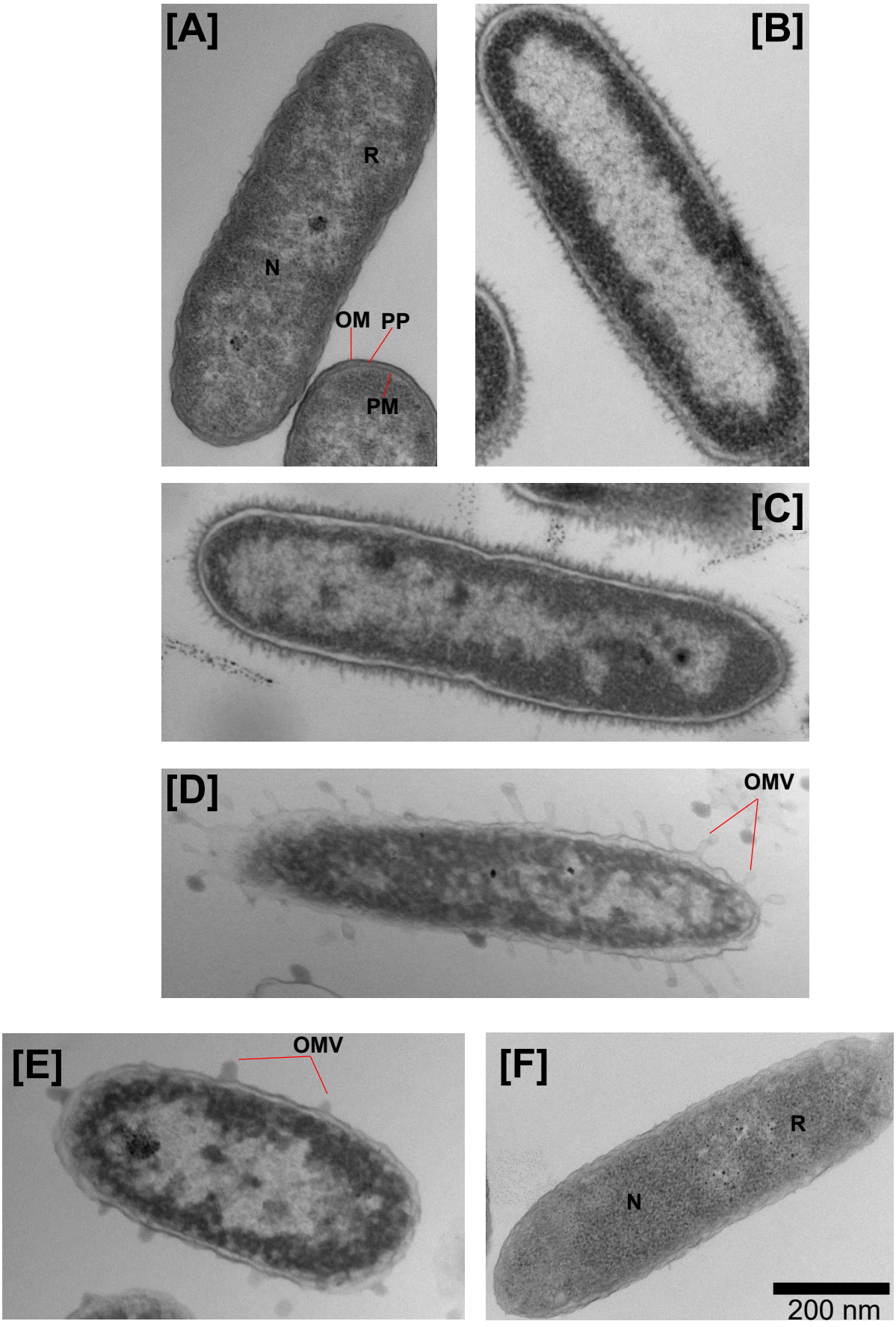
[A]
R
N
OM
PP
PM
[B]
[C]
[D]
OMV
[E]
OMV
[F]
R
N
200 nm

Figure 5.3 TEM images of P. aeruginosa, either untreated or treated with antimicrobials

All images shown were taken at x 20,000 magnification with **[A]** showing untreated *P. aeruginosa*, and 40 minutes post treatment with **[B]** Peptide 5, **[C]** Peptide 7, **[D]** BmKn2, **[E]** Lassioglossin III and **[F]** Ciprofloxacin. OM = outer membrane, PP = periplasm, which encompasses the peptidoglycan and PM = plasma membrane. N = the homogenous central nucleoid, with R showing the electron-opaque ribosomal particles and OMV = outer membrane vesicles. Images were taken using a Hitachi H-7100 Transmission Electron Microscope (TEM) equipped with an AMT 16-megapixel high resolution camera.

5.7 Isothermal microcalorimetry

Overall, from all the experiments performed thus far, peptide 7 has been the best performer. When bacterial species interact with antimicrobial compounds, there are changes concerning their growth and metabolism in response to the change of environment. Isothermal microcalorimetry relies on the heat produced via microbial growth and serves as an indicator for microbial growth and metabolism (Figure 5.4). There is a time-lapse between interaction and setting up the experiments (shaded region), where data is not usable due to equilibration of the Symcel. There was no metabolic activity observed for ciprofloxacin between 0.06 and 1 µg/mL. At sub-MIC levels, ciprofloxacin showed a tendency to delay *P. aeruginosa* growth. However, the concentration of ciprofloxacin was inversely correlated with heat flow. There was approximately 100 µW reduction at 0.03 µg/mL from the peak of the heat flow (compared to untreated), and approximately 200 µW reduction at 0.01 µg/mL, compared to the untreated sample (compared to untreated).

No metabolic activity was observed for peptide 7 between 4 and 64 µg/mL. The sub-MIC concentration of peptide 7 (2 µg/mL) indicated growth suppression of the bacteria. The effect was shown with a peak heat flow of 100 µW after 12.5 hours, compared to 350 µW at 6.5 hours for the untreated *P. aeruginosa*.

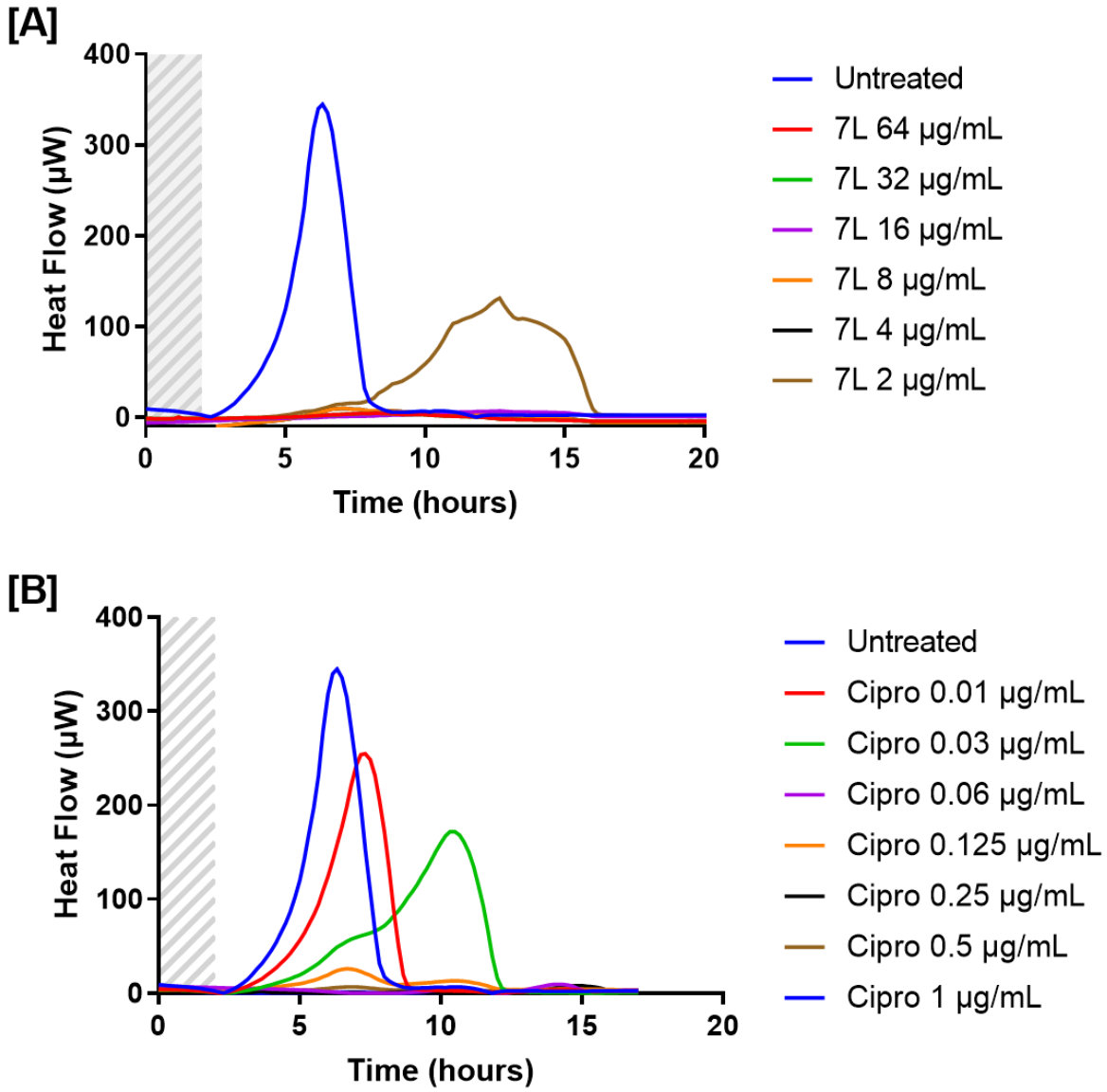

Figure 5.4 Isothermal Microcalimetry of Peptide 7 and Ciprofloxacin

The tested compounds were subjected to the same conditions as that of an MIC. **[A]** Ciprofloxacin and **[B]** Peptide 7 treated *P. aeruginosa* samples were measured for heat flow. The MH Br only served as the baseline reference for all the samples. The heat flow is presented as an average over three independent biological experiments. Shaded region was the equibration period (2 hours), where data was not used.

