Novel approaches to prevent and treat infections involving staphylococcal biofilms

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Work contained in this thesis has been published in the following paper:


Work from the published paper will be included in the following chapter:

Chapter 3: Development of antibiofilm compounds for medical implants

The candidate performed MIC screens for the paper, and contributed to accompanying text. The other named authors performed all other laboratory work and contributed all other written text.

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In the words of Rick and Morty, “Science, huh? Ain’t it a thing”.
Abstract

Bacteria can be found as surface adhered, structured colonies that generate an extracellular matrix, known as a biofilm. Biofilms are recalcitrant to antibiotics. Therefore, treatments of biofilm infections are limited, especially in terms of indwelling medical devices. The major causes of biofilm-associated infections on medical implants are *S. aureus* and *S. epidermidis*. This work sought to identify and characterise compounds with antistaphylococcal antibiofilm activity.

Initial studies were performed as a part of the COATIM consortium. The antibacterial and antibiofilm activity of 56 compounds provided by members of the consortium were assessed against bacterial and fungal organisms. The top five (4-45, CIM008405, P1a-PEP1, P2-5 Tocris 2611) compounds were selected for further characterisation based on their antibiofilm activity and toxicity. The MOA of the five compounds were characterised in *S. aureus*. All five compounds caused membrane perturbation, but Tocris 2611 was the only one to exhibit some prokaryote selectivity. In addition, Tocris 2611 eradicated preformed staphylococcal biofilms by sterilising the biofilm cells and had low resistance potential. The five compounds were then coated on titanium substrates (representing dental/orthopaedic implants) and tested for their ability to prevent biofilm formation *in vitro* and *in vivo* against biofilm colonisation in vivo and in vitro. No useful antibiofilm activity was observed.

Finally, three chemical libraries of biologically active small molecules were screened for adjuvants of antibiotics ciprofloxacin and rifampicin against *S. aureus* biofilms, and rifampicin, fusidic acid or linezolid against planktonic *E. coli*. No potentiators were determined against *S. aureus* biofilms at the tested concentrations. However, several synergistic interactions were initially identified against *E. coli*, but had limited antibacterial activity against multi-drug resistant (MDR) pathogens.

Due to the limitations of current antibiofilm strategies and the ability of bacteria to adapt continuously, it is important to consider combining multiple approaches to target biofilms successfully.
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Abbreviations

ABM - antibiofilm molecule
BHA - Brain Heart Infusion agar
BSAC - British Society for Antimicrobial Chemotherapy
C - Celsius
CBD - Calgary biofilm device
CFU - colony forming unit
CLSI - Clinical and Laboratory Standards Institute
DMSO - dimethylsulphoxide
DRaCALA - differential radial capillary action of ligand assay
DNA - deoxyribonucleic acid
EPS - extracellular polymeric substance
FIC - fractional inhibitory concentration
HEPES - 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
MBEC - minimum biofilm eradication concentration
MIC - minimum inhibitory concentration
MDR - multiple drug resistance
MHA - Mueller-Hinton agar
MHB - Mueller-Hinton broth
MMS - macromolecular synthesis
MOA - mode of action
MRSA - methicillin-resistant *Staphylococcus aureus*
MSCRAMMS - microbial surface components requiring adhesive matrix molecules

ND - not determined

OD - optical density

PIA - polysaccharide intercellular adhesin

PBS - phosphate-buffered saline

PCR - polymerase chain reaction

pH - potential hydrogen

PMBN - polymyxin B nonapeptide

PNAG - poly-N-acetyl glucosamine

SDS - sodium dodecyl sulphate

TAE - Tris-acetate-EDTA

TCA - trichloroacetic acid

TE - Tris-EDTA

WGS - whole genome sequencing

WHO - World Health Organisation

w/v - weight per volume
Chapter 1

Introduction

1.1 Introduction to bacterial biofilms

The evolution of antimicrobial resistance is a natural phenomenon that is exacerbated by the exposure of bacteria to antimicrobial drugs. Due to the natural phenomenon of antimicrobial resistance and the misuse of available antimicrobial treatments, antibiotic resistance is now a worldwide problem. An estimated 50% of all prescribed antibiotics are not needed or fully effective as prescribed and unless action is taken to improve infection prevention and antibiotic use, the world is set to enter a post-antibiotic era (CDC, 2013). Presently, a serious threat to human welfare is multi-drug resistant (MDR) bacteria, such as Gram-positive organism meticillin-resistant Staphylococcus aureus. Individuals infected with MRSA are estimated to be 64% more likely to die from infection than those with a non-resistant strain (WHO, 2014). An additional problem in treating antibacterial infections and MDR bacteria is the formation of bacterial biofilms.

Biofilms are bacteria within multicellular, structured communities adhered to an inert or biological surface and encased in a self-produced extracellular matrix (Lopez et al, 2010). It is the most common form of bacterial culture found in the natural environment, with an estimated 99% of microorganisms living in these communities (Carvalho et al, 2007). Biofilms can develop in a range of different environmental conditions, including water supply systems, hydrothermal vents and living tissue (Carvalho et al, 2007).

Bacteria within the biofilm are phenotypically distinct from those in the planktonic state (Lopez, 2010). Notably, biofilm-associated bacteria exhibit reduced susceptibility to antibacterial treatment (particularly due to the presence of slow-or non-growing (SONG) bacteria, including persister cells) (O’Neill, 2011). Biofilms high recalcitrance to antibiotic treatment is especially problematic in the clinical environment, where biofilm formation is estimated to be involved in
80% of all bacterial infections in humans, such as osteomyelitis, endocarditis and infections of implanted medical devices (Römling and Balsalobre, 2012).

Numerous bacterial species form biofilms, but some of the most frequent causes of biofilm-associated infections include *Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae* and *Enterococcus faecalis*. The most significant of these in terms of biofilm-associated infections and healthcare costs are *S. aureus* and *S. epidermidis* (Costerton et al., 1999). Indeed, they are considered to be the cause of up to 70% of biofilm infections on catheters (Treter and Macedo, 2011), and up to 50% of biofilm infections on prosthetic heart valves (Nagpal et al, 2012). Since these types of infection can be untreatable, they are especially problematic in the clinical setting, often resulting in the failure and subsequent surgical removal of the implanted medical device (Johnjulio et al, 2012 and Carvalho et al, 2007). This type of revision surgery is stressful for patients, increases the duration of hospital stays and adds to hospitalization costs (Carvalho et al, 2012). Biofilm-related corrective surgery costs in Europe are estimated at €800 million per year, and the reduced susceptibility of biofilms to antibacterial killing is driving and directing research into the development of new antibacterial agents, in order to tackle this growing problem (O’Neill, 2011).

However, it is also important to acknowledge that not all biofilms are detrimental to health and that some biofilms are beneficial and necessary for our survival. For example, biofilms formed by the host microbiota can act as a defence mechanism by protecting against foreign pathogens (Belkaid et al, 2014). An example of this is the gut microflora which protects against food borne pathogens which could potentially colonise the gut and cause infection (Lee et al., 2000).

1.1.1 Biofilm formation

Although biofilm formation has been found to differ between bacterial species, and is impacted by both genetic and environmental factors, there are two steps that are considered to be universal in all biofilm-forming bacteria; attachment, followed by maturation. However, these
can be further broken down into five general stages of development; reversible attachment, irreversible attachment, growth, maturation and finally dispersal (Figure 1.1).

**Figure 1.1** Five steps of biofilm formation, from planktonic culture to structured biofilm community. Reversible attachment (1): planktonic bacteria adhere to an inert or living surface; Irreversible attachment (2): affixed bacteria begin to form a biofilm, resulting in irreversible attachment; Growth (3): the biofilm structure begins to grow and mature. Bacteria will now be protected from environmental stresses by the extracellular matrix; Maturation (4): continued development and further maturation of the biofilm; Dispersal (5): planktonic bacteria are dispersed from the biofilm, which can result in the spread of the infection. Image adapted from Monroe (2007).

For a biofilm to form, planktonic bacterial cells must first attach to a biological or inert surface (Figure 1.1). Often for attachment to occur, trace organic or inorganic molecules (bridging molecules) must be absorbed onto the surface (Lindsay et al, 2006). This is known as surface conditioning, and is thought to neutralize any charge or free energy that may have inhibitory effects. Surface coated bridging molecules, together with forces such as Van de Waals, electrostatic and hydrophobic, have been suggested to cause an initial attraction between the bacterial cells and the surface (Lindsay et al, 2006). Attachment also requires bacteria to be within close range of the surface. Existing structural features of the bacterial cells, such as flagella, can be used to reach the surface (Carvalho et al, 2007). Otherwise bacteria can rely on mechanical processes like shear force. Primary attachment is weak, and as a consequence reversible (Garrett
et al, 2008). Bacteria can therefore dissociate from the surface and revert to non-adhered, planktonic cells.

Bacterial cells become irreversibly bound to the surface by either of two pathways; production of EPS or surface receptor protein (Monds et al, 2009). Subsequently, attached bacteria begin to divide, and accumulate with one another, sometimes travelling across the surface to join existing accumulations of cells (Aparna et al, 2008 and Monds et al, 2009). As biofilms enter the growth phase, newly formed colonies of bacteria continue to divide, and there is a release of signalling molecules, a process known as quorum sensing (del Pozo et al, 2007). Quorum sensing is a population density dependent programming system used by bacteria to coordinate activity and gene expression amongst the community (Miller et al, 2001). The levels of these signalling molecules increase in a cell density dependent manner until a threshold is passed, and are detected by receptors on the bacterial surface (Albuquerque et al, 2014). When the chemical molecules bind to the specific receptors it causes the transcription of particular genes in the majority of the cells at roughly the same time (Ng et al, 2015). Bacteria are therefore releasing, detecting and reacting to the chemical signals in response to the dynamic environment and thus regulating specific genes which control important biological functions (Miller et al, 2001). Quorum sensing therefore allows bacteria to assess their surrounding conditions and adjust gene expression accordingly to increase bacterial survival (Withers et al., 2001 and Ng et al, 2015).

As the biofilm matures, a multicellular structure is formed, including further assembly of the exopolysaccharide matrix. At this stage, other species of bacteria or fungi can also become associated with the surface, forming a polymicrobial biofilm. For example, in clinical settings, \textit{S. aureus} is thought to be the third most prevalent bacterium found in polymicrobial biofilms with \textit{Candida albicans} (Peters et al., 2012).

The final stage of formation is dispersal (Kaplan et al, 2010). This is where the mature biofilm can release bacterial cells, which return to the surrounding planktonic community. Dispersal contributes to the spread of infections and colonization of new surfaces (Kaplan et al, 2010).
1.1.2 Biofilm composition/structure

As with biofilm formation, biofilm structure varies between different bacterial species and is affected by environmental factors, such as the source of nutrients (Hall-Stoodley et al, 2004). It is currently accepted that there is a general stratified structure consisting of three defined sections common to all biofilms (Donlan, 2002). The bottom layer of the structure attaches the biofilm to the surface. The next ‘layer’ is composed of colonies of either multiple or single species of bacteria, and finally a surface film (or extracellular matrix) encompasses and embeds the bacteria (Aparna et al, 2008). The extracellular matrix is a hydrated polyanionic complex, which encompasses and contains DNA, proteins and extracellular polymeric substance (EPS) or exopolysaccharides, which are secreted by the bacteria. This EPS component can comprise between 50% and 90% of the biofilm’s organic carbon (del Pozo et al, 2012). Each component of the EPS plays an important role in the biofilm. For example, the extracellular DNA (eDNA) functions as an intercellular connector, whilst enzymes break down EPS biopolymers to molecules, such as carbon, which can be used as an energy source for the bacteria within the biofilm. Another common structural feature found within biofilms are mushroom shaped microcolonies (Aparna et al, 2008). However, this is not the only type of architecture the biofilm can assume, as the biofilm structure can also be flat, depending on the nutrient source of the bacteria (Hall-Stoodley et al, 2004). Additionally, to ensure that there is not an accumulation of waste material, which could potentially be toxic to the bacterial cells, there are also water channels which occur between the microcolonies in the biofilm which, it has been suggested, allow the community to clear any toxic metabolites and also provide a route for mineral and nutrient uptake (Aparna et al, 2008).

1.2 Staphylococcal biofilms

One of the primary bacterial genera of interest with regards to biofilm infections are the Gram-positive staphylococci (Darouiche et al, 2004 and Zimmerli et al., 2004). There are at least 40 species of staphylococci, most of which are ordinarily harmless, inhabiting mammalian mucous
membranes and the skin (Kiedrowski et al, 2011). *S. aureus* is estimated to be in the anterior nares of 20% of the population (Rongpharpi et al, 2013), whilst *S. epidermidis*, which is coagulase negative, is present on most of the population (Becker et al, 2014). Furthermore, *S. aureus* and *S. epidermidis* are the most common cause of hospital-acquired infections, in addition to being the leading cause of biofilm-associated infections, particularly on indwelling medical devices (Kiedrowski et al, 2011).

The ability of staphylococci to form a biofilm provides a survival advantage in the ordinarily adverse environments of the human host, in addition to the production of virulence factors, such as toxins, catalase and coagulase (Tenover et al, 2000). Indeed, most staphylococci infections are biofilm-associated (Otto et al, 2008).

### 1.2.1 Biofilm formation in staphylococci

Staphylococci bind to surfaces using microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Patti et al, 1994). There are approximately 20 different MSCRAMM proteins (Walsh et al, 2008). These surface proteins associate with serum proteins and host extracellular matrix molecules mediating attachment, such as fibronectin binding protein A and B (FnBPA and FnBPB) which chiefly bind to the large glycoprotein fibronectin (Sepziale et al, 2014). These adhesins are attached to the staphylococcal cell wall, and are able to bind host proteins, including fibronectin, collagen and elastin (Speziale et al, 2014). The general overall structure of MSCRAMMs can be broken into three domains (Foster et al, 2014). Firstly, the domain is either covalently or non-covalently bound to the surface of the bacteria, followed by the cell-wall spanning domain and the final domain that is exposed for binding (Foster et al, 2014). In addition to the role of MSCRAMMs in staphylococcal surface adhesion, the type of surface can also influence the attachment process (Otto et al, 2010). It has been reported that staphylococci show preferential attachment to plastic surfaces, which may be a factor in the significance of staphylococcal biofilm infection on implanted medical devices (Sousa et al, 2008).
Following attachment, staphylococci begin to produce substances such as extracellular DNA, proteins, teichoic acid and polysaccharide intercellular adhesin (PIA) that form the extracellular matrix (Garrett et al, 2008). Furthermore, PIA, which consists chiefly of poly-N-acetylglucosamine (PNAG), is the primary compound that causes intercellular adhesion, a major part of staphylococcal biofilm maturation (Rohde et al., 2010). PIA is a partially de-acetylated $\beta$ 1-6-linked $N$-acetylglucosamine homopolymer, which is generated through the intercellular adhesion ($ica$) operon (consisting of biosynthetic gene $icaADBC$, as well as transcriptional repressor IcaR) and accessory membrane proteins (Arciola et al 2015). However, the generation of PIA is not a requisite for biofilm development in staphylococci, as PIA-independent biofilm formation can also occur (Valle et al, 2003). It has been suggested that adhesive proteins will replace PIA during PIA-independent biofilm formation (Otto et al, 2015).

The major transcriptional regulators of genes involved in biofilm formation apart from IcaR include alternative sigma factor (SigB), which functions to increase the generation of proteins at the cell surface which promotes the formation of the biofilm, the accessory gene regulator ($agr$), which functions in the dispersal of the biofilm, staphylococcal regulator ($sarA$), which like SigB is involved in biofilm formation, ArlR-ArlA two component system (TCS), which again is involved in biofilm formation, and finally the teicoplanin-associated locus regulator (TcaR) which is involved in the regulating the expression of the $ica$ operon (Cerca et al, 2008).

After surface attachment, and intercellular aggregation, the maturation phase of staphylococcal biofilm development includes the 3-dimensional structuring of the biofilm. As mentioned previously (section 1.1.2), this includes mushroom-shaped towers and water channels, providing oxygenation removal of waste (Tilahun et al, 2016). Finally, bacterial cells may be released from the mature biofilm, known as dispersal. These dispersed cells may travel to and attach to new surfaces, resulting in biofilm formation of that surface (Kaplan et al, 2010).

1.3 Biofilm recalcitrance to antimicrobials

Biofilm infections are rarely able to be resolved by the host immune system, or with antibiotics. Biofilm formation is therefore central to the pathogenesis of many bacteria and resistance can be
through more than one mechanism (del Pozo and Patel, 2007). The reasons that biofilms display such a high level of recalcitrance to antimicrobials are thought to be through inherent mechanisms of resistance or the transfer of genetic material (Norrby, 1991, O'Neill, 2011).

1.3.1 Inherent mechanisms of antibiotic resistance in biofilms

1.3.1.1 Stress Response/restricted penetration

It has been suggested that, as the bacteria within biofilms are enclosed in an exopolysaccharide matrix, the diffusion and penetration of antimicrobial drugs could be restricted. For example, the positively-charged aminoglycosides are prevented from penetrating the negatively charged extracellular matrix, due to binding to the negatively charged matrix polymers (Aparna and Yadav, 2008, del Pozo and Patel, 2007, Lewis, 2001, Shigeta et al., 1997). Mature biofilms are also thought to contain altered micro-environments, for example due to the build-up of waste products, low oxygen concentrations, and pH, which may further inhibit the activity of the antimicrobial agent (del Pozo and Patel, 2007). However, restricted penetration has been shown not to apply for most classes of antibiotics. For example, fluoroquinolones easily travel across the extracellular matrix of the biofilm (del Pozo and Patel, 2007, Lewis, 2001). Alternatively the antimicrobial agent may penetrate the biofilm, but quickly become bound and inactivated by enzymes produced by the bacteria against the active drug (del Pozo and Patel, 2007). Also, when bacteria are exposed to conditions which do not favour growth they are able to up-regulate genes, known as stress-response genes, resulting in rapid adaptation (del Pozo and Patel, 2007). These genes cause a change in the bacterial phenotype which is more resistant to the surrounding environmental stress (Waters and Storz, 2009). For example, the ndvB gene in Pseudomonas aeruginosa, has been found to be required for the production of periplasmic glucans, which prevent tobramycin from reaching its target through direct interaction (del Pozo and Patel, 2007).
1.3.1.2 Evasion of host immune defence

Not only can biofilms display decreased susceptibility to antimicrobial killing, they can also display a decreased susceptibility to the host immune system. This can be through decreased expression of significant antigens, increased resistance to phagocytosis or resistance to killing by polymorphonuclear leukocytes (Bradley et al., 2003). This resistance is thought to be conferred by the EPS, which forms a physical barrier against the attacking components of the host immune system, decreasing the efficiency of host immune killing compared to killing of single bacterial cells and secreting molecules which chemically inactivate attacking immune cells (Foster, 2005). For example, polysaccharide intercellular adhesins (PIA) found in *S. epidermidis*, are charged homopolymers which hold cells together in the biofilm but also contribute to evading the host immune defence by promoting resistance to antimicrobial defensin peptides (Foster, 2005). *S. epidermidis* mutants lacking PIA have been shown to be increasingly predisposed to killing by neutrophils and host peptides such as LL-27, β-defensin 3 and dermicidin (Foster, 2005).

1.3.1.3 Antibiotic survival in biofilms

Antibiotic survival can be defined as tolerance to killing by bactericidal antibiotics, whilst still being sensitive to an antibiotics inhibitory mechanism, i.e. the bacteria can survive in the presence of antibiotics, but not grow (O'Neill, 2011). By contrast ‘resistance’ is described by the World Health Organisation as “…recalcitrance of a microorganism to an antimicrobial medicine to which it was previously sensitive.” (WHO, 2012), which is caused by heritable genetic mutations. Antibiotic survival in biofilms is generally through two main mechanisms; drug indifference and persistence (Figure 4) (Hall-Stoodley et al., 2004). Drug indifference is considered to be a phenotypic state of quiescent (non-dividing) bacteria, which the majority of antibacterial agents are unable to kill (Levin and Rozen, 2006, McDermott, 1958, O'Neill, 2011). The more established an infection is, the more non-growing, stationary phase cells are
present and the less effective the antibiotic agent will be (Levin and Rozen, 2006). Therefore, if cells are not undergoing active metabolism, the non-growing cells become ‘indifferent’ to the antibacterial agents because of a lack of ‘target corruption’. Target corruption is where the antibiotic kills the cell, not by directly inhibiting the target to which it binds, but through altering its function resulting in the production of toxic derivatives. It is these downstream by-products which lead to cell death (Lewis, 2007). For example, aminoglycosides cause cell death by causing the cell to make misfolded toxic proteins, via disruption of translation (Lewis, 2007).

