Antibacterial activity and mechanism of action of lipophilic antioxidants

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The candidate confirms that the work submitted is 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 within this thesis has been published in the following paper:

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

Chapter 3: Antibacterial activity of *tert*-butylhydroquinone (TBHQ) and its oxidation product, *tert*-butylbenzoquinone (TBBQ)

Chapter 4: Investigations into the mode of action of TBBQ

All laboratory work was carried out by the candidate. The paper was written by the candidate in conjunction with other authors, who offered intellectual and editorial input.

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Abstract

The emergence and spread of antibiotic resistance hampers effective treatment of bacterial infections. This is particularly the case for infections involving a biofilm component, as the activity of existing antibacterial drugs against these surface-attached communities is limited. The work presented in this thesis sought to identify and characterise compounds with antibacterial and antibiofilm activity against the important pathogen, *Staphylococcus aureus*.

Antistaphylococcal activity was assessed for 16 antioxidants that are used in cosmetics, traditional medicines or as food additives, and which have been reported previously to have some antibacterial activity. Initial experiments with *tert*-butylhydroquinone (TBHQ) showed that activity that had previously been ascribed to the antioxidant, was a consequence of its conversion to *tert*-butylbenzoquinone (TBBQ) under culture conditions. TBBQ displayed innate bactericidal activity against *S. aureus* that was effected through perturbation of the bacterial membrane. The other antioxidants also inhibited staphylococcal growth through perturbation of the cytoplasmic membrane, and compounds that displayed selective action against bacterial membranes were identified. Of the agents with bacterial specificity, TBBQ, celastrol and nordihydroguaiaretic acid (NDGA) also eradicated staphylococcal biofilms; a rare property amongst antibacterial agents. Although these antioxidants exhibited a similar membrane-damaging mode of action, their mechanisms of antibiofilm activity differed. TBBQ eradicated preformed biofilms through sterilisation of slow-growing and persister cell populations, whilst celastrol and NDGA caused physical disruption of the biofilm. All three antioxidants acted synergistically with gentamicin against biofilms, eradicating surface attached populations at concentrations that did not cause irritation or visible damage to a human skin equivalent.

The potent and selective antibacterial activity, and low resistance potential upon extended subculture, suggest that these compounds could be used topically in combination with gentamicin to treat infected wounds.

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Abbreviations

ATP - adenosine triphosphate

BHA - Brain Heart Infusion agar

BLAST - basic local alignment search tool

BSAC - British Society for Antimicrobial Chemotherapy

C - Celcius

CA-MRSA - community-acquired meticillin-resistant *Staphylococcus aureus*

CBD - Calgary biofilm device

CCCP - carbonyl cyanide m-chlorophenyl hydrazone

cDNA - complementary deoxyribonucleic acid

CFU - colony forming unit

CLSI - Clinical and Laboratory Standards Institute

CTAB - cetyltrimethylammonium bromide

D-ala - D-alanine

DCCD - dicyclohexylcarbodiimide

D-lac - D-lactate

DMSO - dimethylsulphoxide

DNA - deoxyribonucleic acid

FIC - fractional inhibitory concentration

HA-MRSA - hospital-acquired meticillin-resistant *Staphylococcus aureus*

HEPES - 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

MBC - minimum bactericidal concentration

MBEC - minimum biofilm eradication concentration

ME - mosaic ends

MF - major facilitator

MHA - Mueller-Hinton agar

MHB - Mueller-Hinton broth

MIC - minimum inhibitory concentration

MIC_{90} - minimum inhibitory concentration required to inhibit the growth of 90% of isolates tested

MMS - macromolecular synthesis

Mn-SOD - manganese superoxide dismutase

MOA - mechanism of action

mRNA - messenger ribonucleic acid

MRSA - meticillin-resistant *Staphylococcus aureus*

MSCRAMMs - microbial surface components recognising adhesive matrix molecules

MSSA - meticillin-sensitive *Staphylococcus aureus*

NAD^{+} - Nicotinamide adenine dinucleotide

NADH - reduced nicotinamide adenine dinucleotide

ndc - no-drug control

NDGA - nordihydroguaiaretic acid

OD - optical density

PBP - penicillin binding protein

PBS - phosphate-buffered saline

PCR - polymerase chain reaction

pH - potential hydrogen

PIA - polysaccharide intercellular adhesin

PMBN - polymyxin B nonapeptide

PNAG - poly-*N*-acetyl glucosamine

rRNA - ribosomal ribonucleic acid

RNA - ribonucleic acid

ROS - reactive oxygen species

rpm - revolutions per minute

SCV - small colony variant

SDS - sodium dodecyl sulphate

SOD - superoxide dismutase

TAE - Tris-acetate-EDTA

TBBQ - *tert*-butylbenzoquinone

TBHQ - *tert*-butylhydroquinone

TCA - trichloroacetic acid

TE - Tris-EDTA

Tn - transposon

tRNA - transfer ribonucleic acid

VISA - vancomycin-intermediate *Staphylococcus aureus*

v/v - volume per volume

WGS - whole genome sequencing

WHO - World Health Organization

w/v - weight per volume

Chapter 1

Introduction

1.1 The burden of bacterial infections

Infectious diseases cause approximately 25% of deaths worldwide (Taylor *et al.*, 2001), and in 2011, accounted for the loss of ∼6.8 million lives (WHO, 2013). In the USA alone, communicable diseases cost $120 billion annually (NIAID, 2013). Approximately 1400 species of infectious organisms contribute to morbidity and mortality, including approximately 500 bacterial species (Taylor *et al.*, 2001). Amongst the bacteria that contribute to this loss of life are *Mycobacterium tuberculosis*, *Neisseria meningitidis*, and *Vibrio cholerae*. Of the bacterial pathogens, *M. tuberculosis* is the organism that leads to the greatest loss of life, and caused approximately 1.4 million deaths in 2011 alone (WHO, 2013). *M. tuberculosis*, *N. meningitidis*, and *V. cholerae* are community-acquired organisms, primarily contributing to loss of life in developing countries. However, in the industrialised world these organisms contribute less to the burden of infection, and instead hospital-acquired infections, caused by bacteria such as the Gram-positive staphylococci, are a major problem (WHO, 2013; Lindsay & Holden, 2004). Staphylococci are human skin commensals, with *Staphylococcus aureus* being recovered from the anterior nares of about 25% of the population (Kluytmans *et al.*, 1997; Lindsay *et al.*, 2012). However,

under certain conditions, *e.g.* following surgical procedures or in the immunosuppressed, these organisms are also capable of causing infections (Klein *et al.*, 2007). In 2005, *S. aureus* was estimated to cause around half a million hospitalisations in the USA, costing $9.5 billion to treat and causing ∼11,400 deaths (Klein *et al.*, 2007; Noskin *et al.*, 2005). *S. aureus* is the most common cause of hospital-acquired infections (Lindsay & Holden, 2004), and conditions caused by this organism include skin and soft tissue infections, impetigo, infective endocarditis, septicaemia, pneumonia and toxic shock syndrome (David & Daum, 2010; Noble, 1998). The coagulase-negative organism *Staphylococcus epidermidis* causes fewer severe infections in healthy hosts than *S. aureus*, but frequently infects individuals with an indwelling medical device, or who are immunocompromised (McCann *et al.*, 2008).

Treatment of bacterial infections is heavily dependent upon antibiotics, one of the most successful classes of chemotherapeutic agent to have been developed (Davies & Davies, 2010; Wright, 2007). However, there are problems with the antibiotics currently deployed clinically, which drives the need to develop new antibacterial agents. As will be discussed in this chapter and thesis, difficulties in treating bacterial infections result from the presence of antibiotic-resistant bacteria, cells in different growth states, and bacteria growing as biofilms. A major aspect of this study will be assessing antioxidants and their utility as antibacterial and antibiofilm agents. Therefore, information pertaining to the use of antioxidants as novel antibacterial agents will be reviewed. This chapter will focus on research carried out with *S. aureus*, since the aim of this investigation was to identify agents with useful antistaphylococcal activity. It should also be noted that whilst the strict historical definition of ‘antibiotic’ refers to natural products with antibacterial properties (Clardy *et al.*, 2009), the terms ‘antibiotic’ and ‘antibacterial agent’ are frequently used interchangeably in scientific literature and also throughout this thesis.

1.2 The causes of antibiotic treatment failure

1.2.1 The problem of antibiotic resistance

Although antibiotics are often successful in treating bacterial infections, there are situations in which antibiotics fail (Corey, 2009). Drug-resistant bacteria are a significant contributing factor to treatment failure, elevated care costs and mortality rates (Gandhi *et al.*, 2010). The prevalence of bacteria that are able to resist the effects of commonly prescribed antibiotics has increased over the last decade (EARS-Net, 2013), necessitating the development of agents to which bacteria are not yet resistant. However, bacterial resistance is not a new phenomenon; resistance to penicillin was identified in *S. aureus* within a year of its introduction to clinical use (Barber & Rozwadowska-Dowzenko, 1948). Subsequently novel antibiotics were discovered including aminoglycosides and tetracyclines, but as was the case with penicillin, resistance was identified soon thereafter (within 2 years of their FDA approval) (Bush, 2004). In recent years, antibiotic resistance has become more widespread due to multiple factors, including increased antibiotic consumption, veterinary practice, and improper use of antibiotics (Coates *et al.*, 2011; Davies, 2006). This has created a situation in which first-line antibiotics are no longer effective against some bacteria and too few new compounds are being approved in order to replace antibiotics that can no longer be used in a clinical setting (Coates *et al.*, 2011). These circumstances have prompted the World Health Organization to declare antibiotic resistance 'one of the three greatest threats to human health' and signals 'an end to modern medicine as we know it' (WHO, 2013).

The rise of multidrug resistant meticillin resistant *S. aureus* (MRSA) has been well documented in the media, and recent research estimates that MRSA accounts for up to 54% of clinical *S. aureus* isolates and is found in up to 24% of hospital patients (Dulon *et al.*, 2011). Indeed, MRSA is the most frequently isolated organism in nosocomial settings (Wang & Barrett, 2007). However, in the UK, reductions in the number of

infections caused by MRSA since 2006 has been attributed to introduction of successful infection control measures, such as improved hand hygiene and patient screening and decolonisation (Edgeworth, 2011). In addition to β-lactam resistance, MRSA strains often also carry genes that confer resistance to other antibiotics including macrolides, aminoglycosides, and tetracyclines (Lindsay & Holden, 2004). Concerns regarding MRSA were initially centered around its presence in the healthcare setting (HA-MRSA). However, more recently community acquired-MRSA (CA-MRSA) has been shown to cause infections in healthy individuals and may have more severe outcomes (David & Daum, 2010). CA-MRSA often contains pore-forming toxins encoded by the Panton-Valentine Leukocidin genes, and generally causes skin and soft tissue infections (Baba *et al.*, 2002; David & Daum, 2010). There is high genetic diversity within MRSA, and different strains predominate in different countries (Lindsay, 2010). MRSA is not necessarily more pathogenic than meticillin sensitive *S. aureus*, but it does cause more deaths, longer hospitalisation and increased care costs due to initial treatment failure (Cosgrove, 2006). Where β-lactams can be prescribed, due to infections caused by susceptible *S. aureus*, these antibiotics are the frontline treatment. However, if the infection is caused by MRSA, vancomycin or daptomycin are the recommended treatment options for patients with bacteraemia or infective endocarditis (Liu *et al.*, 2011).

1.2.2 Antibiotic resistance mechanisms

Bacteria are genetically plastic and can evolve to become resistant to one or a number of antibacterial agents. Classical inherited resistance to antibiotics may arise through endogenous (mutational) or exogenous means (by horizontal gene transmission) (Silver, 2011). In staphylococci, resistance may result from acquisition of mobile genetic elements through transformation, conjugation, or transduction (Lindsay, 2010). Indeed, mobile genetic elements account for 15-20% of the staphylococcal genome, with staphylococcal cassette chromosome *mec* (SCC*mec*) and plasmids generally carrying

resistance genes (Lindsay *et al.*, 2012).

Resistance to antibiotics can result through a number of means (Figure 1.1) (Wright, 2010). Bacteria may evade the effects of antibiotics by down regulating porin production, increasing expression of efflux pumps or through acquisition of mutations that increase pump efficiency (Webber & Piddock, 2003; Piddock, 2006). Efflux pumps may transport a specific substrate or a range of substrates (Webber & Piddock, 2003), and the genome of *S. aureus* suggests that the organism may be capable of producing $\sim$17 multidrug-resistance efflux pumps (Kaatz *et al.*, 2003). These are subdivided into groups based upon their structure, and in staphylococci, the majority are members of the major facilitator (MF) superfamily, which traverse the membrane. Their substrates include macrolides, tetracyclines, and fluoroquinolones along with biocides and dyes (Kaatz *et al.*, 2003). Although overexpression of efflux pumps rarely causes high-level resistance to clinically relevant antibiotics, it may augment development of antibiotic target site mutations (Webber & Piddock, 2003).

Resistance to tetracycline has been well characterised and occurs via both efflux and target protection in staphylococci. Tetracycline perturbs bacterial protein synthesis by preventing binding of aminoacyl-tRNA in the peptidyltransferase centre of the 30S ribosomal subunit, and in the process inhibits bacterial growth (Chopra & Roberts, 2001). Bacteria may carry plasmid-located *tet*(K) and *tet*(L) that code for specific pumps, which efflux tetracycline from the cell in exchange for a proton. Alternatively, resistance is due to ribosomal protection proteins, which are coded for by resistance determinants such as *tet*(M) and *tet*(O). When translated these proteins bind to the ribosome and allosterically prompt release of tetracycline from the ribosome (Schmitz *et al.*, 2001; Roberts, 2005).

Other resistance mechanisms involve modification of the antibacterial target. In the case of vancomycin, the antibiotic target is the pentapeptide linker of peptidoglycan (Courvalin, 2006). *S. aureus* contains the bacteria-specific polymer, peptidoglycan, which is composed of repeated units of *N*-acetyl glucosamine-*N*-actyl muramic acid

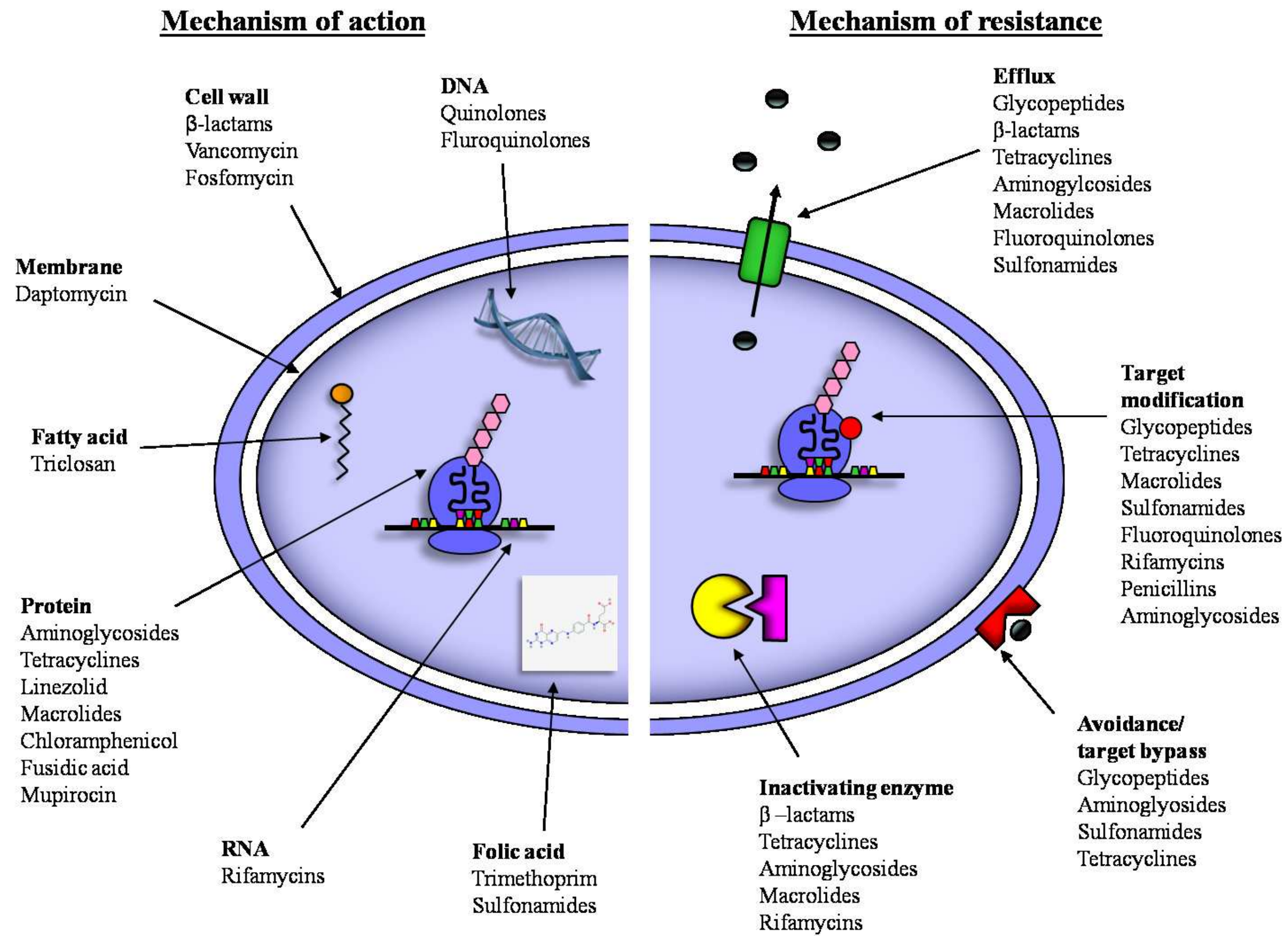

Figure 1.1: **The major sites of antibiotic action, and mechanisms of bacterial resistance.** Image adapted from Wright (2010).