5.8 Serum and pH Stability

AMPs tend to reduce bioactivity or degrade in the presence of human serum. This is due to proteolytic degradation via proteases and potentially other components, such as albumin. It has been one of the hindrances of advanced and developed peptides. Peptides 7, 7D, Cyc-7 and cyclotide-7 were incubated in 100% serum for 24-hours (Figure 5.5) by Johannes Koehbach from the University of Queensland. Peptides 7 and Cyc-7 were wholly degraded within 3 hours. Cyclotide-7 had approximately 70% of its peptide remaining in the same period, reducing to about 30% after 24 hours. Peptide 7D generally remained intact after 24 hours, with approximately 80% of the peptide remaining. The half-life for peptides 7, Cyc-7 and cyclotide-7 was 13, 9 and 435 minutes, respectively.

As mentioned earlier, the pH stability was assessed (also by Johannes Koehbach) at 24 hours (Figure 5.6) for the peptides. The water pH was adjusted to low pH 1.2 to mimic the pH in gastrointestinal fluids and higher pH 7.5. There was no discernible influence on the presence of any peptides. There was a slight degradation of cyclotide-7 after 24 hours in low pH conditions, but overall, the vast majority remained intact.

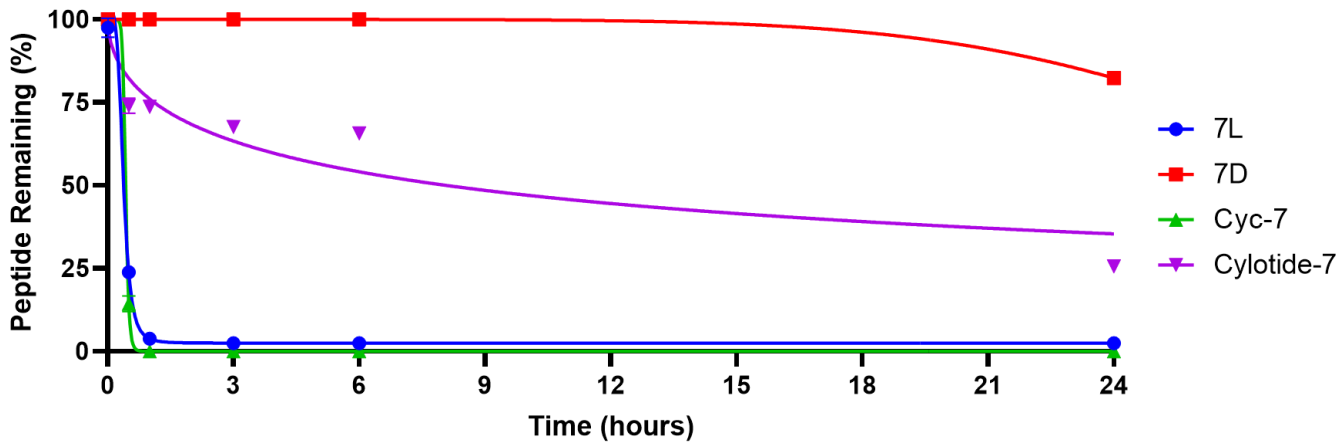

Figure 5.5 Human Serum Stability

The amount of peptide remaining (%) was determined via a Shimadzu UPLC-MS. Experiments were performed in duplicate at least over 24 hours (in University of Queensland), with data presented as the mean average with SEM bars.

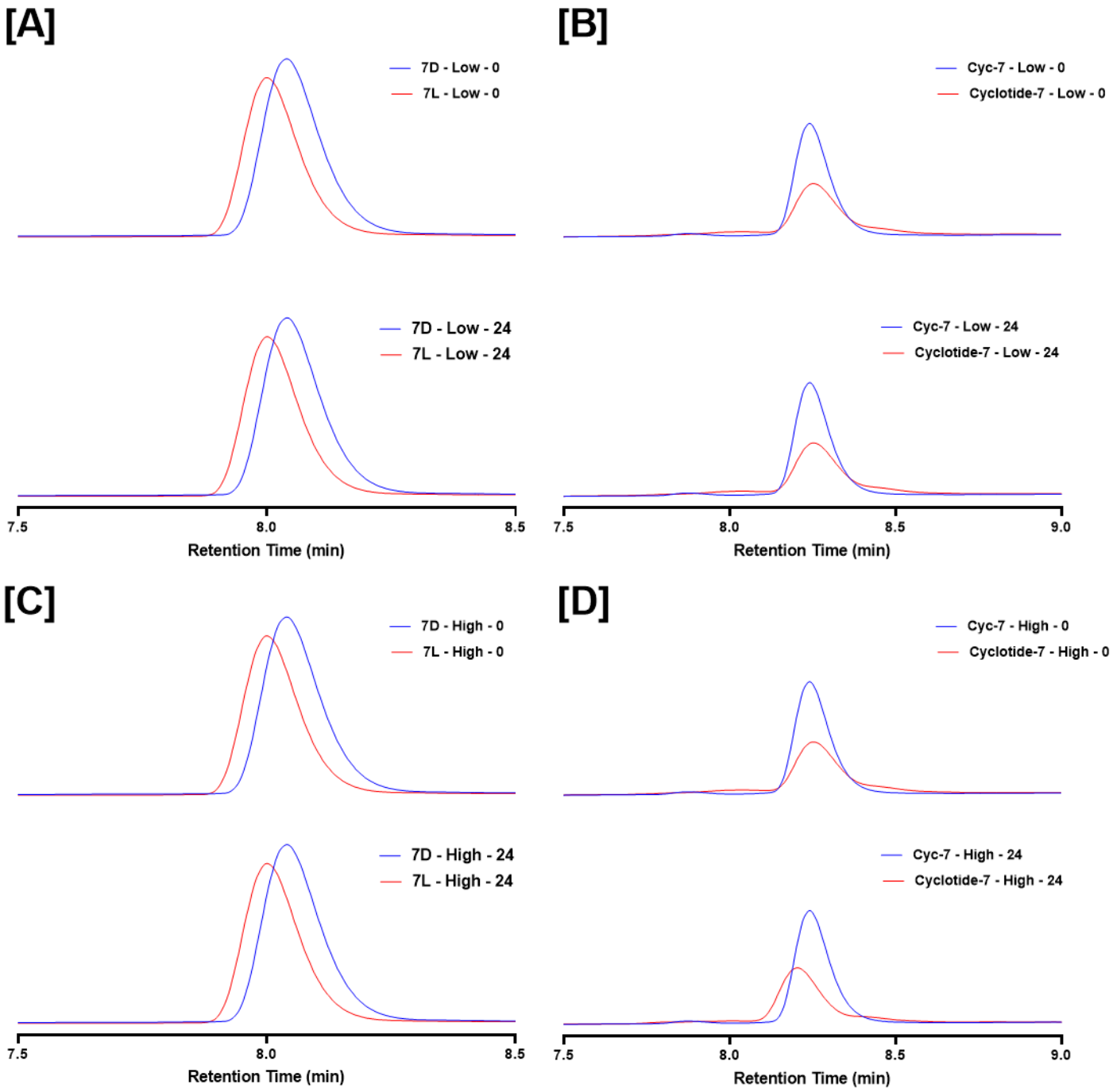

Figure 5.6 pH stability

Peptide 7 variants were incubated in low (pH 1.2) and high (pH 7.5) conditions over a 24-hour period at 20 µM by Johaness Koehbach at the University of Queensland. Stability was monitored via a Shimadzu UPLC. Chromatograms represent the peak intensity from samples analysed at 0 and 24 hours.

5.9 Multistep Resistance Profile

A multistep resistance assay (Figure 5.7) was performed to evaluate the proclivity of *P. aeruginosa* to evolve and develop resistance to antibiotic gentamicin and peptide 7 (see 2.15). After three passages, it was observed that there was a twofold increase in MIC for peptide 7. The same fold increase was also observed after four passages for gentamicin. The MIC of peptide 7 remained stable at 8 µg/mL (i.e. a twofold increase from initial MIC) until the completion of the experiment at 25 passages. In contrast, the MIC of gentamicin demonstrated an eightfold increase, 2 µg/mL, by 15 passages. Moreover, a 128-fold increase in MIC by 25 passages was observed, with a final concentration of 64 µg/mL of gentamicin being recorded.

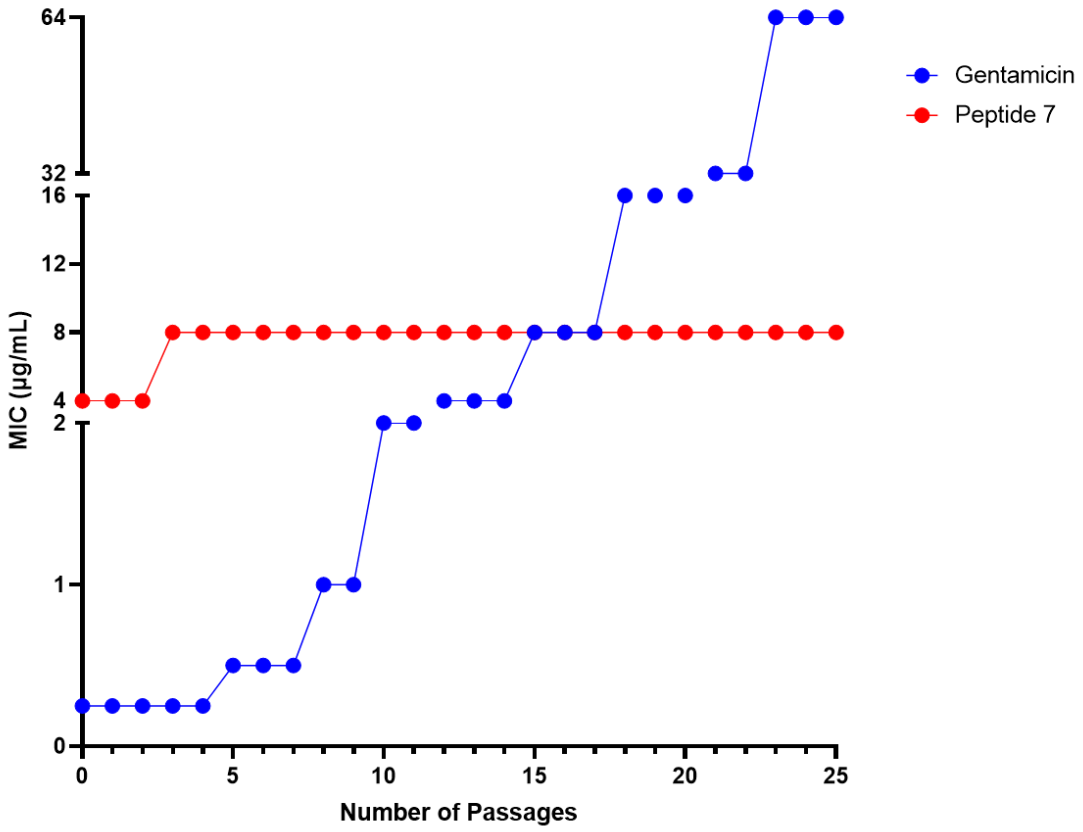

Figure 5.7 Multi-passage resistance profile

P. aeruginosa cultures in MH Br were incubated with gentamicin and peptide 7 overnight, and the bacteria exhibiting growth in the highest concentration were repassaged into sub-MIC levels for each antimicrobial for 25 consecutive days. Development of resistance was confirmed if *P. aeruginosa* was growing continuously at increasing concentrations. Evaluations were performed in duplicate.