Persistence (adaptive resistance, phenotypic tolerance) is described as a subpopulation of a multiplying bacterial population which bactericidal antibiotics are unable to eliminate (Kiedrowski and Horswill, 2011, Gotz, 2002). Ineffective elimination is due to the persistent cells undergoing a much slower growth rate compared to the remainder of the population (Lewis, 2007). The reduced growth rate is suggested to be through numerous metabolic genes being down-regulated. This allows these quiescent cells to endure high levels of antibiotics, without displaying classic resistance mechanisms. Persistent cells also have the ability to reform biofilms following antibiotic cessation (Miller and Bassler, 2001). Persisters are therefore another important factor to consider in biofilm resistance (Figure 1.2).

However, it is still uncertain whether persister cells are undergoing a completely separate state to those undergoing drug indifference, or if they are just a subpopulation of more antibiotic tolerant cells.
Figure 1.2 Model of antibiotic survival due to drug indifference/persistence. Panel (a): Biofilm with no challenge from antibiotic agent, and unsuccessful immune attack; Panel (b): Antibiotic chemotherapy kills the non-tolerant (grey) bacterial cells within the biofilm, whilst the host immune system targets and kills both the tolerant (red) and non-tolerant (grey) planktonic cells; Panel (c): Following antibiotic cessation remaining bacterial cells due to drug indifference/persistence repopulate the biofilm, causing the infection to recur. Adapted from Lowy (1998)

1.3.2 Gene transfer
Pathogenic bacteria have developed numerous strategies to resist the action of antibiotics, including modification and inactivation of the drug, exclusion of the antibiotic and alteration of the target (Kumar et al., 2012). The origin of antibiotic resistance genes in pathogenic bacteria is unclear, but it is thought that horizontal gene transfer (HGT) of this genetic material is largely
 responsible for the increasing incidence of antibiotic resistant infection worldwide (Davies J., 2010). HGT, which includes transduction, transformation and conjugation (Rees et al., 2009), has been found to occur at a higher frequency in biofilms than bacteria in the planktonic state (Madsen et al, 2012). Thus, plasmids that code for antimicrobial resistance (or virulence factors) can be transferred between cells, enabling previously non-resistant bacteria cells to start exhibiting resistance or increased virulence (Schroeder et al., 2017). For example, in biofilms formed by *Klebsiella pneumoniae*, genes encoding β-lactamase can be transferred between cells, which allow them to quickly inactivate β-lactams, such as ampicillin (del Pozo and Patel, 2007). Resistance has a clinical impact on patients, leading to more treatment failures, higher mortality, prolonged length of hospital stays and higher costs (Warnes SL., 2012). Emphasizing the importance of efforts to limit their emergence and spread.

### 1.4 Antibiofilm surfaces

Biofilms can form on indwelling medical devices (e.g. cardiac pacemakers, joint prosthesis) due to the surfaces of these necessary implants providing an ideal scaffold for bacterial attachment and biofilm formation. The consequent highly resistant infection ultimately results in the requirement for surgical removal of the implanted device. However, this is costly, stressful for the patient and not always immediately possible, such as in the case of joint prosthesis or in critically unstable patients for whom surgery may put their lives at risk (Kiedrowski and Horswill, 2011). Thus, the need for alternative treatments is crucial. One such alternative is the development of anti-biofilm coatings to cover indwelling medical devices. Currently, medical implants which have been developed have a porous surface, enabling rapid osseointegration. However, it has recently been discovered that such surfaces pose an enhanced risk of infection from biofilms (Darouiche, 2004).

All current antibiofilm coatings function by impeding bacterial attachment and consequent biofilm maturation, through the incorporation of conventional antibiotics, or through the delivery of silver (or occasionally zinc) ions. Although these methods display some positive
results, such as reduced biofilm formation, they also result in side effects that include toxicity, hypersensitivity and the development of bacterial resistance to antibiotics (Dror et al., 2009). When evaluating antibiofilm coatings for medical implants, it is important to consider that even with the same type of antibiofilm surface, different types of implants may display different level of effectiveness in the disruption/prevention of biofilm formation (Dror et al., 2009). For example, urinary catheters impregnated with silver were found to increase the numbers of staphylococcal bacteriuria infections, whilst endotracheal tubes impregnated with silver reduced the incidence of ventilator associated pneumonia (VAP). There is therefore a need for the development, improvement and standardisation of antibiofilm coatings, focusing on either complete eradication of the biofilm, or prevention of biofilm formation (Dror et al., 2009). One novel approach to biofilm eradication is to disrupt the biofilm maturation process through interference with autoinducer signalling pathways (Brözel et al., 1995, Davies et al., 1993, Sauer and Camper, 2001). For example, the organic chemical compounds furocoumarins are able to inhibit cellular autoinducer signalling, which results in the inhibition of biofilm formation, in organisms such as *E. coli* O157:H7, *Salmonella typhimurium* and *Pseudomonas aeruginosa* (Brözel et al., 1995).

Although the development of antibiofilm surfaces is an exciting approach to biofilm treatment it is also important to consider alternatives for those infections which are unable to be treated in this way. For example, the use of bacteriophages. Bacteriophages were originally discovered by Ernest Hanbury Hankin in the late 1800s (Abedon et al., 2011), and the potential of these bacterial antagonists in treating human disease was almost immediately recognized and have been used to treat a range of bacterial diseases (Davies et al., 2010). Bacteriophages have been found to generate polysaccharide lyases capable of depolymerising the biofilm exopolysaccharide matrix (Maszewska, A, 2015). Following degradation of the matrix, the destruction of the bacteria within is the same process seen in planktonic cells. i.e. the phage infects the host bacterium, the nucleic acid is delivered into the cell and the phage DNA or RNA is then transcribed within the host cell by hijacking the host machinery, and the phage is
reproduced quickly and in high numbers (Kawa et al., 2012). The new phages then lyse the cell and are released, resulting in host cell death and further infection of host cells (Azeredo and Sutherland, 2008, Beckmann et al., 2005). This method of bacteriophage infection has only so far been used in cystic fibrosis patients against *Pseudomonas aeruginosa* biofilms, but could potentially be adapted to tackle infection with medical devices, and also be modified genetically to target a wider range of bacterial species (Beckmann et al., 2005, Hughes et al., 1998).

However, bacteria have evolved a diverse repertoire of phage defence mechanisms, including both innate defence systems and, the more recently discovered, CRISPR RNA-guided adaptive immune systems (Sorek R., 2013). Despite this, the development of CRISPR-mediated immune systems has not eradicated phages, suggesting that viruses have evolved mechanisms to subvert CRISPR-mediated protection (Wiedenheft B., 2013). Recently, Bondy-Denomy and colleagues (2013) discovered several phage-encoded anti-CRISPR proteins that may prove valuable in augmenting phage therapy by enabling targeted suppression bacterial immune systems.

Whilst the use of naturally-occurring phages to treat bacterial infection has been controversial, phage-based antimicrobials has advanced well beyond traditional methods (Lin et al., 2017). Novel technologies such as bioengineered chimeras of phage-derived lytic proteins show potential as a new class of antibacterial pharmaceuticals (Lin et al., 2017), specifically against multidrug-resistant bacterial infections. Suggesting phage therapy has the potential to be used as either an alternative or a supplement to antibiotic treatments.

**1.5 Research objectives**

Despite obvious advances in the area of biofilm prevention and eradication, the current strategies are not entirely effective (del Pozo and Patel, 2007). This leaves a significant amount of development needed in the area of antibiofilm coatings and antibiofilm strategies in general, specifically for medical implants. This work aims to tackle the increasing problem of biofilm infections on medical devices in collaboration with nine other research institutes, SMEs and
companies. Using a combinatorial approach the consortium aims to develop a new generation of medical implant surfaces/coatings, containing novel antibiotic molecules (ABMs), such as peptides (COATIM, 2012). Studies that were carried out as a member of this partnership screened a number of antibiofilm molecules (provided by partners of the consortium) for activity against staphylococci and established their mode of action in S. aureus. The top five ABMs were then attached onto a porous titanium layer, which would ultimately be used coated dental and orthopaedic implants. Next the ABM coated implants were evaluated for in vitro activity against resisting microbial biofilm infections, without effecting osseointegration. Separately, the ABMs were screened by other members of the consortium for activity against different species of bacteria and fungi, in vivo activity of the ABM coated titanium surface, ABM toxicity, and for the feasibility of upscaling the production of the ABM coated implants.

In addition, this study aimed to identify antibiotic adjuvants for currently available antibiotics ciprofloxacin and rifampicin against S. aureus biofilms, and rifampicin, fusidic acid or linezolid against planktonic E. coli. Screening will use collections of compounds that have either been previously FDA-approved for a different indication or have had some existing pre-clinical analysis. Any potential combinations were then analysed for their spectrum of antibacterial activity the ESKAPE pathogens.
Chapter 2

Materials and Methods

2.1 Bacterial strains, growth media and growth conditions

Bacterial and fungal strains used in this study are described in Table 2.1. Staphylococci were routinely grown in Mueller-Hinton broth (MHB) or Mueller-Hinton agar (MHA) (Oxoid), with the exception of biofilms grown in Nunc™ Microwell™ 96-well microplates, which were grown in Tryptic Soy Broth (TSB), and for the evaluation of surface-immobilised antibiofilm compounds, cultures were grown in Luria-Bertani broth (LBB) (Oxoid). For studies with daptomycin, 183.42 μg/mL CaCl₂ was added to culture media. LBB or Luria-Bertani agar (LBA) (Oxoid) was used for culturing Escherichia coli (E. coli) and Pseudomonas aeruginosa (P. aeruginosa). Candida albicans (C. albicans) were cultured in Yeast Extract-Peptone-Dextrose (YPD) medium or Roswell Park Memorial Institute (RPMI) medium. All organisms were grown at 37 °C with aeration, with the exception of C. albicans which was grown at 30 °C.

Table 2.1 Bacterial and fungal strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Comments</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cubist 581217</td>
<td>NDM-1</td>
<td>Gift from Cubist</td>
</tr>
<tr>
<td>Candida albicans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC MYA-2876</td>
<td>Human clinical isolate</td>
<td>(Fonzi and Irwin, 1993)</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cubist 583750</td>
<td>CTX-M-15, ACT/MIR, NDM-1, OXA-48</td>
<td>Gift from Cubist</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1411</td>
<td>K-12 strain</td>
<td>(O’Neill et al., 2002)</td>
</tr>
<tr>
<td>MG1655</td>
<td>K-12 strain</td>
<td>(Xiao et al., 1991)</td>
</tr>
</tbody>
</table>
Table 2.1 (continued) Bacterial and fungal strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Comments</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG1</td>
<td>K-12 strain</td>
<td>(Biriukova et al., 2010)</td>
</tr>
<tr>
<td>Cubist 449334</td>
<td>CTX-M-15, IMP-1</td>
<td>Gift from Cubist</td>
</tr>
<tr>
<td>Cubist 449742</td>
<td>CTX-M-15, TEM-52</td>
<td>Gift from Cubist</td>
</tr>
<tr>
<td>Cubist 586030</td>
<td>CTX-M-15, CMY-2, NDM-4</td>
<td>Gift from Cubist</td>
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<tr>
<td>Cubist 605879</td>
<td>CTX-M-15, NDM-6</td>
<td>Gift from Cubist</td>
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<td>Cubist 657524</td>
<td>CTX-M-32</td>
<td>Gift from Cubist</td>
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<tr>
<td>Cubist IHMA 659048</td>
<td>KPC-2, CTX-M-15, AmpC, CMY+</td>
<td>Gift from Cubist</td>
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<tr>
<td>Cubist IHMA 684850</td>
<td>KPC-2, SHV-12</td>
<td>Gift from Cubist</td>
</tr>
<tr>
<td>Cubist 702452</td>
<td>CTX-M-97</td>
<td>Gift from Cubist</td>
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<tr>
<td><em>Klebsiella oxytoca</em></td>
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<td></td>
</tr>
<tr>
<td>Cubist 683079</td>
<td>CTX-M-14b</td>
<td>Gift from Cubist</td>
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<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cubist 581436</td>
<td>CTX-M-15, CMY-2, DHA, NDM-1</td>
<td>Gift from Cubist</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA01</td>
<td>Clinical isolate</td>
<td>(Stover et al., 2000)</td>
</tr>
<tr>
<td>PA14</td>
<td>Clinical isolate</td>
<td>(Rahme et al., 1995)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH1000</td>
<td>Derivative of strain 8325-4, with repaired defect in rsbU</td>
<td>(Horsburgh et al., 2002)</td>
</tr>
<tr>
<td>UAMS-1</td>
<td>Methicillin-sensitive clinical strain. Proficient biofilm former</td>
<td>(Gillaspy et al., 1995)</td>
</tr>
<tr>
<td>USA3000</td>
<td>Community acquired MRSA</td>
<td>(Tenover and Goering, 2009)</td>
</tr>
<tr>
<td>Cubist ACC A790662</td>
<td>Human clinical isolate</td>
<td>Gift from Cubist</td>
</tr>
<tr>
<td>ATCC6538</td>
<td>Human clinical isolate</td>
<td>(Connolly et al., 1994)</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 35984</td>
<td>Proficient biofilm former</td>
<td>(Ryder et al., 2012)</td>
</tr>
</tbody>
</table>
2.2 Antimicrobial compounds, reagents and chemicals

All antibiotics and chemicals used in this study were from Sigma-Aldrich (Poole, UK), with the exception of P1a-PEP1, P2-5, CIM008405 and 4-45 (COATIM), Tocris 2611 and Tocriscreen Total (Tocris Biosciences, Bristol, UK), vancomycin (Dehefa Biochemie, Haarlem, The Netherlands), nisin (NBS biologicals, Cambridgeshire, UK), XF-73 (Destiny Pharma, Brighton, UK), National Institute of Health (NIH) clinical collection (NIH, Maryland, USA), Spectrum Collection (Microsource Discovery System Inc, Gaylordsville, USA), Live/Dead BacLight™ kit and Disc3(5) (Invitrogen, Paisley, UK), the radiolabelled chemicals [methyl-³H]thymidine (70–95 Ci/mmol), [5,6-³H]uridine (31–56 Ci/mmol) and L-[G-³H]glutamine (20 –50 Ci/mmol) (Perkin Elmer, Cambridge, UK), defibrinated equine blood (Oxoid Ltd, Cambridge, UK), human plasma and foetal bovine serum (Sera laboratories International, West Sussex, UK). Daptomycin was a gift from Cubist Pharmaceuticals (Lexington, MA, USA). Phospholipids were purchased from Avanti Polar Lipids. Table 2.2 shows all antimicrobial agents used in this study, along with their solvents.

Table 2.2 Antimicrobial agents and their solvents used in this study. SDS: sodium dodecyl sulphate. dH20: deionised water.

<table>
<thead>
<tr>
<th>Antimicrobial compound</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>dH20</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>20 mM HCl</td>
</tr>
<tr>
<td>CIM008405</td>
<td>DMSO</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>dH20 (+ 183.42 µg/mL CaCl₂)</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>50% ethanol</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>dH2O</td>
</tr>
<tr>
<td>Linezolid</td>
<td>DMSO</td>
</tr>
<tr>
<td>Nisin</td>
<td>20 mM HCl</td>
</tr>
<tr>
<td>P1a-PEP1</td>
<td>dH20</td>
</tr>
<tr>
<td>P2-5</td>
<td>dH20</td>
</tr>
</tbody>
</table>
Table 2.2 (continued) Antimicrobial agents and their solvents used in this study. SDS: sodium dodecyl sulphate. dH20: deionised water.

<table>
<thead>
<tr>
<th>Antimicrobial compound</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>DMSO</td>
</tr>
<tr>
<td>SDS</td>
<td>dH2O</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>dH2O</td>
</tr>
<tr>
<td>Tocris 2611</td>
<td>DMSO</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>dH2O</td>
</tr>
<tr>
<td>XF-73</td>
<td>dH2O</td>
</tr>
<tr>
<td>4-45</td>
<td>DMSO</td>
</tr>
</tbody>
</table>

2.3 Antimicrobial susceptibility determinations

Antibiotic minimum inhibitory concentrations (MICs) were determined using 2-fold serial dilutions of antibiotic in MHB according to the Clinical Laboratory Standards Institute (CLSI) guidelines (Cockerill et al., 2012). Overnight cultures were added to the antibacterial agent at an inoculum of $5 \times 10^5$ CFU/mL, and incubated for 18-24 hours at 37 °C. The MIC was identified as the lowest concentration of drug which inhibited visible bacterial growth.

Biofilm MICs (bMICs) and minimum biofilm eradication concentrations (MBECs) were determined using the Calgary Biofilm Device (CBD) (Nunc) as described previously (Ceri et al., 1999). Overnight cultures were diluted 1/100 in fresh MHB, and incubated with pins of the CBD for 24 hours at 37 °C. CBD grown biofilms can reach an inoculum of $\sim 10^7$ CFU/peg. The bMIC was identified as the lowest concentration of drug at which no bacterial growth was observed. For MBEC determination, the pegs from the bMIC were washed twice in saline solution and immersed in wells containing drug free MHB and incubated for 24 hours at 37 °C. The MBEC was identified at the concentration of antibacterial agent that resulted in complete eradication of the biofilm. As biofilms grown on the CBD are limited for more in depth studies, a second biofilm model was used in which biofilms were grown in the wells of 96-well plates. To aid biofilm growth, 20 % human plasma in 0.05 M carbonate buffer was added to the wells.
and incubated at 4 °C overnight. Human plasma was then removed prior to addition of *S. aureus* (1/100 in TSB) before further incubation for 24 hours at 37 °C. Biofilms were washed once in saline solution and exposed to antibacterial agent in fresh MHB. Cultures were maintained at 37 °C for 24 hours. Biofilms were washed once in saline solution and incubated with proteinase K (100 µg/mL in 20 mM tris and 100 mM NaCl, pH 7.5) for 1 hour. Dispersed biofilm cells were collected, washed and resuspended in PBS then cultured on MHA and incubated at 37 °C overnight for viable counting. The MBEC was determined as the lowest concentration of antimicrobial drug that prevented the recovery of viable cells.

### 2.4 Time-dependent killing studies

Standard time-kill methodology was used to study the killing kinetics of Tocris 2611 and comparator agents against early exponential-phase cultures of SH1000 as previously described (Ooi et al., 2009, Randall et al., 2013b). Briefly, bacteria were incubated at 37 °C to optical density (OD)\(_{600}\) of 0.2, exposed to either 4 x MIC of comparator agent or 0.5, 8 and 64 µg/mL of Tocris 2611 (4 xMIC, CBD MBEC and 96-well plate MBEC, respectively). Aliquots of culture were taken regularly over 24 hours for determination of viable counts.

Viable counts were determined over the course of 24 hours by diluting in PBS as necessary, culturing onto MHA, and incubating for 18-24 hours at 37 °C.

Time-kills against stationary phase SH1000, were performed as described, except overnight cultures were centrifuged at 5500 rpm for 10 minutes at 37 °C and resuspended in supernatant to OD\(_{600}\) of 0.2 before being exposed to antibacterial agents. Persister time-kills were performed according to previously described methodology (Keren et al., 2004, Wiuff et al., 2005). Briefly, bacterial cultures were incubated to early exponential phase (OD\(_{600}\) of 0.2). To half of the culture 10 x MIC ciprofloxacin was added, and to the other, 10 x MIC ampicillin was added and cultures were incubated at 37 °C for 24 hours. After 24 hours, remaining viable cells were collected by centrifugation, washed, and resuspended to the same volume in MHB and exposed
to antibacterial agents at 37 °C. Aliquots of culture were taken regularly over 24 hours for determination of viable counts.

2.5 Identification of antibiotic adjuvants from chemical libraries

Three compound libraries of biologically active small molecules were evaluated for antibiotic adjuvants; the NIH Clinical Collection (727 compounds), Tocriscreen Total collection (1120 compounds), and the Spectrum Collection (2320 compounds). All library compounds were supplied pre-dissolved in DMSO at a concentration of 10 mM. Duplicates of each library were made at 1 mM and individually screened in combination with clinically available antibiotics ciprofloxacin or rifampicin against *S. aureus* SH1000 biofilms, or rifampicin, linezolid or fusidic acid against planktonic cultures of *E. coli* 1411. All screens were performed in 96-well plates and library compounds were tested at 10 µM.

*S. aureus* biofilms were grown in 96-well plates as described in section 2.3. After 24 hours growth, biofilms were washed once in saline solution and exposed to 198 µL of MHB containing either 50 µg/mL ciprofloxacin or rifampicin, before 2 µL aliquots of library compounds were added to a final concentration of 10 µM and incubated for 24 hours at 37 °C. Biofilms were washed once in saline solution and stained with 200 µL 10 % crystal violet solution for 30 minutes. Biofilms were washed twice more in deionised water and antibiofilm activity was determined as loss or eradication of the biofilm visualised by crystal violet staining.