that are cross-linked by pentapeptides (Silhavy *et al.*, 2010). The precursor of peptidoglycan crosses the cell membrane and is attached to the cell wall polysaccharide via transglycosylation. Cross linking of the pentapeptide with other cell wall peptides occurs by enzyme-assisted transpeptidation (Courvalin, 2006). The glycopeptide vancomycin inhibits growth by binding to the D-Ala-D-Ala termini of the pentapeptide, stericially hindering transglycosylation (Courvalin, 2006). The consequent disruption to the peptidoglycan structure results in weakening of the cell wall and cell lysis (Tomasz, 1979). In *S. aureus* VanA-type resistance is mediated through a dehydrogenase (VanH) that reduces pyruvate, forming D-Lac, and a ligase (VanA), which forms a bond between D-Ala and D-Lac. This results in conversion of pentapeptide termini from D-ala-D-Ala to D-Ala-D-Lac in the pentapeptide linkers. Additionally, a D,D-dipeptidase (VanX) hydrolyses existing D-Ala-D-Ala, and a D,D-carboxypeptidase (VanY) removes the terminal D-Ala that will be replaced by D-Lac via VanA. This modifies the vancomycin binding pocket, and allows pentapeptides to undergo transglycosylation (Courvalin, 2006). The presence of enterococcal derived *vanA* affords the carrier high level resistance to vancomycin, but has been identified in only a small number of *S. aureus* strains (Courvalin, 2006), and infections caused by vancomycin-resistant *S. aureus* remain rare to date (Melo-Cristino *et al.*, 2013). Vancomycin resistant *S. aureus* also avoid bactericidal effects of the antibiotic via thickening of the peptidoglycan layer, titrating out the effects of vancomycin (Cui *et al.*, 2003).

Similarly, rifampicin- and quinolone-resistant strains evade the inhibitory effects of these drugs by target modification. Rifampicin is an RNA synthesis inhibitor that binds to the β subunit of the transcriptional catalyst RNA polymerase, blocking elongation of RNA (Chopra, 2007). Mutations in *rpoB* (coding for the RNA polymerase β subunit), provide rifampicin resistant strains with a means to evade antibiotic effects (O'Neill *et al.*, 2000). The binding of quinolones to DNA gyrase and topoisomerase IV (responsible for DNA supercoiling and separation of chromosomes) causes conformational changes that inhibit progression of the replication fork and stall DNA replication, leading to chromosome

fragmentation and cell death (Blondeau, 2004; Collin *et al.*, 2011). Resistance to quinolones is conferred by mutations in *grlA*/*gyrA* and *grlB*/*gyrB* (coding for subunits of topoisomerase IV/DNA gyrase) (Collin *et al.*, 2011; Blondeau, 2004). These genetic alterations reduce drug affinity for the primary target, and may act in concert with efflux mechanisms in quinolone resistant staphylococci (Collin *et al.*, 2011; Blondeau, 2004; Piddock, 2006).

S. aureus are resistant to antibiotics, such as meticillin, due to target bypass. β-lactams inhibit peptidoglycan synthesis by covalently binding to the active site of transpeptidases, also known as penicillin-binding proteins (PBPs), which inhibits cell wall cross linking. The product of *mecA*, located within the mobile genetic element SCC*mec*, is an alternative penicillin-binding protein (PBP2a) that displays reduced affinity for β-lactams (Lindsay, 2010; Alekshun & Levy, 2007).

Finally, antibiotics may be enzymatically inactivated by hydrolysis, group transfer or redox reactions (Wright, 2005). A classic example of resistance caused by enzymatic modification is bacterial production of β-lactamase, an enzyme that cleaves the β-lactam ring of penicillins, rendering them inactive. More than 1000 β-lactamases have been discovered (Bush & Fisher, 2011), and can be grouped into four classes. Types A, C and D are serine β-lactamases and B contains zinc metallo enzymes (Rubtsova *et al.*, 2010). Similarly, aminoglycoside resistance primarily occurs in staphylococci through the presence of aminoglycoside modifying enzymes (Schmitz *et al.*, 1999). These antibiotics exert their effect upon the 30S subunit of the bacterial ribosome by inhibiting translocation of tRNA from the A-site to the P-site, and there is evidence to suggest that aminoglycosides also cause membrane damage and incorporation of mistranslated proteins into lipid bilayers (Kotra *et al.*, 2000; Davis, 1987). The most common aminoglycoside modifying enzymes have acetyltransferase, adenylyltransferase and phosphotransferase activity, and alter aminglycosides at amino or hydroxyl groups, inducing loss of ribosome-binding and protein synthesis inhibition (Schmitz *et al.*, 1999).

1.2.3 Impact of bacterial growth state on antibiotic susceptibility

Antibiotic resistance contributes to difficulties in treating bacterial infections, but bacteria displaying altered growth states also appear to affect the outcome of antibiotic treatment. Hard-to-treat infections include those where small colony variants (SCVs) are present (Proctor *et al.*, 2006). SCVs form small, non-pigmented, non-haemolytic colonies and display unique gene expression patterns. They commonly have altered toxin production, adhesion and respiratory pathways and are linked to chronic infection (Proctor *et al.*, 2006). Staphylococcal SCVs are either defective in thymidine biosynthesis, or have a malfunctioning electron transport chain and altered membrane potential as a consequence of defective menadione or haemin biosynthesis (Proctor *et al.*, 2006). SCVs have been implicated in persistent osteomyelitis, sinusitis, arthritis, and device-related bloodstream infections (Seifert *et al.*, 2003; Proctor *et al.*, 2006). It has been suggested that the generation of SCVs is a normal aspect of the pathogenesis of infection, and contributes to aminoglycoside treatment failure. Aminglycoside uptake is dependent upon the cytoplasmic membrane potential, which is reduced in SCVs with a defective electron transport chain, rendering the antibiotic unable to access intracellular targets (Proctor *et al.*, 2006).

Tolerant bacteria (exhibiting a bacteriostatic response to antibacterial challenge that is bactericidal for the majority of the population) can contribute to difficulties in eradicating bacterial infections, and like SCVs, have a mutation/mutations that cause the phenotype (Proctor *et al.*, 2006; Griffiths & O'Neill, 2012). In tolerant bacteria these genetic alterations cause a shift from a bactericidal to a bacteriostatic response to antibiotics (Griffiths & O'Neill, 2012). Tolerance is defined as a minimum bactericidal concentration: minimum inhibitory concentration (MBC:MIC) ratio $\geq$32 (Bizzini *et al.*, 2007), where MBC is a 99.9% kill (3 $\log_{10}$ CFU/mL reduction in viable cells after 24 hours) (Bizzini *et al.*, 2009). This response has been identified in staphylococci and streptococci and is associated with exposure to β-lactams and glycopeptides (Griffiths

& O'Neill, 2012). The genetic basis of tolerance in several *S. aureus* strains is due to mutations in GdpP, a putative signaling protein (Griffiths & O'Neill, 2012). Tolerant cells are clinically relevant, with β-lactam tolerant *S. aureus* infections requiring long treatment regimens, frequently causing treatment failure and patient relapses (Rajashekaraiah *et al.*, 1980).

Bacteria may also become refractory to antibiotics due to non-inherited resistance mechanisms (Levin & Rozen, 2006). This is achieved by drug indifference, where the whole bacterial population is resistant to killing due to metabolic inactivity, or due to the presence of persisters; a sub-population of cells that are killed by antibiotics at a slower rate. Persistence (also known as phenotypic tolerance) is a pre-existing property of bacteria in which metabolic inactivity is transitory, and persisters are not genetically different from other cells within the population (Levin & Rozen, 2006). Persisters tolerate the presence of antibiotics due to down regulation of cellular processes that are targeted by the majority of antibacterial agents (Keren *et al.*, 2004). Antibiotics that target biosynthetic pathways are less effective against cells that are not actively growing, as there are fewer active targets that can be corrupted (Keren *et al.*, 2004). Therefore, compounds that are under development as antibacterial agents would ideally display activity against these resilient populations of cells, in addition to avoiding existing antibiotic resistance mechanisms.

1.2.4 Bacterial biofilms: a recalcitrant mode of growth

Arguably biofilms and the infections that are associated with this mode of growth pose the greatest challenge to therapy and biofilms include some of the types of antibiotic insusceptible cells discussed in the previous section. Bacterial biofilms are found throughout nature, existing in thermal springs, corroding pipes, and are even found on the international space station (Weidler *et al.*, 2007; Juhna *et al.*, 2007; Kim *et al.*, 2013). In a clinical setting, biofilms are implicated in the development of infections

such as dental caries, endocarditis, sinusitis, cystic fibrosis pneumonia and infections of indwelling medical devices (Fux *et al.*, 2005; Del Pozo & Patel, 2007; Brady *et al.*, 2008; Bendouah *et al.*, 2006). In staphylococci, environmental stimuli may initiate the conversion from a planktonic mode of growth to biofilm formation; for example, under iron-limited conditions and in the presence of ethanol (Johnson *et al.*, 2005; Lim *et al.*, 2004). Staphylococcal cells adhere to surfaces with the assistance of ∼20 different proteins known as MSCRAMMs (microbial surface components recognising adhesive matrix molecules) (Walsh *et al.*, 2008). These include fibronectin binding proteins A and B, which are attached to peptidoglycan in the *S. aureus* cell wall (Greene *et al.*, 1995). Attached cells generate extracellular matrix that contains: proteins; teichoic acid; extracellular DNA; and polysaccharide intercellular adhesin (PIA), which is primarily made of poly-*N*-acetyl glucosamine (PNAG) (Izano *et al.*, 2008; Mack *et al.*, 1996). Maturation of staphylococcal biofilms is dependent upon PIA generated via the intercellular adhesion (*ica*) operon (Heilmann *et al.*, 1996), and is aided by a number of surface proteins, such as accumulation-associated protein (*S. epidermidis*) and biofilm-associated protein (*S. aureus*) (Hussain *et al.*, 1997; Cucarella *et al.*, 2001).

Regulation of biofilm-associated genes is primarily through transcriptional regulators including the transcriptional repressor IcaR, staphylococcal accessory regulator (*sarA*), accessory gene regulator (*agr*), and the alternative sigma factor (SigB) (Beenken *et al.*, 2003; Boles & Horswill, 2008; Beenken *et al.*, 2004; Rachid *et al.*, 2000; Nicholas *et al.*, 1999). The *ica* operon contains the negative regulator IcaR and biosynthetic genes *icaADBC*. Repression of IcaR transcription enhances *icaADBC* expression, leading to PIA production and generation of biofilms (Jefferson *et al.*, 2003). However, biofilm formation is multifactorial and may occur in an *ica*-independent fashion; for example *S. aureus* UAMS-1 has mutations in the *ica* operon but is still able to form biofilms (Beenken *et al.*, 2004). The *sigB* operon is also involved in biofilm regulation, enhancing production of cell surface proteins that aid initial biofilm formation (Rachid *et al.*, 2000; Nicholas *et al.*, 1999). Likewise, the global regulator *sarA* is necessary for biofilm

formation (Beenken *et al.*, 2003), whilst *agr* plays a crucial role in biofilm dispersal (Boles & Horswill, 2008). Therefore, the regulation of biofilm-associated genes involves a complex network, the subtleties of which are not yet fully understood.

Following the attachment of cells to a surface, formation of cell-cell bridges, and release of extracellular matrix (Lindsay & Von Holy, 2006), the biofilm increases in structural complexity. Mature forms contain mushroom-like structures and water channels to allow water and oxygen to penetrate the biofilm, and diffusion of waste products (Massol-Deya *et al.*, 1995). The extracellular matrix protects bacteria within the biofilm from the host's immune system (Hall-Stoodley *et al.*, 2004), and some bacteria are dispersed from the mature biofilm as planktonic cells that can colonise new surfaces (Kaplan, 2010).

The cells contained within the biofilm experience a range of environments and consequently display stratified anabolic activity. Those near the surface of the biofilm have sufficient nutrients and oxygen (Rani *et al.*, 2007). However, for those bacteria deep within the biofilm, low availability of metabolites and oxygen, and the buildup of waste products creates conditions inhibitory to growth. Under these circumstances bacteria may exhibit a beneficial survival strategy: cessation of active growth. Thus, biofilms contain bacteria in a range of metabolic states: growing aerobically; growing anaerobically/fermentatively; non-growing and dead cells (Rani *et al.*, 2007; Mah & O'Toole, 2001).

Bacteria contained within biofilms are 10-1000x less susceptible to antibiotic action than planktonic counterparts (Mah & O'Toole, 2001), and biofilm recalcitrance to antibiotic action is a consequence of multiple mechanisms (Figure 1.2). Antibiotics that are used clinically were selected for development on the basis of their activity against planktonic cells, without consideration for antibiofilm activity. As such, agents that are capable of eradicating biofilms may display different characteristics to established antibiotics. For example, antibiofilm agents may need to retain activity against tolerant bacteria, which are present in biofilms and contribute to antibiotic insusceptibility (Nguyen *et al.*,

2011). Tolerance in biofilms is generated by nutrient starvation that causes physiological changes following induction of the stringent response (Nguyen *et al.*, 2011). The presence of dormant or persister cells alongside metabolically active bacteria also contributes to difficulty in eradicating biofilms. Ciprofloxacin has been shown to kill growing cells at the edges of *P. aeruginosa* biofilms and colistin is active against metabolically inactive cells at the centre, but the compounds cannot eradicate whole biofilms when applied individually (Høiby *et al.*, 2010). Therefore, monotherapy may simply kill a portion of the cells, leaving persister cells to repopulate the biofilm upon removal of antibiotic (Lewis, 2006). As such, biofilms may be most successfully treated with synergistic combinations of antibacterial agents (Høiby *et al.*, 2010).

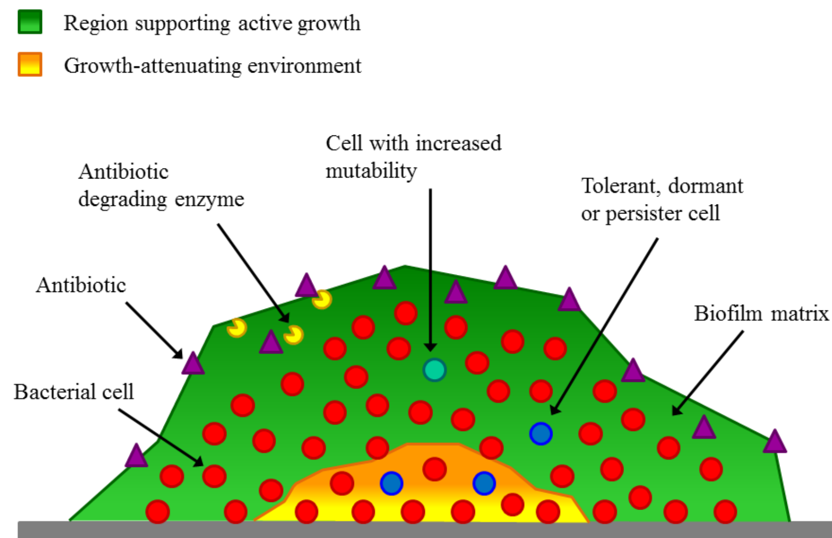

Figure 1.2: **Mechanisms by which biofilms can resist antibacterial agents.** The matrix limits access of some antibiotics to the centre of the biofilm, where tolerant, persister and dormant cells display reduced drug susceptibility. Bacterial cells display altered gene expression and produce antibiotic degrading enzymes. Antibiotic resistance arises due to enhanced mutability of biofilm cells, and can be rapidly disseminated.