5.10 Discussion

Highly Concentrated Bacteria

The evaluation of antimicrobial activity usually occurs with the bacteria concentration in the 10^5 - 10^6 CFU/mL range, as shown with MICs and time-kill studies. However, more complex and material dense applications require increasing bacteria concentration for further experimentation. Bacteria with 10^5 - 10^6 CFU/mL did not allow adequate pellets to be formed upon centrifugation, thus making it difficult to conduct any subsequent MOA studies; therefore, 10^8 CFU/mL was necessary. Expectedly there was a marked increase in the MICs against *P. aeruginosa* for the more concentrated bacteria.

An increased number of cells results in fewer peptide molecules per cell, thus leading to less effective antimicrobial activity. This has previously been reported for antimicrobial peptides with *E. coli*, where increasing inoculum has coincided with an increase in MICs (Loffredo *et al.*, 2020). A similar observation was made with colistin with different inoculums of *P. aeruginosa* (Bulitta *et al.*, 2010). Furthermore, a denser population of bacterial cells invariably leads to reduced cell division due to the amount of nutrients available in the MH Br solution and a shorter period to saturation of microbial growth. This can affect antimicrobial agents that require active microbial growth to enable them to kill the bacteria, such as antibiotics like beta-lactams and aminoglycosides (Breidenstein *et al.*, 2011).

Killing Kinetics

Bactericidal is defined as causing >99.9 % reduction in viable cells (Pankey and Sabath, 2004). By that definition, all peptides, except peptide 5.3, were observed as bactericidal at the concentrations tested within 4 hours. However, bombolottin IV treated bacteria recovered to the original bacterial starting concentration after 24 hours. This indicates a biphasic mechanism of action for bombolottin IV. The biphasic mechanism is when there is a large reduction in the bacterial burden initially, followed by a rebound of bacteria back to a high burden. This phenomenon has also been reported in antibiotics such as colistin and ACH-702, a novel isothiazoloquinolone, related to the fluoroquinolone family with activity towards

P. aeruginosa (Li *et al.*, 2001; Pucci *et al.*, 2011). Bacterial regrowth and static activity for peptides 5.3 and bombolottin IV could be explained by heteroresistance. Heteroresistance is defined as a subpopulation of bacteria that exhibit greater tolerance to antimicrobial compounds than the rest of the population within the culture (El-Halfawy and Valvano, 2015). It has been observed amongst the carbapenems class of antibiotics towards *P. aeruginosa* (He *et al.*, 2018). Furthermore, with time, the peptides could have been gradually digested by the proteases produced by *P. aeruginosa*, which have been observed with peptide LL-37 (Strömstedt *et al.*, 2009). Rapid killing does not allow the bacteria adequate time to develop resistance, thus serving as an advantage and an essential feature for antimicrobial drug development. The bacteriocidal peptides can rapidly inhibit growth of bacteria due to their initial electrostatic interactions with the bacterial membrane (Zhang *et al.*, 2020). There have been reports where the killing of bacteria has taken place within seconds of contact with AMPs (Loeffler *et al.*, 2001), while this chapter has shown burden reduction after minutes, and others have shown hours (Strömstedt *et al.*, 2017).

BioSAXS and TEM

Evaluating the morphological impact of antimicrobial agents on *P. aeruginosa* was assessed via BioSAXS. To date, this technique has only been used to classify the MOA of antibiotics and peptides on *E. coli* (von Gundlach *et al.*, 2016) and two synthetic antimicrobial peptides for *E. coli* and MRSA (von Gundlach *et al.*, 2019). Despite an extensive literature search, this technique was not used with any antimicrobial agents and *P. aeruginosa*.

The scattering curves generated by the BioSAXs were analysed by Cristoph Rumancev for their first two PCAs and generated a “fingerprint”, which was plotted. The “fingerprint” is a representation of the ultrastructural changes that take place following antimicrobial agent treatment. This technique was able to distinguish clearly between conventional antibiotics and antimicrobial peptides. Furthermore, BioSAXs was sensitive enough to detect differences within *P. aeruginosa* treated with peptides and antibiotics. Antimicrobial agents from similar classes had a proximity of “fingerprints” near each other.

Such was observed with membrane destabilisation peptides lasioglossin III, CM-15 and BmKn2 (Moghaddam *et al.*, 2012; Mishra *et al.*, 2013). The synthetic peptides have an unknown mechanism of action; however, there is some indication of membrane disruption and perhaps intracellular MOA per the transmission electron microscopy – a finding also supported by works from von Gundlach and colleagues (Von Gundlach *et al.*, 2016; von Gundlach *et al.*, 2019). Antibiotics tobramycin and gentamycin, both from the aminoglycoside class, share the same MOA and are also nearby on the PCA plot (Mingeot-Leclercq *et al.*, 1999). Beta-lactam antibiotics meropenem and cefepime also showed proximity on the PCA plot and correlated with their MOA (Tipper, 1979).

To ascertain a more detailed morphological and ultrastructural impact of antimicrobial treatment of *P. aeruginosa,* TEM was performed. Following treatment with short synthetic peptides 5 and 7, there was a visible expansion of the nucleoid, suggesting that these peptides were causing some form of DNA damage, whether direct or indirect, with it being more pronounced following peptide 5 treatment. Both peptides 5 and 7 showed non-lytic activity with the cell wall intact, hence pointing toward an intracellular mechanism. In contrast, a loss of nucleoid material was observed for two synthetic peptides, T14 and T69, with *E. coli* upon treatment and another short nine amino acid short peptide (von Gundlach *et al.*, 2016, 2019). Furthermore, T14 and T69 correlated well with their proximity with *P. aeruginosa*. Peptides with similar amino acid profiles as 5 and 7 have shown partitioning into lipid domains instead of membrane acting (Scheinpflug *et al.*, 2015).

Peptides BmKn2 and lasioglossin III are from the venom of scorpions and bees, respectively. Their MOA shows membrane destabilisation, followed by intracellular disruption prevalent in electron micrographs, with intracellular contents leakage (Battista *et al.*, 2021). Similar morphological observations compared to this chapter for BmKn2 was observed with *E. coli* treated with a BmKn2 analogue, Kn2-7 (Cao *et al.*, 2012). However, the original BmKn2 did not show much morphological change compared to the untreated bacteria, albeit it was only at 1 x MIC (Cao *et al.*, 2012). Ciprofloxacin mechanism of action involves inhibition of the DNA gyrase (LeBel, 1988). However, complete structural integrity was observed after 40

minutes, similar to the untreated bacteria. The untreated *P. aeruginosa* had a high electron density, with most membrane components still visible and distinguishable.

TEM also acted as a supporting study for the BioSAXS for the different classes of antimicrobials showing similar MOA. It was seen with BmKn2 and lasioglossin III in close proximity in the BioSAXs and morphological changes with TEM. Moreover, a similar correlation was seen with the novel peptides and ciprofloxacin. A similar correlation was also observed by von Gundlach *et al*., using BioSAXS and TEM for different antibiotic classes (von Gundlach *et al.*, 2016).

Isothermal microcalorimetry and Serum/pH stability

The metabolism of microbes can be assessed in real-time using isothermal microcalorimetry. Furthermore, isothermal microcalorimetry comes with the added advantage of the lack of labelling, thus considering pure and unadulterated interaction. This technique has been used extensively to determine MICs and antimicrobial resistance (Von Ah *et al.*, 2009; Butini *et al.*, 2019; Tellapragada *et al.*, 2020). It can also be used to determine the onset of antimicrobial action and the time taken to kill more accurately, which is of great importance for subsequent *in vivo* evaluation. However, it should be noted that due to an approximate 1-hour equilibration time, it may not be suitable for fast-acting compounds. The use of IMC does allow for antimicrobial profiling of novel compounds at different concentrations to make a more well-informed decision to proceed with any novel compounds of interest. It would not be possible to compare a short time frame and fast-acting compounds to a time-kill. However, over a more extended period using slower acting compounds, it can be compared to a time-kill. A reduction of CFU/mL can be correlated with a decrease in heat flow in real-time, which is not feasible with the time-kill assay.

One of the significant hindrances surrounding antimicrobial peptide development has been the issue of proteolytic degradation by proteases present in both bacteria and the blood serum of humans (Strömstedt *et al.*, 2009). Many strategies such as in-cooperating D-amino acids (Lu *et al.*, 2020), cyclisation and scaffolds have been explored to rectify this issue (Clark

et al., 2005; Pränting *et al.*, 2010). Peptide 7 degraded very soon after incubation in human serum. However, the degradation of cyclic-7 was unexpected within such a short space of time and displayed a shorter half-life than the linear peptide 7. Cyclic-7 underwent head-to-tail cyclisation, which should have had more proteolytic resistance compared to the side-chain cyclisation strategy (Hayes *et al.*, 2021). Cyclotide-7 fared better, with about 30 % of the peptide present after 24 hours. This phenomenon contrasts with what has been extensively previously reported regarding the stability of cyclotides (Gould *et al.*, 2011; Camarero, 2017). It may be the case that an insertion in this particular peptide sequence caused the scaffold to be more inclined to proteolytic degradation. It has been reported that using the same cyclotide used in this chapter, MCoTI-II, which has a peptide inserted within, has demonstrated promising activity against leukaemia (D'Souza *et al.*, 2016). Peptide 7D demonstrated the best overall stability with about 20 % degradation over 24 hours. It is a widely used strategy to overcome proteolytic degradation and has shown greater antimicrobial activity (Hamamoto *et al.*, 2002; Manabe and Kawasaki, 2017; Lu *et al.*, 2020).