Planktonic cultures of *E. coli* were tested according to CLSI guidelines for broth MICs (section 2.3) with the exception that antibacterial agent rifampicin was tested at fixed concentration 0.5 µg/mL, whilst linezolid and fusidic acid were tested at 50 µg/mL. As before, library compounds were tested at a final concentration of 10 µM. Activity was identified in the same way as broth MIC’s; inhibition of bacterial growth.
2.6 Identification of synergistic interactions

Combinations of antibacterial agents were assessed for synergistic interactions against planktonic cultures of *E. coli*, following the checkerboard methodology (Pillai et al., 2005). The following equation was used to calculate the fractional inhibitory concentration (FIC) index:

\[
\text{FIC index} = \left(\frac{\text{lowest concentration A}}{\text{MIC A}}\right) + \left(\frac{\text{lowest concentration B}}{\text{MIC B}}\right)
\]

A FIC index of <0.5 is considered a synergistic interaction, an index of 1 is taken to indicate an additive interaction, whilst ≥2 is an antagonistic interaction.

2.7 Antibacterial mode of action studies

2.7.1 Macromolecular synthesis assay

Alterations in macromolecular synthesis as a consequence of exposure to Tocris 2611 and comparator agents was investigated by quantifying incorporation of radiolabelled precursors into DNA ([methyl-\(^3\)H]thymidine), RNA ([5,6-\(^3\)H]uridine) and protein (L-[\(^5\)G-\(^3\)H]glutamine), in early exponential-phase cultures of *S. aureus* SH1000 in MHB (culture absorbance 0.2 units at 600 nm; \(\sim 10^8\) cfu/mL) (Cherrington et al., 1990). At culture OD, precursors were added to 1\(\mu\)Ci/ml and incubated at 37 °C. After incubation for 10 minutes, 100 \(\mu\)L of each radiolabeled culture was mixed with 100 \(\mu\)L of ice-cold 10 % trichloroacetic acid (TCA) and stored on ice. Remaining cultures were treated with 4 x MIC antibacterial agents. After 10 minutes incubation, 100 \(\mu\)L of the cultures were mixed with an equal amount of 10 % TCA and kept on ice for 30 minutes. TCA precipitates were collected under vacuum using UniFilter GF/B plates (Perkin-Elmer), and filters onto which [5,6-\(^3\)H]uridine-labelled samples had been deposited were washed twice with 100 \(\mu\)L unlabeled uridine. Individual filters were then washed twice with 200 \(\mu\)L of 10 % TCA, and twice with 200 \(\mu\)L of acetic acid (Hilliard et al., 1999). Filter plates were dried, and 25 \(\mu\)L scintillant (Microscint 20, Perking-Elmer) was added to each well, and radioactivity was measured using a Chameleon multilabel plate scintillation counter (Hidex).
2.7.2 BacLight™ assay

The BacLight™ assay was used to measure staphylococcal membrane integrity following exposure to antimicrobial agents at 4 x MIC. *S. aureus* SH1000 was grown to OD$_{600}$ 0.5-0.6 (Hilliard et al., 1999, Ooi et al., 2013). Cells were harvested, washed in sterile deionised water and resuspended to double the original volume. Compounds were added at 4 x MIC, with the exception of SDS which was used as a 5 % (w/v) solution, and incubated at 37 °C for 10 minutes. Cells were washed twice in sterile deionised water and resuspended to equal volumes. 50 µL of suspensions were dispensed to individual wells of a black 96-well plate (Greiner Bio-One). In the dark, 150 µL of BacLight™ reagent was added to the wells and incubated for 15 minutes at room temperature. Red (emission 620-650 nm) and green (emission 510-540 nm) fluorescence were measured using a FLUOstar Omega (BMG Labtech) plate reader (excitation wavelength of 470nm). The red : green fluorescence ratio was calculated and percentage membrane integrity relative to cells treated with 5% (w/v) SDS was calculated. If membrane integrity was <70%, compounds were considered membrane damaging.

2.7.3 DiSC$_3$(5) assay

The membrane potential of staphylococci exposed to antimicrobial agents was determined using the fluorescent dye DiSC$_3$(5) as previously described (Hobbs et al., 2008). Cultures of SH1000 cells were grown to OD$_{600}$ 0.2, washed twice, and resuspended in 5 mM HEPES and 5 mM glucose buffer (pH 7.2). In the absence of light, 100 mM KCl and 2 µM DiSC$_3$(5) were incubated with cells at 37 °C for 30 minutes to allow the dye to be taken up. Cultures were then exposed to antibacterial agents at 4x MIC. At necessary time points, samples were taken and processed as follows; centrifugation to collect cells, followed by 1 mL of supernatant added to 1 mL DMSO. A further 1 mL DMSO was added to the remaining pellet to lyse the cells. After 10 minutes incubation, 1 mL of lysed sample was mixed with 1 mL of HEPES/glucose buffer (pH 7.2). Extracellular and intracellular fluorescence were read on the LS 45 luminescence spectrometer (Perkin-Elmer) at an excitation of 622 nm and an emission of 670 nm. The Nernst equation (Silverman et al., 2001) was used to calculate membrane potential;
\[ \Delta = -\frac{RT}{F} \ln \left( \frac{\text{Disc}_5(5)_{\text{inside}}}{\text{Disc}_5(5)_{\text{outside}}} \right) \]

\( \Delta \) is the membrane potential, \( R \) is the universal gas constant (8.3144598 J mol\(^{-1}\) K\(^{-1}\)), \( T \) is the absolute temperature and \( F \) is the Faraday constant (96485.33289 Coulomb mol\(^{-1}\)).

### 2.7.4 Liposome integrity

Carboxyfluorescein (CF) loaded liposomes matching the lipid composition of staphylococcal (60% [wt/wt] phosphatidylglycerol, 40% cardiolipin) or mammalian (50% phosphatic acid [wt/wt], 50% phosphatidylcholine) cytoplasmic membranes were prepared and tested as previously described (Randall et al., 2013a, StGelais et al., 2007). Briefly, lipid mix was pre-dried using non-oxygen gas Argon, and further dried for two hours under vacuum. The dried lipid mix was resuspended at room temperature and vortexed thoroughly. Homogenised lipids were then extruded at 37°C and ultracentrifuged at 100000g for 15min (three times). The following equation was used to determine liposomes were intact and measure liposome concentration (mM) at OD\textsubscript{570}:

\[
\frac{2.75\text{mM (average lipid molarity)}}{\text{OD}^{570} \text{ pre extrusion sample} \times \text{ dilution (1:10)} \times \text{OD}^{570} \text{ liposomes}}
\]

For the assay itself, 50 µM of liposomes were treated with compounds at 4 x MIC and leakage of carboxyfluorescein was monitored over 3 hours by measuring fluorescence. Percent liposome integrity was calculated relative to the positive control, 0.5% Triton X-100. Fluorescence was measured at excitation 485 nm and emission 520 m, in fluorescence microtitre plates (Greiner Bio-One) using the FLUOstar Omega (BMG Labtech).
2.7.5 Haemolysis assay

Haemolysis of equine erythrocytes was measured to evaluate the ability of compounds to damage mammalian membranes (Ooi et al., 2013, Oliva et al., 2003). Compounds were tested at 4 x MIC. Whole blood containing lithium heparin was centrifuged at 1000 x g for 10 minutes, at 4 °C. The supernatant was removed from the buffy coat and erythrocytes were washed three times in 1/20 10 mM Tris-HCl, 0.9% NaCl, pH 7.4 (kept at 4 °C). Erythrocytes were diluted 1/25 in buffer and before use were incubated at 37 °C for 15 minutes. Compounds and erythrocytes were incubated for 1 hour at 37 °C before being centrifuged at 3000 x g for 5 minutes. Haemolysis was measured at OD₅₄₀ and expressed as a percentage relative to the positive control (5 % SDS).

2.7.6 Selection of Tocris 2611 resistant mutants

Mutational resistance of S. aureus to Tocris 2611 and control agent rifampicin was initially determined by assessing spontaneous mutation frequencies (Ryder et al., 2012, O'Neil et al., 2001). Overnight cultures of S. aureus SH1000 were plated onto MHA containing 4 x MIC of antibacterial agent and incubated for 48 hours at 37 °C. Mutation frequencies were determined as the number of drug resistant colonies as a percentage of the total population determined on drug-free MHA.

Exposure to Tocris 2611 did not cause resistance from spontaneous mutation. Therefore, an alternative protocol for resistance selection was used; the extended gradient MIC (Randall et al., 2013b, Friedman et al., 2006). Cultures of SH1000 were continuously exposed to an extended concentration range of Tocris 2611 and control agent daptomycin over 25 passages. Methodology was carried out in accordance with the methodology for broth MICs (Section 2.3), with the exception that cultures were exposed to a wider range of concentrations within the dilution series. Each new passage was inoculated by the cells which grew at the highest concentration of antibacterial agent in the preceding passage.
2.8 Quantification of biofilm material

To determine the effect of Tocris 2611 on biofilm structure, the proportion of matrix and cells present after 6 or 24 hours exposure to Tocris 2611 was quantified. Biofilms were grown and exposed to antibacterial agents using the 96-well plate format (Section 2.3). Proteinase K was added at 100 µg/mL in 20 mM tris and 100 mM NaCl, pH 7.5. Tocris 2611 was tested at 0.5, 8 and 64 µg/mL (4 x MIC, CBD MBEC, and well MBEC, respectively). After 6 or 24 hours biofilms were washed in deionised water and stained in the dark for 30 minutes with SYPRO® Ruby containing 0.167 µM SYTO® 9. Biofilms were washed again in deionised water and fluorescence was read using the FLUOstar Omega (BMG Labtech) at an excitation of 480 nm and emission of 620 nm (matrix) and 520 nm (cells). Red : green fluorescence ratios were calculated to determine matrix : cell ratio.

2.9 Analysis of surface immobilised antibiofilm molecules

2.9.1 In vitro activity analysis of antibiofilm compounds immobilised on smooth titanium surfaces

P. aeruginosa PA14, E. coli TG1 and S. aureus (ATCC6538 or SH1000) were cultured overnight in LBB at 37 °C. Simultaneously, smooth titanium disks were incubated overnight in foetal bovine serum at 37 °C. Disks were washed once in PBS and transferred to wells of a 96-well plate. 200 µL of overnight culture of P. aeruginosa or E. coli diluted 1/100 in 1/20 TSB, or S. aureus diluted 1/100 in LBB were added to disks and incubated for 24 hours at 37 °C. Disks were individually washed once in PBS and transferred to a fresh 1 mL PBS, before being vigorously vortexed for 1 minute, sonicated for 10 minutes and vortexed again for 1 minute to remove adherent biofilms. Serial dilutions in PBS were then made to quantify biofilm formation on the disks, before plating onto MHA and incubating for 18-24 hours at 37 °C. Biofilm inhibition was calculated by enumerating colonies and expressing as a percentage of the negative control (compound free disks).
2.9.2 In vitro activity analysis of antibiofilm compounds immobilised on open porous titanium surfaces

Antibiofilm activity of antibacterial compounds immobilised to open porous titanium disks were analysed using the same methodology described in section 2.9.2 with the following exceptions; instead of using a 96-well plate, disks were wrapped in parafilm and silicone tubing (VWR International) leaving only the drug coated side of the disk exposed. Parafilm and silicone tubing were previously sterilized using 70 % ethanol. Overnight bacterial cultures were diluted 1/10000 in 1/20 TSB and 200 µL was added to the surface of the disk, before being incubated for 24 hours at 37 °C. In sterile conditions, disks were removed from the parafilm and silicone tubing before being washed, vortexed and enumerated as before (Section 2.9.2).

2.10 DNA manipulation

2.10.1 Genomic DNA extraction

Preparation of high-purity genomic DNA from S. aureus was carried out using the PurElute™ bacterial genomic kit (EdgeBio, Maryland, USA) following manufacturer’s instructions, with the following additions; spheroplast buffer was supplemented with 100 µg/ml lysostaphin and cells incubated for 60 minutes at 37 °C, and a 15 minute incubation with 100 µg/ml of proteinase K at 37 °C was included before the addition of Advamax 2 beads. Whole genome sequencing and bioinformatic analysis was carried out by the Leeds Institute of Molecular Medicine, University of Leeds.

2.10.2 Polymerase chain reaction (PCR)

Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Massachusetts, USA) was used to amplify genomic DNA according to manufacturer’s instructions. All primers were synthesised by Eurofins MWG Operon and reaction conditions followed those suggested by the manufacturer. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN,
Manchester, UK) according to manufacturer’s instructions and sequenced by Beckman Coulter Genomics (Essex, UK). Oligonucleotide primers are listed in Appendix A.

2.10.3 Agarose gel electrophoresis

Amplified PCR products were visualised by agarose gel electrophoresis (Sambrook and Russell, 2001). Gels were 0.8% (w/v) agarose dissolved in Tris-acetate-EDTA (TAE) buffer containing 1:10000 SYBR® safe gel stain (Invitrogen, ThermoFisher Scientific, Massachusetts, USA). To determine the molecular weight of the analysed PCR product, either DNA Hyperladder I or II (Bioline Reagents) were run alongside the sample. A potential difference of 90 V was applied across the gel for 30 minutes. DNA was visualised using a Syngene, Genegnious Bioimaging gel documentation system.

2.10.4 DNA quantification

To determine DNA concentration, the absorbance of samples was read using Nanophotometer® (Geneflow, Lichfield, UK), at 260 nm. The ratio of absorbance values at 260 nm : 230 nm and 260 nm : 280 nm reported on sample purity. A 1.8 ratio reflected a pure sample (Desjardins and Conklin, 2010).
Chapter 3

Development of antibiofilm compounds for medical implants

3.1 Abstract

Biofilm associated infections are responsible for \(\sim 25\%\) of medical device implant (MDI) failures. The most recent generation of MDIs with open porosity facilitate osseointegration, but also present increased risk of biofilm formation as bacteria preferentially adhere to surfaces such as these. This work therefore aimed to identify and characterise five novel ABMs, for development as dental and orthopaedic implants coatings. The activity and potential toxicity of 56 novel ABMs was assessed and the best five were identified: 4-45, CIM008405, P1a-PEP1, P2-5 and Tocris 2611. P1a-PEP1 and P2-5 were most effective against fungal pathogens, whilst 4-45, CIM008405 and Tocris 2611 exhibited more potent activity against bacterial pathogens. Tocris 2611 was the only ABM to possess the ability to eradicate preformed staphylococcal biofilms and its MOA is consequently discussed in Chapter 4 instead. Since the preclinical evaluation of new drugs includes MOA studies, initial experiments investigated whether the antibacterial activity of the ABMs was through inhibition of one of the major macromolecular synthesis pathways (DNA, RNA or protein). None of the four ABMs (4-45, CIM008405, P1a-PEP1, P2-5) caused preferential inhibition any of the specific pathways tested, instead targeting the bacterial membrane, resulting in damage and loss of potential. All four ABMs caused destruction of staphylococcal and mammalian liposomes, as well as complete or near complete lysis of equine erythrocytes, indicating a direct interaction with the phospholipid bilayer, and a lack of prokaryotic specificity. \textit{In vitro} testing of the five ABMs attached to smooth and open porous surfaces impacted biofilm formation, but had no effect on biofilm formation \textit{in vivo}. Despite this, work presented here successfully developed a useful approach to implant coatings.
3.2 Introduction

Biofilm formation is estimated to be involved in 80% of all bacterial infections in humans and biofilm-related corrective surgery costs in Europe are estimated at €15,000 per patient and €800 million per year (Davies, 2003, Fux et al., 2005). Medical implants that require rapid osseointegration, such as joint prostheses, have been developed to include porous surfaces, which further increases the risk of biofilm related infections due to enhanced bacterial attachment. Alternative treatment approaches to prevention are therefore crucial. One such alternative approach is the development of antibiofilm surfaces to cover indwelling medical devices. To reduce biofilm-associated infections on implants, biocidal coatings can be applied based on the use of metal ions like silver, which is toxic when accumulated, or the release of conventional antibiotics to prevent infection at the site of the implant. A serious concern regarding such continuous antibiotic pressure however is an increased incidence of clinical drug resistance, such as observed for the methicillin-resistant Staphylococcus aureus (MRSA). In this respect, the development of antibiotic resistant infections can lead to devastating effects in the absence of any valid medical treatment to control the infection, and has become a serious public health problem. Another important challenge of biocidal implant coatings is to achieve antimicrobial activity without impairing osseointegration caused by general cytotoxicity.

It is therefore important to elucidate the mechanism of action of compounds for clinical applications such as these. To determine a compound’s MOA there are a wide variety of experiments that can be performed. For the work described in this chapter, studies to identify an agents cellular target were initially performed, by measuring alterations in macromolecular synthesis (MMS) pathways (DNA, RNA and protein) as a consequence of exposure to the compound. Although not performed in the following sections, initial MOA studies could also include, but are not limited to, bacterial cytological profiling and the use of whole-cell B. subtilis biosensors.

This work therefore aims to develop antibiofilm coatings on porous titanium layers coated implant material using novel small molecules and peptides with inhibitory activity against
microbial biofilms, which are associated with partners of this project (COATIM). Research focuses on implant coatings that do not release the antibiofilm compounds, representing longer-lasting antibiofilm and topical activity. In addition, studies will assess the effects of the ABMs on osseointegration, as well as characterise the MOA of the top five ABMs.

3.3 Aims and objectives

Work described in this chapter was intended to identify five novel antibiofilm molecules (ABMs), elucidate their mode of action (MOA) against S. aureus and assess their ability to prevent biofilm formation when immobilised to a titanium surface. This work was performed as part of an EU consortium, collectively known as COATIM. COATIM involves the collaboration of four SME’s, one company, and three universities aiming to develop the next generation of antibiofilm coatings for medical implants.

3.3 Results and Discussion

3.3.1 Identification of the five top novel antibiofilm molecules

In COATIM, 56 ABMs (identified in previous drug screening programs conducted by the partners) were assessed in order to determine the best five. These molecules were selected for inhibitory activity against bacterial and/or fungal biofilms (either inhibiting their formation or eradicating biofilms including persisters). The five ABMs were selected based on their ability to inhibit biofilm formation of different bacterial and fungal species, toxicity against nematodes, and toxicity profiles for different human primary cell types, which are relevant for implant fixation. In parallel, a biofilm mouse colonization model was developed and the mode of action of these ABMs was unraveled. Finally, the ABM-coated implants were evaluated for in vitro and in vivo activity in resisting microbial infection without compromising osseointegration. The work presented here, performed as a member of the COATIM consortium, focuses on
characterization of the five best ABMs, in the Gram-positive organism *Staphylococcus aureus*; the leading cause of post-operative infections.

56 ABMs screened for activity against biofilms of *S. epidermidis, S. aureus, S. mutans, P. aeruginosa, C. albicans, E. coli* and *S. typhimurium.*

56 ABMs tested for non-toxicity of the multicellular nematode worm *C. elegans.*

Based on these data, the following 25 ABMs were selected for further testing:

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Antibacterial agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 8339</td>
<td>- 202611</td>
</tr>
<tr>
<td>- 27458</td>
<td>- 4-49</td>
</tr>
<tr>
<td>- 42170</td>
<td>- 4-32</td>
</tr>
<tr>
<td>- P2-1</td>
<td>- 4-122</td>
</tr>
<tr>
<td>- P2-5</td>
<td>- 5-21</td>
</tr>
<tr>
<td>- P2-8</td>
<td>- 5-59</td>
</tr>
<tr>
<td>- P2-14</td>
<td>- 5-102</td>
</tr>
<tr>
<td>- P1a-PEP1</td>
<td>- P1a-CIM02</td>
</tr>
<tr>
<td>- OSIP10</td>
<td>- P1a-PEP7</td>
</tr>
<tr>
<td></td>
<td>- CIM006387</td>
</tr>
<tr>
<td></td>
<td>- CIM006387</td>
</tr>
<tr>
<td></td>
<td>- CIM007844</td>
</tr>
<tr>
<td></td>
<td>- CIM008405</td>
</tr>
<tr>
<td></td>
<td>- CIM003592</td>
</tr>
<tr>
<td></td>
<td>- BCF-AA022</td>
</tr>
<tr>
<td></td>
<td>- BCF-AA045</td>
</tr>
</tbody>
</table>

The ability of the 25 ABMs to prevent biofilm formation of *P. gingivalis* and polymicrobial biofilms.

The effects of the ABMs on cell viability, and functional behaviour (differentiation potential) were tested, to determine that they are not cytotoxic for bone tissue and do not negatively effect osseointegrative potential of the implant.