Additionally, biofilms accelerate both the emergence and dissemination of antibiotic resistance. Staphylococcal biofilms display increased mutability, which enables development of resistance through spontaneous mutation at an increased rate (Ryder *et al.*, 2012). Furthermore, dramatic increases in conjugative transfer of plasmids enable rapid dissemination of plasmid-borne resistance determinants within surface-adhered populations (Savage *et al.*, 2013).

Bacteria in biofilms alter their gene expression, enhancing production of efflux pumps and antibiotic modifying enzymes (Høiby *et al.*, 2010). Indeed, upregulation of efflux pumps and transporters accounts for $\sim$20% of all the genes upregulated in biofilms (Kvist *et al.*, 2008). Increased levels of β-lactamases are found near the surface of the biofilm, where the concentration of antibiotic is highest. This response is mediated via quorum sensing-regulated mechanisms, in which organisms sense a critical concentration of bacteria within in a limited space and respond by altering gene expression (Høiby *et al.*, 2010). For those compounds that are not degraded enzymatically, exclusion from the biofilm may reduce antibiotic activity. For example, oxacillin, cefotaxime and vancomycin have retarded penetration through the staphylococcal matrix, whilst ciprofloxacin and amikacin diffusion is unaffected (Singh *et al.*, 2010).

The continual emergence of antibiotic resistant bacteria, and ability of organisms to avoid the lethal effects of antibiotics when growing under certain conditions contributes to the urgent need for discovery of new antibacterial agents. Since a large proportion of infections involve bacterial biofilms (Davies, 2003), it is particularly important to discover new antibacterial agents that can eradicate biofilms. Furthermore, to overcome problems associated with antibiotic resistance, compounds would ideally have a novel MOA, as resistance to these agents would be less likely to arise through alterations to an existing resistance mechanism (O'Neill & Chopra, 2004). There are numerous methods that can be employed to identify novel inhibitors and antibiotic discovery strategies are considered the following section.

1.3 A brief history of antibiotic discovery

Antibacterial drug discovery programmes aim to identify compounds with targets that are essential to the bacteria, have no human homologues, and have low potential to generate resistance (Silver, 2011). There are a number of approaches that can be taken to achieve these goals. Historically, the majority of existing antibiotics have been discovered through empirical screens of fermentation products, and modification of compounds with known antibacterial activity. Early research into antibiotics include Duschense's identification of bactericidal products from fungi in the 1890s (Duckett, 1999), and Fleming's detection of penicillin from mould in 1929 (Fleming, 1929; Conly & Johnston, 2005). During the 1930s, sulphonamides were discovered (Conly & Johnston, 2005), and in the following decades numerous classes of antibiotics were identified. This era was termed as the 'golden age' of antibiotic discovery, during which time aminoglycosides, tetracyclines, macrolides, quinolones and cephalosporins were discovered and brought into use (see Figure 1.3) (Silver, 2011; Conly & Johnston, 2005).

Subsequently, the impetus moved to target based screening in order to avoid rediscovery of the same compounds from natural product libraries. This approach had limited success, but yielded some compounds that reached the market, such as trimethoprim and fosfomycin (Silver, 2011). By the 1970s, antibiotic discovery had slowed significantly. In recent times, rational/structure-based drug design has increased in popularity, though as yet no compounds have been successfully developed as antibiotics (O'Neill & Chopra, 2004; Silver, 2011). Additionally, large-scale chemical screens have been carried out by pharmaceutical companies, but have been fruitless, in part due to inadequate or unsuitable chemical libraries (Silver, 2011). While compounds with antibacterial activity are readily discovered, they are rarely suitable for development due to inhibitory activity in mammalian cells. Difficulties in delivering chemical inhibitors of bacterial targets into cells and avoiding efflux contribute to low discovery rate. This is a problem particularly

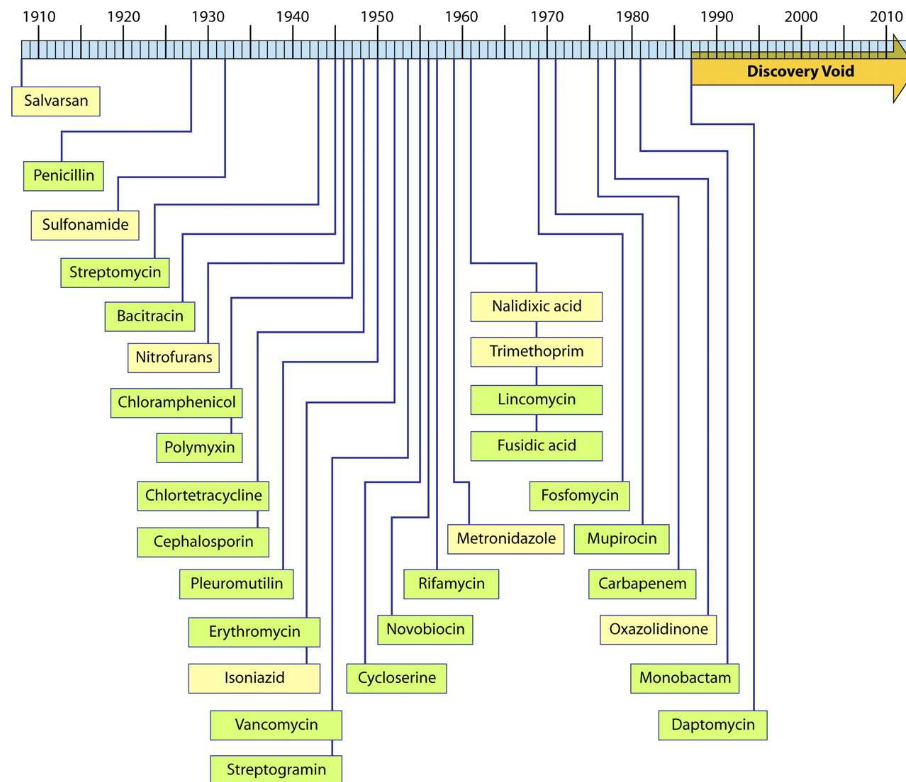

Figure 1.3: **Timeline of antibiotic discovery.** Image reproduced from Silver (2011).

for Gram-negative antibacterials, for which the outer membrane is a significant challenge (Silver, 2011). In the last 40 years, although analogues of existing antibiotics have been developed, very few novel antibiotic classes have been discovered and successfully developed for human use. This 'discovery void' directly contributes to current difficulties in treating bacterial infections, as there are reducing numbers of effective antibiotics (Silver, 2011; Coates *et al.*, 2011).

1.4 Antioxidants and antibacterial activity

Due to the dearth of successful development of novel antibiotics, investigating underexploited compounds has become increasingly common (Chopra, 2003). Antibiotics such as daptomycin and retapamulin, that had previously been discovered but were not developed for clinical use, have been successfully revisited (Hawkey, 2008; Kirst, 2012). Repurposing of compounds that are already in human use, for example in healthcare products, may be a rewarding approach to discovery of new antibacterial agents. Therefore, the present study sought to investigate the antibacterial properties of antioxidants that were already known to have some antibacterial activity. This project arose from research carried out at Evocutis (formerly Syntopix), the aim of which had been to identify antioxidants that could resolve acne due to activity against *Propionibacterium acnes*. Several antioxidants displayed potentially useful activity against Gram-positive bacteria, and patents held by Evocutis cover the use of *tert*-butylhydroquinone (TBHQ) and AO 2246 as topical acne treatments (Seville & Wilkinson, 2008; Jones *et al.*, 2008a). From the screens carried out at Evocutis (susceptibility testing of propionibacteria and staphylococci to antioxidants in broth), 16 phenylic antioxidants were identified that had antistaphylococcal activity (see Figure 1.4 for chemical structures). A number of these antioxidants are in use as cosmetics additives or food preservatives (Seville & Wilkinson, 2008; Merrifield & Yang, 1965; Fung *et al.*, 1985), whilst others derive from plants that have been used medicinally (thymoquinone from *Nigella sativa* (Chaieb *et al.*, 2011), NDGA from *Larrea tridentate* (Martins *et al.*, 2013), celastrol from *Tripterygium wilfordii* (Chen *et al.*, 2011) and bakuchiol from *Psoralea corylifolia* (Katsura *et al.*, 2001). These antioxidants have been used in medicinal applications or are approved for human consumption, suggesting that they do not have overt eukaryotic toxicity. Table 1.1 contains a list of phenylic antioxidants with known antibacterial activity and information pertaining to their mechanism of action.

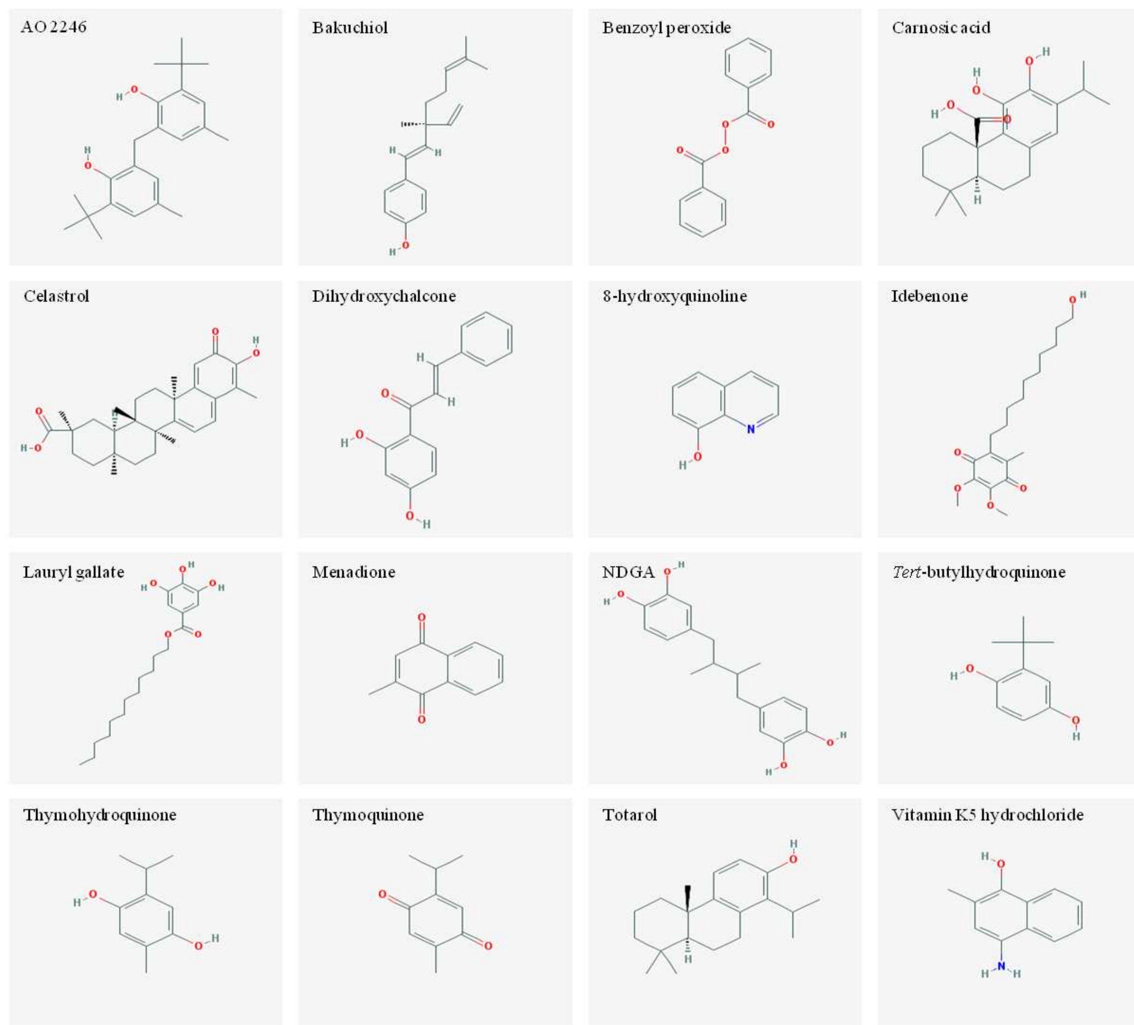

Figure 1.4: **Chemical structures of phenylic antioxidants** All structures obtained from PubChem (http://pubchem.ncbi.nlm.nih.gov/).

Compound	MIC for a range of bacteria (μg/mL)	Mechanism of action	Reference
Antioxidant 2246	0.98-31.25	-	Seville & Wilkinson (2008)
Bakuchiol	1-32	In *Streptococcus mutans* bakuchiol causes membrane damage	Reddy *et al.* (2010) Katsura *et al.* (2001)
Benzoyl peroxide	64-512	-	Eady *et al.* (1994)
Carnosic acid	2-30	In *S. aureus* carnosic acid increases membrane permeability and affects efflux	Moreno *et al.* (2006) Horiuchi *et al.* (2007)
Celastrol	0.3-1.3	-	Moujir *et al.* (1990), Ankli *et al.* (2000)
Dihydroxychalcone	0.1-100	In *S. aureus* dihydroxychalcone may damage the cell envelope or affect efflux	Zampini *et al.* (2005) Tran *et al.* (2012)
8-hydroxyquinoline	0.9-15.6	In *B. subtilis* 8-hydroxyquinoline inhibits DNA synthesis	Çolak *et al.* (2009) Mariner *et al.* (2011)
Idebenone	1.6-100	In *Helicobacter pylori* idebenone inhibits respiration and decreases intracellular ATP	Inatsu *et al.* (2006)

Table 1.1: **Antibacterial activity and MOA of antioxidants .**

Compound	MIC for a range of bacteria (μg/mL)	Mechanism of action	Reference
Lauryl gallate	6.25-100	In *P. aeruginosa* and *S. aureus* lauryl gallate disrupts the membrane, inhibits the respiratory chain and causes ROS generation. ROS are not the sole cause of death	Kubo *et al.* (2003)
Menadione	2-3.2	In *S. aureus* ROS generation contributes to antibacterial activity, but is not the sole cause of death	Inatsu *et al.* (2006), Chaudhuri *et al.* (2010) Schlievert *et al.* (2013)
NDGA	125-500	-	Martins *et al.* (2013)
TBHQ	0.5-31	In *E. coli* TBHQ may induce ROS production that could damage DNA or membranes	Raccach & Henningsen (1982) Jones *et al.* (2008a), (Malone *et al.*, 2008)
Thymohydroquinone	4-125	-	Toama *et al.* (1974)
Thymoquinone	8-512	In *P. aeruginosa* and *S. aureus* thymoquinone increases membrane permeability or affects efflux	Chaieb *et al.* (2011) Kouidhi *et al.* (2011)
Totarol	0.4-3.2	In *B. subtilis* and *S. aureus* totarol increases membrane permeability through membrane depolarisation and inhibits the respiratory chain	Kubo *et al.* (1992) Nicholas *et al.* (1999) Foss *et al.* (2013)
Vitamin K5 hydrochoride	64-1024	-	Miranda *et al.* (2011)

Table 1.1 (continued): **Antibacterial activity and MOA of antioxidants.**

Little or nothing is known about the antibacterial MOA of approximately half of the antioxidants under investigation here (Table 1.1). For the remaining compounds, studies suggest that antioxidants either induce membrane damage (Katsura *et al.*, 2001; Horiuchi *et al.*, 2007; Tran *et al.*, 2012; Kubo *et al.*, 2003; Kouidhi *et al.*, 2011; Foss *et al.*, 2013), or kill bacteria through generation of reactive oxygen species (ROS) (Kubo *et al.*, 2003; Malone *et al.*, 2008; Schlievert *et al.*, 2013). Aerobic respiration causes the continual production of ROS through the electron transport chain (Cabiscol *et al.*, 2000). In bacteria oxidation of respiratory chain components transfers electrons to O_2, generating superoxide. This reactive species is converted to hydrogen peroxide, which undergoes the Fenton reaction, involving cycling of transition metals such as Fe^{2+}, eventually forming hydroxyl radicals, water and Fe^{3+} (Cabiscol *et al.*, 2000; Farr & Kogoma, 1991).

Excess production of ROS can exert deleterious effects on bacteria by interacting with cell components and may contribute to antioxidant MOA (Farr & Kogoma, 1991; Kubo *et al.*, 2003; Malone *et al.*, 2008; Schlievert *et al.*, 2013). DNA can be damaged by hydroxyl radicals at either the sugar or base by extraction of a hydrogen atom from the deoxyribose ring. This leads to its collapse and creation of single- and double-strand breakage (Farr & Kogoma, 1991). Protein function can be lost due to superoxide or hydrogen peroxide induced structural modifications, including peptide fragmentation and side chain alterations (Cabiscol *et al.*, 2000). Iron-sulphur cluster-containing enzymes are particularly susceptible to oxidative damage leading to enzyme inactivation and degradation (Farr & Kogoma, 1991). Hydroxyl radicals can also damage polyunsaturated fatty acids in membranes and cause lipid peroxidation. This disrupts membrane function by reducing fluidity, due to shortening of fatty acid chains, and disturbance of membrane bound proteins (Farr & Kogoma, 1991).