The peptide 7 variants, while not all stable in serum (except peptide 7D), showed remarkable stability in very low pH. it was particularly impressive given that pH 1.2 practically represents gastrointestinal conditions. pH can affect the activity of many peptides, and many peptides can be dependent upon particular pH conditions to elicit their activity (Malik *et al.*, 2016).

Resistance Assay

P. aeruginosa has displayed intrinsic resistance to almost all antibiotic classes (Pang *et al.*, 2019). As such, it was important to establish the propensity of *P. aeruginosa* resistance to AMPs, and in particular, peptide 7. This assay demonstrated strong resistance to gentamicin, an aminoglycoside that inhibits protein synthesis, with repeated exposure to *P. aeruginosa*. Gentamicin resistance has been reported numerous times, with the underlying reasons ranging from increased cell permeability to inactivating enzymes (Brzezinska *et al.*, 1972; Bryan *et al.*, 1976). Peptide 7 did not induce any bacterial resistance compared to conventional antibiotic gentamicin.

In conclusion, this chapter demonstrated that a range of antimicrobial peptides from the novel and naturally occurring sources exhibited bactericidal activity in the majority of cases. Furthermore, using BioSAXS, peptides and conventional antibiotics were well-differentiated and even further differentiated within their respective groups, i.e. aminoglycosides within antibiotics and short synthetic peptides within the peptide class. It is possible to weave out potentially new modes of action. The morphological impact of natural peptides lasioglossin III and Bmkn2 indicated a membrane-acting action. The novel peptides 5 and 7 indicated it could potentially be dual-action, acting on both the membrane and intracellularly. Peptide 7 variants showed excellent pH stability but varying degrees of human serum stability. Finally, peptide 7 showed an excellent propensity to avoid *P. aeruginosa* resistance.

6 Predicting *in vivo* activity of antimicrobial peptides using *in vitro* parameters and evaluating efficacy in a *Galleria melenolla* model with *P. aeruginosa*

6.1 Introduction

A plethora of AMPs has shown potent antimicrobial activity in *in vitro* conditions. However, very few have been further evaluated within *in vivo* models, including insect or rodent models. Those that have tended to be mainly for skin infection models (Pfalzgraff *et al.*, 2018; Chen and Lu, 2020). This suggests a gap in understanding how a peptide may behave *in vivo* systemically, considering its expected activity *in vitro*. Understanding how peptides behave in the presence of human serum, albumin, and serum components may be paramount to elucidating patterns, enabling more robust prediction *in vivo*. Moreover, and more importantly, this particular element is a gap in the developmental path for AMPs.

Many peptide families have demonstrated potent *in vitro* activity, such as the bombolitin and dominulin (shown in Chapter 4). However, there has not been any optimisation or subsequent testing within an animal model. On the other hand, some of the well-known peptide families, such as apidaecins and mastoparan, have been optimised and tested in murine models (Knappe *et al.*, 2019; Silva *et al.*, 2020). Therefore, using early *in vitro* predictive conditions could potentially make the development of peptides more efficient and cost-effective.

All drugs that are tested in an animal model usually involve rodents as part of the preclinical testing (Tängdén *et al.*, 2020). However, rodent trials are lengthy, require extensive ethical consideration and come at a high cost (Van Norman, 2019). An alternative strategy,

particularly for antimicrobial agents, is the use of *Galleria mellonella* (Tsai *et al.*, 2016). *G. mellonella* has an analogous innate immune system to humans (Tsai *et al.*, 2016). Moreover, using this model is more time-efficient, cost-effective, and attracts less ethical considerations than rodent models (Parthuisot *et al.*, 2018). *G. mellonella* has been used widely in multiple infection models, including *P. aeruginosa* (Andrejko *et al.*, 2009; Paškevičius *et al.*, 2017; Gorr *et al.*, 2019).

It is crucial to establish if the *G. mellonella* model is suitable for demonstrating the efficacy of antimicrobial peptides. To date, there have been very few published articles pertaining to this model as a choice for antimicrobial therapy against *P. aeruginosa*.

6.2 Aims of This Study

This chapter aims to assess if *in vitro* activity determination can be used as a tool to predict how a peptide may behave *in vivo* by proposing using "pre-*in vivo*" testing. "Pre-*in vivo*" testing incorporates physiological *in vitro* parameters to simulate an *in vivo* model. This chapter's "pre-*in vivo*" parameters primarily include testing within different blood components, namely serum, albumin, and ions. Peptides that have already been assessed in a murine model (and published), novel and naturally occurring peptides were tested in different *in vitro* conditions. Selected candidates were tested in a *G. mellonella* model.

Specific Objectives

1. Evaluate MICs of AMPs in simulated physiological conditions
2. Assess the toxicity of selected peptides towards *G. mellonella*
3. Assess *P. aeruginosa* pathogenesis towards *G. mellonella*
4. Evaluate selected peptides efficacy in a *P. aeruginosa* challenged *G. mellonella* model

6.3 In vivo Prediction

6.3.1 Selection of Peptides

Peptides from different categories were selected for further assessment (Table 6.1). They were selected on the basis that they had already been tested in mice, naturally occurring peptides and novel peptides. The peptides tested in mice were chosen based on demonstrating protecting or reducing bacterial burden within the murine infection model. The peptides were primarily injected intraperitoneally, thus representing systemic treatment. Moreover, the peptides selected have shown strong *in vitro* activity against Gram-negative bacteria and, in particular, *P. aeruginosa*. The natural peptide category is comprised of the better performing peptides used in chapter 4. The novel peptides are mainly from chapter 3, except peptide ab_A03 or ak_E01, which are in-house optimised novel peptides from the Hilpert Laboraty. The entire library of peptides chosen for this chapter was called "Pre-in-*vivo* Library (PIVL)" and encompasses peptides tested in mice, naturally occurring peptides and novel peptides.

Table 6.1 Pre In Vivo Library peptides

The PIVL peptides (unless stated) were synthesised using an Intavis Multipep RSI. The *in vivo* tested peptides have been subjected to murine testing as per their relevant publication. # = Acquired from Bachem, * = Supplied by Hoffmann Lab. Lower case letters indicate D-amino acids. N/A = not applicable

Peptide	Sequence	Status	Reference
5	RVKWWIRVR	Novel	N/A
7	KRRVRWIIW	Novel	(Koehbach *et al.*, 2021)
ak_E01	KLWIYIRRR	Novel	N/A
ab_A03	RIIWRIRIR	Novel	N/A
5D#	rvkwwirvr	Novel	N/A
7D#	krrvrwiiw	Novel	(Koehbach *et al.*, 2021)
ak_E01_D#	klwiyirrr	Novel	N/A
ab_A03_D#	riiwririr	Novel	N/A
Temporin Pta	FFGSVLKLIPKIL	Natural	(Conlon *et al.*, 2008)
Aurein 1.2	GLFDIIKKIAESF	Natural	(Rozek *et al.*, 2000)
Indolicidin	ILPWKWPWWPWRR	Natural	(Selsted *et al.*, 1992)
Lasioglossin III	VNWKKILGKIIKVVK	Natural	(Čeřovský *et al.*, 2009)
BmKn2	FIGAIARLLSKIF	Natural	(Zeng *et al.*, 2004)
Bombolitin IV	INIKDILAKLVKVLGHV	Natural	(Argiolas and Pisano, 1985)
Pleurocidin	GWGSFFKKAAHVGKHVGKAALTHYL	Natural	(Cole *et al.*, 1997)
NLF20	NLFRKLTHRLFRRNFGYTLR	*In vivo* tested	(Papareddy *et al.*, 2016)
Clavanin-MO	FLPIIVFQFLGKIIHHVGNFVHGFSHVF	*In vivo* tested	(Silva *et al.*, 2016)
HHC10	KRWWKWIRW	*In vivo* tested	(Cherkasov *et al.*, 2009)
HHC36	KRWWKWWRR	*In vivo* tested	(Cherkasov *et al.*, 2009)
P3	VNFKLLSHSLLVTLASHL	*In vivo* tested	(Q. Zhang *et al.*, 2015)
Api88*	GNNRPVYIPQPRPPHPRL	*In vivo* tested	(Schmidt *et al.*, 2017)
Api137*	ONNRPVYIPRPRPPHPRL	*In vivo* tested	(Schmidt *et al.*, 2017)
Onc72*	VDKPPYLPRPRPPROIYNO	*In vivo* tested	(Schmidt *et al.*, 2016)
Onc112*	VDKPPYLPRPRPPRrIYNr	*In vivo* tested	(Schmidt *et al.*, 2016)

6.3.2 Antimicrobial Activity against P. aeruginosa in MH Br

All the peptides chosen for this study were initially subjected to an MIC assay in standard and diluted MH broth (Table 6.2). Within the standard MH Br conditions, the novel peptides had a MIC range from 4 – 16 µg/mL, with peptides 7 and 7D showing the lowest, while ak_E01 showed the highest MIC value. The range for the natural peptides was between 4 and 256 µg/mL, with lasioglossin III and BmKn2 showing the lowest MIC and indolicidin showing the highest. Among the *in vivo* tested peptides, HHC10 showed the lowest MIC. The inactive with peptides Api88, Api137, Onc72, Onc112, clavanin-MO and P3 at > 256 µg/m, with the highest MIC.

Within diluted media (20 % MH broth) conditions, the novel peptides demonstrated an MIC range between 1 – 4 µg/mL. Lasioglossin III and BmKn2 remained at 4 µg/mL among the natural peptides, which was still the lowest. Aurein 1.2 was the worst performer at 64 µg/mL. Despite the media dilution, the *in vivo* assessed peptides clavanin-MO and P3 remained at > 256 µg/mL. In contrast, Api88, Api137, Onc72 and Onc112 ranged from 8 -16 µg/mL upon the media dilution. HHC10 demonstrated the lowest MIC at 1 µg/mL in diluted media conditions. Antibiotics ciprofloxacin and gentamicin MICs were minimally affected by media dilution.