The 25 ABMs were tested for their remaining activity upon gamma-irradiation (the technique which will be used to sterilize the implants).

Based on these results, the following 5 ABMs were selected:

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Antibacterial agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>- P2-5</td>
<td>- 202611</td>
</tr>
<tr>
<td>- P1a-PEP1</td>
<td>- 4-45</td>
</tr>
<tr>
<td></td>
<td>- CIM008405</td>
</tr>
</tbody>
</table>

Mode of action studies of the top 5 ABMs (including propensity of microbials to develop resistance to the agents).

*In vitro* activity of ABM-coated smooth and open porous disks.

*In vivo* activity of ABM-coated smooth and open porous disks.

All 5 ABMs are found to be membrane damaging agents.

ABMs coated on smooth disks exhibited stronger antimicrobial activity than on open porous disks.

None of the ABMs exhibited *in vivo* antibiofilm activity.


Figure 3.1 A selection of 56 antibiofilm molecules (ABMs) were screened for antibacterial and antifungal activity, as well as cytotoxicity. Based on these results, the top five ABMs were grafted on small titanium implant substrates, as a model for dental and orthopaedic implants. Next, the ABM-coated implants were evaluated for in vitro and in vivo activity in resisting microbial infection without compromising osseointegration. In parallel, the antibiofilm mode of action of the ABMs was unraveled. This work aimed to develop the next generation of implant coatings containing novel potent proprietary antibiofilm molecules (ABMs) with inhibitory activity against microbial biofilms.

3.3.1.2 Susceptibility of monospecies staphylococcal strains to novel antibiofilm molecules

The susceptibility of three different S. aureus strains were used: SH1000, UAMS-1 and USA300, and one strain of S. epidermidis: RP62A, was determined against the 56 novel antimicrobial agents (Appendix B). SH1000 is a strain extensively studied because it forms robust biofilms in vitro (Geoghegan et al., 2010). It has a repaired defect in rsbU, which encodes a positive regulator of the alternative sigma factor Sigma(B), which positively increases biofilm formation (Jonsson et al., 2004). UAMS-1 is a widely used MSSA strain that was isolated from an osteomyelitic patient, and is a prolific biofilm former (Gillaspy et al., 1995). USA300, which is the most prevalent community acquired MRSA strain. RP62A, a commonly used reference strain, is also biofilm producing and was isolated during the 1979 to 1980 Memphis, Tennessee, outbreak of catheter-associated sepsis (Christensen et al., 1987). In terms of in vitro biofilm composition, SH1000 and UAMS-1 biofilms are polysaccharide dominant (Beenken et al., 2004), and USA300 is a protein adhesion type (Pozzi., 2012). Whilst biofilm formation in RP62A is PIA-dependent (Izano et al, 2005). All compounds were tested in µM (up to 100 µM) to account for the differences in molecular weight.

Approximately half of compounds tested against planktonic cultures of SH1000, USA300, UAMS-1 or RP62A (22 of 56), exhibited no activity (MICs greater than 100 µM) (Appendix B).
Most compounds (34 of 56) were unable to inhibit the shedding of cells from the biofilm in all four staphylococcal strains, with bMICs greater than 100 µM (Appendix B). Of the remaining compounds tested, MICs and bMICs ranged from 6.25-100 µM, with the exception of Tocris 2611, which exhibited an MIC and bMIC of less than 0.4 µM (Appendix B). By comparison, established antibiotics ciprofloxacin and gentamicin exhibited MICs and bMICs ranging from 6.25-100 µM. With the exception of Tocris 2611, which had MBECs ranging from 6.25 µM to 12.5 µM, none of the compounds (including control agents ciprofloxacin and gentamicin) were able to eradicate pre-formed staphylococcal biofilms (Appendix B).

Although staphylococci are one of the most common causes of biofilm-related infections, it is also important to consider other pathogens frequently associated with these types of infection, such as *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Candida albicans* (*C. albicans*). Furthermore, agents developed as clinical treatments must exhibit selective toxicity against microbial cells. Consequently, all 56 novel ABMs were screened by other members of the COATIM consortium, against *Streptococcus mutans* (*S. mutans*), *Escherichia coli* (*E. coli*), *Salmonella Typhimurium* (*S. Typhimurium*), *P. aeruginosa*, *Porphyromonas gingivalis* (*P. gingivalis*) and *C. albicans*, and their toxicity was assessed against *Caenorhabditis elegans* (*C. elegans*). *C. elegans* is regularly used as toxicity model, since many of the basic physiological processes and stress responses that are observed in higher organisms, such as humans, are conserved in *C. elegans*. Accordingly, 14 of 56 compounds were selected for further testing based on the potency of their antimicrobial activity and non-toxicity for *C. elegans*. These were 4-29, 4-45, 4-122, 5-21, 5-59, 5-102, BS-342, CIM003592, CIM006387, CIM007844, CIM008405, P1a-CIM02, P1a-PEP1 and Tocris 2611.

### 3.3.1.2. Susceptibility of multispecies staphylococcal biofilms to antibiofilm compounds

*In vitro* biofilm research is performed predominantly using single species of microorganisms, despite the fact that the majority of biofilm infections involve polymicrobial communities.
Subsequently, pre-formed polymicrobial staphylococcal biofilms of *S. aureus USA300* and *S. epidermidis* RP62A were exposed to the 14 ABMs that were selected for further testing (Table 3.1). Five of the 14 compounds exhibited no inhibition of biofilm shedding, with bMICs greater than 100 µM. With the exception of Tocris 2611, bMICs for the remaining 9 compounds ranged from 25-100 µM. Tocris 2611 exhibited comparable activity against polymicrobial and monospecies biofilms, with a bMIC of < 0.4 µM, and an MBEC of 6.25 µM (Table 3.1). None of the other 14 compounds were able to eradicate pre-formed polymicrobial *S. aureus* and *S. epidermidis* biofilms, with MBECs > 100 µM (Table 3.1). In addition, control agents ciprofloxacin and gentamicin were also unable to eradicate an established *S. aureus/S. epidermidis* biofilm, with MBECs greater than 100 µM (Table 3.1).

The ability of the 14 novel antibiofilm molecules to prevent formation of other multi-species biofilms was determined by other COATIM partners. Polymicrobial biofilms tested were *C. albicans* and *S. epidermidis, C. albicans* and *S. aureus, C. albicans* and *E. coli,* and *E. coli* and *P. aeruginosa.* Furthermore, the effect of the 14 antibiofilm compounds on the three most relevant cell types represented in bone tissue (osteoblasts, bone marrow derived stem cells and endothelial cells) was also assessed by the COATIM consortium, to determine if the ABMs negatively affect the osseointegrative potential of the implant. Cytotoxicity tests were determined in these cell types since it is important the antibiofilm molecules to not negatively impact the osseointegrative potential of the coated implant, or the surrounding bone tissue.
Table 3.1 Antibiofilm activity of novel antibacterials agents and control agents (ciprofloxacin and gentamicin) against *S. aureus* USA300 and *S. epidermidis* RP62A polymicrobial biofilms.

<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>Biofilm minimum inhibitory concentration (bMIC) (µM)</th>
<th>Minimum biofilm eradication concentration (MBEC) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. aureus</em> USA300 and <em>S. epidermidis</em> RP62A</td>
<td><em>S. aureus</em> USA300 and <em>S. epidermidis</em> RP62A</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4-29</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4-45</td>
<td>25</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4-122</td>
<td>50</td>
<td>&gt;100</td>
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<tr>
<td>5-21</td>
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<td>&gt;100</td>
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<td>5-59</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5-102</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>BS-342</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CIM003592</td>
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<tr>
<td>CIM007844</td>
<td>100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CIM008405</td>
<td>25</td>
<td>&gt;100</td>
</tr>
<tr>
<td>P1a-CIM02</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>P1a-PEP7</td>
<td>50</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Tocris 2611</td>
<td>&lt; 0.4</td>
<td>6.25</td>
</tr>
</tbody>
</table>

Based on these parameters, the five best ABMs were selected from the 14 that underwent further investigation. These were 4-45, CIM008405, P1a-PEP1, P2-5 and Tocris 2611.

However, as Tocris 2611 exhibited eradication activity of preformed staphylococcal biofilms, its MOA will no longer be presented in this chapter, instead it is presented in Chapter 4.
3.3.2 Elucidation of the mode of action of the four antibiofilm molecules

3.3.2.1 Effects of the four antibiofilm molecules on major macromolecular synthesis pathways

Initial investigations to establish the MOA of the four ABMs (4-45, CIM008405, P1a-PEP1 and P2-5) monitored alterations in macromolecular synthesis following exposure to 4 x MIC of compound. This was done by quantifying incorporation of radiolabeled precursors into the macromolecules DNA, RNA and protein (Hobbs et al., 2008, Ooi et al., 2009, Randall et al., 2013b). This method is commonly used as it can provide insights into the MOA, especially since some antibiotics often exhibit activity which is specific to one macromolecular pathway, with little or no effect on other macromolecular biosynthesis pathways (O'Neill and Chopra, 2004, Ooi et al., 2013, Randall et al., 2013b).

Against S. aureus, at 4 x MIC, none of the four ABMs caused preferential inhibition of DNA, RNA or protein synthesis within 10 minutes (Figure 3.1). In comparison, control agents ciprofloxacin, rifampicin and tetracycline specifically inhibit the synthesis of DNA, RNA, and protein, respectively (Figure 3.1). This type of non-specific response is indicative of compounds that have antimicrobial activity against S. aureus by disrupting the cytoplasmic membrane (O'Neill and Chopra, 2004, Ooi et al., 2009, Randall et al., 2013b).
Figure 3.1 Effects of control agents and four ABMs on major macromolecular biosynthesis pathways in *S. aureus* SH1000. Percentage incorporation of $^3$H thymidine, uridine and glutamine into SH1000 DNA, RNA and protein synthesis. Means of at least three independent replicates; error bars show standard error.
3.3.2.2 Effects of the four antibiofilm molecules determined in membrane damaging assays

To further assess whether the MOA of the four ABMs is through disruption of the cytoplasmic membrane, the BacLight™ assay was used to measure integrity of the *S. aureus* membrane. This method measures membrane integrity using two dyes (SYTO® 9 and propidium iodide). Both dyes stain nucleic acid, but propidium iodide can only enter cells which have undergone damage to the membrane, whilst SYTO® 9 is able to enter both damaged and undamaged cells. The ratio of the dyes is therefore directly proportional to the amount of membrane damage as a consequence of exposure to the drug.

*S. aureus* cultures were exposed to 4 x MIC of the antibiofilm molecules and control agents (tetracycline and nisin) for 10 minutes. Tetracycline is known to inhibit protein synthesis, and to have no effect on the bacterial membrane. Nisin is a known membrane damaging compound which forms pores in the membrane (McAuliffe et al., 2001). The four antibiofilm molecules caused a loss in membrane integrity (Table 3.2); compounds 4-45, CIM008405 and P2-5 resulted in complete loss of membrane integrity after 10 minutes, comparable to that observed for membrane damaging compound nisin (Table 3.2). Whilst *S. aureus* cells exposed to P1a-PEP1 and tetracycline, retained 71% and 98 % membrane integrity, respectively (Table 3.2). However, compounds that reduce the membrane integrity by ≥ 25% in this assay, are still considered to exert their antibacterial effects through membrane disruption.

The four antibiofilm molecules were then assessed in a second membrane damaging assay, that utilize the membrane potential-sensitive fluorescent dye DiSC₃(5), and offers a more sensitive measure of membrane perturbation. *S. aureus* cells are hyperpolarised and incubated with DiSC₃(5), causing the dye to concentrate in the membrane and become self-quenching. Subsequent membrane damage causes a loss of membrane potential and release of DiSC₃(5), which is then measured by fluorescence. All compounds were tested at 4 x MIC and nisin and tetracycline were again used as comparator agents.

All four ABMs caused a rapid loss in membrane potential (Table 3.2). Within 1 hour, there was a 100 % reduction in membrane potential of cells exposed to 4-45, CIM008405 and P2-5, a
result comparable to that observed for the known membrane damaging agent, nisin (Table 3.2). The loss of membrane potential caused by P1a-PEP1 was 57% after one hour, a less pronounced effect than that caused by four other ABMs (Table 3.2). Similarly, a less significant effect was observed for P1a-PEP1 in the BacLight™ assay and quantification of macromolecular synthesis pathways, which may indicate that the antimicrobial activity of this compound is time or concentration dependent.

Table 3.2 Effects of the four antibiofilm molecules and comparator agents at 4 x MIC on S. aureus SH1000 cellular membranes. Values are the means of at least three (Randall et al., 2013a) biological replicates (± SD). S. aureus SH1000 cells were treated with antibacterial agents for 10 minutes to determine membrane integrity, and 60 minutes to determine membrane potential.

<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>% S. aureus membrane integrity (10 min)</th>
<th>% S. aureus membrane potential (60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 0</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>4-45</td>
<td>2 ± 1</td>
<td>0 ± 4</td>
</tr>
<tr>
<td>CIM008405</td>
<td>4 ± 3</td>
<td>0 ± 4</td>
</tr>
<tr>
<td>Nisin</td>
<td>5 ± 1</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>P1a-PEP1</td>
<td>71 ± 4</td>
<td>43 ± 4</td>
</tr>
<tr>
<td>P2-5</td>
<td>0 ± 5</td>
<td>0 ± 7</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>98 ± 6</td>
<td>97 ± 6</td>
</tr>
</tbody>
</table>

Taken together, the MMS, BacLight™ and DiSC₃(5) data suggest that all four ABMs exert their antimicrobial activity against S. aureus by disrupting the cell membrane. To further resolve the
MOA, the ability of the four ABMs to compromise the integrity carboxyfluorescein-filled staphylococcal liposomes was assessed. This assay was used to determine if membrane damage was a consequence of direct disruption of the phospholipid bilayer. Staphylococcal liposomes had a composition analogous to the phospholipid bilayer of the *S. aureus* cytoplasmic membrane (approximately 60% [wt/wt] phosphatidylglycerol, 40% cardiolipin) (Randall et al., 2013a). *S. aureus* liposomes (50 µM) were challenged for 10, 60 and 180 min with 4 x MIC ABMs (Table 3.3). The leakage of carboxyfluorescein from the liposomes was monitored and percent liposome integrity was calculated relative to liposomes challenged with 0.5% Triton X-100 (corresponding to 100% liposome damage [0% liposome integrity]). As carboxyfluorescein leaks out of the liposomes, it goes from a quenched to non-quenched state, and is therefore directly proportional to fluorescence. All four ABMs and the known membrane disruptor, SDS, caused damage by targeting the phospholipid component of the membrane. After 10 minutes, a greater than 50% loss of staphylococcal liposome integrity was observed, and ~ 90% or greater after an hour (Table 3.3). Results may therefore indicate that the membrane interaction of 4-45, CIM008405, P2-5 and P1a-PEP1 is surfactant-like, directly disrupting the staphylococcal lipid bilayer.

Antibacterial agents that exert their antibacterial effects through membrane perturbation have often been shown to lack prokaryotic specificity. Subsequently, the ability of the four ABMs to compromise the integrity of carboxyfluorescein-filled liposomes made of a phospholipid content matching that of mammalian cell membranes was assessed, to determine if their effect was specific to prokaryotic phospholipids. Mammalian liposomes were composed of approximately 50% [wt/wt] phosphatidylcholine, 50% phosphatic acid, following the same procedure employed for staphylococcal liposomes. The four ABMs, which caused carboxyfluorescein leakage from staphylococcal liposomes exhibited comparable effects on mammalian liposomes. After 10 minutes a loss of greater than 40% integrity was observed, and 100% integrity was lost after an hour (Table 3.3). Since the ABMs exhibited equivalent damage
to both mammalian and staphylococcal liposomes, it would suggest that these agents have a non-specific interaction with the membrane, targeting both prokaryotic and eukaryotic cells.

**Table 3.3** Effect of the four ABMs (4-45, CIM008405, P1a-PEP1, P2-5) and comparator agents on % *S. aureus* liposomaliposome and % mammalian liposome integrity after 10 minutes, 60 minutes and 180 minutes challenge at 4 x MIC (± SD). Values are the means of at least three biological replicates.

<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>10 minutes</th>
<th>60 minutes</th>
<th>180 minutes</th>
<th>10 minutes</th>
<th>60 minutes</th>
<th>180 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-45</td>
<td>47 ± 9</td>
<td>0 ± 9</td>
<td>0 ± 10</td>
<td>31 ± 10</td>
<td>0 ± 10</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>CIM008405</td>
<td>32 ± 9</td>
<td>12 ± 8</td>
<td>0 ± 10</td>
<td>1 ± 10</td>
<td>0 ± 10</td>
<td>0 ± 7</td>
</tr>
<tr>
<td>P1a-PEP1</td>
<td>12 ± 9</td>
<td>7 ± 10</td>
<td>5 ± 10</td>
<td>37 ± 8</td>
<td>0 ± 9</td>
<td>0 ± 10</td>
</tr>
<tr>
<td>P2-5</td>
<td>0 ± 10</td>
<td>0 ± 9</td>
<td>0 ± 10</td>
<td>0 ± 5</td>
<td>0 ± 10</td>
<td>0 ± 7</td>
</tr>
<tr>
<td>SDS</td>
<td>41 ± 10</td>
<td>0 ± 10</td>
<td>0 ± 9</td>
<td>0 ± 8</td>
<td>0 ± 8</td>
<td>0 ± 7</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>100 ± 4</td>
<td>100 ± 5</td>
<td>99 ± 9</td>
<td>100 ± 3</td>
<td>100 ± 6</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>98 ± 7</td>
<td>98 ± 10</td>
<td>97 ± 9</td>
<td>99 ± 5</td>
<td>99 ± 7</td>
<td>99 ± 5</td>
</tr>
</tbody>
</table>

Since mammalian liposomes represent only the phospholipid component of the membrane, the four ABMs were also tested for their ability to cause haemolysis of mammalian erythrocytes (Table 3.4). Prokaryotic specific agents tetracycline and vancomycin caused ≤3% loss in erythrocyte integrity in one hour (Table 3.4). In comparison, agents that are known not to be prokaryotic specific, such as SDS, induced complete haemolysis of erythrocytes (Table 3.4). The four ABMs (4-45, CIM008405, P1a-PEP1, P2-5) that damaged both staphylococcal and
mammalian liposomes caused complete or near complete lysis of erythrocytes, further suggesting a lack of prokaryotic specificity (Table 3.4).

Table 3.4 Effect of the four ABMs (4-45, CIM008405, P1a-PEP1, P2-5) and comparator agents on erythrocytes at 4 x MIC (± SD). Values are the means of at least three biological replicates.

<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>% Erythrocyte integrity (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>4-45</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>CIM008405</td>
<td>0 ± 8</td>
</tr>
<tr>
<td>Nisin</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>P1a-PEP1</td>
<td>14 ± 10</td>
</tr>
<tr>
<td>P2-5</td>
<td>0 ± 10</td>
</tr>
<tr>
<td>SDS</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>97 ± 8</td>
</tr>
</tbody>
</table>

Results therefore indicate that the antibacterial target of the four ABMs (4-45, CIM008405, P1a-PEP1 and P2-5) is the staphylococcal membrane, specifically the phospholipid bilayer. However, this effect is not specific, causing comparable damage to eukaryotic cells.

3.3.3 Analysis of surface immobilized antibiofilm molecules

To determine if the five ABMs were still active upon covalent binding to smooth and open porous titanium disks, their in vitro activity profile was determined. Originally compound 4-45 was selected as one of the five best ABMs. However, the toxicity profile of this compound was
less favorable, and was therefore replaced by the structural analogue LC0024, which exhibited similar antibiofilm activity as compared to 4-45, but reduced toxicity against OB, MSC and EC cells. Therefore, this compound was used as a replacement of the originally selected 4-45 for in vitro and in vivo activity tests described below. Since each compound exhibited species-specific activity, compound-disk substrates were tested against the bacterial or fungal organism against which they were most active. Titanium disks were 0.5-1 cm wide, and were coated in either smooth or open porous surfaces. Open porous surfaces promote osseointegration on dental and orthopaedic implants, but are also at a higher risk of biofilm associated infections, as a consequence of increased bacterial attachment. Smooth surfaces were therefore also assessed.

The five ABMs (CIM008405, LC0024, P1a-PEP1, P2-5, Tocris 2611) were immobilised onto either smooth or open porous titanium disks. Briefly, titanium surfaces were functionalized with an amino-group by treatment with Fmoc-protected 3-aminopropyl-triethoxy silane, followed by deprotection (Carpino, 1987). Functionalized disks were then placed in a hydrolysis vessel containing a solution (1 mL/disc) of n-heptane/hexamethylene diisocyanate (85:15) for 3 hours at room temperature. Samples were rinsed with n-heptane and placed in a vessel containing compounds dissolved in 100 mL dimethyl sulfoxide. After 16 hours, the disks were rinsed with demineralized, pyrogen-free water and subsequently with acetone, after which disks were allowed to dry (Figure 3.3b). This work was performed by the COATIM partner, Hemoteq (Aachen, Germany).