A number of the antioxidants are reported to damage the bacterial membrane, which is a property that is often associated with antibiofilm activity (Hurdle *et al.*, 2011). Additionally, the antioxidants thymoquinone, benzoyl peroxide and bakuchiol were found

to inhibit growth of bacterial biofilms, although eradication of preformed surface-attached communities has not been documented to date (Chaieb *et al.*, 2011; Coenye *et al.*, 2007; Reddy *et al.*, 2010). Therefore, there are indications that these antioxidants are suitable for investigation as antibacterial agents, and the possibility of antioxidants displaying antibiofilm activity is of particular interest.

1.5 Objectives of this study

Due to the increasing prevalence of multidrug-resistant bacteria and the critical lack of novel antibiotics in development, this study aimed to identify potential candidates for antistaphylococcal treatments. In order to avoid some of the pitfalls of recent attempts to identify new antibacterial agents (Silver, 2011), studies were carried out with compounds that were already known to have whole-cell antibacterial activity. Studies focused on inhibitors of *S. aureus*, a nosocomial and community-acquired pathogen of major clinical significance (Livermore, 2009). A number of antioxidants were screened for activity against both planktonic and biofilm cultures, and synergistic interactions of compounds with existing antibiotics were assessed. If compounds had detectable inhibitory activity against biofilms, the basis of this rare property was investigated by studying effects on components of the biofilm.

Characterisation of the MOA of antibacterial agents is a critical aspect of novel drug development (O'Neill & Chopra, 2004). Therefore, the killing kinetics and MOA of promising antioxidants were determined. MOA experiments were used to identify the effects of compounds on common antibacterial targets: the synthesis of macromolecules and the bacterial membrane. The development of resistance to agents was investigated *in vitro* in order to provide information about the likelihood of appearance of high-level resistance in the clinic. Following identification of resistant mutants, the genetic basis of altered susceptibility was studied via genome sequence determination and the

contribution of any identified mutations was confirmed through complementation studies. This provided further information regarding the cellular target of the compound. Finally, compounds with useful antibacterial activity were assessed for bacterial specificity and effects on eukaryotic cells. It is hoped that this approach has identified a lead/several lead compounds that could be developed as antibacterial products.

Chapter 2

Materials and Methods

2.1 Bacterial strains, plasmids, and growth conditions

Laboratory strains of bacteria used in this study are listed in Table 2.1. Clinical *S. aureus* isolates used for susceptibility testing were part of a culture collection belonging to the Antimicrobial Research Centre, University of Leeds. Plasmid pEPSA5 was used in complementation studies (Forsyth *et al.*, 2002). Staphylococci and *Pseudomonas aeruginosa* were propagated in Mueller-Hinton broth (MHB) at 37°C with aeration and on Mueller-Hinton agar (MHA) (Oxoid). Strains of *E. coli* and *B. subtilis* were grown in Luria-Bertani broth at 37°C with aeration and on Luria-Bertani agar (Oxoid). For studies with daptomycin and valinomycin, culture media were supplemented with calcium (50 μg/mL, in the form of $CaCl_2$) and potassium chloride (0.1 M), respectively. For biofilm penetration experiments using cellulose-ester membrane filter discs (Millipore), biofilms were grown on Brain Heart Infusion agar (BHA) (Oxoid), and staphylococcal biofilms in microtitre plates were grown in Tryptic Soy Broth (TSB) (Oxoid). Small colony variants were generated by transduction of *hemB*::*ermB* and *menD*::*ermC* from *S. aureus* 8325-4 (Bates *et al.*, 2003) into *S. aureus* SH1000 using phage ϕ11 (Section 2.8.1) (Foster, 1998).

Strain	Comments	References/Source
Staphylococcus aureus		
SH1000	Standard laboratory strain (*S. aureus* 8325-4 with functional *rsbU*)	Horsburgh *et al.* (2002), O'Neill (2010)
ATCC 23723	Antibiotic susceptibility testing strain	BSAC (1991)
ATCC 29213	Antibiotic susceptibility testing strain	BSAC (1991)
UAMS-1	Prolific biofilm-forming strain	Gillaspy *et al.* (1995)
KC043	SH1000 with defective *ahpC/katA*	Cosgrove *et al.* (2007)
MHK11AM	SH1000 with defective *sodA/sodM*	Karavolos *et al.* (2003)
SH1000 *hemB*::*ermB*	Small colony variant	This study
SH1000 *menD*::*ermC*	Small colony variant	This study
RN4220	Restriction deficient derivative of *S. aureus* 8325-4	Fairweather *et al.* (1983)
CAJ192 C9	Part of the University of Leeds Tn library	This study
B7	-	This study
C3	-	This study
Staphylococcus epidermidis		
RP62A	Prolific biofilm-forming strain (ATCC 35984)	Ryder *et al.* (2012)

Table 2.1: **Bacterial strains used in this study.**

Strain	Comments	References/Source
Bacillus subtilis		
1S34 (pS77)	DNA synthesis biosensor	Urban *et al.* (2007)
1S34 (pS63)	RNA synthesis biosensor	Urban *et al.* (2007)
1S34 (pS72)	Protein synthesis biosensor	Urban *et al.* (2007)
1S34 (pS107)	Cell wall synthesis biosensor	Urban *et al.* (2007)
1S34 (pNS14)	Fatty acid synthesis biosensor	Fischer *et al.* (2004)
Escherichia coli		
1411	K-12 strain	O'Neill *et al.* (2002)
SM1411	1411 with defective *acrAB*	O'Neill *et al.* (2002)
AB734	K-12 strain	Shapiro & Baneyx (2002)
ES100	AB734 with defective *tolC*	Shapiro & Baneyx (2002)
DH5α	Standard cloning strain	Invitrogen
Pseudomonas aeruginosa		
PAO1	Standard laboratory strain	Stover *et al.* (2000)

Table 2.1 (continued): **Bacterial strains used in this study.**

2.2 Chemicals, reagents and antibacterial compounds

Antibiotics and chemicals were from Sigma-Aldrich unless otherwise stated. Ampicillin was purchased from Fisher Scientific, cefotaxime from MP Biomedicals, ciprofloxacin from Bayer, carbonyl cyanide m-chlorophenylhydrazone (CCCP) from Millipore, daptomycin from Cubist Pharmaceuticals, dicyclohexylcarbodiimide (DCCD) from Alfa Aesar, flucloxacillin from CP Pharmaceuticals, linezolid from Pfizer, meropenem from AstraZeneca, moxifloxacin from Bayer, SEP155342 from Sunovion Pharmaceuticals, triclosan from LG Life sciences, vancomycin from Duchefa Biochemie, D-luciferin from Melford, ethanol and nuclease-free water from Fisher Scientific, and the radiolabeled chemicals [*methyl*-^{3}H]thymidine (70-95 Ci/mmol), [5,6-^{3}H]uridine (31-56 Ci/mmol), and L-[G-^{3}H]glutamine (20-50 Ci/mmol) from GE Healthcare. XF-73 and XF-70 were gifts from Destiny Pharma and antioxidants were gifts from Evocutis. SYPRO® Ruby, 3,3'-dipropylthiadicarbocyanine iodide (DiSC$_3$(5)), the Live/Dead *Bac*Light™ kit, ATP Determination Kit and SYBR safe gel stain were from Invitrogen. The PCR nucleotide mix was from Promega and phospholipids were from Avanti Polar Lipids. Antimicrobial agents and their solvents are described in Table 2.2.

Compound	Solvent	Compound	Solvent
Ampicillin	Water	Linezolid	Water
AO2246	DMSO	Menadione	DMSO
Bakuchiol	DMSO	Meropenem	Water
Benzoyl peroxide	Ethanol	Moxifloxacin	Water
Carnosic acid	Ethanol	Mupirocin	50% (v/v) ethanol
CCCP	DMSO	NDGA	Ethanol
Cefotaxime	Water	Nisin	20 mM HCL
Celastrol	DMSO	Norfloxacin	Water
Chloramphenicol	50% (v/v) ethanol	Oxacillin	Water
Chlorhexidine	DMSO	Rifampicin	DMSO
Ciprofloxacin	20 mM HCL	SDS	Water
Clofazamine	50% (v/v) ethanol	SEP155342	DMSO
CTAB	Water	Spectinomycin	Water
Daptomycin	Water	TBBQ	Ethanol
DCCD	Ethanol	TBHQ	Ethanol
Dihydroxychalcone	DMSO	Tetracycline	Water
Erythromycin	50% (v/v) ethanol	Thymohydroquinone	DMSO
Flucloxacillin	Water	Thymoquinone	Ethanol
Fosfomycin	Water	Totarol	DMSO
Fusidic acid	50% (v/v) ethanol	Triclosan	Ethanol
Gentamicin	Water	Valinomycin	DMSO
8-hydroxyquinoline	Ethanol	Vancomycin	Water
Idebenone	Ethanol	Vitamin K5	Ethanol
Kanamycin	Water	XF-70	Water
Lauryl gallate	Ethanol	XF-73	Water

Table 2.2: **Antimicrobial agents and their solvents used in this study.** CCCP: carbonyl cyanide m-chlorophenyl hydrazone; CTAB: cetyltrimethylammonium bromide; DCCD: dicyclohexylcarbodiimide; NDGA: nordihydroguaiaretic acid; SDS: sodium dodecyl sulphate; Vitamin K5: Vitamin K5 hydrochloride.

2.3 Stability studies with TBHQ and TBBQ

Extinction coefficients for TBHQ (2945.1 $M^{-1}cm^{-1}$) and TBBQ (16274.3 $M^{-1}cm^{-1}$) were determined at their absorbance maxima (290nm and 252nm, respectively) (Li *et al.*, 2002) using known concentrations of both compounds in PBS. Stability studies were performed at 37°C with shaking using starting concentrations of TBHQ and TBBQ for which absorbance readings could be taken without requiring dilution of the solution (absorbance values of <0.8); for TBHQ, the concentration used was 385 μM (64 μg/mL), and for TBBQ, 97 μM (16 μg/mL). The Beer-Lambert law was used to calculate the concentrations of TBHQ and TBBQ present in solution over time (Cammack *et al.*, 2006). The stability of TBHQ/TBBQ was measured in PBS (pH 6, 6.5 or 7) under aerobic and anaerobic conditions, and following addition of ascorbic acid up to 512 μg/mL.

2.4 Antibacterial activity testing

2.4.1 Standardised susceptibility testing

MICs were determined in broth by exposing bacteria to serial dilutions of antibacterial agents in MHB according to Clinical and Laboratory Standards Institute (CLSI) broth microdilution guidelines (Cockerill *et al.*, 2012). Minimum Bactericidal Concentrations (MBCs) were determined by enumerating bacteria surviving challenge with concentrations of antibacterial agents above the MIC; bacterial colonies were counted after incubation at 37°C for 18-24 hours on MHA, and the MBC defined as the minimum concentration of antibacterial agent that caused 99.9% kill (Barry *et al.*, 1999). Biofilm MICs (bMICs) and Minimum Biofilm Eradication Concentrations (MBECs) were determined using *S. aureus* SH1000 biofilms grown using the Calgary Biofilm Device (CBD), which supports $\sim 10^7$ CFU/peg (Nunc, Roskilde, Denmark) (Ceri *et al.*, 1999;

Miller *et al.*, 2005). Biofilms were washed twice in saline before being exposed to antibacterial agents in MHB at 37°C for 24 hours. The bMICs were determined as the lowest concentration of compound that inhibited planktonic growth of bacteria shed from the biofilm. The CBD pegs were washed twice in saline and transferred to drug-free MHB. Following incubation at 37°C for 24 hours, the MBEC was determined as the lowest concentration of compound that effectively sterilised the biofilm, allowing no further planktonic growth. Susceptibility testing was conducted on a minimum of three independent occasions to ensure reproducibility.

2.4.2 Identification of synergism

Synergistic interactions of antioxidants with antibiotics were evaluated for planktonic cultures and biofilms grown on the CBD using the checkerboard method (Pillai *et al.*, 2005). In planktonic synergism experiments, compound concentrations ranged from 2x MIC to 1/32x MIC. In biofilm synergism experiments, antioxidant concentration ranged from 2x MBEC to 1/32x MBEC and antibiotic concentrations $\leq$256 μg/mL. The fractional inhibitory concentration (FIC) index was calculated as follows:

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

An FIC value $\leq$0.5 represents a synergistic interaction, a value of 1 is an additive interaction and $\geq$2 is an antagonistic interaction.

2.4.3 Time-dependent killing studies

Time-kill assays were performed using a method adapted from Oliva *et al.* (2003). Briefly, bacteria were cultured to early exponential phase (OD_{600} of 0.2) in MHB and challenged with antibacterial agents at 4x MIC. In some instances, bacteria were cultured to early exponential phase, washed and resuspended in PBS or 5 mM HEPES buffer (pH 7.2) supplemented with 5 mM glucose pre-warmed to 37°C, before exposure to antibacterial agents. Bacterial viability was monitored by dilution in PBS and plating cultures onto MHA, and enumerating colonies after incubation at 37°C for 18-24 hours.

Time-kill experiments were also performed with bacterial cells in non-growing states. Overnight cultures of SH1000 were harvested by centrifugation and cells were resuspended in the supernatant media to an OD_{600} of 0.2 before exposure to antibacterial agents. SH1000 biofilm cells were liberated from 48-hour-old biofilms grown on cellulose discs by incubation in cellulase (1 mg/ml in 0.05 M citrate buffer pH 5.0) (Ryder *et al.*, 2012), before being washed and resuspended to OD_{600} of 0.2 in HEPES and glucose buffer.

2.4.4 Susceptibility of persisters to antimicrobial agents

Persister cells were generated by growing SH1000 to early exponential phase, then exposing the cells to ampicillin or ciprofloxacin at 10x MIC for 24 hours at 37°C. Cells were then washed and resuspended to the same volume in MHB, and surviving persisters were challenged with antioxidants at 10x MIC (Wiuff *et al.*, 2005; Wu *et al.*, 2012). Bacterial viability was monitored following exposure to antioxidants by plating cultures onto MHA, and enumerating colonies after incubation for 18-24 hours at 37°C.

2.4.5 Identification of bacterial lysis

To detect bacterial lysis following challenge with antibacterial agents at 4x MIC or 256 μg/mL, the culture turbidity of early exponential phase cultures at 37°C was monitored by absorbance measurements at 600nm (Oliva *et al.*, 2003).

2.5 Doubling time determinations

Overnight cultures were diluted 1:100 in MHB and incubated at 37°C with aeration until reaching an OD_{600} of $\sim$0.1. The absorbance at 600nm was measured every 4 minutes until the culture reached an OD_{600} of $\sim$0.2. Log_{10} OD values were plotted against time (minutes) and a line of best fit was calculated. Doubling times were determined as follows:

$$\text{Doubling time (minutes)} = \frac{\log_{10}(2)}{\text{gradient of line of best fit}}.$$

2.6 Antibacterial MOA studies

2.6.1 Quantification of macromolecular biosynthesis

Antibacterial agents commonly exert their activity through inhibition of macromolecular biosynthesis pathways (O'Neill & Chopra, 2004). Therefore, the effect of TBBQ on biosynthesis of DNA, RNA and protein was monitored in early exponential phase cultures of *S. aureus* SH1000 (OD_{600} of 0.2) by following the incorporation of the radiolabeled precursors [*methyl*-^{3}H]thymidine, [5,6-^{3}H]uridine and L-[G-^{3}H]glutamine into macromolecules (Wilson *et al.*, 1995; Ooi *et al.*, 2009). Cultures were grown

to early exponential phase (OD_{600} 0.2) in MHB, then radiolabels were added to 1 μCi/mL. Cultures were incubated with precursors for 10 minutes at 37°C with aeration prior to addition of compounds at 4x MIC. Ciprofloxacin was used as the positive control for DNA synthesis inhibition experiments, rifampicin for RNA, and tetracycline for protein. Samples were taken before adding compounds to cultures and were sampled again following a 10 minute incubation. Samples (500 μL) were processed by dilution in 5 mL of ice cold 10% TCA, and maintaining on ice for 30 minutes to precipitate macromolecules. For RNA experiments, MF 100 glass microfiber filters (Fisher Scientific) were washed with 2.5 mL of 10 mM unlabelled uridine before samples were filtered using a vacuum pump. Samples on filters were washed twice with 5 mL 10% TCA and 5% acetic acid before being transferred to 5 mL Emulsifier-Safe™ scintillation cocktail (PerkinElmer) in scintillation counter vials (Greiner Bio-One) for quantification using a Packard Tri-Carb 2100TR liquid scintillation analyser (Packard Bioscience).