Table 6.2 MICs in standard and diluted media

The PIVL peptides were subjected to MICs in standard MH broth and diluted 20 % MH broth. The MICs are reported as the mode of three independent experiments and observered visually via eye.

Peptide	Sequence	Status	MIC (µg/mL)	
			Standard	Diluted
5	RVKWWIRVR	Novel	8	2
7	KRRVRWIIW	Novel	4	1
ak_E01	KLWIYIRRR	Novel	16	4
ab_A03	RIIWRIRIR	Novel	8	4
5D	rvkwwirvr	Novel	8	2
7D	krrvrwiiw	Novel	4	1
ak_E01_D	klwiyirrr	Novel	8	2
ab_A03_D	riiwririr	Novel	8	2
Temporin Pta	FFGSVLKLIPKIL	Natural	128	32
Aurein 1.2	GLFDIIKKIAESF	Natural	128	64
Indolicidin	ILPWKWPWWPWRR	Natural	256	16
Lasioglossin III	VNWKKILGKIIKVVK	Natural	4	4
BmKn2	FIGAIARLLSKIF	Natural	4	4
Bombolitin IV	INIKDILAKLVKVLGHV	Natural	64	16
Pleurocidin	GWGSFFKKAAHVGKHVGKAALTHYL	Natural	4	4
NLF20	NLFRKLTHRLFRRNFGYTLR	*In vivo* tested	32	4
Clavanin-MO	FLPIIVFQFLGKIIHHVGNFVHGFSHVF	*In vivo* tested	>256	>256
HHC10	KRWWKWIRW	*In vivo* tested	2	1
HHC36	KRWWKWWRR	*In vivo* tested	8	2
P3	VNFKLLSHSLLVTLASHL	*In vivo* tested	>256	>256
Api88	GNNRPVYIPQPRPPHPRL	*In vivo* tested	>256	8
Api137	ONNRPVYIPRPRPPHPRL	*In vivo* tested	>256	16
Onc72	VDKPPYLPRPRPPROIYNO	*In vivo* tested	>256	16
Onc112	VDKPPYLPRPRPPRrIYNr	*In vivo* tested	>256	8
Gentamicin	-	Antibiotic	0.125	0.125
Ciprofloxacin	-	Antibiotic	0.06	0.03

6.3.3 Antimicrobial Activity against P. aeruginosa in the presence of Human Serum

The PIVL peptides were subjected to MIC assays in the presence of diluted human serum at 10 % and 25 % (*v/v*), respectively (Table 6.3). These concentrations were chosen so that the bacteria had adequate nutrients to grow from MHBr. The L-peptides from the novel peptides showed an MIC between 32 µg/mL (peptide 7) and 256 µg/mL (peptide ak_E01) in 10 % serum, with all of them > 256 µg/mL upon interaction in the presence of 25 % serum. The tested D-novel peptides 5D and 7D showed 8 and 16 µg/mL in 10 % serum, respectively. However, the MIC substantially increased 256 and 64 µg/mL in the presence of 25 % serum. At lower serum concentrations, most natural peptides showed either 32 µg/mL or > 256 µg/mL. All peptides tested in standard MH broth showed > 256 µg/mL upon higher serum concentrations except Bmkn2 (256 µg/mL). The peptides tested in diluted media showed a four to sixteen-fold increase in the MIC in 25 % serum, compared to 10 %. Antibiotics gentamicin and ciprofloxacin also increased in the MIC when serum concentration was increased, with a two and fourfold increase, respectively.

Table 6.3 The PIVL Peptides MIC in Human Serum

The PIVL peptides were subjected to MICs against *P aeruginos*a in the presence of two different human serum concentrations. The MICs are reported as the mode of three independent experiments. * = performed in 20 % MH Br.

Peptide	Sequence	Status	MIC (µg/mL) in Human Serum	
			10%	25%
5	RVKWWIRVR	Novel	128	>256
7	KRRVRWIIW	Novel	32	>256
ak_E01	KLWIYIRRR	Novel	256	>256
ab_A03	RIIWRIRIR	Novel	128	>256
5D	rvkwwirvr	Novel	16	256
7D	krrvrwiiw	Novel	8	64
ak_E01_D	klwiyirrr	Novel	NT	256
ab_A03_D	riiwririr	Novel	NT	>256
Temporin Pta	FFGSVLKLIPKIL	Natural	>256	>256
Aurein 1.2	GLFDIIKKIAESF	Natural	>256	>256
Indolicidin	ILPWKWPWWPWRR	Natural	>256	>256
Lasioglossin III	VNWKKILGKIIKVVK	Natural	32	>256
BmKn2	FIGAIARLLSKIF	Natural	32	256
Bombolitin IV	INIKDILAKLVKVLGHV	Natural	>256	>256
Pleurocidin	GWGSFFKKAAHVGKHVGKAALTHYL	Natural	32	>256
NLF20	NLFRKLTHRLFRRNFGYTLR	*In vivo* tested	>256	>256
Clavanin-MO	FLPIIVFQFLGKIIHHVGNFVHGFSHVF	*In vivo* tested	>256	>256
HHC10	KRWWKWIRW	*in vivo* tested	64	>256
HHC36	KRWWKWWRR	*In vivo* tested	256	>256
P3	VNFKLLSHSLLVTLASHL	*In vivo* tested	>256	>256
Api88	GNNRPVYIPQPRPPHPRL	*In vivo* tested	8*	128*
Api137	ONNRPVYIPRPRPPHPRL	*In vivo* tested	32*	128*
Onc72	VDKPPYLPRPRPPROIYNO	*In vivo* tested	16*	64*
Onc112	VDKPPYLPRPRPPRrIYNr	*In vivo* tested	8*	64*
Gentamicin	-	Antibiotic	0.125	1
Ciprofloxacin	-	Antibiotic	0.06	0.125

6.3.4 Antimicrobial Activity in the presence of Albumin

The PIVL peptides were subjected to MICs against *P. aeruginosa* in the presence of Human Serum Albumin (HSA) at 10 mg/mL, which is equivalent to the amount present in 25 % human serum. HSA directly from humans and recombinant HSA expressed in *Pichia pastoris* was assessed (Table 6.4). All peptides tested in human HSA had an MIC of > 64 µg/mL. In contrast, novel peptides tested in the presence of recombinant HSA showed MICs of either 16 or 32 µg/mL. The natural peptides had MICs of 16 or 64 µg/mL, and the *in vivo* tested peptides had a range of MICs from 4 to > 64 µg/mL. On the other hand, gentamicin demonstrated a twofold decrease in MIC from *Pichia pastoris* expressed HSA to direct HSA.

Table 6.4 MICs against P. aeruginosa the presence of Human Serum Albumin

The PIVL peptides were subjected to MICs in the presence of two different sources of human serum albumins, with the final albumin concentration of 10 mg/mL. The MICs are reported as the mode of three independent experiments. 1 = Albumin directly from human plasma, 2 = Human Serum Albumin expressed in *Pichia pastoris*, * = performed in diluted media, i.e. 20 % MH broth.

Peptide	**Sequence**	**Status**	MIC (µg/mL) in Human Serum Albumin		
			NO HSA	**HSA[1]**	**HSA[2]**
5	RVKWWIRVR	Novel	8	>64	16
7	KRRVRWIIW	Novel	4	>64	16
ak_E01	KLWIYIRRR	Novel	16	>64	32
ab_A03	RIIWRIRIR	Novel	8	>64	32
5D	rvkwwirvr	Novel	8	>64	16
7D	krrvrwiiw	Novel	4	>64	16
ak_E01_D	klwiyirrr	Novel	8	>64	32
ab_A03_D	riiwririr	Novel	8	>64	16
Temporin Pta	FFGSVLKLIPKIL	Natural	128	>64	NT
Aurein 1.2	GLFDIIKKIAESF	Natural	128	>64	NT
Indolicidin	ILPWKWPWWPWRR	Natural	256	>64	NT
Lasioglossin III	VNWKKILGKIIKVVK	Natural	4	>64	16
BmKn2	FIGAIARLLSKIF	Natural	4	>64	16
Bombolitin IV	INIKDILAKLVKVLGHV	Natural	64	>64	64
Pleurocidin	GWGSFFKKAAHVGKHVGKAALTHYL	Natural	4	>64	16
NLF20	NLFRKLTHRLFRRNFGYTLR	*In vivo* tested	32	>64	16
Clavanin-MO	FLPIIVFQFLGKIIHHVGNFVHGFSHVF	*In vivo* tested	>256	>64	>64
HHC10	KRWWKWIRW	*in vivo* tested	2	>64	4
HHC36	KRWWKWWRR	*in vivo* tested	8	>64	16
P3	VNFKLLSHSLLVTLASHL	*In vivo* tested	>256	>64	>64
Api88	GNNRPVYIPQPRPPHPRL	*In vivo* tested	8*	>64*	8*
Api137	ONNRPVYIPRPRPPHPRL	*In vivo* tested	16*	>64*	>64*
Onc72	VDKPPYLPRPRPPROIYNO	*In vivo* tested	16*	>64*	64*
Onc112	VDKPPYLPRPRPPRrIYNr	*In vivo* tested	8*	>64*	32*
Gentamicin	-	Antibiotic	0.125	0.5	1
Ciprofloxacin	-	Antibiotic	0.06	NT	NT

6.3.5 Antimicrobial Activity in the presence of Ions

Different ions are present in human serum, potentially affecting the AMP antimicrobial effect. It has been previously shown that particular ions can hinder the antimicrobial effect of compounds. Selected ions were supplemented in media at the physiological concentrations (See 2.9.3), and each tested stand-alone and all mixed (Table 6.5). Novel peptides tended to lose activity primarily in the presence of calcium and sodium, with the calcium-only addition causing the most significant MIC drop. The novel peptides tended to retain antimicrobial activity in most other ions. The natural peptides tended to be affected the least, except temporin Pta, aurein 1.2, and indolicdin. These three peptides were already weak in their activity in standard MH broth regardless of the addition of ions. The *in vivo* peptides that were also tested were minimally affected by the introduction of ions with a maximum of a fourfold drop in activity for Bmkn2 in the presence of magnesium. The gentamicin MIC significantly dropped with the addition of calcium and magnesium.