### 3.3.3.1 In vitro activity of ABM-coated smooth disks

With the exception of Tocris 2611, all other work described in this section was performed by other COATIM members.

To promote biofilm formation, the ABM-coated smooth titanium disks were treated with foetal bovine serum overnight, before being exposed to inoculated media for 24 hours. Biofilm inhibition was calculated as a percentage of the negative control (disks containing no ABM
Compounds P1a-PEP1 and P2-5 were found to be most active against fungal organisms, and therefore disks coated with these ABMs were tested against *C. albicans*. P1a-PEP1 and P2-5 caused a reduction in *C. albicans* biofilm growth relative to the uncoated control of approximately 70 and 85 % respectively (Table 3.5). Compared to control antifungal agent (caspofungin) which reduced biofilm growth by greater than 99 % (Table 3.5). However, no P1a-PEP1 could be detected upon HMDI coating. As such, it is unclear if the observed antibiofilm activity is due to P1a-pep1 concentrations below the minimal detection limit or due to the coating procedure. No further studies were therefore performed with P1a-PEP1. ABMs LC0024 and Tocris 2611 reduced biofilm growth by approximately 25-35 % in *S. aureus* ATCC6538 and *S. aureus* SH1000 respectively. Antibacterial ABM CIM008405 (KU Leuven) exhibited no prevention in *P. aeruginosa* PA14 biofilm formation relative to the uncoated control (Table 3.5), whilst control antibacterial agent vancomycin reduced the formation of *S. aureus* ATCC6538 biofilms by 87 % (Table 3.5).
Table 3.5 Effects of smooth titanium surface immobilised ABMs (CIM008405, LC0024, P1a-PEP1, P2-5, Tocris 2611) and comparator agents on biofilm formation. ND – not determined.

<table>
<thead>
<tr>
<th>Compound on smooth titanium disk</th>
<th>Test strain</th>
<th>Concentration (pmol/cm²)</th>
<th>% Inhibition of biofilm growth after 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspofungin</td>
<td>C. albicans SC5314</td>
<td>2191.4</td>
<td>&gt; 99.5</td>
</tr>
<tr>
<td>CIM008405</td>
<td>P. aeruginosa PA14</td>
<td>54.8</td>
<td>0</td>
</tr>
<tr>
<td>LC0024</td>
<td>S. aureus ATCC6538</td>
<td>ND</td>
<td>25</td>
</tr>
<tr>
<td>P1a-PEP1</td>
<td>C. albicans SC5314</td>
<td>0</td>
<td>69</td>
</tr>
<tr>
<td>P2-5</td>
<td>C. albicans SC5314</td>
<td>315.2</td>
<td>&gt; 85</td>
</tr>
<tr>
<td>Tocris 2611</td>
<td>S. aureus SH1000</td>
<td>104.3</td>
<td>34</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>S. aureus ATCC6538</td>
<td>0/35.2</td>
<td>87</td>
</tr>
</tbody>
</table>

3.3.3.2 In vitro activity profile of ABM coated open porous disks

With the exception of Tocris 2611, all other work described in this section was performed by other COATIM members.

Experiments to determine the in vitro activity of the ABM coated open porous disks were equivalent to those used for the smooth disks, with the exception that inhibition of biofilm growth was determined for the ABM coated side of the disks only (as opposed to the whole disk, including both ABM coated and uncoated surfaces) and a lower inoculum was used for bacterial cultures. These changes were implemented due to the inoculum used for tests involving smooth titanium disks being significantly higher than that exhibited in vivo, and the
consideration that there might be an increase in activity observed if the non ABM-coated surfaces of disks are excluded.

Immobilised onto an open porous titanium surface, antifungal ABM P2-5 (KU Leuven) was unable to prevent the formation of *C. albicans* SC5314 biofilms relative to the uncoated control (Table 3.6). Antifungal control agent caspofungin also exhibited poor biofilm prevention activity against *C. albicans* SC5314 biofilms, with only 6% inhibition of biofilm growth relative to the uncoated control (Table 3.6). Similarly, ABM CIM008405 (KU Leuven) also displayed poor biofilm prevention activity, preventing the formation of *P. aeruginosa* PA14 biofilms by 13% (Table 3.6). Antibacterial ABM LC0024 and control agent vancomycin had comparable activity, preventing *S. aureus* ATCC6538 biofilm formation by 29% (Table 3.6) (KU Leuven). The reduction in antibiofilm activity observed on open porous surfaces may be due to their being favourable for biofilm formation as a consequence of increased bacterial adherence to the irregularity of the surface.

On open porous surfaces, ABM Tocris 2611 exhibited the most potent antibiofilm activity, preventing the formation of *S. aureus* SH1000 biofilms by 53% relative to the uncoated control. Although Tocris 2611 exhibited improved biofilm prevention activity on the open porous surface, this could be due to the increased concentration of surface bound Tocris 2611 achieved, compared with that on the smooth titanium disks (approximately 10 x as much).
Table 3.6 Effects of five ABMs (CIM008405, LC0024, P1a-PEP1, P2-5, tocris 2611) and comparator agents on biofilm formation when immobilised to an open porous titanium surface.

<table>
<thead>
<tr>
<th>Compound on open porous titanium disk</th>
<th>Test strain</th>
<th>Concentration (pmol/cm²)</th>
<th>% Inhibition of biofilm growth after 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspofungin</td>
<td><em>C. albicans</em> SC5314</td>
<td>5468.26</td>
<td>6</td>
</tr>
<tr>
<td>CIM008405</td>
<td><em>P. aeruginosa</em> PA14</td>
<td>390.05</td>
<td>13</td>
</tr>
<tr>
<td>LC0024</td>
<td><em>S. aureus</em> ATCC6538</td>
<td>ND</td>
<td>29</td>
</tr>
<tr>
<td>P2-5</td>
<td><em>C. albicans</em> SC5314</td>
<td>2126.11</td>
<td>1</td>
</tr>
<tr>
<td>Tocris 2611</td>
<td><em>S. aureus</em> SH1000</td>
<td>1011.38</td>
<td>53</td>
</tr>
<tr>
<td>Vancomycin</td>
<td><em>S. aureus</em> ATCC6538</td>
<td>169.87</td>
<td>29</td>
</tr>
</tbody>
</table>

3.3.4 *In vivo* activity profile of ABM coated smooth and open porous disks

All work described in this section was carried out by other COATIM members.

As part of the development of the ABM coated surfaces for medical implants, the titanium substrates were tested using a subcutaneous *in vivo* biofilm model system in mice. Experiments were performed using smooth titanium disks only, due to several problems with the open porous surfaces, such as the animals exhibiting clear signs of pain and difficulties in removing the disks. The antibiofilm activity of the antibacterial ABMs (CIM008405, LC0024, Tocris 2611, vancomycin) was assessed against *S. aureus* SH1000, whilst *C. albicans* SC5314 was used to test antifungal ABM, caspofungin. P2-5 did not undergo *in vivo* assessment, due to poor *in vitro* activity. 24 hours after implantation of the ABM-coated disks, *S. aureus* or *C. albicans* were
injected subcutaneously at $1 \times 10^7$ and $1 \times 10^8$ cells respectively. Mice infected with *S. aureus* were then sacrificed 48 hours post injection, whilst mice infected with *C. albicans* were sacrificed 96 hours after infection. Following animal sacrifice, disks were removed and percentage biofilm inhibition relative to the uncoated control was determined.

Against *S. aureus* SH1000 biofilms CIM008405, LC0024 and Tocris 2611 did not influence *in vivo* biofilm development. However, in comparison to the control (non-coated) disk, vancomycin-coated disks resulted in a statistically significant ($p<0.05$) reduction in biofilm formation. Titanium disks coated in antifungal control agent, caspofungin, also significantly prevented ($p<0.05$) *in vivo* biofilm development, of *C. albicans*, compared to the uncoated control.

### 3.4 Conclusions

The five best ABMs (4-45, CIM0008405, P1a-PEP1, P2-5 and Tocris 2611) were identified from a panel of 56, based on their spectrum of activity and toxicity profiles against *C. elegans* and the three most relevant bone tissue cell types; osteoblasts, bone marrow derived stem cells and endothelial cells. P1a-PEP1 and P2-5 were most active against fungal pathogens such as *C. albicans*, whilst 4-45, CIM008405 and Tocris 2611 target bacterial pathogens such as *S. aureus* and *P. aeruginosa*. The MOA of 4-45, CIM008405, P1a-PEP1 and P2-5 was determined in *S. aureus* SH1000 cultures. None of the four ABMs caused preferential inhibition of DNA, RNA or protein synthesis, but all caused loss of membrane integrity and membrane potential. These findings clearly indicate that all of the compounds exert their antibacterial effects through membrane perturbation. The ABMs also caused destruction of both staphylococcal and mammalian liposomes and complete or near complete lysis of equine erythrocytes, indicating a direct effect of these compounds on the phospholipid bilayer, and a lack of prokaryotic specificity. It can therefore be considered that the phospholipid bilayer of the cytoplasmic membrane in *S. aureus* is the cellular target. All agents tested exhibited more promising biofilm prevention activity when immobilised to smooth titanium surface, then when immobilised to
an open porous titanium surface. However, smooth and open porous disks were assessed following alternate methodology. With exception of control compounds vancomycin and caspofungin, none of the ABMs exhibited any effect on biofilm formation *in vivo*. However, this work provides a novel approach for the coating medical implants, to which other, more successful antibiofilm compounds could be applied.
Chapter 4

Anti-staphylococcal activity and mechanism of action of Tocris 2611

4.1 Abstract

In search of novel antibiofilm molecules, research performed by the COATIM project identified compound Tocris 2611, which exhibited potent antibacterial activity against staphylococci, including staphylococcal biofilms. Consequently, Tocris 2611 may have potential for use in the healthcare setting as an anti-staphylococcal agent. As part of the development of novel antibacterial agents, pre-clinical assessment must involve elucidation of the mechanism of action. Investigations therefore attempted to identify the antibacterial target of Tocris 2611 against the Gram-positive pathogen *Staphylococcus aureus*. Initial studies revealed that Tocris 2611 causes simultaneous and non-preferential inhibition of DNA, RNA and protein biosynthesis, a signature often seen for compounds that damage the bacterial membrane. Further investigations demonstrated that Tocris 2611 caused a substantial loss of membrane integrity and complete loss of membrane potential, but not as a consequence of direct interaction with the phospholipid component of the cell membrane. Subsequently, Tocris 2611 was shown to exhibit potent bactericidal activity against staphylococci independent of their growth state, including cell types ordinarily present in biofilms. These results imply that Tocris 2611 exerts its antibacterial effects through perturbation of the bacterial membrane, enabling eradication of biofilms as a consequence of bacterial killing regardless of growth state. Additionally, low level resistance potential was observed for Tocris 2611, generated only by continuous selection. Results indicate that Tocris 2611 warrants further investigation as a candidate for the treatment of staphylococcal, biofilm-associated infections.
4.2 Introduction

Biofilms provide protection for bacteria from the host immune system and are recalcitrant to antibiotics (particularly due to slow-or non-growing (SONG) bacteria, including persister cells present in biofilms) (Lebeaux et al., 2014, Lewis, 2001). Consequently, currently available antibiofilm therapies are largely ineffective, often making these types of infections untreatable. This is especially problematic in the clinical environment, particularly when associated with indwelling medical devices. Indeed, it is estimated that 15-25% of implant failures are due to infections involving a biofilm component.

Therefore, as discussed in Chapter 3, the COATIM project assessed a number of novel antibiofilm molecules (ABMs) for their ability to prevent biofilm formation when adhered to a titanium surface. Surfaces were representative of those used for implanted medical devices, with the aim of addressing the current issues of implant failure due to infection attributed to biofilm formation. Based on their spectrum of activity and relative toxicity against eukaryotic cells, five ABMs (4-45, CIM008405, P1a-PEP1, P2-5, Tocris 2611) were selected for further evaluation. The phospholipid bilayer of the cytoplasmic membrane was identified as the cellular target for four (4-45, CIM008405, P1a-PEP1, P2-5) ABMs, resulting in membrane perturbation. With the exception of Tocris 2611, none of the ABMs were able to eradicate pre-formed biofilms in vitro. Furthermore, Tocris 2611 was the only ABM to exhibit potent anti-staphylococcal activity, which is a vital characteristic given that staphylococci are the primary cause of hospital-acquired and indwelling medical device infections, frequently involving biofilm formation (Otto, 2008, Weinstein, 2001).

Tocris 2611 was originally identified due to its biological activity against HMC-1 and breast cancer cells, leading to cycle arrest and apoptosis, in addition to its effects on cardiomyocytes, reducing damage caused by myocardial ischemia/reperfusion (Onai et al., 2004, Tanaka et al., 2005, Tanaka et al., 2006). Subsequently, the O’Neill laboratory in collaboration with the Collins group, identified Tocris 2611 as a potential inhibitor of bacterial RNA polymerase (RNAP), using in silico docking (Mariner, 2011). However, further experimentation revealed
that Tocris 2611 was not active against RNAP in vitro (Mariner, 2011), but nevertheless possessed potent anti-staphylococcal activity, and the rarely seen ability to eradicate pre-formed staphylococcal biofilms. A more extensive analysis was therefore required to elucidate the MOA of Tocris 2611 against both planktonic and biofilm communities of S. aureus SH1000.

4.3 Aims and objectives

Work described in this chapter aimed to characterise the mechanism of antibacterial activity of Tocris 2611 against planktonic and biofilm populations of S. aureus. Studies also intended to identify the potential for bacterial resistance to emerge against this novel compound.

4.4 Results and Discussion

4.4.1 Anti-staphylococcal activity of Tocris 2611

The activity of Tocris 2611 was determined against four staphylococcal strains; S. aureus SH1000, USA300, UAMS-1 and S. epidermidis RP62A. Testing different strains of staphylococci is important as they have distinct properties, such as differences in the composition of the biofilm matrix (Jorgensen and Ferraro, 2009, Olson et al., 2002).

Tocris 2611 exhibited activity against the four individual staphylococcal strains comparable to established antimicrobial agents, with an MIC of 0.125 µg/mL. Whilst, in contrast to the majority of current antimicrobial agents, Tocris 2611 also exhibited antibiofilm activity, which was assessed in two separate biofilm models; the Calgary Biofilm Device (CBD) and 96-well microtitre plate. The CBD enables high-throughput screening for preliminary evaluation of compounds, but supports the growth of only low cell density biofilms, which are likely immature. An alternative biofilm model was therefore used to assess the effects of Tocris 2611, where biofilms were grown in the wells of a 96-well plate, achieving a higher cell density. Tocris 2611 eradicated staphylococcal biofilms at 8 µg/mL and 64 µg/mL in the CBD and 96-
well microtitre plate, respectively. Although a reduction in activity was observed against mature biofilms, Tocris 2611 still retained eradication properties. This effect was not seen for any other antimicrobials tested, which were unable to eradicate biofilms on either device at concentrations \( \leq 256 \mu g/mL \). Furthermore, compounds which exhibit activity against established biofilms are predominantly bactericidal, which may indicate that Tocris 2611 possesses bactericidal activity (Otto, 2014).

4.4.2 Elucidation of the mode of action of Tocris 2611 against planktonic *S. aureus*

4.4.2.1 Effects of Tocris 2611 on the major macromolecular synthesis pathways

As discussed in Chapter 3, the antibacterial effect of a compound is often due to specific inhibition of one of the major biosynthetic pathways (e.g. DNA, RNA or protein) (O'Neill and Chopra, 2004). To establish whether Tocris 2611 inhibits one or more of these pathways, macromolecular synthesis was monitored by measuring incorporation of radiolabeled precursors into DNA, RNA, and protein. At 4 x MIC Tocris 2611 caused non-preferential inhibition of DNA, RNA, or protein synthesis within 10 minutes, a signature often seen for compounds that exert their antibacterial effect through perturbation of the bacterial membrane (Figure 4.1) (O'Neill and Chopra, 2004, Ooi et al., 2009). In comparison, ciprofloxacin, rifampicin and tetracycline, which are known to inhibit only DNA, RNA and protein synthesis, respectively, resulted in a specific response (Figure 4.1).
Figure 4.1 Effects of Tocris 2611 and control agents on the relative incorporation of radiolabelled ($^3$H) thymidine, uridine and glutamine into DNA, RNA and protein, respectively. Error bars show standard deviation from the means of at least three independent experiments.
4.4.2.2 Assays to determine if Tocris 2611 targets the staphylococcal membrane

To explore further whether the MOA of Tocris 2611 is through disruption of the cytoplasmic membrane, the BacLight™ and DiSC₃(5) assays were used to measure membrane integrity and measure membrane potential, respectively. In a 10 minute BacLight™ assay, *S. aureus* cells exposed to nisin (a known membrane damaging compound which forms pores in the membrane (Ruhr and Sahl, 1985)) and Tocris 2611, resulted in a membrane integrity of 5% and 45%, respectively (Table 4.1). Tocris 2611 also caused a 100% decrease in membrane potential in 1 hour (Table 4.1). This result is comparable to the surfactant sodium dodecyl sulfate (SDS) which caused 100% loss of membrane potential (Table 4.1). SDS causes membrane perturbation and depolarisation by forming micelles that target the membrane lipids. Results may therefore suggest that the antibacterial MOA of Tocris 2611 is through disruption of the bacterial membrane and subsequent leakage of intracellular components.

Compounds that target the bacterial membrane frequently demonstrate the same effect against mammalian cells, which is an undesirable characteristic for an antimicrobial drug candidate. Tocris 2611 was therefore tested for its ability to cause haemolysis of mammalian erythrocytes at 4 x MIC (Table 4.2). As expected, the prokaryote specific agents tetracycline and vancomycin caused <3% loss in erythrocyte integrity in 1 hour. In comparison, agents which are known to not be prokaryote specific and also cause membrane damage, such as SDS, induced complete haemolysis of erythrocytes. Tocris 2611 resulted in a decrease in erythrocyte integrity of approximately 40%, an effect also demonstrated by the membrane damaging compound nisin (Table 4.2).
Table 4.1 Effect of Tocris 2611 and comparator agents at 4 x MIC on *S. aureus* SH1000 cellular membranes, and erythrocyte integrity. Values are the means of at least three biological replicates (± SD). ND indicated not determined. NDC indicated no drug control.

<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>% <em>S. aureus</em> membrane integrity (+ SD) (10 min)</th>
<th>% <em>S. aureus</em> membrane potential (+ SD) (60 min)</th>
<th>% Erythrocyte integrity (+ SD) (60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDC</td>
<td>100 ± 0</td>
<td>100 ± 5</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Nisin</td>
<td>5 ± 1</td>
<td>0 ± 3</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>SDS</td>
<td>0 ± 0</td>
<td>0 ± 2</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>98 ± 6</td>
<td>97 ± 6</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>Tocris 2611</td>
<td>45 ± 2</td>
<td>0 ± 7</td>
<td>62 ± 7</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>100 ± 4</td>
<td>ND</td>
<td>97 ± 8</td>
</tr>
</tbody>
</table>

As demonstrated in Chapter 3, the antibacterial effects of membrane damaging compounds can be a consequence of direct interaction with the phospholipid bi-layer; therefore the ability of Tocris 2611 to compromise the integrity the phospholipid bi-layer was assessed. Carboxyfluorescein-filled liposomes with a composition analogous to the phospholipid bi-layer of the *S. aureus* CM (approximately 60% [wt/wt] phosphatidylglycerol, 40% cardiolipin) were challenged with 4 x MIC Tocris 2611. Damage was measured at 10, 60 and 180 minutes (Table 4.2). After 180 minutes *S. aureus* liposomes only exhibited a loss of 9% integrity. In contrast, SDS caused a substantial (59%) loss in integrity after only 10 minutes, and completely degraded liposomes (100% loss in integrity) after 60 minutes (Table 4.2). These results strongly suggest that the MOA of Tocris 2611 does not involve targeting the phospholipid component of the cell membrane. In order to investigate whether the previously observed haemolytic effect of Tocris 2611 results from direct interaction of the drug with the mammalian phospholipid bilayer, mammalian liposomes were synthesised and challenged with the Tocris 2611.
liposomes (50 % [wt/wt] phosphatidylcholine, 50% phosphatidic acid) were therefore challenged with 4 x MIC Tocris 2611. No appreciable activity was observed, with a loss of integrity of 3 % after 180 minutes (Table 4.2).