2.6.2 *Bacillus subtilis* biosensors

The action of TBBQ was also investigated using a suite of pathway-specific biosensor strains of *B. subtilis* that have previously been described for use in MOA studies (Mariner *et al.*, 2011; Urban *et al.*, 2007). Bacteria were grown to an OD_{600} of 0.2, and were then exposed to a range of concentrations of TBBQ or control antibiotic above and below the MIC (0.005 - 20 μg/mL TBBQ). Samples (100 μL) of culture containing compounds were incubated at 37°C with aeration for 1 hour (cell wall biosensor), 1.5 hours (RNA biosensor) and 3 hours (protein, DNA and fatty acid biosensors). Subsequently, 60 μL of 0.8 mM luciferin in 0.1M citrate buffer (pH 5) was added to cultures and luminescence measured in white luminescence microtitre plates (Greiner Bio-One) using a FLUOstar Omega plate reader (BMG, LABTECH). Luminescence was compared with a drug-free control, and induction was identified as a $>$2 fold increase in relative luminescence.

2.6.3 Assays to detect perturbation of membranes

2.6.3.1 The *Bac*Light™ assay

The effect of antibacterial compounds on the integrity of the staphylococcal membrane was assessed using the *Bac*Light™ assay (Ooi *et al.*, 2009; Hilliard *et al.*, 1999). *S. aureus* SH1000 was grown to OD_{600} 0.5-0.6. Volumes of cultures were washed in sterile deionised water and resuspended to twice the volume. Cells were incubated with 5% SDS or test compounds at 4x MIC for 10 minutes at room temperature with aeration, and then were washed and resuspended in water. Samples (500 μL) were diluted in a further 1.5 mL of water and mixed with 6 μL of *Bac*Light™ reagent. Samples were incubated in the dark for 15 minutes and fluorescence measured at an excitation of 485 nm and emission of 530 nm or 645 nm using a PerkinElmer LS 45 luminescence spectrometer. The ratio of green:red fluorescence was determined and percentage membrane integrity was calculated with respect to the SDS positive control. Compounds were considered membrane damaging if they caused $>$30% loss of membrane integrity.

2.6.3.2 Measurement of cytoplasmic membrane potential

Membrane potential and leakage of intracellular ions from cells resuspended in HEPES and glucose buffer was determined following exposure to antibacterial agents over a 3-hour period at 4x MIC (Hobbs *et al.*, 2008). Cultures of *S. aureus* SH1000 were grown to exponential phase (OD_{600} 0.2), washed twice and resuspended in HEPES and glucose buffer. For membrane potential measurements, cells were incubated with 0.1 M KCl and 2 μM $DiSC_3(5)$ for 30 minute at 37°C to allow uptake of dye. Subsequently, cultures were exposed to compounds at 4x MIC and samples removed at appropriate time points. Cells were pelleted and 1 mL of supernatant was mixed with 1 mL DMSO. The pellet was lysed in DMSO for 10 minutes, and was then added to an equal volume of HEPES and glucose buffer. Extracellular and intracellular fluorescence was measured on a LS 45

luminescence spectrometer (PerkinElmer) at an excitation of 622 nm and an emission of 670 nm. Membrane potential was calculated using the Nernst equation (Silverman *et al.*, 2001),

$$\Delta\psi = -\frac{RT}{F}\ln\left(\frac{\text{DiSC}_3(5)\text{ inside}}{\text{DiSC}_3(5)\text{ outside}}\right),$$

and was expressed as a percentage of the initial value. $\Delta\psi$ is the membrane potential in mV, R is the universal gas constant, T is the absolute temperature and F is the Faraday constant.

2.6.3.3 Measurement of ionic leakage

For ionic leakage experiments, cells resuspended in HEPES and glucose buffer were exposed to compounds at 4x MIC at 37°C with aeration for 3 hours. Samples (5 mL) were removed at regular intervals, filtered through a sterile 0.2 μm filter and stored at -20°C until they could be analysed by flame atomic absorption spectroscopy. The positive controls for potassium leakage experiments were cells that had been boiled for 10 minutes prior to filtration. For magnesium leakage experiments, cells were incubated at 37°C with 100 μg/mL lysostaphin for 3 hours before filtration. Potassium standards (0.05, 0.1, 0.2, 0.5, 1, 1.5, 2, 2.5 and 3 μg/mL) and magnesium standards (0.01, 0.025, 0.05, 0.075, 0.1, 0.2, and 0.3 μg/mL) were made in HEPES and glucose buffer, and a Perkin Elmer AAnalyst 100 spectrometer was used to generate standard curves from which ionic contents of samples were calculated (Ioannou *et al.*, 2007).

2.6.3.4 Haemolysis assay

The ability of compounds to damage mammalian membranes was examined by measuring haemolysis of equine erythrocytes (Oliva *et al.*, 2003). Briefly, lithium heparin-treated whole equine blood (Matrix Biologicals) was centrifuged at 1000 x g for 10 minutes at 4°C, and the supernatant and buffy coat discarded. The erythrocyte pellet was washed and resuspended to 5% v/v in 10 mM Tris-HCl buffer containing 0.9% NaCl (pH 7.4) at 4°C. Erythrocytes were diluted 25-fold in buffer, and pre-incubated at 37°C for 15 minutes prior to use. Erythrocytes were exposed to a concentration of antibacterial agent equivalent to 4x MIC against *S. aureus* SH1000 for 1 hour at 37°C. Mixtures were centrifuged at 3000 x g for 5 minutes, then the extent of haemoglobin leakage was measured at OD_{540}, and expressed as a percentage of the positive control (5% SDS).

2.6.3.5 Liposome disruption

Disruption of staphylococcal liposomes was measured at 4x MIC following 3 hour incubation with compounds as previously described (StGelais *et al.*, 2007; Randall *et al.*, 2013). Briefly, carboxyfluorescein-containing liposomes were generated with a phospholipid content analogous to the *S. aureus* cytoplasmic membrane: 40% cardiolipin (wt/wt) and 60% phosphatidylglycerol (Avanti Polar Lipids). *S. aureus* liposomes (50 μM) were exposed to compounds at 4x MIC for 3 hours and leakage of carboxyfluorescein was measured in a fluorescence microtiter plate (Greiner Bio-One) at 485 nm using a FLUOstar Omega plate reader. Liposome integrity was calculated as a percentage of the positive control, treated with 0.5% Triton X-100.

2.6.4 Quantification of ATP pools

Total concentrations of ATP were monitored in exponential phase cultures of *S. aureus* SH1000 using a luciferin-luciferase chemiluminescence assay (O'Neill *et al.*, 2004). Cultures were grown to OD_{600} 0.2 before being washed twice, resuspended in HEPES and glucose buffer, and exposed to compounds at 4x MIC. Samples were processed at regular intervals by pelleting cells and transferring supernatant to an equal volume of DMSO. Cell pellets were lysed in DMSO for 10 minutes, and an equal volume of HEPES and glucose buffer was added. Samples (10 μL) of extracellular or intracellular samples were transferred to white luminescence microtitre plates (Greiner Bio-One) and 90μL of ATP standard solution, made according to the manufacturer's instructions, was added using a repeater pipette immediately before taking luminescence measurements using a FLUOstar Omega plate reader. Total ATP levels were calculated from intracellular and extracellular samples. ATP remaining was calculated as a percentage of the starting ATP.

2.6.5 Determining the mechanism of antibiofilm activity

2.6.5.1 Penetration of antibacterial agents through biofilms

To assess the penetration of compounds into staphylococcal biofilms, biofilms were grown on cellulose discs for 48 hours (Ryder *et al.*, 2012), and were then transferred to BHA containing antioxidants or vancomycin at 256 μg/mL. A 13 mm cellulose disc was placed on the biofilm, on top of which was placed a 6 mm antibiotic disc (Oxoid) moistened with PBS (Figure 2.1). Following incubation at 37°C for 24 hours, the antibiotic disc was transferred to a petri dish containing MHA spread with SH1000. Following incubation at 37°C for 24 hours, the diameter of zone of inhibition was measured and compared with a calibration curve generated using discs impregnated with known concentrations of antibiotics. The concentration of test compound in each disc and percentage penetration

was calculated with respect to a control assembly containing no biofilm (Anderl *et al.*, 2000; Singh *et al.*, 2010).

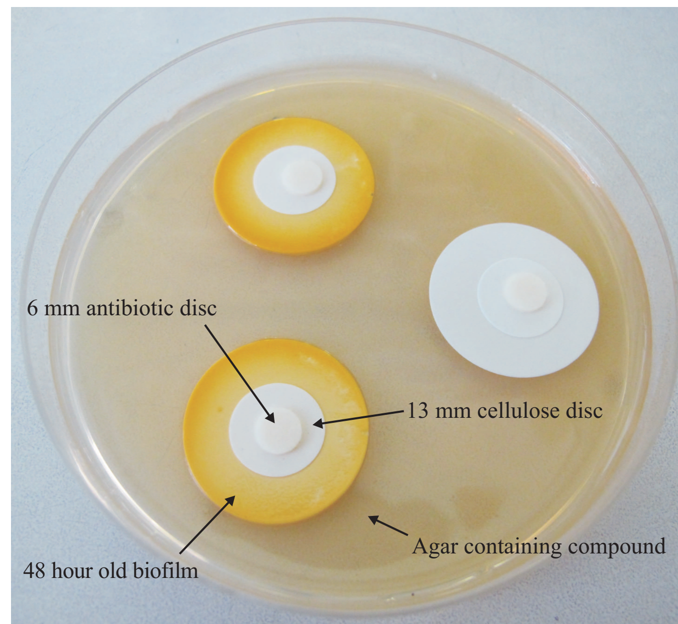

Figure 2.1: **Experimental setup for biofilm penetration experiments.**

2.6.5.2 Quantification of adherent biofilm material

Alterations in biofilm structure following challenge with antibacterial agents were detected by quantifying matrix material and adhered cells by staining with SYPRO® Ruby and SYTO® 9, respectively (Frank & Patel, 2007). Microtitre plates were preconditioned with 20% normal pooled human plasma (Sera Laboratories International) in 0.05 M carbonate buffer overnight at 4°C. Subsequently, SH1000 biofilms were grown in wells for 24 hours, and exposed to antioxidant at 256 μg/mL in MHB, or proteinase K (100 μg/mL in 20 mM tris pH7.5 and 100 mM NaCl), for 1 or 24 hours (Frank & Patel, 2007). Biofilms were washed in water before being stained with SYPRO® Ruby containing 0.167 μM SYTO® 9 for 30 minutes. Following a further wash in water,

absorbance was measured at an excitation of 480 nm and emission of 620 nm (matrix) and 520 nm (cells) (Frank & Patel, 2007). In parallel experiments, total biofilm viability was measured following exposure to compounds for 1 hour. Detached cells were collected, and adherent cells were dispersed by incubation with proteinase K (100 μg/mL) in buffer for 1 hour. All cells were washed in PBS before being plated onto MHA, and enumeration of colonies was carried out after incubation for 18-24 hours at 37°C.

2.6.6 Identification and characterisation of a transposon insertion mutant exhibiting hypersusceptibility to TBBQ

A near-saturation transposon library containing ~20500 transposon mutants (Blake & O'Neill, 2012) was screened for hypersusceptibility to TBBQ. The library was generated at the University of Leeds by random mutagenesis of *S. aureus* SH1000 with the Tn InsTet^{G+}2^{Cm} (Blake & O'Neill, 2012). Samples (0.2 μL) of overnight culture were transferred to MHA in square petri dishes containing doubling dilutions of TBBQ. Each square petri dish contained inocula from 6 microtitre plates. The concentration of TBBQ used to identify hypersusceptibility was determined in a pilot study. Briefly, 6 transposon library plates were chosen at random and were used to inoculate agar containing doubling dilutions of TBBQ at or below the MIC. The concentration at which 1-4 strains per library plate displayed altered susceptibility was selected for the full screen (14 μg/mL). Putative hypersusceptible transposants were re-screened twice and altered susceptibility was confirmed by broth MIC according to CLSI methodology (Section 2.4.1). The Tn insertion site of mutants was identified by direct sequence determination of genomic DNA using an outward facing promoter, Tnp_out2 (Appendix A) (Bertram *et al.*, 2005). The link between genotype and hypersusceptibility was confirmed by demonstrating that transduction of the Tn insertion into SH1000 resulted in co-transfer of the phenotype (Section 2.8.1).

2.6.7 Selection of antioxidant resistant mutants

Spontaneous mutation frequencies were determined for antioxidants and the control agent, rifampicin, as described previously (O'Neill *et al.*, 2001). Briefly, *S. aureus* overnight cultures were spread on drug-free MHA and MHA containing antimicrobial compounds at 4x MIC, and were incubated at 37°C for 48 hours. Mutation frequencies were expressed as the number of drug resistant mutants as a proportion of the total population.

For compounds where mutants were not identified from mutation frequency determinations, attempts were made to select resistant mutants using the extended spectrum MIC method (Friedman *et al.*, 2006). Cultures of *S. aureus* SH1000 were challenged with antibacterial agents using broth MIC methodology (Section 2.4.1) with the exception that the dilution series had smaller increments between concentrations (doubling-dilutions from 256, 192 and 160 μg/mL). Following 24 hour incubation at 37°C, the MIC was recorded and cells from the well containing the highest concentration of drug that permitted growth were used as the inoculum for the next MIC. The process was repeated for 40 passages, during which cells were continually exposed to selective compounds. Putative resistance was confirmed by standard broth MIC methodology.

2.7 Effects of antioxidants on human LabSkin™

To test whether antioxidants have potential as topical antistaphylococcal agents, the effect of compounds on a living skin equivalent was assessed. Fully differentiated, 28-day-old LabSkin™ and maintenance medium were provided by Evocutis. At the start of the experiment, spent LabSkin™ medium was removed and stored at -80°C until analysis. Medium was replaced with fresh LabSkin™ maintenance medium and 100 μL of test compound at 10 or 4x MIC in sterile deionised water (0.2% v/v solvent

load) was spread across the surface of each individual LabSkin™ using an inoculation loop. The drug-free control was exposed to deionised water and solvent alone, and the positive control was incubated with 5% SDS. LabSkin™ was incubated at 37°C, 5% CO_2, >95% relative humidity for 6 hours (Holland *et al.*, 2008), then medium was removed and stored at -80°C, and replaced with fresh maintenance medium. LabSkin™ was incubated for a further 18 hours before medium was sampled again. Tissue was fixed in 10% neutral buffered formalin for 24 hours before being embedded in wax and carrying out hematoxylin and eosin staining and visual inspection of tissue sections (Holland *et al.*, 2008). To identify potential skin irritation following application of compounds, IL-1α ELISA analysis was carried out on sampled medium (Bernhofer *et al.*, 1999; Rasmussen *et al.*, 2010). Interleukin-1α ELISAs were performed using a Human IL-1 alpha/IL-1F1 Quantikine ELISA Kit (R&D systems) and were performed according to the manufacture's instructions. IL-1α was quantified at 450nm using a FLUOstar Optima plate reader, and compared with the positive control.

2.8 DNA manipulation

2.8.1 Bacteriophage transduction

Overnight cultures of donor strains, containing the gene of interest, were grown in phage broth (20 g/L Oxoid nutrient broth No. 2). Cultures were diluted 1:100 in phage broth supplemented with 10 mM calcium chloride. Approximately 10^5 phage ϕ11 particles were added to 300 μL of cells and incubated at room temperature for 30 minutes. Volumes (10 mL) of molten phage top agar (phage broth and 3.5 g/L oxoid agar No.1) supplemented with 10 mM calcium chloride was mixed with cells and poured over two plates containing phage base agar (phage broth and 7 g/L oxoid agar No.1) supplemented with 10 mM calcium chloride. Plates were incubated at 37°C in a sealed bag for

24-48 hours. The phage top agar of plates with the highest dilution that gave confluent lysis was centrifuged, and the phage-containing supernatant was filtered twice through 0.45 μm filters and was stored at 4°C (Foster, 1998).

Recipient bacteria were grown overnight in 20mL TSB, then were centrifuged and resuspended in 1 mL TSB. Samples (500 μL) of recipient cells and 500 μL phage were incubated in 1 mL LB supplemented with 10 mM calcium chloride at 37°C statically for 25 minutes, then with agitation (200 rpm) for a further 15 minutes. Cells were washed and resuspended in 1 mL ice-cold 20 mM sodium citrate before incubation on ice for 2 hours. Phage-containing recipient cells were spread on TSA supplemented with 0.05% sodium citrate and selection antibiotic, and were incubated at 37°C overnight. To ensure loss of phage, single colonies containing the gene of interest were streaked on TSA containing sodium citrate and selection antibiotic (Foster, 1998).