Table 6.5 MICs in the Presence of Ions

The PIVL peptides were tested against *P. aeruginosa*, with ions supplemented into MH broth at their respective concentrations (*w/v*). The MIC value was determined by identifying the lowest peptide concentration required to inhibit bacterial growth via visual assessment of the microtitre plate. All data are presented as the modal value from three independent repeats unless stated. * = performed in diluted media i.e. 20 % MH broth, # = performed twice and NT

Peptide	MIC (µg/mL) in Ions						
	Standard Media	Calcium (2.5 mM)	Sodium (150 mM)	Zinc (8 µM)	Iron (4 µM)	Magnesium (1 mM)	Potassium (4 mM)
5	8	128	32	8	8	16	8
7	4	64	16	4	4	8	4
ak_E01	16	128	32	8	8	16	8
ab_A03	8	128	32	8	8	16	8
5D	8	32	16	2	2	8	2
7D	4	16	8	1	1	8	1
ak_E01_D	8	64	32	2	2	16	8
ab_A03_D	8	64	16	2	2	16	8
Temporin Pta	128	>256	>256	128	>256	>256	128
Aurein 1.2	128	>256	>256	128	128	>256	128
Indolicidin	256	>256	>256	256	256	>256	256
Lasioglossin III	4	4	4	4	4	8	4
BmKn2	4	8	4	4	4	16	4
Bombolitin IV	64	128	64	64	64	128	64
Pleurocidin	4	4	4	4	4	4	4
NLF20	32	64	32	32	32	64	32
Clavanin-MO	>256	NT	NT	NT	NT	NT	NT
HHC10	2	8	4	2	2	8	8
HHC36	8	32	8	8	8	128	8
P3	>256	NT	NT	NT	NT	NT	NT
Api88	8	8	8	8	8	8	8
Api137	16	16	16	16	16	16	16
Onc72	16	16	16	16	16	16	16
Onc112	8	8	8	8	8	8	8
Gentamicin	0.125	1	0.25	0.25	0.25	>2	0.25

6.4 Galleria mellonella model

6.4.1 P. aeruginosa pathogenesis

The pathogenicity of *P. aeruginosa* was evaluated in a *G. mellonella* model (Figure 6.1). Inoculation with most concentrations of bacteria led to death by day 1. The lowest concentration of bacteria at 1 x 10^2 CFU/mL led to 100 % death at day 2 (log-rank Mantel-Cox test, p = < 0.05). Thus, *G. mellonella* are highly susceptible to *P. aeruginosa*.

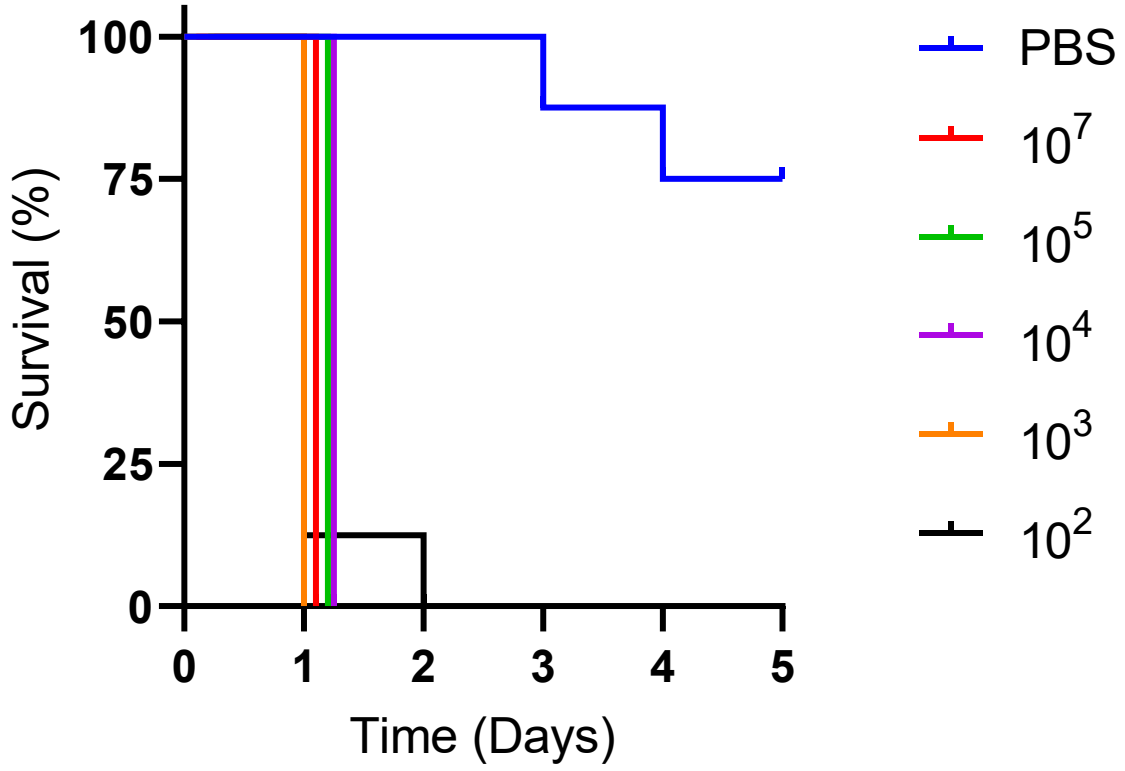

Figure 6.1 Pathogenicity of P. aeruginosa in G. mellonella larvae

Survival of *G. mellonella* inoculated with various concentrations of *P. aeruginosa* is shown on the Kaplan-Meier plot. The larvae were injected with 20 µL of bacteria in PBS into the last left proleg and incubated for up to 5 days at 37 ˚C, with a daily survival count recorded. Larvae were considered dead if there was melanisation or no response to physical stimuli. *n* = 8 for each inoculum.

6.4.2 Antimicrobial Peptides toxicity towards G. mellonella

Peptide 7 and its D-enantiomer were used to assess toxicity. These two peptides were found to be the most favourable consistent in bioactivity. The D-enantiomer was used to rule out any potential degradation within the haemolymph of the *G. mellonella*. It was found that there was not a dose-dependent effect of the peptides on the toxicity (Figure 6.2). Overall, there was mild toxicity of the peptides towards the larvae. Peptide 7 (L-form) and gentamicin yielded a survival rate of 25 % after four days, irrespective of concentration. However, Peptide 7 fared better than gentamicin after three days, with a 75 % survival rate, compared to 50 % for gentamicin in the same period. Meanwhile, peptide 7D was slightly more toxic with no survival after four days.

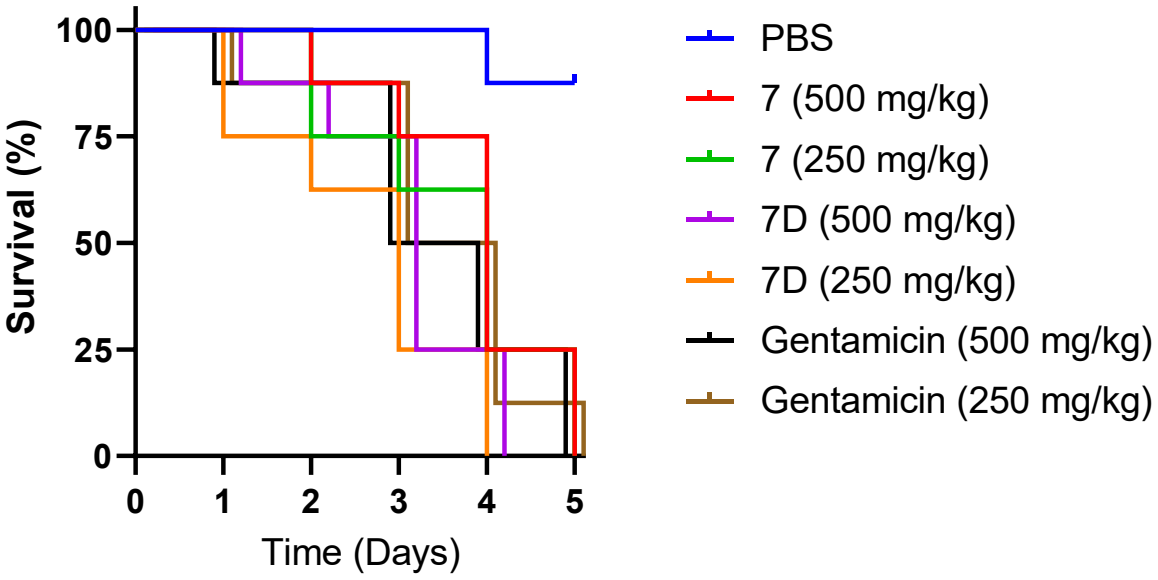

Figure 6.2 AMP administered toxicity towards G. mellonella

A Kaplan-Meier plot denoting the toxicity of peptides 7 and 7D and gentamicin towards *G. mellonella*. Daily survival counts were recorded. Larvae were considered dead if there was melanisation or no response to physical stimuli. *n* = 8 for each peptide and concentration.

6.4.3 Protective Efficacy of Antimicrobial Peptides against *P. aeruginosa* infection

Since all concentrations of *P. aeruginosa* were lethal, a 1 x 10^3 CFU/mL inoculum was administered to larvae, followed by antimicrobial treatment (Figure 6.3). Peptide 7 treated larvae led to 33 % of the survival into day two. Peptide 7D and PBS treated larvae showed no survival after day one. Gentamicin was 50% efficacious after three days (log-rank Mantel-Cox test, p = < 0.05). Overall, peptides failed to improve the survival of larvae infected with a lethal dose of *P. aeruginosa*.