Failure to observe activity against staphylococcal liposomes implies that Tocris 2611 likely exerts its antibacterial effect through interaction with one or more membrane proteins. Therefore, to provide further insight into the membrane component targeted by Tocris 2611, attempts were made to generate staphylococcal proteoliposomes. However, despite some experimental progress, studies could not be included in this work due to time constraints.
Table 4.2 Effect of Tocris 2611 and comparator agents on % *S. aureus* liposome integrity after 10 minutes, 60 minutes, 180 minutes challenge at 4 x MIC (±SD). Values are means of at least three biological replicates. NDC indicates no drug control.

<table>
<thead>
<tr>
<th>Antibacterial Agent</th>
<th>% Staphylococcal integrity (±SD)</th>
<th>% Mammalian liposome integrity (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 minutes</td>
<td>60 minutes</td>
</tr>
<tr>
<td>NDC</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>SDS</td>
<td>41 ± 10</td>
<td>0 ± 10</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>100 ± 4</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Tocris 2611</td>
<td>97 ± 6</td>
<td>92 ± 7</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>98 ± 7</td>
<td>98 ± 10</td>
</tr>
</tbody>
</table>
4.4.3 Antibiofilm activity of Tocris 2611

Tocris 2611 demonstrated potent anti-staphylococcal activity against both planktonic and biofilm cultures, and the MOA against planktonic cultures was determined to be through membrane perturbation. Investigations therefore sought to evaluate the mechanism by which Tocris 2611 eradicates biofilms.

4.4.3.1 Activity of Tocris 2611 against slow or non-growing staphylococcal cells

Compound-mediated eradication of biofilms may occur through two possible mechanisms; disruption of the biofilm matrix or comprehensive killing (sterilisation) of bacteria within the biofilm. Since biofilm communities are highly recalcitrant to antimicrobial therapy due to slow or non-growing (SONG) cells, the ability of Tocris 2611 to kill both these cell types was assessed. Initially, the effect of Tocris 2611 and comparator agents on the viability of exponential and stationary phase cells was determined over a period of 24 hours (Figure 4.2).
Figure 4.2 Killing kinetics of Tocris 2611 at 4 x MIC (0.5 µg/mL), 8 µg/mL (Calgary MBEC) and 64 µg/mL (well MBEC) and comparator agents (daptomycin, tetracycline and XF-73) at 4 x MIC, against exponential phase and early stationary phase cultures of S. aureus SH1000 over 24 hours. Panel (a): exponential phase cultures; Panel (b) stationary phase cultures. Values are the means of at least three biological replicates; error bars show standard deviations.
At 4 x MIC, Tocris 2611 was bacteriostatic against exponentially growing cells, demonstrating a <1 log drop in cell viability over 24 hours, a result comparable to tetracycline (Figure 4.2a). At 8 µg/ml (CBD MBEC), Tocris 2611 became bactericidal against exponentially growing cells, with a log drop in cell viability of >3 over 24 hours, which is comparable to daptomycin (Figure 4.2a). No significant bactericidal activity was observed against stationary phase cells at these concentrations (Figure 4.2b). In contrast, Tocris 2611 at 64 µg/mL (well MBEC) was highly effective at killing both stationary phase and exponentially growing cells, achieving sterilisation after 6 hours (limit of detection 10 cfu/mL), and 3 hours respectively (Figure 4.2a and Figure 4.2b). Whereas, daptomycin was essentially inactive against stationary phase cultures (Figure 4.2b).

Subsequent investigations assessed the activity of Tocris 2611 against populations of persister cells. At 64 µg/mL (well MBEC), exposure to Tocris 2611 resulted in a complete loss of cell viability after 24 hours, an effect not seen by any of the other agents tested, with the exception of XF-73, a potent anti-staphylococcal agent which exerts its antibacterial effect through membrane disruption, and possesses bactericidal activity (Figure 4.3).
Figure 4.3 Effect of Tocris 2611 and comparator agents on the survival of S. aureus SH1000 persister cell cultures isolated by selection with exposure to 10 x MIC ciprofloxacin or 10 x MIC ampicillin. Tocris 2611 was added at 4 x MIC (0.5 µg/mL), 8 µg/mL (Calgary MBEC) and 64 µg/mL (well MBEC), whilst comparator agents were added at 4 x MIC. Values are the means of at least three biological replicates.

4.4.3.2 Effect of Tocris 2611 on biofilm structure

Since it has been demonstrated that Tocris 2611 retains antibacterial activity against SONG cells found in biofilm communities, the antibiofilm activity of Tocris 2611 is most likely through the sterilisation of viable cells within the biofilm. Furthermore, visual assessment of the
biofilm after treatment with biofilm eradicating concentrations of Tocris 2611, followed by staining with 5% crystal violet, suggested that the matrix/superstructure of the biofilm was largely unaffected. This finding was corroborated by quantifying the proportion of matrix and cells after 24 hours exposure to Tocris 2611 and comparator agents (Figure 4.4). Quantitative analysis used the matrix-specific stain SYPRO Ruby, and bacterial cell stain SYTO 9. After 24 hours exposure to biofilm eradicating concentrations of Tocris 2611, the reduction in cell viability was far greater than the effects observed on the matrix, with a log drop in cell viability of twice as much as that in matrix (Figure 4.4). However, there was still a notable loss of matrix material in comparison to untreated biofilms. To determine whether dispersion of the biofilm, resulting in a reduction of matrix material, is an indirect effect of bacterial killing by Tocris 2611, quantification of matrix and cells was repeated following six hours exposure to Tocris 2611 (Figure 4.4). Tocris 2611 achieves sterilisation of stationary phase cultures within six hours, therefore providing a more representative effect on the biofilm structure. After six hours, Tocris 2611 caused essentially no loss of adherent material, whilst leading to a substantial loss of bacterial viability, comparable to that observed after 24 hours (Figure 4.4). This suggests that destructuring of the biofilm is a consequence of cell death, and the antibiofilm activity of Tocris 2611 does not involve biofilm disruption (Figure 4.4).
Figure 4.4 Effects of Tocris 2611 and comparator agents on the proportion of matrix and cells of S. aureus SH1000 biofilms. Panel (a): after 24 hours exposure to proteinase K (100 µg/ml) and Tocris 2611 64 µg/ml (96-well MBEC); Panel (b) after 24 hours exposure to proteinase K (100 µg/ml), Tocris 2611 4 x MIC, 8 µg/ml and 64 µg/ml (Calgary Biofilm Device and 96-well MBEC respectively); Panel (c) after 6 hours exposure to proteinase K (100 µg/ml) and Tocris
2611 64 µg/ml (96-well MBEC). Values are the means of at least three biological replicates; error bars show standard deviations.

Taken together, results suggest that Tocris 2611 eradicates both planktonic and biofilm cultures of *S. aureus* by killing viable cells (including SONGs) through perturbation of the bacterial membrane. Indeed, there is a growing recognition of membrane damaging agents due their frequently seen ability to eradicate biofilms. The bacterial membrane is a fundamental requisite of both growing and SONG cells, and therefore serves as an ideal antibacterial target (assuming specificity for the bacterial membrane can be achieved). Since disruption of the membrane is not dependent on active biosynthetic pathways, membrane damaging agents are therefore often more effective than non-membrane damagers in the treatment of biofilm associated infections. However, not all agents known to target the bacterial membrane are able to eradicate biofilms, for example daptomycin has only limited antibiofilm activity against some types staphylococcal biofilm infections. Loss of effective antibiofilm activity may be attributed to the existence of different subpopulations within the biofilm, and varying compositions of the membrane between subpopulation, interfering with the interaction between the compound and the membrane. Therefore, although membrane damaging agents may provide a more effective alternative in the treatment of biofilm infections, targeting the bacterial membrane does not necessarily indicate that a compounds will possess antibiofilm activity.

### 4.4.4 Investigating the propensity of *S. aureus* to develop resistance to Tocris 2611

For clinical use an advantageous property for antimicrobials is a low potential for bacteria to develop resistance to the agent of interest. To determine if resistance readily develops as a consequence of exposure to Tocris 2611, both spontaneous resistance and resistance as consequence of prolonged exposure was assessed.
4.4.4.1 Selection of bacterial Tocris 2611 resistance

Spontaneous resistance to Tocris 2611 of *S. aureus* SH1000 was evaluated by plating saturated cultures onto MHA containing 4 x MIC Tocris 2611. No resistant mutants were isolated (limit of detection <5.0 X 10^{-9}), indicating that bacterial resistance to Tocris 2611 requires multiple mutational steps. As such Tocris 2611 may be considered to exhibit a low resistance potential.

Instead, resistance following prolonged selection with Tocris 2611 was investigated to determine whether a reduction in susceptibility would arise under continuous exposure to Tocris 2611. This was done by continuously exposing five individual lineages of SH1000 to an extended concentration range of Tocris 2611 over 25 days (extended gradient MIC method, section 2.6.7). Resistance to Tocris 2611 arose in all five lineages, and mutants were 14 x less susceptible after 25 days (Figure 4.4). In contrast, three days of selection was sufficient to select clinically relevant levels of daptomycin resistance (≥2 µg/mL) (Figure 4.4). Although *S. aureus* mutants resistant to Tocris 2611 were selected over 25 days of continuous exposure, the level of resistance that was generated was still less than 1 µg/mL. Compared with daptomycin, the control antibiotic used in this study, for which up to 36 µg/mL resistance was selected in *S. aureus*. Daptomycin is currently used clinically to treat many multi-drug resistant strains of *S. aureus*, particularly for complicated skin and soft tissue infections. Furthermore, the MIC of 1 µg/mL exhibited by the Tocris 2611 resistant strains is 8 and 64-fold lower than the biofilm eradicating concentrations of Tocris 2611 against biofilms grown the Calgary Biofilm Device, and 96-well plate respectively. Taken together, results may indicate that although bacterial resistance can emerge for Tocris 2611, it is low level and requires prolonged selective pressure. Therefore, it is reasonable to suggest that Tocris 2611 resistance would be unlikely to effect the development of this compound for use as an antibiofilm treatment.
4.4.4.2 Characterisation of Tocris 2611 resistant strains

To establish which genes impact the susceptibility of *S. aureus* to Tocris 2611, the most resistant strain (T2), generated by prolonged exposure to Tocris 2611, was analysed using whole-genome sequencing (WGS). WGS enables the identification of the genetic changes responsible for resistance. Seven mutations were confirmed, which are potentially involved in Tocris 2611 resistance (Table 4.5). Tocris resistant strain T2 exhibited an MIC of 1 µg/mL.
Table 4.3 Mutations in the DNA sequence of Tocris 2611 resistant strain T2 compared with wild-type *S. aureus* SH1000. Whole genome sequencing was used to identify genomic changes.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Function</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intergenic region upstream of SAOUHSC_00198</td>
<td>Putative long-chain acyl-CoA synthetase</td>
<td>G39A</td>
<td>-</td>
</tr>
<tr>
<td>SAOUHSC_00467</td>
<td><em>pur</em> operon repressor</td>
<td>G88T</td>
<td>V30L</td>
</tr>
<tr>
<td>SAOUHSC_01070</td>
<td>Regulatory protein YlfF</td>
<td>T78A</td>
<td>Y26X</td>
</tr>
<tr>
<td>SAOUHSC_01359</td>
<td>Membrane modifying protein MprF</td>
<td>C1711T</td>
<td>L571F</td>
</tr>
<tr>
<td>SAOUHSC_01621</td>
<td>Antitermination protein NusB</td>
<td>C365T</td>
<td>G122D</td>
</tr>
<tr>
<td>SAOUHSC_02485</td>
<td>DNA-directed RNA polymerase, alpha subunit</td>
<td>G19T</td>
<td>P7T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G43C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Deleted T46</td>
<td></td>
</tr>
<tr>
<td>Intergenic region upstream of SAOUHSC_02659</td>
<td>Putative transcriptional regulator AcrR</td>
<td>G47C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G50A</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T51C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C52A</td>
<td>-</td>
</tr>
</tbody>
</table>

To determine if these mutations were universal for causing Tocris 2611 resistance, the genes and intergenic regions containing the mutations outlined in Table 4.5 were sequenced in the four other resistant strains generated. The same regions were also sequenced in the resistant strain T2, but at the five and ten day stage of Tocris 2611 exposure, to determine if an increase in the level of resistance observed could be attributed to a particular mutation. In addition to resistant strain T2, the alteration in DNA sequence of SAOUHSC_00467 (*pur* operon repressor) was identified in the 10 day T2 strain, and resistant strain T5. The mutation identified in membrane
modifying protein MprF (SAOUHSC_01359) was also identified in ten day T2, and the point mutation at 219191 was identified in both the five and ten day T2. Since reduced susceptibility to Tocris 2611 cannot be directly attributed to a specific mutation, it would suggest that the MOA of Tocris 2611 involves disruption of multiple targets, an effect which has been previously reported for membrane-active compounds.

Mutations in mprF have also been identified in daptomycin, nisin and vancomycin resistant strains of S. aureus. Multiple peptide resistance factor (MprF) is a membrane protein, which adds L-lysine to phosphatidylglycerol (a phosphatidylglycerol lysyltransferase). The addition of L-lysine increases net positive charge of the bacterial cell surface, which is known to decrease the binding of daptomycin, and may have a similar effect on Tocris 2611. Mutations in DNA-directed RNA polymerase have also been identified in daptomycin and vancomycin resistant strains of S. aureus. However DNA-directed RNA polymerase mutations in response to daptomycin and vancomycin exposure occur in the β-subunit or C-subunit, whereas mutation in the Tocris 2611 resistant strain was present in the α-subunit. It has been suggested that these mutations result in altered fluidity, surface charge and permeability of the cell membrane, and may therefore indicate that similar membrane alterations are involved in conferring reduced susceptibility to Tocris 2611. Furthermore, a mutation in long chain acyl-CoA synthetase, which is required for fatty acid biosynthesis, may also affect the membrane structure, causing reduced susceptibility to Tocris 2611. Three of the mutations (nusB, ylbF, and the region upstream of acrR) can be grouped into those that may impact the regulatory processes within the cell. Finally, mutations in the pur operon repressor are a frequently encountered artefact of selecting resistance in S. aureus. It has been suggested that the mutation allows for better growth in culture media.

Results indicate that reduced susceptibility to Tocris 2611 occurs as consequence of the acquisition of multiple mutations, as opposed to one single point mutation. However, further work would be required to determine the cause of the reduced susceptibility of S. aureus to Tocris 2611(see Chapter 6).
4.5 Conclusions

Tocris 2611 is a potent anti-staphylococcal agent which is able to eradicate *S. aureus* planktonic cells and biofilms (including community acquired MRSA USA300, and prolific biofilm former UAMS-1 and indeed *S. epidermidis* RP62A). In SH1000, it has been elucidated that its mechanism of action is through disrupting the cytoplasmic membrane. Although Tocris 2611 also has some effect on eukaryotic cells, it only resulted in modest lysis of erythrocytes, demonstrating a degree of selective toxicity. Tocris 2611 also demonstrated a low resistance potential. Therefore, Tocris 2611 makes an interesting candidate as an anti-staphylococcal, antibiofilm agent.
Chapter 5

Screening chemical libraries for potentiators of established antimicrobial agents

5.1 Abstract

Due to antibiotic resistance seen in both biofilm and non-biofilm bacterial infections, there is a pressing need for new antibacterial agents. However, it could take over 10 to 20 years before new antibiotics are accessible. One potential strategy to address this problem is repurposing existing drugs as antibiotic adjuvants, potentiating the activity of available antibiotics against resistant bacteria. Work described in this chapter screened 3 chemical libraries to identify adjuvants of the antibiotics ciprofloxacin and rifampicin against S. aureus SH1000 biofilms and rifampicin, fusidic acid or linezolid against planktonic E. coli 1411. No compounds were found to enhance the efficacy of ciprofloxacin or rifampicin against SH1000 biofilms, but many were shown to have a synergistic interaction with rifampicin and/or linezolid against E. coli 1411. A number of compounds also exhibited antibacterial activity alone. Further evaluation showed that the compounds and compound-antibiotic combinations, identified from this screen, had limited antibacterial activity against the clinically relevant ESKAPE pathogens. Although no antibiotic adjuvants were determined that could be considered for clinical use, drug repurposing and adjuvant therapy remain important approaches to antimicrobial drug discovery and development. In addition, these strategies may provide a temporary solution to the antibiotic resistance crisis, whilst adequate numbers of new antibiotics are made available.

5.2 Introduction

Bacterial resistance to available antibiotics is rapidly increasing, and poses a serious threat to human health. With the exception of the recently described Gram-positive antibacterial
teixobactin, that is considered to belong to a new class of antibiotic, no new classes have been discovered since 1987, and none have been introduced against Gram-negative bacteria for more than 40 years. Furthermore, the available treatments for biofilm-associated infections are limited and largely ineffective. As a consequence, the need for novel antibiotics and alternative therapeutic options for both biofilm and non-biofilm infections has now become critical. However, the discovery, and subsequent development required for new compounds to reach FDA-approval can take up to, or even more than, 20 years.

One alternative approach to this antibiotic pipeline is to repurpose existing drugs as antibiotic adjuvants, thereby enhancing the antibacterial activity of clinically available antibiotics. Adjuvants such as these are preferably non-antibiotic compounds (have no reported antibacterial activity), which are either FDA-approved for an alternative indication, or have undergone some pre-clinical evaluation. Two antibiotics can also be adjuvants if they interact synergistically.

There are several properties that potential antibiotic adjuvants could possess. First, adjuvants could have direct antibacterial activity, which may or may not be used clinically. Second, adjuvants could overcome antibiotic resistance mechanisms. Adjuvants that are used to suppress bacterial resistance include, but are not limited to, efflux pump inhibitors, outer membrane permeabilisers and enzyme inhibitors, such as inhibitors against β-lactamase. In clinical use, agents such as flavones have been used successfully in combination with fluoroquinolone ciprofloxacin, by preventing efflux by NorA. Outer membrane permeabilisers have also been previously researched for use as antibiotic adjuvants, although none have been introduced into the clinic. Problems such as a lack of prokaryotic specificity render these compounds unsuitable for use in humans. Agents such as clavulanic acid, sulbactam and tazobactam, which are β-lactamase inhibitors, have been co-administered with the penicillins for three decades. However, these examples are directed at non-biofilm infections, whilst approximately 80% of infections grow as a biofilm. It has recently been demonstrated that peptide 1018, a new class of adjuvant, synergizes with antibiotics ceftazidime, tobramycin, imipenem and ciprofloxacin, as well as exhibiting broad-spectrum antibiofilm activity.
Finally, adjuvants could help clear infections by activating the host immune/defence mechanisms, such as promoting autophagy. Although any one of these properties would make a compound potentially useful as an antibiotic adjuvant, those that possessed more than one may be of greater value. Furthermore, the ability to achieve plasma concentrations that are comparable to the minimum inhibitory concentration required for antibacterial activity is also important. In addition, antibiotic/adjuvant combinations that have different molecular targets are often found to be more effective.

Employing antibiotic adjuvants may therefore enable the use of antibiotics against bacteria which had either developed resistance, or to which were previously insensitive. Furthermore, they may decrease the rate of resistance developing. In addition, using repurposed drugs can reduce the cost of bringing a drug to market by up to 40 %, as well as the time spent in drug development being significantly reduced. Finally, chemicals libraries provide an ideal platform form for drug discovery, providing an extensive and diverse number of biologically relevant compounds.

In view of this, three compound libraries of biologically active small molecules were evaluated for antibiotic adjuvants; the NIH Clinical Collection, Tocriscreen Total collection and the Spectrum Collection.

The NIH clinical collection (727 compounds) consists almost entirely of drugs that have been used in phase I-III clinical trials and have not been represented in other arrayed collections. These compounds also have favourable attributes for inclusion in a screening collection, such as purity, solubility and commercial availability for re-supply (Cao et al., 2015). The collection was assembled by the NIH through the Molecular Libraries Roadmap Initiative as part of its mission to enable the use of compound screens in biomedical research (Austin et al., 2004). The clinically tested compounds in the NCC are highly drug-like with known safety profiles (Cao et al., 2015). This collection provides an excellent source of compounds which may be appropriate for direct human use in new disease areas.
Tocriscreen Total is a library of 1120 biologically active, well-characterised small molecule inhibitors (Dittmar et al., 2015). The collection covers a wide range of pharmacological targets (>300) and research areas, such as cancer and immunology and including a range of therapeutic classes, such as cardiovascular and nervous systems (Dittmar et al., 2015).