2.8.2 DNA extraction, PCR and DNA sequence determination

Genomic DNA was extracted from *S. aureus* using the PurElute™ bacterial genomic kit (EdgeBio). Briefly, 2 mL of *S. aureus* culture was pelleted and washed in 1 mL TE buffer. Cells were resuspended in 400 μL of spheroplast buffer supplemented with 100 μg/mL lysostaphin, and were incubated at 37°C for 1 hour. DNA was purified from samples according to the manufacturer's instructions, with the addition of incubation with proteinase K (100 μg/mL) at 37°C for 15 minutes prior to addition of Advamax 2 beads.

Plasmid DNA was purified from bacteria using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Purified DNA was stored at -20°C.

Polymerase chain reactions were performed with Phusion® High-Fidelity DNA Polymerase (New England Biolabs) according to standard methodology (Sambrook & Russell, 2001). Oligonucleotide primers were designed using OLIGO 6.0 software (Molecular Biology Insights Inc.), and manufactured by Eurofins MWG Operon. PCR

conditions were based upon the manufacturer's recommendations (Appendix A).

PCR products were visualised by gel electrophoresis (Sambrook & Russell, 2001). Agarose gels contained SYBR safe gel stain (1:10000) in 0.8% agarose (w/v) in Tris-acetate-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA). Hyperladder I and II (Bioline Reagents) were used alongside DNA samples to determine the molecular weight of PCR products. A potential difference of 90 V was applied across gels for 30-40 minutes. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions, and were stored at -20°C.

The sequence of PCR products was determined by Beckman Coulter Genomics, and direct genomic sequence determination for locating transposon insertion sites was carried out by Microsynth AG. DNA sequence determination data were analysed using Sequencher software, version 4.9 (Gene Codes Corporation). Whole genome sequencing and analysis of resistant mutants was carried out by Leeds Institute of Molecular Medicine, University of Leeds.

2.8.3 Molecular cloning

The vector pEPSA5 (Forsyth *et al.*, 2002) and DNA fragments for ligation were modified using restriction enzymes purchased from New England Biolabs. Ligations were performed using the Quick Ligation™ Kit (New England Biolabs), and both restriction digests and ligation reactions were carried out according to the manufacturer's instructions.

Chemically competent *E. coli* DH5α cells were prepared according to the Inoue method (Sambrook & Russell, 2001). *E. coli* were transformed by incubating 100 μL of competent cells and 10 μL of ligation reaction on ice for 1 hour. Cells were mixed with 1 mL of SOC media (New England Biolabs) and incubated at 37°C with aeration for 1 hour before spreading onto LBA containing selection antibiotic.

Electrocompetent *S. aureus* cells were generated using a method modified from Schenk & Laddaga (1992). A 25 mL culture was grown to OD_{600} 0.6 in TSB with 2.5% yeast extract. Cells were harvested by centrifugation and were washed 3 times in dH_2O, before a final wash in 10% glycerol. Cells were incubated in 10% glycerol for 15 minutes, pelleted, and resuspended to 800 μL in 10% glycerol and stored at -80°C (Schenk & Laddaga, 1992). Cells (60 μL) were transformed with 2 μL of vector in a GenePulser 0.1 cm electrode gap cuvette at 2.3 kV, 100 Ω, 25 μF in a GenePulser XCell Electroporation System (Bio-Rad). Transformed cells were resuspended in 390 μL TSB+YE immediately. Cells were incubated at 37°C with aeration for 1 hour before spreading onto TSA containing selection antibiotic (Schenk & Laddaga, 1992).

Chapter 3

Antibacterial activity of *tert*-butylhydroquinone (TBHQ) and its oxidation product, *tert*-butylbenzoquinone (TBBQ)

The work contained within this chapter has been published in part in Ooi *et al.* (2013).

N. Ooi, I. Chopra, A. Eady, J. Cove, R. Bojar and A. J. O'Neill (2013) Antibacterial activity and mode of action of *tert*-butylhydroquinone (TBHQ) and its oxidation product, *tert*-butylbenzoquinone (TBBQ). Journal of Antimicrobial Chemotherapy, 68 (6): 1297-1304.

3.1 Abstract

The antioxidant TBHQ is a food additive reported to have antibacterial activity, and may therefore have application in the healthcare setting. Work reported in this chapter was undertaken to characterise the antimicrobial activity of TBHQ and its oxidation product, TBBQ. TBHQ underwent oxidation in buffer to form TBBQ in a pH- and oxygen-dependent manner. When oxidation was prevented, TBHQ lacked useful antibacterial activity. In time-kill experiments TBHQ (4x MIC) induced reductions in bacterial viability only after 2 hours, which coincided with the commencement of TBBQ formation. These experiments indicate that TBBQ is responsible for the activity previously attributed to TBHQ. Susceptibility testing was performed against a number of medically relevant organisms. TBBQ was most effective against Gram-positive bacteria, and displayed activity against both planktonic (MIC 8 μg/mL) and biofilm cultures (MBEC 64 μg/mL) of *S. aureus* SH1000. TBBQ was rapidly and extensively bactericidal, causing $>$4-$\log_{10}$ reduction in cell viability within 6 hours at 4x MIC, but was non-lytic. TBBQ retained cidal activity against slow-growing cells, sterilising stationary phase and persister cultures following 24 hour incubation with the compound. The antioxidant did not induce dispersal of staphylococcal biofilms. Therefore, the ability of TBBQ to eradicate preformed biofilms is due to activity against slow- and non-growing cells contained within the biofilm.

3.2 Introduction

3.2.1 The phenolic antioxidant TBHQ

Within the last 25 years, the rate of discovery of antibiotics from novel classes has declined dramatically (Silver, 2011). Therefore, revisiting existing chemicals with known

antibacterial activities may be a rewarding approach to drug discovery. The lipophilic antioxidant *tert*-butylhydroquinone (TBHQ) is a sterically-hindered hydroquinone with a phenol ring at its core (Figure 3.1a). TBHQ was approved for use in the USA as a food preservative in 1972 (Fung *et al.*, 1985; Przybylski *et al.*, 1998; Raccach & Henningsen, 1982), and has been shown to possess antibacterial activity (Fung *et al.*, 1985). TBHQ has predominantly been employed for the stabilisation and preservation of fats/foods with high fat content owing to its radical-scavenging activity (Fung *et al.*, 1985; Kashanian & Dolatabadi, 2009).

An important consideration when seeking to characterise the biological properties of antioxidants is their stability in solution, as they can undergo oxidation to form other chemical species. Since the biological properties of an antioxidant and its breakdown products may differ, care must therefore be taken to relate observed properties to the chemical species present. As TBHQ has most often been used as an additive for vegetable oils (Kashanian & Dolatabadi, 2009), previous studies have examined its stability at temperatures reached in the frying process. At such temperatures (175°C-185°C) TBHQ is oxidised primarily to *tert*-butylbenzoquinone (TBBQ) (Figure 3.1a), although smaller quantities of a number of other compounds have been detected, including dimerised TBHQ (Kim & Pratt, 1990; Hamama & Nawar, 1991) and free radical species that decompose to alternate oxidation products (Hamama & Nawar, 1991). The degradation of TBHQ to TBBQ at high temperatures is summarised in Figure 3.1b. TBBQ that has formed as a consequence of TBHQ degradation may then be reduced back to TBHQ, generating a redox cycling system in the presence of a source of reducing equivalents (Kim & Pratt, 1990).

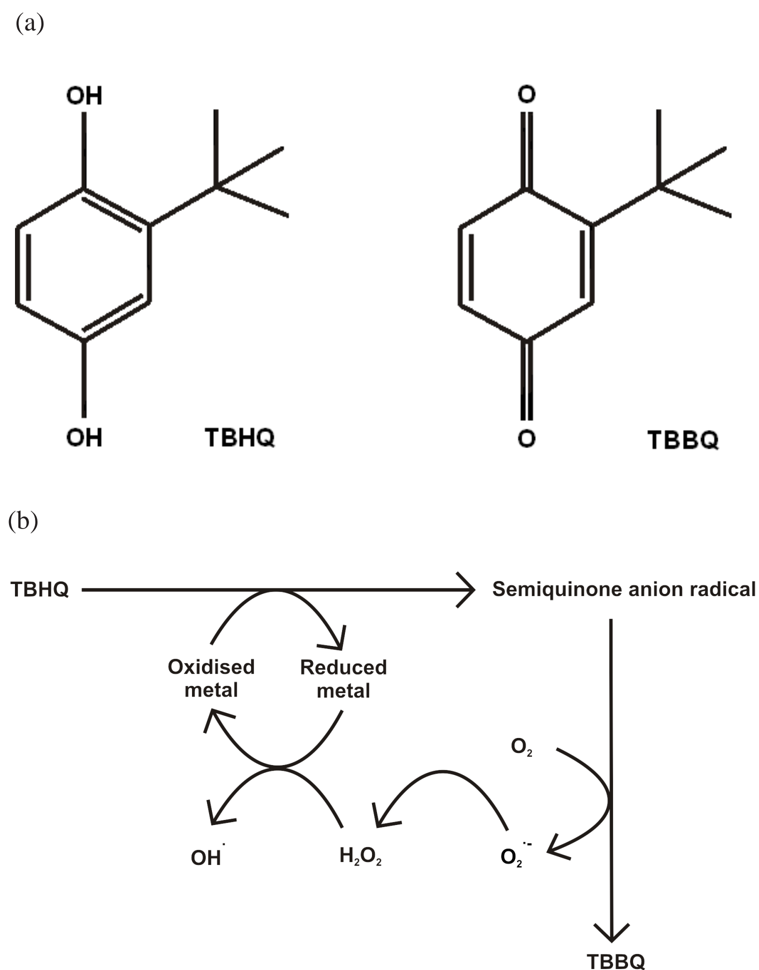

Figure 3.1: **Chemical structures of TBHQ and TBBQ, and oxidative conversion of the former to the latter.** Panel (a): The chemical structure of *tert*-butylhydroquinone (TBHQ) and *tert*-butylbenzoquinone (TBBQ). Panel (b): A schematic for the transition metal assisted oxidation of TBHQ to TBBQ with the associated production of superoxide, hydrogen peroxide and hydroxyl radicals (Li *et al.*, 2002).

TBHQ has a relatively broad spectrum of antimicrobial activity that encompasses bacterial species responsible for human disease, including *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Streptococcus mutans*, *Streptococcus agalactiae* and *Pediococcus pentosaceus* (Raccach & Henningsen, 1982; Fung *et al.*, 1985; Kupp *et al.*, 1985; Ogunrinola *et al.*, 1996; Davidson *et al.*, 1981; Rico-Munoz & Davidson, 1984; Chew *et al.*, 1985). Fung *et al.* exposed 8 Gram-positive and 16 Gram-negative species to TBHQ (Fung *et al.*, 1985), and found that Gram-positive organisms were generally more susceptible to the antibacterial effects of TBHQ, but sensitivity varied by species. In agar susceptibility testing TBHQ inhibited approximately 90% of the species at a concentration of 500 ppm ($\sim$500 μg/mL) (Fung *et al.*, 1985; Davidson *et al.*, 1981).

Studies with *S. aureus* highlight the necessity for standardised methodology for TBHQ susceptibility testing; different studies have reported varying inhibitory concentrations, in part due to different starting inocula. Until the methodology has been standardised using a specified inoculum the efficacy of TBHQ cannot be assessed properly. However, it is clear that TBHQ is active against strains of *S. aureus* (Table 3.1).

In view of its history of safe use in foods and its antibacterial activity, TBHQ has been under consideration for use in healthcare products by Evocutis (Fitzgerald *et al.*, 2008). Therefore, work reported in this chapter was undertaken to characterise the antibacterial activity of TBHQ.

S. aureus strain	Response	TBHQ concentration	Cells/mL	Reference
z-88	No inhibition	50 μg/mL	$1.2x10^3$	Raccach & Henningsen (1982)
A100	>$2log_{10}$ reduction in viability	100 ppm	10^7	Rico-Munoz & Davidson (1984)
100	Bacteriostatic	30 μg/mL	$3x10^3$	Raccach & Henningsen (1982)
-	Bacteriostatic	25 ppm	$2.4x10^5$	Davidson *et al.* (1981)
Newbould 305	Bactericidal	250 ppm	10^4	Chew *et al.* (1985)
100	Bactericidal	100 μg/mL	$3x10^3$	Raccach & Henningsen (1982)

Table 3.1: **Reported inhibitory effects of TBHQ against *S. aureus* strains.**

3.2.2 Aims and Objectives

Work described in this chapter aims to determine the stability of TBHQ and TBBQ in solution at temperatures relevant to the study of their antimicrobial activity. The relationship between compound stability and antibacterial activity will be investigated, and killing kinetics determined. The spectrum of activity of the compounds will be assessed, with a particular emphasis on their antistaphylococcal and antibiofilm activity. Compound effects on the structure of *S. aureus* biofilms, and activity against slow-growing or persister cells that are located within biofilms will be investigated.

3.3 Results and discussion

3.3.1 Stability studies with TBHQ and TBBQ

Although degradation of TBHQ has been observed at high temperatures ($\sim$180°C), stability has not been examined at temperatures relevant to the study of its antibacterial activity. TBHQ maintained in PBS at 37°C was found to be unstable, degrading in a pH- and oxygen-dependent manner (Figure 3.2a, 3.2b). At neutral pH, this degradation proceeded rapidly, with only $\sim$50% of the starting concentration remaining after 6 hours (Figure 3.2a). Concentrations of TBHQ decreased and TBBQ increased concurrently, indicating that TBHQ was primarily being oxidised to form TBBQ. The rate of degradation of TBHQ was slower at more acidic pH (Figure 3.2a), and degradation did not occur under anaerobic conditions (Figure 3.2b). Addition of the antioxidant ascorbic acid, which has previously been shown to inhibit decomposition of TBHQ at 100 μg/mL (Tsuji *et al.*, 2005), was able to inhibit the degradation of TBHQ at pH 7 in a concentration-dependent manner (Figure 3.2c).

Since TBHQ undergoes rapid oxidation to TBBQ in aqueous solutions, experiments were performed to assess whether this oxidation product is stable in solution. Under aerobic conditions at 37°C, TBBQ was found to degrade only slowly and in a pH-independent manner, with 90% of TBBQ remaining after 6 hours (Figure 3.3a). However, and in contrast with TBHQ, TBBQ was unstable under anaerobic conditions and its degradation could not be prevented by concentrations of $\leq$512 μg/mL of ascorbic acid (Figure 3.3b and 3.3c). The reduction in TBBQ concentration was not accompanied by an increase in TBHQ, indicating that TBBQ must be undergoing conversion to an alternative product. Since addition of ascorbic acid to this product prompted regeneration of TBHQ (Figure 3.3c), the TBBQ breakdown product is likely to be the intermediary semiquinone anion radical (Figure 3.1b).

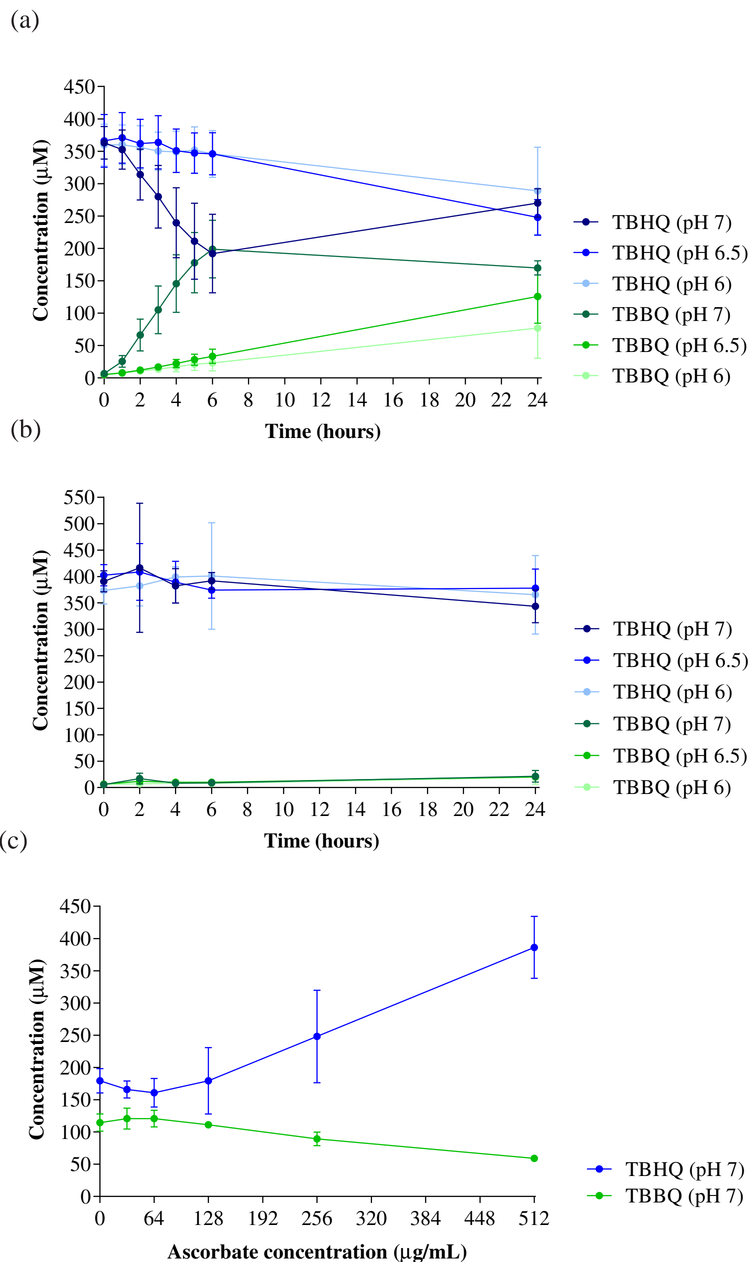

Figure 3.2: **Stability of TBHQ at 385 μM (64 μg/mL) in PBS.** Panel (a): aerobic stability; Panel (b): anaerobic stability; Panel (c): stability following a 24 hour incubation in the presence of ascorbic acid. Concentration of compound in PBS is plotted against the y-axis (mean of at least three independent replicates; error bars show standard deviations).