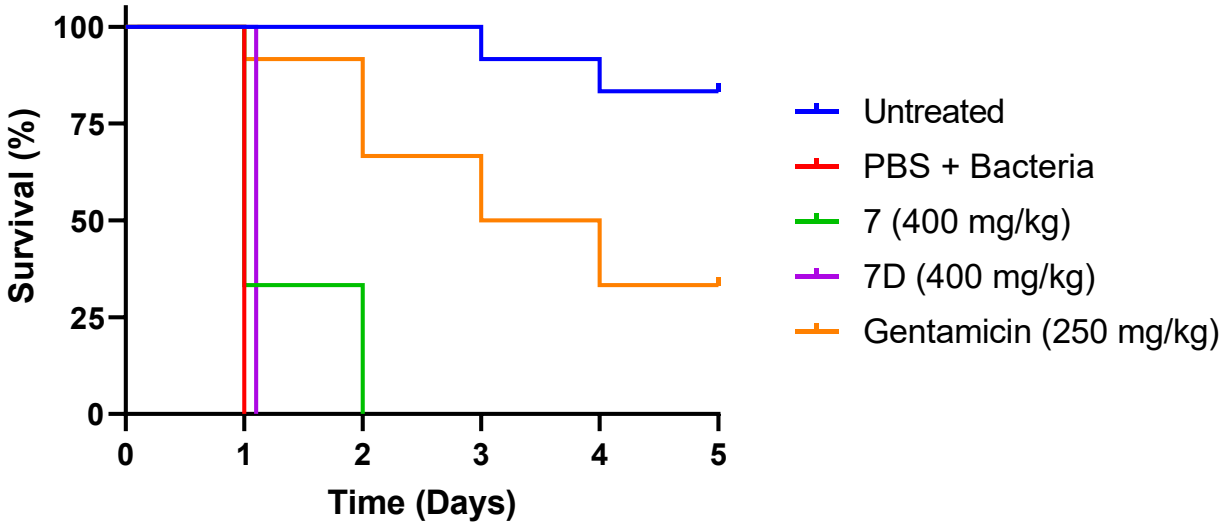

Figure 6.3 Efficacy of AMPs in a G. mellonella model

A Kaplan-Meier plot showing the efficacy of peptide 7 and its derivates towards *G. mellonella*. Survival counts were recorded daily. Larvae were considered dead if there was melanisation or no response to physical stimuli. *n* = 12 for each condition.

6.5 Discussion

In vivo prediction

Thousands of AMPs have been tested and characterised in *in vitro* conditions (Wang *et al.*, 2015; Waghu *et al.*, 2016; Kang *et al.*, 2019). However, it is essential for clinical development to establish how they may behave in situations that resemble their eventual intended use. The PIVL peptides were tested in murine models that served as an ideal basis for comparison of prediction. Peptide NLF20 was shown to reduce *P. aeruginosa* burden in the murine infection model (Papareddy *et al.*, 2016). Moreover, it showed an MIC of 2.5 – 20 µM across the different strains of *P. aeruginosa*, equivalent to MICs of 6.5 – 52 µg/mL, which agrees with the observed results for activity in standard media. On the other hand, peptide clavanin-MO would be classified as inactive in the MICs performed, which disagrees with the published literature (Silva *et al.*, 2016). Clavanin-MO was observed to have an MIC of 3 µM (10 µg/mL equivalent) after a 12-hour incubation (Silva *et al.*, 2016). The same was seen for peptide P3, which was deemed inactive in this chapter, but has a published MIC of 25 µg/mL (Q. Zhang *et al.*, 2015). Peptides HHC-10 and HHC-36 showed low MICs of 2 and 8 µg/mL, respectively.HHC10 and HHC-36 demonstrated equally low MICs *in vitro* and protected murine in an *S. aureus* infection model (Cherkasov *et al.*, 2009). In addition, HHC-10 showed almost a 96 % reduction of *Mycobacterium bovis* BCG in the lungs of mice (Llamas-González *et al.*, 2013). Api88, Api137, Onc72 and Onc112 have all shown differing levels of efficacy within a murine infection model (Czihal *et al.*, 2012; Knappe *et al.*, 2012; Schmidt *et al.*, 2017). This chapter demonstrates that the peptides tested *in vitro* only work in diluted media. They have also been published with their activity expressed in diluted media (Holfeld *et al.*, 2018). The aforementioned has shown that peptides behave differently, depending on the concentration or type of media. Thus media alone is not ideal for predicting how a peptide may behave in a mouse model.

It was found that all peptide antimicrobial activity decreases markedly in the presence of increasing human serum concentrations. It does raise concerns regarding systemic applications of antimicrobials. Human serum has long been a hindrance to antimicrobial peptide development, with the propensity to cause proteolytic degradation (Nguyen *et al.*, 2010). Alternative strategies such as D-amino acids, cyclisation and unnatural amino acid introduction have been employed to overcome serum degradation (Mirski *et al.*, 2018; Lu *et al.*, 2020). The D-amino acid strategy was effective in this chapter, with most D-peptides showing antimicrobial activity in the presence of serum, albeit slightly. Peptide PV3 is one of the very few published L-peptides to demonstrate antimicrobial activity reduction when in the presence of serum and eradicate bacterial burden *in vivo* (Memariani *et al.*, 2016; Memariani, Shahbazzadeh, J. M. Sabatier, *et al.*, 2018). Memariani *et al*. assessed PV3 against various strains of *P. aeruginosa* and found MICs between 0.5 and 4 µg/mL in MH broth, and MICs between 2 – 8 µg/mL in 25 % serum, thus a 4 – 16-fold reduction in activity (Memariani *et al.*, 2016). Moreover, topically applied PV3 eradicated MDR *P. aeruginosa* in a burn model (Memariani *et al.*, 2018). Human serum should also not be used as a sole predictor of in vivo activity, just as with broth.

Albumin is the most abundant protein within blood plasma, and peptides have shown decreased antimicrobial activity in its presence (Svenson *et al.*, 2007; Maisetta *et al.*, 2008). Human Beta Defensin 3 showed a reduction of its antimicrobial activity against *A. bauumanii* and total activity abolishment with *S. aureus* in the presence of human serum albumin (Maisetta *et al.*, 2008). Furthermore, Svenson *et al*. found that MICs were increased 10-fold for many peptides against *S. aureus* in the presence of bovine serum albumin (Svenson *et al.*, 2007). Albumin is a negatively charged protein and could likely compete with negatively charged lipids on the bacterial membrane surface for cationic AMPs for binding, which inevitably leads to a reduction or total loss of antimicrobial activity (Svenson *et al.*, 2007). Peptides tested in the presence of human serum albumin from human plasma produced a more substantial decrease in antimicrobial activity than recombinant HSA. It could result from slight differences, such as recombinant HSA existing primarily as mercaptalbumin (a reduced

form of albumin), higher than human plasma-derived albumin (Watanabe *et al.*, 2001). HSA expressed in *P. pastoris* is protease-free, mostly glycosylated, and has higher free thiol content, depending on how it is produced (Mallem *et al.*, 2014; Radoman *et al.*, 2021). On the other hand, plasma-derived HSA is not always free of proteases, fatty acids and steroids and is mostly (circa 90%) non-glycosylated (Shaklai *et al.*, 1984; He and Carter, 1992). As such, this potentially results in peptide binding being altered, which could explain the difference in antimicrobial activity observed. Furthermore, steric hindrance could have also potentially affected the binding capacity of peptides and competition for bacterial surfaces.

Ions form a vital makeup of components within the blood, potentially affecting the antimicrobial activity of peptides. This chapter examined salts present in the blood at their physiological concentrations (Maisetta *et al.*, 2008). The novel peptides tended to be sensitive to sodium and magnesium, particularly calcium, thus explaining the significant antimicrobial activity drop. Other short novel AMPs, which have some overlapping similarities in the sequences to the novel peptides in this study, have shown a decline in antimicrobial activity in the presence of salts, particularly sodium (Saravanan *et al.*, 2014; Mohanram and Bhattacharjya, 2016). Although, with some changes, improvements to antimicrobial activity were observed in the presence of sodium (Saravanan *et al.*, 2014; Mohanram and Bhattacharjya, 2016). Most of the natural and animal peptides that demonstrated antimicrobial activity remained tolerant to the different additions to salts. The most was a four-fold reduction in MIC for BmKn2 in magnesium , while pleurocidin retained same activity regardless of the ion condtions. Histidine rich peptides such as pleurocidin, may be resistant to ions due to a histidine-mediated response, which protects them from inhibition (Xian *et al.*, 2022)

Testing in the presence of salts solely is also not an ideal indicator of peptide *in vivo* behaviour. However, correlation with *in vivo* tested peptides (the ones which demonstrated strong antimicrobial activity) fared better than serum and albumin. The attempt of *in vivo* activity prediction by using *in vitro* parameters did not correlate to the published *in vivo* activity. Therefore, this indicates that fine-tuning or alternative strategies should be explored.

In vivo activity

It is essential to assess the host toxicity of any new antimicrobial compound early into the developmental phase. This chapter could not elicit robust parameters *in vitro* to determine the potential antimicrobial activity in a murine model, as seen with PIVL peptides. The peptides that showed the most promising activity across many experiments were chosen. The peptides were tested for their *in vitro* toxicity against mammalian cell lines (as shown in previous chapters). They were found to be largely minimally toxic or non-toxic.

Peptide 7 and 7D, alongside gentamicin, was used to evaluate toxicity and efficacy in *G. mellonella*. The *G. mellonella* model has been used extensively to evaluate antimicrobial compounds (Desbois and Coote, 2011; Tsai *et al.*, 2016; Ignasiak and Maxwell, 2017; Zheng *et al.*, 2017). Furthermore, there has been a positive correlation of virulence of *P. aeruginosa* between mice and *G. mellonella*; therefore, it was a suitable pre-murine model to evaluate (Jander *et al.*, 2000).

P. aeruginosa proved to be lethal at all concentrations tested. Such observation has agreed with other studies, with as little 2 CFUs causing rapid death (Hill *et al.*, 2014; Beeton *et al.*, 2015; Paškevičius *et al.*, 2017). Overall, it can be concluded that the larvae tolerated peptides 7 and 7D at high concentrations. Peptide toxicity was slightly higher with peptide 7D, with reduced survival. The reasoning behind the slight increase in toxicity is yet to be determined; however, one possible explanation could be that the D-peptide was protected from degradation thus remained within the haemolymph for an extended period. In comparative studies for D-peptides, Dean *et al*. reported no ill effects to the larvae with a D-enantiomer version of LL-37, a human cathelicidin, albeit at a very low dose of 10 µg (Dean *et al.*, 2011). It translates to about 40 mg/kg, which is more than ten-fold less concentrated than the highest concentration of 7D tested in this chapter.