The Spectrum Collection presents 2320 compounds of bioactive compounds and natural products, and includes all of the compounds in the US and International Drug Collections, together with our Natural Product and Discover libraries (Rochester et al, 2017). Compounds were selected by medicinal chemists and biologists to provide a wide range of biological activities and structural diversity for screening programs (Rochester et al, 2017). Sixty per cent of the library is made up of drugs that have been introduced in the US (~1280) and ~320 that are limited to use in Europe. A further 25% of the collection consists of natural products (~640) with unknown biological properties, derived from sources worldwide. (Rochester et al., 2017) These compounds were selected on the basis of chemical class and structural diversity. The final 15% of the collection are non-drug enzyme inhibitors, receptor blockers, membrane active compounds and cellular toxins (Rochester et al., 2017). Many of these 420 compounds have either not reached development, or were dropped for toxicological or other reasons. Also included are representatives of marketed pesticides and herbicides for comparative purposes (Rochester et al., 2017).

Each library was individually screened in combination with clinically available antibiotics ciprofloxacin or rifampicin against *S. aureus* SH1000 biofilms, or rifampicin, linezolid or fusidic acid against planktonic cultures of *E. coli* 1411. Both ciprofloxacin and rifampicin were selected since they possess bactericidal activity, a property that is necessary for the successful eradication of biofilms (Wu et al., 2015). Indeed, previous reports suggest rifampicin does exhibit some activity against staphylococcal biofilms, although this is limited (Sanchez et al., 2015). Furthermore, both compounds have alternate mechanisms of action, enabling a wider range of interactions to be assessed during screening. Rifampicin was also screened for potentiators against planktonic *E. coli* since this compound possesses potent broad-spectrum
antibacterial activity. However, due to a high resistance potential, rifampicin is only used in combination therapy, customarily for the treatment of tuberculosis (Campbell et al., 2001). Rifampicin resistance is most frequently due to a mutation in the β subunit of the RNA polymerase, which changes the structure (Campbell et al., 2001, Wehrli, 1983). Finally, fusidic acid and linezolid were screened potentiators against planktonic E. coli. Both fusidic acid and linezolid are narrow-spectrum antibiotics, possessing activity against Gram-positive organisms only (Dobie and Gray, 2003, Sztanke et al., 2004). They are also bacteriostatic, and kill bacteria through the inhibition of protein synthesis (Cundliffe, 1972, Sztanke et al., 2004). However, they interfere with bacterial protein synthesis in alternate mechanisms. Fusidic acid binds to bacterial protein elongation factor G (EF-G), trapping it to ribosome and preventing further elongation of the peptide (Dobie and Gray, 2003, McLaws et al., 2011). Linezolid inhibits protein synthesis by binding to the 50S subunit of the ribosome, preventing it from complexing with the 30S subunit, and other factors necessary to form the initiation complex (Livermore, 2003, Sztanke et al., 2004). Following the initial screen with the NIH clinical collection, fusidic acid was substituted with linezolid, as it cannot be assumed that efflux is always the principal mechanism leading to the lack of Gram-negative activity. Whereas efflux is considered the primary cause of Gram-negatives reduced susceptibility to linezolid.

5.3 Aims and Objectives

Work described in this chapter aims to screen three chemical compound libraries (NIH Clinical Collection, Tocriscreen Total and Spectrum Collection) for antibiotic adjuvants, which potentiate the activity of ciprofloxacin or rifampicin against S. aureus SH1000 biofilms and rifampicin, linezolid or fusidic acid against planktonic E. coli 1411.
5.4 Results and Discussion

5.4.1 Antibacterial properties of antibiotics screened in combination with chemical libraries

To determine the appropriate concentrations at which the four antibiotics (ciprofloxacin, rifampicin, linezolid and fusidic acid) should be screened at, MICs and/or 96-well plate MBECs against standard laboratory strains *S. aureus* SH1000 or *E. coli* 1411 were determined. The four antibiotics (ciprofloxacin, fusidic acid, linezolid and rifampicin) exhibited MICs ranging from 0.008-2 µg/mL against planktonic cultures of *S. aureus*, and 4 - >256 µg/mL against planktonic cultures of *E. coli*. None of the compounds were able to eradicate *S. aureus* biofilms grown in 96-well plates (Table 5.1). Consequently, as no activity against staphylococcal biofilms was observed with ciprofloxacin and rifampicin, they were screened against SH1000 well grown biofilms at 50 µg/mL in combination with the three compound libraries. Similarly, fusidic acid and linezolid displayed no activity against planktonic *E. coli* 1411, and were therefore also screened at 50 µg/mL. Rifampicin exhibited an MIC of 4 µg/mL against planktonic *E. coli* 1411 and was subsequently screened in combination with the three libraries at the sub-MIC concentration of 0.5 µg/mL. The three compound libraries were tested at 10 µM.
Table 5.1 Minimum inhibitory concentrations (MICs) and 96-well plate minimum biofilm eradication concentrations (well MBEC) of antibacterial agents ciprofloxacin, fusidic acid, linezolid and rifampicin, against planktonic and biofilm *S. aureus* SH1000 and planktonic *E. coli* 1411. ND indicates not determined.

<table>
<thead>
<tr>
<th>Antibacterial Agent</th>
<th><em>S. aureus</em> SH1000 planktonic MIC</th>
<th><em>S. aureus</em> SH1000 well MBEC</th>
<th><em>E. coli</em> 1411 planktonic MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>2</td>
<td>&gt;256</td>
<td>ND</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>0.25</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Linezolid</td>
<td>2</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.008</td>
<td>&gt;256</td>
<td>4</td>
</tr>
</tbody>
</table>

5.4.2 Screen for *S. aureus* biofilm eradication

The three compound libraries were screened in combination with ciprofloxacin and rifampicin against *S. aureus* SH1000 biofilms, to identify compounds that potentiated their activity against staphylococcal biofilms. *S. aureus* SH1000 has been used extensively in previous studies and forms a biofilm (Ooi et al., 2009, Ooi et al., 2010, Randall et al., 2013b).

This screen identified no potentiators of ciprofloxacin or rifampicin which resulted in *S. aureus* biofilm eradication. The absence of any anti-staphylococcal antibiofilm activity observed could, in part, be attributed to the highly refractory nature of biofilms to most antimicrobials. Conceivably, higher concentrations of agents might have yielded more positive results.

5.4.3 Screen for antibiotic adjuvants against *E. coli*

The compound libraries in were screened for agents able to potentiate the activity of rifampicin, fusidic acid or linezolid against *E. coli* 1411 grown in planktonic culture.

5.4.3.1 NIH clinical collection in combination with rifampicin or fusidic acid
No potentiators of rifampicin or fusidic acid activity against *E. coli* 1411 were discovered from the screen of the NIH clinical collection. However, the screen did identify 26 compounds which were active against planktonic *E. coli* 1411 at 10 µM. 25 of these were found to be pre-existing antibacterials and therefore underwent no further analysis. These were azithromycin, cefaclor, cefinir, cefixime trihydrate, cefotaxime sodium salt, cefuroxime, demeclocycline, enrofloxacin, floxuridine, gatifloxacin, levofloxacin, micocycline hydrochloride, moxifloxacin hydrochloride, norfloxacin, ofloxacin, ormetoprim, oxytetracycline hydrochloride, pazufloxacin, pefloxacin, pefloxacin mesylate, piperacillin sodium salt, rifabutin, rifampicin, terazosin, tosofloxacin tosylate, and triclosan.

Flecainide acetate was the sole compound discovered that exhibited unanticipated antibacterial activity. This drug is a class 1c antiarrhythmic agent used in the prevention and treatment of tachyarrhythmias, and works by blocking the Nav1.5 sodium channel in the heart, slowing the upstroke of the cardiac action potential and conduction of the electrical impulse within the heart. It has also been shown to inhibit ryanodine receptor 1 (RyR2), a major regulator of sarcoplasmic release of stored calcium ions. It can reduce calcium sparks and thus arrhythmogenic calcium waves in the heart.

Following the initial demonstration that flecainide acetate possessed antibacterial activity against planktonic *E. coli* at 10 µM, it was further evaluated using standard procedures. Against planktonic cultures of *S. aureus* SH1000 and *E. coli* 1411 flecainide acetate exhibited MICs of 0.5 µg/mL and 1 µg/mL respectively. When used for its clinically approved indication as an oral antiarrhythmic agent, flecainide acetate achieves a maximum serum concentration (C<sub>max</sub>) of 0.2-1 µg/mL. The MIC values determined against *S. aureus* SH100 and *E. coli* 1411 are therefore within this therapeutic index.

Newly discovered antimicrobial agents that exhibit potent, broad-spectrum activity are frequently membrane damaging. To determine if this was a property of flecainide acetate, the integrity of the *S. aureus* SH1000 membrane was assessed using the BacLight<sup>TM</sup> assay. At 4 x MIC, flecainide acetate caused a loss of 25% to SH1000 membrane integrity within 10 minutes.
(Table 5.3). In the BacLight™ assay, a compound is considered to be membrane damaging when a $\geq 30 \%$ loss of membrane integrity is observed, only 5% greater than that seen for flecainide acetate. Since this assay does not reliably detect subtle membrane disruption, or perturbation occurring over longer than a 10 minute window, additional assays would need to be performed to elucidate the mechanism of action.

When resupplied with flecainide acetate from the NIH, none of the results outlined in above could be reproduced. To determine the active component from the original stock of flecainide acetate, a sample was sent for analysis by mass-spectrometry (MS) and high-performance liquid chromatography (HPLC) (performed by Dr Martin McPhillie). Results suggested the presence of a compound that would have existed as an intermediate in the synthesis of flecainide acetate. To determine the specific structure of the unknown intermediate, nuclear magnetic resonance (NMR) would need to be performed. However, due to insufficient quantities, NMR could not be performed, and further investigations could not be continued.

5.3.2.1 Tocriscreen Total in combination with rifampicin or linezolid

Tocriscreen Total chemical library was screened in combination with rifampicin and linezolid against planktonic E. coli 1411. No potentiators of rifampicin were determined. One compound was identified which potentiated the activity of linezolid; the protein kinase A inhibitor, H89 dihydrochloride (H 89). In prokaryotic physiology, protein kinases fulfil purposes similar to those in eukaryotes, transducing signals to the bacterial chromosome, although they are far less complex. The inhibitory action of H89 dihydrochloride is non-specific, and inhibits several other kinases, including but not limited to, ribosomal protein S6 kinase beta-1 and mitogen/stress activated protein kinase. Subsequently, the ability of H 89 dihydrochloride to potentiate the activity of linezolid against planktonic E. coli 1411 could possibly be a result of the inhibitory activity of H 89, acting on the efflux pumps, directly compromising their function, or indirectly preventing their up-regulation.
In addition, this screen identified to two compounds which exhibited antibacterial activity alone at the tested concentration of 10 µM; the potent multi-enzyme inhibitor, diphenyleneiodonium chloride, and the competitive bradykinin B$_2$ receptor antagonist, WIN 64338 hydrochloride. Diphenyleneiodonium chloride is a eukaryotic agonist of G-protein coupled receptor 3, a member of the G-protein coupled receptor family (Ye et al., 2014). It also binds strongly to flavoproteins, inhibiting a number of enzymes, including NO synthase, NADPH oxidases and NADPH cytochrome P450 oxidoreductase (Stuehr et al., 1991, Tew, 1993, Yea et al., 1990, Wang et al., 1993). Diphenyleneidonium chloride also induces Ca$^{2+}$ mobilization and β-arrestin receptor internalization, and inhibits platelet aggregation. Due to the activity of this compound affecting multiple enzymes, it is likely that the antibacterial effect observed against planktonic E. coli 1411 is due to non-specific effects. As a consequence, potential antibacterial properties of diphenyleneiodonium chloride were not investigated further in this study.

As mentioned above, WIN 64338 hydrochloride is identified as a mammalian, non-peptide, competitive bradykinin B$_2$ receptor antagonist (Hu et al., 2004, Scherrer et al., 1995, Marceau et al., 1994). In mammals, the bradykinin receptor family is a G-protein coupled receptor that stimulates phospholipase C, increasing intracellular calcium and inhibiting adenylate cyclase. It is involved in many pathways, such as inflammation, vasodilation and smooth muscle relaxation. Furthermore, as WIN 64338 hydrochloride is not a peptide, it is not subject to poor bioavailability and metabolism that would limit other peptide molecules (Sawutz et al., 1994).

To investigate the properties of H 89 dihydrochloride and WIN 64338 hydrochloride further, MICs were determined against S. aureus SH1000, E. coli 1411 and P. aeruginosa PA01 (Table 5.2). Screening found that H 89 dihydrochloride was able to potentiate the activity of linezolid against planktonic E. coli 1411, but here has also been shown to have antibacterial activity alone, exhibiting MICs of 64 µg/mL, 16 µg/mL and >256 µg/mL, against SH1000, 1411 and PAO1 respectively (Table 5.2). WIN 64338 dihydrochloride had stronger activity, exhibiting MICs of 1 µg/mL, 4 µg/mL and 128 µg/mL against SH1000, 1411 and PAO1 respectively (Table 5.2).
Table 5.2 Minimum inhibitory concentrations (MICs) of H 89 dihydrochloride and WIN 64338 hydrochloride from the Tocriscreen Total collection, against *S. aureus* SH1000, *E. coli* 1411 and *P. aeruginosa* PAO1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>10µM in µg/mL</th>
<th>10µM <em>S. aureus</em> SH1000 planktonic MIC</th>
<th>10µM <em>E. coli</em> 1411 planktonic MIC</th>
<th>10µM <em>P. aeruginosa</em> PAO1 planktonic MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>H 89 dihydrochloride</td>
<td>5.19</td>
<td>64</td>
<td>16</td>
<td>&gt;256</td>
</tr>
<tr>
<td>WIN 64338 hydrochloride</td>
<td>7.83</td>
<td>1</td>
<td>4</td>
<td>128</td>
</tr>
</tbody>
</table>

Although reasonable activity against *S. aureus* SH1000 and *E. coli* 1411 was observed for WIN 64338 hydrochloride, and a possible synergistic interaction between H 89 dihydrochloride and linezolid against *E. coli* 1411, no pre-clinical whole animal studies on these compounds have been performed. Consequently, neither H 89 dihydrochloride nor WIN 64338 was investigated further in this study.

5.3.2.2 Spectrum collection in combination with rifampicin and linezolid

The systematic screening of the Spectrum Collection in combination with rifampicin or linezolid against planktonic *E. coli* 1411 identified 38 compounds that exhibited antibacterial activity alone. There were alexidine hydrochloride, azithromycin, aztreonam, bekamycin sulphate, belomycin, ceftinir, ceftibuten, chloramphenicol, ciprofloxacin, doxycycline hydrochloride, enrofloxacin, furazolidone, gatifloxacin, gemifloxacin mesylate, levofloxacin, lemofoxacin hydrochloride, meclocycline sulfosalicylate, micocycline hydrochloride, mitomycin C, moxifloxacin hydrochloride, nifuroxazide, norfloxacin, ofloxacin, oxytetracycline, pefloxacin mesylate, phenylmercuric acetate, polymixin B sulphate, pyrithioone zinc, sarafloxacin hydrochloride, sucralose, telithromycin, tetracycline hydrochloride, thimersosal, thioguanine, thiogunanosine, triclosan and zidovudine. However of the 38 compounds, only 6 were not previously described antimicrobials. Included in those 6
were 4 antineoplastic agents; bleomycin, mitomycin C, thioguanine and thioguanosine. Antineoplastics are used to inhibit tumours from growing and spreading. The remaining 2 compounds were sucralose, used to sweeten consumables, and the antiretroviral zidovudine, that inhibits viral reverse-transcriptase.

The screen also identified 11 compounds that potentiated the activity of rifampicin. These were cefditoren pivoxil, colistemethate hydrochloride, dirithromycin, doxifluridine, florfenicol, floxuridine, fluorouracil, metaraminol bitartrate, methacycline hydrochloride, spectinomycin hydrochloride and sulfonomethoxine. In addition, 7 compound were identified that potentiated the activity of linezolid against planktonic \textit{E. coli} 1411. These were colistimethate sodium, dirithromycin, doxifluridine, floxuridine, fluorouracil, methacycline hydrochloride and spectinomycin hydrochloride. Only 4 of the 11 rifampicin potentiators were not existing antimicrobials. These included the 3 antineoplastic agents doxifluridine, floxuridine and fluorouracil, and one antihypotensive known as metaraminol bitartrate, used to raise reduced blood pressure. All 7 of the compounds found to potentiate the activity of linezolid were also found amongst the 11 compounds that potentiated the activity of rifampicin. Of these, 3 were the antineoplastic agents doxifluridine, floxuridine and fluorouracil, and the remaining 4 were established antimicrobial agents.

Since the majority of compounds identified in the Spectrum Collection screen were found to be existing antimicrobials, only 10 were investigated further. From the 38 compounds that exhibited antibacterial activity alone sucralose, thioguanine and zidovudine were chosen for additional analysis, and the remaining 7 compounds were selected from those originally determined as being potentiators of rifampicin alone, or both rifampicin and linezolid against \textit{E. coli} 1411. These were dirithromycin, florfenicol, fluorouracil, metaraminol bitartrate, methacycline hydrochloride, spectinomycin hydrochloride and sulfamonomethoxine.

To investigate further the antibacterial activity of these 10 compounds, MICs against \textit{S. aureus} SH1000 and \textit{E. coli} 1411 were determined. Against SH1000, MICs ranged from 0.5 - >256 \( \mu g/mL \), and against 1411, MICs ranged from 0.03125 - >256 \( \mu g/mL \) (Table 5.3). Two of the
three nucleoside analogue inhibitors, thioguanine and zidovudine, were found to have no activity against *S. aureus* SH1000, but displayed potent activity against *E. coli* 1411, exhibiting MICs of 0.5 and 0.03 µg/mL, respectively. The third nucleoside analogue, fluorouracil, exhibited comparable 1411 activity with an MIC of 1 µg/mL, but was also active against SH1000, with an MIC of 8 µg/mL (Table 5.3). Sucralose was one of the compounds that exhibited activity alone against planktonic *E. coli* 1411, however, upon Spectrum resupplying this compound, no antibacterial activity was observed (Table 5.3). Of the remaining 6 compounds, 5 are approved antibacterials; dirithromycin, florfenicol, methacycline hydrochloride, spectinomycin hydrochloride and sulfamonomethoxine. Dirithromycin, methacycline hydrochloride and spectinomycin hydrochloride were all found to potentiate both rifampicin and linezolid activity against planktonic *E. coli* 1411. All three compounds also exhibited antibacterial activity alone. Against SH1000, MICs were 0.5, 1 and 32 µg/mL, for dirithromycin, methacycline hydrochloride and spectinomycin hydrochloride respectively. Against 1411, dirithromycin and methacycline hydrochloride exhibited MICs of 4 and 1 µg/mL respectively, whilst spectinomycin hydrochloride displayed more potent activity against 1411 than SH1000, with an MIC of 4 µg/mL (Table 5.3).

The remaining antibacterial, sulfamethoxine, only potentiated the activity of rifampicin, and exhibited no antibacterial activity against 1411 or SH1000, when resupplied from Spectrum, displaying MICs >256 µg/mL. Finally, metartraminol bitartrate, which was originally identified as a potentiator of rifampicin against planktonic *E. coli* 1411, displayed an MIC against SH1000 of 32 µg/mL, but lacked activity against 1411, exhibiting an MIC of >256 µg/mL (Table 5.3). Metaraminol bitartrate is a eukaryotic α1-adrenergic receptor agonist, used as an antihypotensive.
Table 5.3 Minimum inhibitory concentrations (MICs) of the 10 compounds identified from the Spectrum Collection for preliminary susceptibility studies. MICs were determined against *S. aureus* SH1000 and *E. coli* 1411.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Function</th>
<th>($\text{10\text{\mu}M}$ in $\text{\mu}g/\text{mL}$)</th>
<th><em>S. aureus</em> SH1000 planktonic MIC</th>
<th><em>E. coli</em> 1411 planktonic MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dirithromycin</td>
<td>Antibacterial</td>
<td>8.35</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>Antibacterial</td>
<td>3.58</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Fluorouracil</td>
<td>Antineoplastic and pyrimidine antimetabolite</td>
<td>1.3</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Metaraminol bitartrate</td>
<td>Antihypotensive</td>
<td>1.67</td>
<td>32</td>
<td>$&gt;$256</td>
</tr>
<tr>
<td>Methacycline hydrochloride</td>
<td>Antibacterial</td>
<td>4.79</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Spectinomycin hydrochloride</td>
<td>Antibacterial</td>
<td>4.95</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>Sucralose</td>
<td>Sweetener</td>
<td>3.98</td>
<td>$&gt;$256</td>
<td>$&gt;$256</td>
</tr>
<tr>
<td>Sulfamonomethoxine</td>
<td>Antibacterial</td>
<td>2.80</td>
<td>$&gt;$256</td>
<td>$&gt;$256</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>Antineoplastic and purine antimetabolite</td>
<td>1.67</td>
<td>$&gt;$256</td>
<td>0.5</td>
</tr>
<tr>
<td>Zidovudine</td>
<td>RT transferase inhibitor and antiviral</td>
<td>2.67</td>
<td>$&gt;$256</td>
<td>0.03125</td>
</tr>
</tbody>
</table>

Following initial susceptibility determinations it was decided to focus the subsequent investigations on the 3 three nucleoside analogues fluorouracil, thioguanine and zidovudine. To determine the range of activity of each, susceptibility testing was performed against the so-called ESKAPE pathogens, which represent the bacteria that are recognised to be the major cause of hospital infections in the USA. These are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterbacter species*. Fluorouracil exhibited MICs ranging from 4 – 64 $\mu$g/mL, thioguanine was
inactive against all strains tested, with MICs greater than 256 µg/mL, and zidovudine exhibited MICs ranging from 4−>256 µg/mL (Table 5.4).