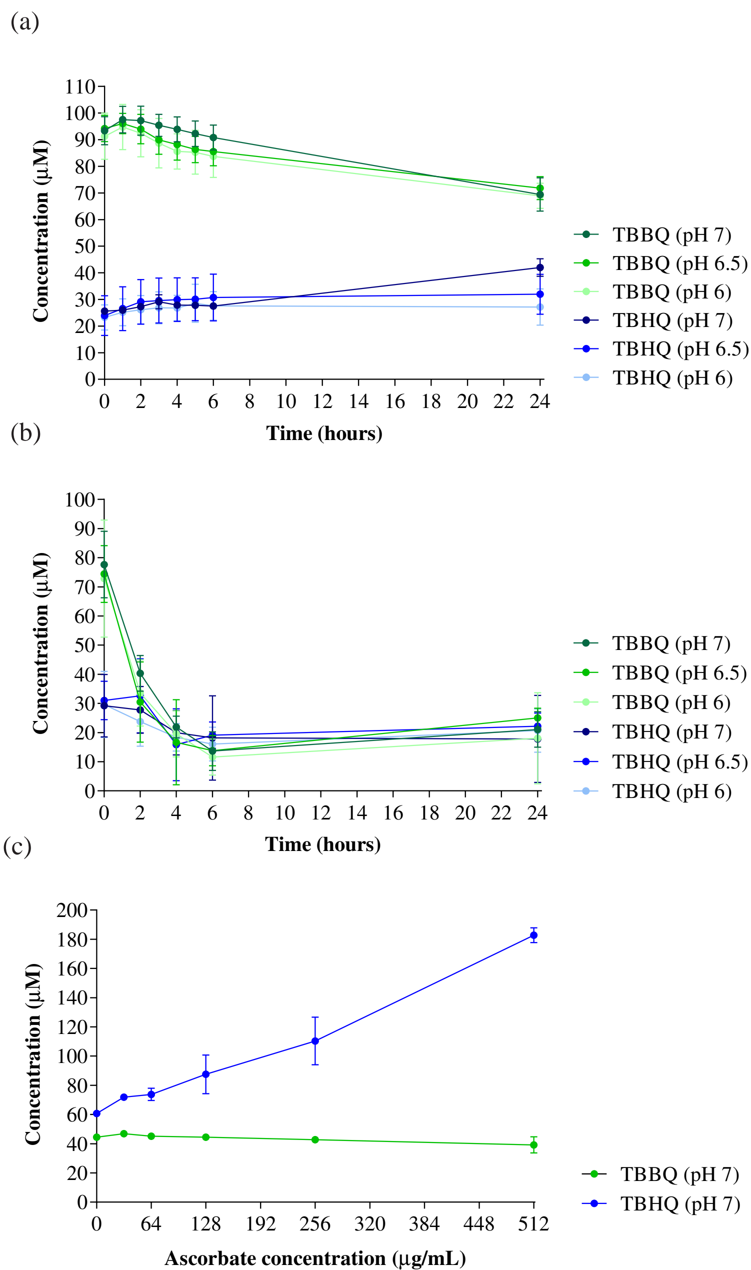

Figure 3.3: **Stability of TBBQ at 97 μM (16 μg/mL) in PBS.** Panel (a): aerobic stability; Panel (b): anaerobic stability; Panel (c): stability following a 24 hour incubation in the presence of ascorbic acid. Concentration of compound in PBS is plotted against the y-axis (mean of at least three independent replicates; error bars show standard deviations).

3.3.2 Antibacterial activity of TBHQ and TBBQ

TBHQ and TBBQ MICs and MBCs were determined against the *S. aureus* laboratory strain SH1000, and two commonly used *S. aureus* susceptibility testing strains (ATCC 23723 and ATCC 29213). TBHQ exhibited an MIC and MBC of 8 μg/mL and 8-16 μg/mL against all three strains. TBBQ had comparable activity, exhibiting an MIC and MBC of 4-8 μg/mL and 8-16 μg/mL. The MIC_{90} of TBHQ and TBBQ against 70 clinical *S. aureus* isolates (which included meticillin-resistant [MRSA] and vancomycin-intermediate [VISA] strains) was 16 μg/mL, with MICs of TBHQ and TBBQ ranging from 8-32 μg/mL and 4-16 μg/mL respectively (Appendix B).

Studies on the stability of TBHQ implied that this agent would undergo extensive conversion during the course of a 24 hour MIC determination (Figure 3.2a), with potential impact on its antibacterial activity. Therefore, the possibility of stabilising TBHQ in solution was investigated. Since both the absence of oxygen and addition of ascorbic acid inhibited degradation of TBHQ (Figure 3.2b and 3.2c), MICs were determined anaerobically and following the addition of ascorbic acid to the culture media. Under anaerobic conditions there was an 8-fold increase in TBHQ MIC against *S. aureus* SH1000, from 8 μg/mL to 64 μg/mL. TBHQ MICs also increased dramatically in the presence of ascorbic acid, reaching 512 μg/mL (64-fold increase) upon addition of ascorbic acid at 1024 μg/mL. Therefore, TBHQ lost useful antibacterial activity when spontaneous oxidation of TBHQ to TBBQ was prevented. In contrast, TBBQ retained activity in the absence of oxygen, with only a 2-fold increase in TBBQ MIC observed, and the addition of 1024 μg/mL of ascorbic acid caused an 8-fold increase in MIC. This suggests that the antibacterial effect associated with TBHQ results either from the process of conversion of TBHQ to TBBQ (*e.g.* through the production of reactive intermediates), or is intrinsic to TBBQ. Since TBHQ and TBBQ are equally effective at inhibiting bacterial growth (MIC of 8 μg/mL against SH1000 in both cases), the antibacterial activity of TBHQ cannot be attributed to an intermediate species arising from conversion of

TBHQ to TBBQ, and must be a property of TBBQ.

The antibacterial activity of a solution containing the breakdown product of TBBQ was also assessed against *S. aureus* SH1000, and displayed an MIC of 128 μg/mL. However, this activity was attributed to the TBBQ remaining in the solution; approximately 10% of the starting TBBQ remained, a concentration of TBBQ that would provide this level of antibacterial activity. Thus, the final breakdown product of TBBQ appears to have little or no antibacterial activity.

Previous studies have suggested that TBHQ has activity against a number of Gram-positive and Gram-negative species (Fung *et al.*, 1985; Raccach & Henningsen, 1982; Kupp *et al.*, 1985; Ogunrinola *et al.*, 1996). To investigate the spectrum of activity of TBBQ, MICs were carried out against a number of bacterial species. TBHQ and TBBQ displayed limited activity against Gram-negative species as reported previously (Fung *et al.*, 1985) (*E. coli* 1411 MIC of 512 μg/mL and *P. aeruginosa* PAO1 MIC of $>$512 μg/mL).

To explore the reason for the poor activity of this compound against Gram-negative bacteria, susceptibility testing was performed with TBHQ and TBBQ against *E. coli* in the presence of an outer membrane permeabilising agent (polymyxin B nonapeptide [PMBN], at a concentration of 4 μg/mL), and against efflux pump-deficient strains (Shapiro & Baneyx, 2002; O'Neill *et al.*, 2002). AcrAB is the major transporter in *E. coli*, and presence of a functional pump reduces susceptibility to multiple antibacterial agents (Okusu *et al.*, 1996). The antibacterial activity of TBHQ and TBBQ increased against *E. coli* strains lacking AcrAB or TolC (256 and 128 μg/mL against strains SM1411 and ES100, respectively), and reached a level of activity comparable with that observed against *S. aureus* (16 μg/mL) in the presence of PMBN (MICs of 32 μg/mL and 16 μg/mL against SM1411 and ES100, respectively). The results suggest that the limited activity of the compounds against *E. coli* is the result both of limited ingress across the outer membrane, and as a consequence of active efflux from the cell by AcrAB-TolC.

3.3.3 Evaluation of bacterial killing and lysis by TBHQ and TBBQ

In order to characterise the antibacterial activity of TBHQ and TBBQ further, the killing kinetics of these compounds were assessed alongside established antibiotics. Daptomycin caused a reduction in cell viability of approximately 3 $\log_{10}$ cfu/mL after 6 hours in PBS, while tetracycline caused only bacteriostasis (Figure 3.4). TBHQ had no effect on bacterial viability for the first 2 hours of the experiment, but killing was observed thereafter (Figure 3.4). The initiation of bacterial killing coincided with the commencement of conversion of TBHQ to TBBQ (Figure 3.5a), and the extent of kill increased with rising TBBQ concentration, ultimately leading to a 4 $\log_{10}$ reduction in cell number after 6 hours (Figure 3.5a). Thus, time-kill studies provided further support for the concept that TBBQ is responsible for the antibacterial activity of TBHQ. TBBQ showed no initial lag in killing activity and was rapidly and extensively bactericidal, causing a >5 $\log_{10}$ reduction in cell numbers within 3 hours, and effectively sterilised the culture within 6 hours. Over this period TBBQ degradation was minimal (Figure 3.5b), indicating that the process of conversion of TBBQ, or a breakdown product of TBBQ, are not responsible for the lethal effect.

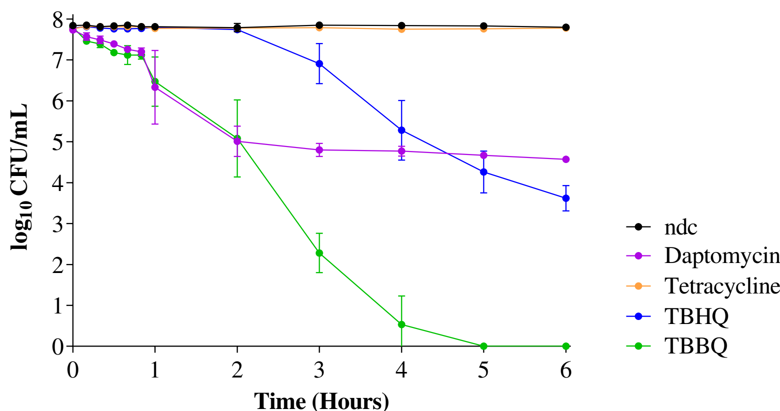

Figure 3.4: **The effect of antibacterial agents at 4x MIC on *S. aureus* SH1000 suspended in PBS.** ndc: no drug control (mean of at least three independent replicates; error bars show standard deviations).

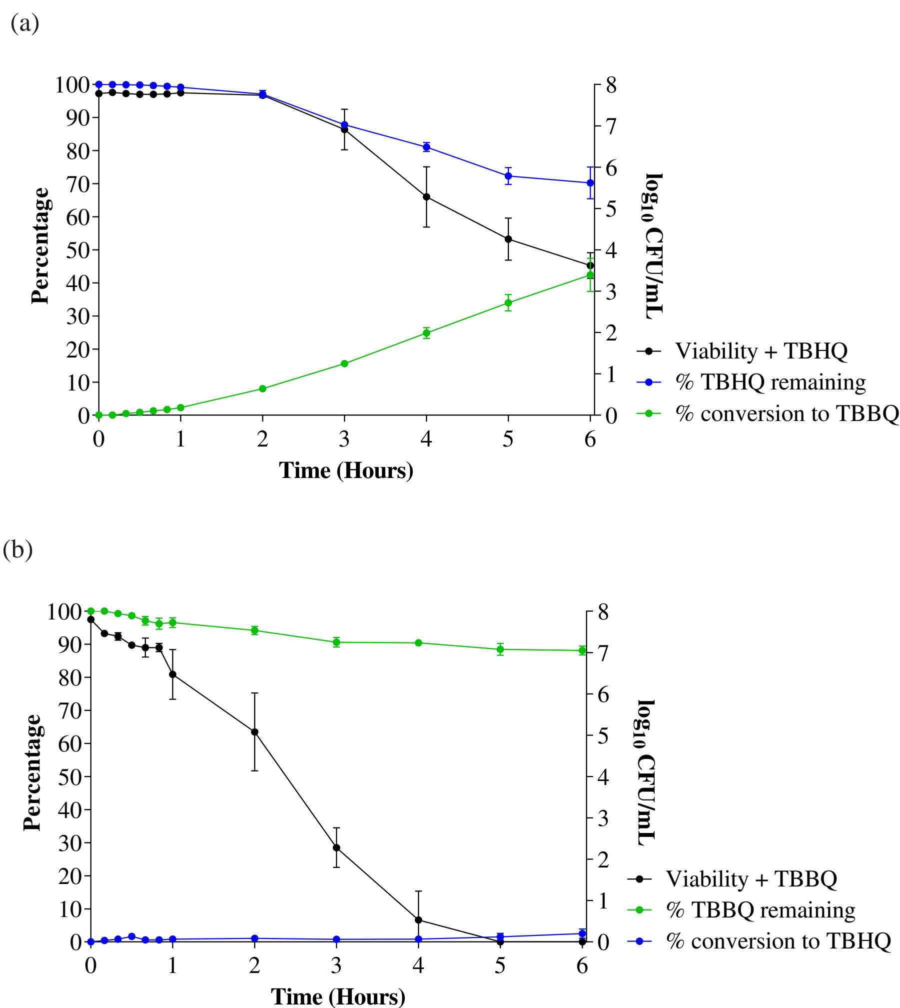

Figure 3.5: **TBHQ/TBBQ stability and killing kinetics against *S. aureus* SH1000 suspended in PBS.** Panel (a): TBHQ; Panel (b): TBBQ. Percentage of the starting concentration of compound that remains or percentage that is converted is plotted against the left y-axis (mean of at least three independent replicates; error bars show standard errors). Viable count is plotted against the right y-axis (mean of at least three independent replicates; error bars show standard deviations).

In nutrient-rich culture medium (MHB), TBHQ and TBBQ displayed reduced activity against *S. aureus* SH1000 compared with that seen in PBS. Nonetheless, TBBQ retained bactericidal activity in MHB, causing >4 $\log_{10}$ reduction in viable cell numbers within 6 hours (Figure 3.6a). At TBHQ or TBBQ concentrations of up to 256 μg/mL, there was little or no reduction in culture turbidity over a 24 hour period (Figure 3.6b), indicating that bacterial killing by TBBQ occurred without concomitant cell lysis. The absence of cell lysis is a beneficial property, as there is reduced risk of releasing immunogenic bacterial molecules into the host's bloodstream (Nau & Eiffert, 2002).