The efficacy of peptides 7 and 7D was considered to be non-efficacious. Furthermore, even the antibiotic was low in efficacy in *G. mellonella*. This raises queries as to whether the model itself, the pathogen or the peptides are ideal for deducing the efficacy of antimicrobial

peptides. Furthermore, the bacterial burden could have been too high, which led to the failure of the trial. Also another possibility could be batch of unhealthy larvae was purchased.

Very few studies have shown peptide treated *P. aeruginosa* infected *G. mellonella* model to be highly efficacious. Ciociola *et al*. reported that peptide SP-E was 50% efficacious six days post-inoculation with *P. aeruginosa* and treatment (Ciociola *et al.*, 2018). Moreover, Zheng *et al*. found that larvae treated peptide with Cecropin A2 alone yielded no survival of *P. aeruginosa* after four days (Zheng *et al.*, 2017). However, in combination with antibiotic tetracycline, the larvae showed 100 % survival after four days and 80 % survival after six days, thus suggesting a synergistic effect (Zheng *et al.*, 2017). The results found in this chapter, alongside other published observations, may question this constraining method of treating the larvae (injecting into the haemolymph). Also, *P. aeruginosa* may not be the most appropriate bacteria to evaluate in this model, given the very low bacteria inoculum required to be lethal towards *G. mellonella*.

In conclusion, it was found that antimicrobial activity for the novel peptides was hampered in the presence of serum, albumin and certain salts. Most of the natural and *in vivo* tested peptides also had vastly reduced activity in serum and albumin, but not too significantly in the presence of salts. More studies are required to fine-tune the "predictive value" of *in vitro* experiments to predict the potential activity of an *in vivo* model. In an insect model, the pathogenicity of *P. aeruginosa* and the lack of efficacy of any peptides suggest that more work is required to determine the suitability of this model.

7 Discussion and Future Work

7.1 Final Discussion

Spot synthesis, screening and validation

The alarming rise of multidrug resistance amongst pathogenic bacteria has led to an urgent need for novel therapeutic agents. Antimicrobial peptides are one of the potential options to fill the void left in the antibiotic pipeline. Thousands have been discovered, and they form a critical component of the innate immune system of mammals. Synthesising thousands of peptides (using Spot synthesis) and screening them using high-throughput harmonised methods is an efficient way to downstream potent candidates.

A 560 peptide library (OL) designed *in silico* arising from a 3000 peptide screen performed by Mikut *et al*. (2016) was synthesised and screened against a luminescent variation of *P. aeruginosa* and human erythrocytes for their antimicrobial and haemolytic activity. In addition, 400 more peptides (NL) from the APD3 database were also screened to broaden the scope of potential potent peptides further. Upon bioinformatic analysis, 11 % of peptides were either similar or more active than the control in the antimicrobial screen from the novel OL. In comparison, 20 % of peptides from the NL were either similar or more active than the control. However, upon the haemolytic screening, 98 % of the NOL peptides were found to be non-haemolytic. In contrast, none of the best performing NL peptides was non-haemolytic. The prediction using *in silico* design developed by Mikut successfully outputted peptides with high therapeutic potential, particularly concerning the haemolytic activity. Previous libraries of Mikut *et al*. were assessed for their therapeutic potential (derived by combining the antimicrobial activity and the hemolytic activity). They found that five different libraries tested contained between 0.3 % - 5.9 % of peptides in the class "strong" for therapeutic potential (unpublished data). However, the peptides from the NOL contained 19 % of peptides in the same category.

The validation of the screen showed MICs between 4 – 16 µg/mL when both streams of peptides were synthesised on resin and subsequently purified. The predictive value of SciXMiner was confirmed with the HC_{50} of the most potent novel peptides between 800 – 1190 µg/mL, while the natural peptides were between 2 – 200 µg/mL, thus a larger therapeutic index than the natural peptides. The comparison of MICs of these peptides to those in clinical is limited to peptide POL7080. POL7080 demonstrated i*n vitro* activity against over 1200 *P. aeruginosa* isolates revealed MICs of 0.12 µg/mL (Sader *et al.*, 2018). Unfortunately, the phase III trial for this peptide was terminated because of patients' high incident rate of acute kidney injury, thus reinforcing the importance of toxicity testing earlier on in the development.

Engineering, Mode of Action, Translation and *in vivo*

In the first instance from the screening and validation, three from the NOL and one from the NL were subjected to engineering with amino acid substitutions. From the screens, no overall better peptide was found. However, other researchers using this technique successfully found substitutions leading to more potent peptides. Knappe *et al*. found that combining two favourable amino acid substitutions for the peptide Oncocin resulted in unmasking an analogue that was 10-fold more potent against *P. aeruginosa* and 100-fold more potent against *S. aureus* (Knappe *et al.*, 2016). Further engineering strategies were only performed on peptide 7, the best overall performer from both streams. Overall, the engineering strategies did not improve the bioactivity, except for D-amino acid and cyclotide insertion, which showed minor improvements in serum.

The mode of action of most peptides showed mainly bactericidal activity. Moreover, the natural peptides assessed showed a membrane action. In contrast, the novel short peptides appeared to show a multimodal mode of action. This study further ascertained that modes of action of many different antimicrobials could be differentiated using BioSAXS, thus allowing efficient development. This sentiment was also supported by von Gundlach *et al*. (2016)

There were no conclusive *in vitro* parameters that could be established to predict *in vivo* activity of AMPs. The peptides selected for the *in vivo* larvae model (*G. mellonella*) showed minimal toxicity but no efficacy or lack of efficacy with the conventional antibiotic. It suggests that the model may not potentially be suitable for antimicrobial development for a *P. aeruginosa* infection model, primarily due to the sensitivity of the larvae to *P. aeruginosa*.

7.2 Future Work

This study has shown much potential; however, it is currently in the preliminary stages of drug development. Some potential further work could have been conducted had time and resources allowed.

Clinical Isolates and Other Pathogens

For this study, the peptides were tested mainly on *P. aeruginosa* PA01 or H174 (luminescent variation). However, clinical isolates and, in particular, multidrug-resistant isolates could have been further explored. Some peptides from the NOL were tested against a few drug-resistant and multidrug-resistant isolates with MICs ranging from 4 – 16 µg/mL (data not shown). Moreover, expanded testing of the AMPs could have been explored in other organisms further (beyond MICs) for the ESKAPE pathogens. A comparison between the bioactivity and mode of action between Gram-positive and Gram-negative bacteria would be insightful.

Engineering Strategies

Although a few engineering strategies were explored in this study, other strategies could potentially yield lead candidates. Strategies include different types of cyclisation such as side-chain-to-tail, head-to-side chain and side chain-to-side chain. Modifications by conjugation could be a useful strategy to increase peptide stability. These include the addition of bovine serum albumin (BSA) or polyethene glycol (PEG) to the peptides of interest. PEGylation, in particular, has shown promising potential with *P. aeruginosa* in some instances (Morris *et al.*, 2012; Ju *et al.*, 2020). However, PEGylation has also shown total activity loss in other cases (Grimsey *et al.*, 2020). Also, a comparison between Spot synthesised peptides and resin can be evaluated because Spot peptides contain an additional glycine to functionalised the membrane.

Synergy Studies

Synergy is defined as two compounds working together to enhance an effect. In this particular case, it would be enhancing antimicrobial peptide activity either with another peptide or compound. There are only two combination preparations on the market for antimicrobials, co-amoxiclav (amoxicillin and clavulanic acid) and Tozacin (piperacillin and tazobactam), both fall under the β-lactam/β-lactamase category.

Studies have shown synergistic effects of AMPs and antimicrobial peptoids (Chongsiriwatana *et al.*, 2011). Also, it has been shown with AMPs and small-molecule antibiotics and even with other peptides (Yan and Hancock, 2001; Cassone *et al.*, 2010; Ruden *et al.*, 2019).

Further Mode of Action Studies

This study had a cursory glance into the mechanistic features of AMPs. A more detailed insight into the mode of action could be achieved by fluorescence-activated cell sorting (FACS). It can show characteristics of the bacterial cells, which may give an insight into the mode of killing employed by the antimicrobial peptide. FACS has been used to differentiate the mode of action of two fluorescently labelled peptides with *E. coli* as the bacteria of interest (Benincasa *et al.*, 2009). Leakages assays can be used to investigate an AMPs ability to disrupt the bilayers using different fluorescent dyes (Benfield and Henriques, 2020). Moreover, the mode of action of AMPs can be assessed via gene regulations of *P. aeruginosa* upon treatment. A transcriptomic analysis after next-generation sequencing can give a deeper insight into the stresses that bacteria undergo and the genes which are up or down-regulated. It has been demonstrated using a short peptide, DM3, and *Streptococcus pneumoniae* (Le *et al.*, 2016).

Application and Murine Models

Although this study aimed at exploring the potential application of systemic infections, particularly blood and serum components, a topical application may be more favourable. *P. aeruginosa* causes many skin and soft tissue related infections (Fergie *et al.*, 1991; Agger and Mardan, 1995). Peptides have been evaluated in a *P. aeruginosa* wound and burn model and demonstrated a reduction in bacterial burden or eradication (Memariani *et al.*, 2018; Yang *et al.*, 2019).

7.3 Conclusion

This study demonstrated that antimicrobial peptide activity and toxicity could be predisicted using machine learning. It demonstrated superior potential to naturally occurring AMPs and those deposited in online repositories. Using a harmonised method for synthesising and screening is economical, timely and effective at unravelling peptide candidates with strong therapeutic potential. Moreover, the *in silico* designed peptide 7 did not induce any bacterial resistance, which is ubiquitous amongst all antibiotics pertaining to *P. aeruginosa*.

While this study has great potential, further study of the promising candidates is required. Should further peptides' activity and toxicity profile be more favourable, there is scope for further optimisation, formulation strategies, and drug delivery methods. Finally, the machine learning platform can continue to learn and generate potentially new potent AMPs for use within *P. aeruginosa* and other infectious diseases.

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