**Table 5.4** Minimum inhibitory concentrations (MICs) of fluorouracil, thioguanine and zidovudine against ESKAPE pathogens.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibacterial Activity (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>Acinetobacter baumannii Cubist 581217</td>
<td>8</td>
</tr>
<tr>
<td>Enterobacter cloacae Cubist 583750</td>
<td>4</td>
</tr>
<tr>
<td>Escherichia coli Cubist IHMA 659048</td>
<td>8</td>
</tr>
<tr>
<td>Klebsiella oxytoca Cubist 683079</td>
<td>16</td>
</tr>
<tr>
<td>Klebsiella pneumoniae Cubist 581436</td>
<td>64</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa PA01</td>
<td>32</td>
</tr>
<tr>
<td>Staphylococcus aureus Cubist ACC A790662</td>
<td>32</td>
</tr>
</tbody>
</table>

Due to the limited activity exhibited by fluorouracil, thioguanine and zidovudine against the ESKAPE organisms, compared to that observed in the preliminary determinations’ against *E. coli*, the susceptibility of 8 further *E. coli* strains was investigated. These included 7 strains that were multi-drug resistant, and the wildtype *E. coli* strain MG1655 (ATCC 700926) (Table 5.5). Fluorouracil exhibited a lower MIC against MG1655, than the multi-drug resistant stains, with MICs of 2 µg/mL and 16-64 µg/mL respectively (Table 5.5). Thioguanine exhibited no activity against any of the 8 *E. coli* strains tested, with MICs >128 µg/mL and >256 µg/mL, which is
comparable the poor activity observed against the ESKAPE pathogens (Table 5.4 and 5.5). Like fluorouracil, zidovudine displayed the lowest MIC against MG1655, at 4 µg/mL. This was also observed against *E. coli* Cubist 657524, an *E. coli* strain possessing enzyme CTX-M-32, which is an extended spectrum β-lactamase. Against the remaining *E. coli* strains, zidovudine MICs ranged from 64->256 µg/mL, which is also comparable to the range of activity against the ESKAPE pathogens (Table 5.4 and 5.5).

Table 5.5 Minimum inhibitory concentrations (MICs) of fluorouracil, thioguanine and zidovudine against 8 strains of *E. coli*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fluorouracil</th>
<th>Thioguanine</th>
<th>Zidovudine</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>2</td>
<td>&gt;256</td>
<td>4</td>
</tr>
<tr>
<td>Cubist IHMA 684850</td>
<td>64</td>
<td>&gt;128</td>
<td>128</td>
</tr>
<tr>
<td>Cubist 449334</td>
<td>128</td>
<td>&gt;128</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cubist 586030</td>
<td>16</td>
<td>&gt;128</td>
<td>128</td>
</tr>
<tr>
<td>Cubist 605879</td>
<td>32</td>
<td>&gt;128</td>
<td>256</td>
</tr>
<tr>
<td>Cubist 702452</td>
<td>32</td>
<td>&gt;128</td>
<td>64</td>
</tr>
<tr>
<td>Cubist 657524</td>
<td>32</td>
<td>&gt;128</td>
<td>4</td>
</tr>
<tr>
<td>Cubist 449742</td>
<td>16</td>
<td>&gt;128</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

The poor activity of fluorouracil, thioguanine and zidovudine against the 8 *E. coli* strains was therefore comparable to that observed against the ESKAPE pathogens. However, when treating infections, it has been frequently suggested that compounds can be used more successfully in combination due to a synergistic interaction. This is referred to as synergy. Subsequently,
attempts were made to determine if combinations of fluorouracil, thioguanine and zidovudine were synergistic, against planktonic cultures of *E. coli* 1411, MG1655 and Cubist 449472 (Table 5.6). *E. coli* strain Cubist 449472 contains β-lactamase enzymes CTX-M-15 and TEM-15. Synergism of fluorouracil with thioguanine was not observed. Indeed, the combination exhibited an antagonistic interaction against MG1655, with an FIC equal to 2, and an additive effect against 1411 and Cubist 449472, both with an FIC of 1.031 (Table 5.6). Similarly, zidovudine with thioguanine displayed antagonistic activity against MG1655, with an FIC of 2, and an additive interaction against 1411 and Cubist 449472, with FICs of 0.56 and 0.625 respectively (Table 5.6). Fluorouracil with zidovudine acted synergistically against all 3 *E. coli* strains, exhibiting an FIC of 0.265 against 1411 and Cubist 449472 and 0.281 against MG1655 (Table 5.6).

**Table 5.6** Synergism of fluorouracil with thioguanine, fluorouracil with zidovudine and zidovudine with thioguanine against planktonic cultures of *E. coli* 1411, MG1655 and Cubist 449472. FIC index < 0.5 is synergistic, equal to 1 is additive and ≥ 2 antagonistic.

<table>
<thead>
<tr>
<th>Compound combinations</th>
<th><em>E. coli</em> 1411</th>
<th><em>E. coli</em> MG1655</th>
<th><em>E. coli</em> Cubist 449472</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorouracil and thioguanine</td>
<td>1.031</td>
<td>2</td>
<td>1.031</td>
</tr>
<tr>
<td>Fluorouracil and Zidovudine</td>
<td>0.265</td>
<td>0.281</td>
<td>0.265</td>
</tr>
<tr>
<td>Thioguanine and Zidovudine</td>
<td>0.56</td>
<td>2</td>
<td>0.625</td>
</tr>
</tbody>
</table>

Fluorouracil and zidovudine were therefore the only combination to exhibit a synergistic interaction, and have the potential to be investigated further as novel antibacterial treatment for *E. coli*.
5.4 Conclusions

Since it takes an average of 10-15 years for a drug to go from discovery to clinical use, drug repurposing is an attractive approach in the search for novel antimicrobial agents, due to a significantly reduced development phase. It is therefore of some significance that this work has identified a number of compounds, either as antibacterial agents alone, or as adjuvants for existing antibiotics, following this method. Based on preliminary assessment, the unidentified flecainide acetate intermediary was the most promising candidate as a broad spectrum antibacterial agent, with MICs against S. aureus SH1000 and E. coli 1411 of 1 µg/mL or less, activity that is comparable to clinically available antibiotics. H 89 dihydrochloride demonstrated the most potential for repurposing as an antibiotic adjuvant of linezolid against the Gram-negative organism E. coli, against which no previous activity has been reported. In addition, fluorouracil in combination with zidovudine also exhibited potential as a novel treatment of E. coli. Although no anti-staphylococcal, antibiofilm agents were determined, this could be attributed to biofilm communities being highly recalcitrant to antimicrobial therapy. Ultimately, this work demonstrates that the application of drug repurposing to the search for novel antimicrobial agents discovery is a feasible approach, and the publication of screening data also holds value in the prevention of repeat investigations.
Chapter 6

General conclusions and future studies

The ability of bacteria to grow in the form of a biofilm provides protection from multiple antimicrobial agents and the host immune system (Donlan and Costerton, 2002). Multiple mechanisms have been demonstrated to contribute to the recalcitrance of biofilms, such as restricted antibiotic penetration and high levels of slow or non-growing (SONG) cells (Lebeaux et al., 2014). This type of infection has been shown to be particularly problematic when associated with indwelling medical devices (Donlan, 2001). Consequently, there is a pressing need for novel antibiofilm treatments. This work therefore focused on strategies in the prevention and eradication of biofilms, primarily on implanted medical devices and the discovery of new antibiofilm molecules.

During the initial stages of this study screening of 56 novel antibiofilm molecules (ABMs) was performed to determine antimicrobial activity and potential toxicity. Based on these parameters, the five most promising ABMs were selected; 4-45, CIM008405, P1a-PEP1, P2-5, Tocris 2611. These five compounds exhibited varying spectrums of antimicrobial activity; 4-45 and Tocris 2611 were predominantly active against Gram-positive species, namely staphylococci. Whilst CIM008405 exhibited preferential activity against Gram-negative organisms, notably \textit{P. aeruginosa}. Finally, P1a-PEP1 and P2-5 had potent anti-fungal activity. As all five ABMs demonstrated antimicrobial activity against \textit{S. aureus}, this organism was used as a model to investigate the MOA of each compound. Initially, radiolabeled precursors were used to assess the effects of the compounds on DNA, RNA and protein biosynthesis. None of the five ABMs caused preferential inhibition of any of the macromolecular synthesis pathways. This type of non-specific response indicative of compounds which have antimicrobial activity against \textit{Staphylococcus aureus} by disrupting the cytoplasmic membrane, a profile consistent with
macromolecular synthesis inhibition described previously (O’Neill and Chopra, 2004, Ooi et al., 2009, Randall et al., 2013b). Indeed, subsequent experimentation revealed all five ABMs significantly compromised the integrity of the membrane and caused rapid membrane depolarisation. The membrane was therefore considered to be the antibacterial target. Assessment of staphylococcal liposomes demonstrated that four (4-45, CIM008405, P1a-PEP1, P2-5) of the five ABMs caused damage as a consequence of direct interaction with the phospholipid component of the membrane. An effect that was comparable in mammalian liposomes. Furthermore, the four ABMs (4-45, CIM008405, P1a-PEP1, P2-5) induced complete or near complete haemolysis of erythrocytes, indicating that these compounds are not prokaryote specific. Tocris 2611 only resulted in partial haemolysis of erythrocytes, which suggests some bacteria-specific activity. Indeed, after 48 hours exposure to > 30 x MIC Tocris 2611, human osteoblasts (OB) and bone marrow derived stem cells (MSC) retained 95% viability, an effect comparable to the solvent control.

Although it was demonstrated that Tocris 2611 disrupts the bacterial membrane, the specific target of this compound has not been established. The successful generation of staphylococcal cell ghosts could enable further elucidation of the MOA of Tocris 2611. Cell ghosts would comprise an intact staphylococcal cell envelope, filled with carboxyfluorescein. Comparable to the assessment of carboxyfluorescein filled liposomes, leakage of the dye from cell ghosts could be monitored upon exposure to Tocris 2611. Since Tocris 2611 did not cause damage to carboxyfluorescein liposomes (comprised of just phospholipid), release of the dye from cell ghosts would indicate direct membrane perturbation, most likely as a consequence of interaction with the protein component. If cell ghosts remained intact, and no dye was released, it may suggest that the compounds interaction with the membrane is dependent on the membrane being energised. As discussed in Chapter 4, the generation of staphylococcal cell ghosts has not been previously reported, however the successful production of *E. coli* cells ghost has been demonstrated through the controlled expression of phage lysis gene E (Langemann et al., 2010). The phage lysis gene E allows the intracellular contents to leave the cell through a
transmembrane tunnel structure, formed in the cell envelope by phage lysis gene E (Langemann et al., 2010). This is now being adapted for staphylococci. To confer staphylococcal activity a chimeric E-L lysis gene is being generated. However, when being used in staphylococci, membrane vesicles are required to reseal the transmembrane pores (Lubitz, 2010). In addition, as indicated in Chapter 4, attempts were made to generate carboxyfluorescein filled staphylococcal cell ghosts in a similar manner to the liposome assay described in this chapter. Briefly, staphylococcal liposomes (generated as outlined in the liposome assay described in this thesis), were mixed in a ratio of 4:1 with staphylococcal membrane extracts, and subjected to freeze thawing, before being purified by column chromatography. However, this method requires further experimentation.

Additional insight into the antimicrobial MOA of Tocris 2611 could be provided by further resolution of resistant mutants. The activity of Tocris 2611 could be investigated against strains in which individual mutations have been introduced, to confirm which alterations in the DNA sequence confer a reduced Tocris 2611 susceptibility. Alternatively, techniques such as markerless gene deletion in S. aureus could be used to investigate which genes specifically resulted in reduced susceptibility to Tocris 2611 (Kato and Sugai, 2011). This could subsequently provide information as to the intracellular bacterial target of Tocris 2611. It was suggested in section 4.4.4.2 that Tocris resistant strain (T2) had phenotypical alterations to the membrane mutations in membrane modifying protein MprF (SAOUHSC_01359), putative long-chain acyl-CoA synthetase (SAOUHSC_00198) and DNA-directed RNA polymerase (SAOUHSCE_02485) as a result of Tocris 2611 exposure. To investigate this further the membrane fluidity, surface charge and fatty acid content could be measured in the wildtype strain S. aureus SH1000, the resistant strain T2, and a strain in which the mutations had been deleted, and then compared to determine that alterations which were specifically responsible for resistance to Tocris 2611.

The method differential radial capillary action of ligand assay (DRaCALA) could also be used to evaluate further the cellular target of Tocris 2611 (Roelofs et al., 2011). However, this
method would require a radio/fluorescent label on the drug. Furthermore, cross-resistance/susceptibility of the Tocris 2611 resistant strain against a range of compounds, including membrane-damaging agents and approved antimicrobials, could be assessed. A change in susceptibility to alternative compounds could indicate mutations associated with common mechanisms of resistance, such as the production of antibacterial degrading enzymes and changes in the permeability of the bacterial membrane.

Tocris 2611 also exhibited the ability to eradicate pre-formed staphylococcal biofilms, as well as prevent their formation. This unusual property was a consequence of the potent bactericidal activity of Tocris 2611 against SONG cells, resulting in their sterilisation. SONG cells are an important component of biofilms, forming sub-populations against which the majority of antimicrobials are ineffective, and are capable of repopulating the biofilm when antibiotic treatment is discontinued. Up to 80% of bacterial infections are believed to include a biofilm component, which contributes to recurrent infections and treatment failure. Therefore, agents with antibiofilm activity are highly sought after.

To determine the efficacy of ABM coated implant surfaces, compounds were covalently bound to titanium surfaces and assessed for their ability to retain biofilm prevention activity in vivo and in vitro. ABM 4-45 was replaced with derivative LC0024 due reduced toxicity to eukaryotic cells, whilst maintaining comparable levels of bacterial killing. Two surface types were generated; smooth and open porous. Open porosity is favourable due to the promotion of osseointegration. These surfaces do however exhibit an increased risk of biofilm-associated infection in patients as a consequence of enhanced bacterial adherence. The ABM-coated titanium substrates (smooth and open porous) displayed varying levels of biofilm inhibition in vitro, but were unable to prevent biofilm formation in vivo. In contrast, control agents vancomycin and caspofungin retained activity both in vitro and in vivo, preventing the formation of S. aureus and C. albicans biofilms, respectively. Despite this, these drugs are amongst the remaining ‘last resort’ antimicrobials. Therefore the use of these agents in implant surfaces would be inadvisable due to the promotion of bacterial resistance, and the subsequent
loss of any valid treatment to control the infection. Since four (4-45, CIM008405, P1a-PEP1, P2-5) of the five ABMs were found to lack prokaryote specificity, and failed to significantly reduce biofilm formation when bound to a surface, these compounds would be unsuitable for use as antimicrobial therapies. The exact cause of the ABMs loss of activity upon surface conjugation it is not yet known, however it could be due to achieving insufficient concentrations of the compound on the surface, rapid accumulation of serum proteins, forming a protective layer between the active compound and the surrounding environment, or a direct consequence of covalent binding of the compound to the surface. To progress these antibiofilm coatings, future experimentation could include the use of alternative linker molecules or surface conjugation via a different site on the compound. Furthermore, surfaces could be engineered to incorporate molecules that prevent the adhesion of serum proteins in order to optimise the antibiofilm effect of the adhered ABM.

Although surface bound Tocris 2611 also lacked antibiofilm activity in vivo, it was demonstrated to have potent anti-staphylococcal, antibiofilm activity, and exhibited partial prokaryote specificity. In addition, S. aureus developed only low-level resistance as a consequence of prolonged exposure to Tocris 2611. This compound would therefore most likely be unsuitable as for use as a systemic antibiotic due to potential toxicity, but may have potential as a topical treatment for biofilm associated staphylococcal infections. However, development of Tocris 2611 for clinical use would require additional in vivo investigations. Preferably, animal models would be used that were representative of both infections caused from surgical entry and those present in chronic wounds.

Whilst the ABM-coated implants, generated as part of the COATIM project, failed to inhibit microbial biofilm formation, no negative effects on osseointegration were observed. Furthermore, a significant reduction in biofilm growth was demonstrated for coatings incorporating vancomycin and caspofungin. Although agents such as vancomycin and caspofungin would be unsuitable for this purpose, this work has established a viable method for the surface immobilisation of molecules through covalent binding with a novel linker.
Therefore, results present a proof of concept approach, contributing to the development of next generation antibiotic coatings, in addition to providing potential for exploitation by industry. Future work could therefore include the application of alternative antimicrobial compounds to implant surfaces using the methodology developed here.

Finally, in attempt to identify additional antibiotic treatments for staphylococcal infections, three small compound chemical libraries were screened for potentiators of known antibiotics ciprofloxacin and rifampicin. Libraries were also screened for potentiators of rifampicin, linezolid and fusidic acid against E. coli, another well-known human pathogen. At the concentrations tested, screening did not reveal any combinations that lead to the eradication of pre-formed S. aureus biofilms. Subsequent screening, against planktonic E. coli, detected a number of compounds with antibacterial activity, or potentiating effects of rifampicin and linezolid. However, further evaluation determined that these agents did not possess useful antibacterial activity. Drug repurposing and the use of antibiotic adjuvants has previously been regarded as an attractive approach to antibiotic drug discovery, due to a decreased risk of resistance, and reduced costs and time associated with development. Although these strategies clearly provide some advantages, results presented here suggest these methods are inefficient, requiring vast numbers of compounds to be screened for limited output. Furthermore, compounds that have undergone a degree of pre-clinical assessment frequently encounter issues regarding intellectual property, inhibiting their use (at least while the patent exists) in alternative therapies. Therefore, strategies such as these cannot be used as the only approach to the discovery and development of novel antibacterial therapeutics, but instead be used alongside other approaches such as natural product discovery.

Since library screening identified no compound or compound/antibiotic combinations that led to the eradication of pre-formed staphylococcal biofilms, it may be beneficial to increase the tested concentration in subsequent experiments. If agents were identified that exhibited unexpected antibacterial activity, further in vitro investigation could be performed to determine their
potential for repurposing as novel treatments of bacterial infections. Experimentation could include, but would not be limited too, extensive susceptibility testing, including antibiofilm activity (both prevention and eradication), evaluation of toxicity (i.e. selective or non-selective towards bacteria) and MOA characterisation, using approaches such as those outlined in Chapters 3 and 4.

In summary, the work described in this thesis has provided several insights that may be of benefit in the development of novel approaches in the treatment and prevention of biofilm associated infections. For example, it is apparent that compounds with membrane damaging effects may be a useful source of antibiofilm agents. Indeed, membrane damager Tocris 2611 makes an interesting candidate as an anti-staphylococcal, antibiofilm agent, and could potentially be utilised in topical creams. However, future studies would need to be performed to elucidate the exact antibacterial target within the cytoplasmic membrane. Despite advances in the development of antibiofilm coatings, no compounds were identified that retained antibiofilm activity when immobilised to a surface. Leaving a significant amount of development needed in the area of antibiofilm coatings and strategies in the healthcare setting. Finally, studies investigating the repurposing potential of agents highlighted that this approach cannot be reliably used as the sole method for the discovery of new antimicrobial drugs. However, collectively this research could facilitate the development of novel approaches in the treatment and prevention of biofilm associated infections.
Appendix A – Primers used in this study

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Appendix B – Activity of novel antimicrobial agents and control agents (ciprofloxacin and gentamicin) against *S. aureus* SH1000, USA300 and UAMS-1 and *S. epidermidis* RP62A.

Biofilm minimum inhibitory concentrations (bMICs) and minimum biofilm eradication concentration (MBEC) determined in the Calgary Biofilm Device. ND indicates not determined.

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<td>12.5 6.25 12.5 12.5</td>
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