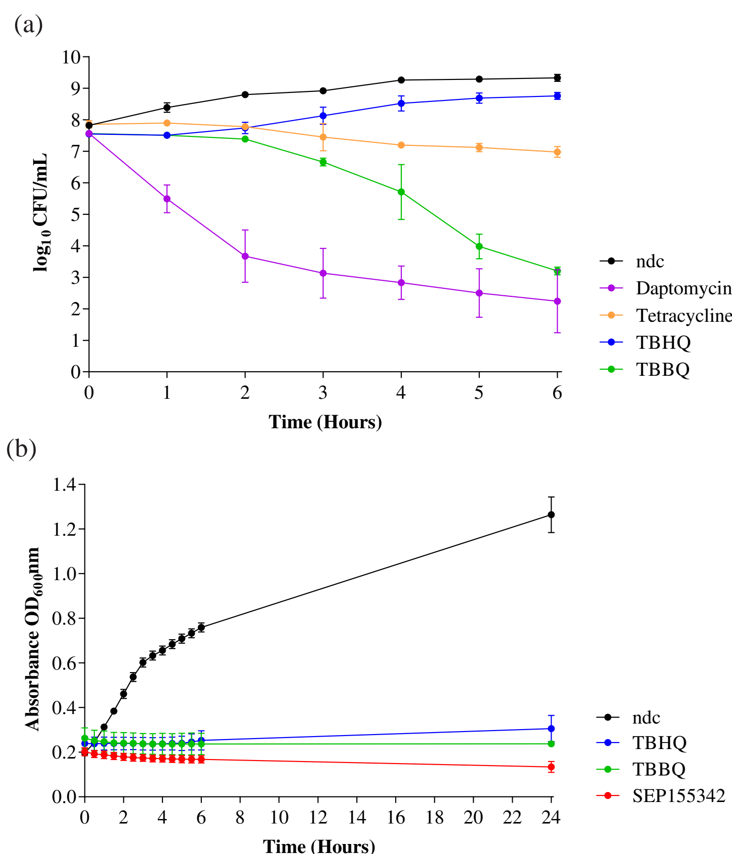

Figure 3.6: **Evaluation of killing and lytic action of TBHQ, TBBQ and comparator agents against *S. aureus* SH1000 in MHB.** Panel (a): killing kinetics of compounds at 4x MIC; Panel (b): absorbance at 600 nm of cultures exposed to TBHQ and TBBQ at 256 μg/mL, or the positive control agent SEP155342 at 4x MIC (8 μg/mL) (Oliva *et al.*, 2003) (mean of at least three independent replicates; error bars show standard deviations).

3.3.4 Antibiofilm activity of TBBQ

Upon demonstrating that TBBQ is responsible for the activity of TBHQ, which itself has little antibacterial activity, experiments were performed to examine whether TBBQ had antibiofilm activity. Pre-formed staphylococcal biofilms were exposed to TBBQ, which inhibited both the shedding of cells from the biofilm (bMIC of 64 μg/mL) and achieved complete eradication of biofilms (MBEC of 64 μg/mL). This property was not shared with the other tested antibacterial agents (Table 3.2). TBBQ also had the ability to eradicate biofilms generated by other staphylococci, including prolific biofilm-forming strains (Ryder *et al.*, 2012) (Table 3.3).

Antibacterial agent	MIC (μg/mL)	bMIC (μg/mL)	MBEC (μg/mL)
Cefotaxime	0.5	4	>256
Chlorhexidine	1	1	>256
Ciprofloxacin	2	4	>256
CTAB	2	2	>256
Daptomycin	1	2	>256
Erythromycin	0.5	0.5	>256
Fosfomycin	16	8	>256
Flucloxacillin	0.125	4	>256
Fusidic acid	0.25	0.5	>256
Gentamicin	0.5	1	>256
Meropenem	0.5	0.5	>256
Mupirocin	0.125	0.25	>256
Nisin	2	>16	>16
Oxacillin	1	1	>256
Rifampicin	0.008	0.02	>256
SDS	256	256	>256
TBBQ	8	64	64
Tetracycline	1	0.5	>256
Vancomycin	1	2	>256

Table 3.2: **Antibiofilm activity of antibacterial agents against *S. aureus* SH1000 biofilms grown on the Calgary Biofilm Device.**

Strain	MIC (μg/mL)	bMIC (μg/mL)	MBEC (μg/mL)
S. aureus SH1000	8	64	64
S. aureus UAMS-1	8	16	16
S. epidermidis RP62A	4	32	64

Table 3.3: **Antibiofilm activity of TBBQ against prolific biofilm-forming strains.**

3.3.5 Effect of TBBQ on biofilm structure

Given that the ability to eradicate biofilms is rare property amongst antibacterial agents, experiments were carried out to determine how TBBQ exerts its antibiofilm effect. The effect of TBBQ on biofilm structural components (adhered matrix and cells) was investigated in staphylococcal biofilms maintained in MHB. Matrix material and biofilm-encapsulated cells were stained with the fluorescent dyes SYPRO® Ruby and SYTO® 9 and quantified. Proteinase K-disrupted biofilms, causing dissociation of all cells and the majority of the extracellular matrix. At concentrations above the MBEC, TBBQ did not cause a significant reduction in the quantity of adhered matrix material or cells (Figure 3.7a and 3.7b), indicating that TBBQ does not induce dissociation of biofilms that are actively growing.

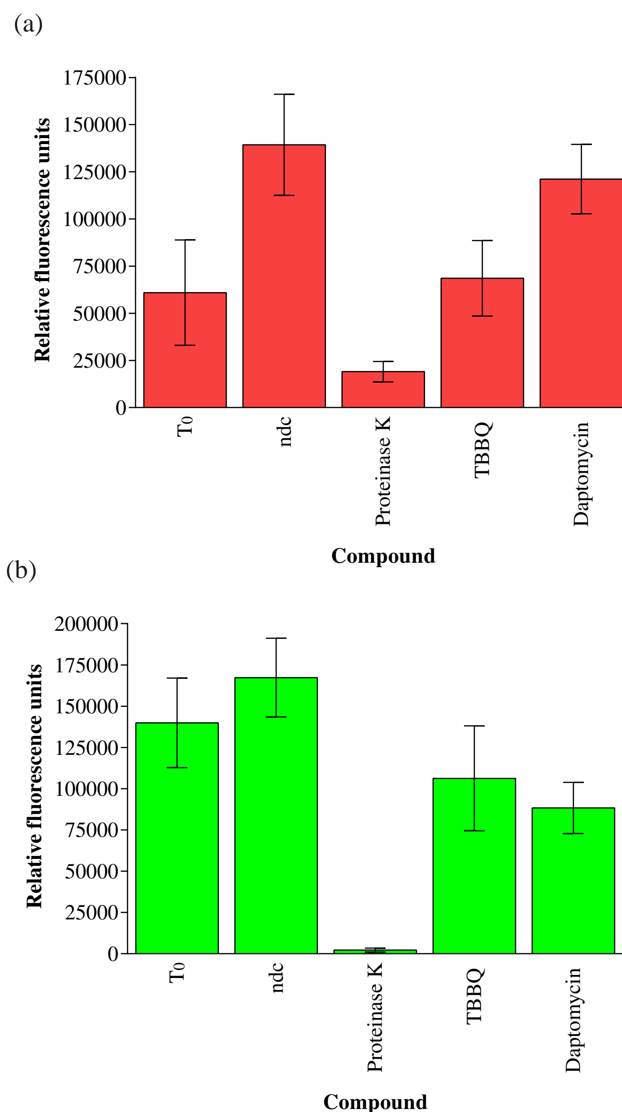

Figure 3.7: **Effect of antibacterial agents on biofilm structure.** Biofilms were exposed to proteinase K at 100 μg/mL, or test compounds at 256 μg/mL for 24 hours. Panel (a): attached matrix; Panel (b): attached cells. The data labeled T_0 shows the quantity of matrix and cells before the addition of compounds. Data are the means of at least three independent replicates, with error bars showing standard deviation.

3.3.6 Effect of TBBQ on slow- and non-growing cells

Biofilm-encapsulated cells display physiological heterogeneity, including slow- or no growth and persistence (Shapiro *et al.*, 2011; Singh *et al.*, 2009). Since TBBQ does not exert antibiofilm activity through disruption and release of biofilms from surfaces, the effects of TBBQ on the mixed population of cells contained within biofilms was investigated (Figure 3.8). Unlike the comparator agent daptomycin, TBBQ retained a small amount of activity against non-dividing cells at 4x MIC, causing ∼1 $\log_{10}$ reduction in viable cells after 6 hours. Thus, TBBQ has some activity against non-growing cells even at concentrations that are below those capable of eradicating biofilms.

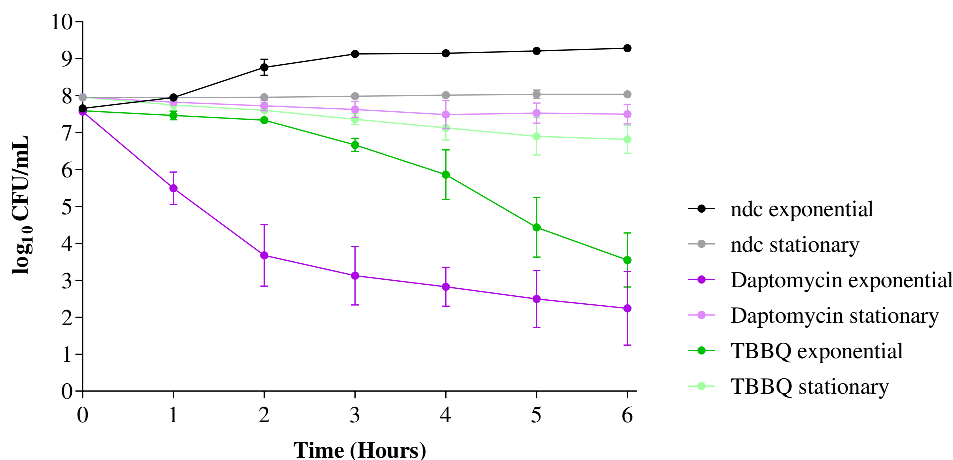

Figure 3.8: **Activity of compounds at 4x MIC against exponential and stationary phase *S. aureus* SH1000 suspended in MHB.** Mean of at least three independent replicates; error bars show standard deviations.

Initial experiments carried out at 4x MIC (32 μg/mL) were not sufficient to display bactericidal activity against stationary phase cells over 6 hours. However, TBBQ eradicates *S. aureus* SH1000 biofilms at 64 μg/mL. Therefore, sterilisation of biofilms may be a consequence of cidal activity against slow-growing cells at higher concentrations

and over longer exposure times than were tested. Further experimentation measured compound activity against exponentially growing and stationary phase cultures incubated with antibacterial agent at 256 μg/mL for 24 hours. At the tested concentration (4x MBEC), TBBQ sterilised cultures of both growing and non-growing cells; a property that was not shared with the comparator agent daptomycin (Figure 3.9).

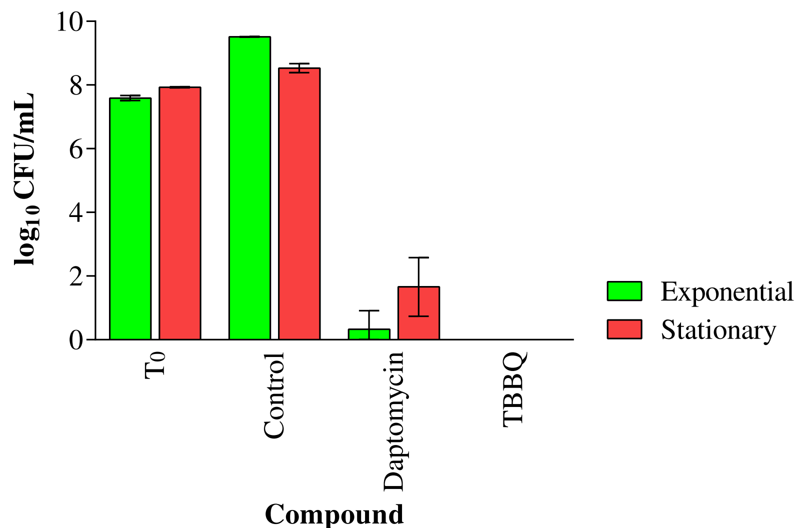

Figure 3.9: **Sterilisation of exponential and stationary phase cultures of *S. aureus* SH1000 at 256 μg/mL.** Mean of at least three independent replicates; error bars show standard deviations.

Biofilms are a reservoir not only for slow-growing cells, but also persisters (Singh *et al.*, 2009). Therefore, compound activity was tested against persisters that were generated by pre-exposure of *S. aureus* to ciprofloxacin or ampicillin at 10x MIC for 24 hours. TBBQ was the only agent that could completely sterilise a population of persister cells (Figure 3.10). Hence, TBBQ may inhibit and eradicate pre-existing biofilms by remaining active against metabolically inactive and persister cells that could otherwise repopulate the biofilm following removal of antibacterial agent.

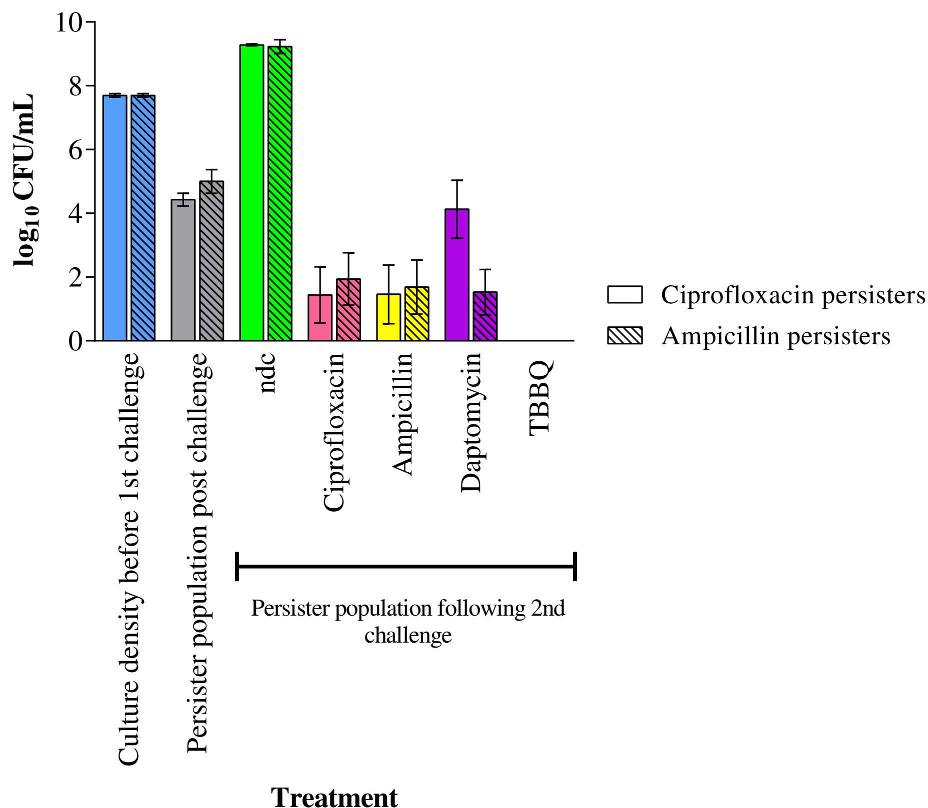

Figure 3.10: **Activity of antibacterial agents against ciprofloxacin- or ampicillin-induced persisters of *S. aureus* SH1000.** Mean of at least three independent replicates; error bars show standard deviations.

3.4 Conclusions

The antioxidant TBHQ was unstable under bacterial culture conditions, and degraded forming TBBQ. Halting the conversion of TBHQ to TBBQ (via the addition of ascorbic acid or incubation under anaerobic conditions) dramatically reduced antibacterial activity, i.e. TBHQ had little intrinsic antibacterial activity when prevented from undergoing oxidation. The oxidative product, TBBQ, possessed inherent antibacterial activity that was then lost upon degradation. TBBQ displayed antibacterial activity against

a number of bacterial species, but was most active against Gram-positive organisms. Potent bactericidal activity against *S. aureus* was not associated with cell lysis up to the maximum tested concentration (256 μg/mL). The compound also eradicated staphylococcal biofilms; a rare property in antibacterial agents. It is proposed that TBBQ sterilises biofilms by retaining activity against slow- and non-growing cells contained within the biofilm.

Chapter 4

Investigations into the antibacterial mechanism of action of TBBQ

The work contained within this chapter has been published in part in Ooi *et al.* (2013).

N. Ooi, I. Chopra, A. Eady, J. Cove, R. Bojar and A. J. O'Neill (2013) Antibacterial activity and mode of action of *tert*-butylhydroquinone (TBHQ) and its oxidation product, *tert*-butylbenzoquinone (TBBQ). Journal of Antimicrobial Chemotherapy, 68 (6): 1297-1304.

4.1 Abstract

MOA studies are an important aspect of preclinical evaluation of antibacterial agents. Therefore, initial studies attempted to identify whether the bactericidal compound TBBQ acts through inhibition of pathways commonly targeted by antibiotics. TBBQ did not display preferential inhibition of any single macromolecular synthetic pathway, but disrupted the staphylococcal membrane. The antioxidant induced loss of membrane

potential and leakage of intracellular ions, without causing damage to staphylococcal liposomes or inducing haemolysis. Additionally, intracellular ATP was hydrolysed and ROS were generated within cells exposed to TBBQ. Only low level TBBQ resistance could be selected using the extended spectrum MIC method, and a hypersusceptible mutant was identified by screening a near-saturation Tn library. Mutations were identified in the resistant (B7) and hypersusceptible (CAJ192 C9) strains by PCR and the role of the genes in TBBQ susceptibility was confirmed by complementation. The potential roles of these alterations are discussed in this chapter, but require further investigation. B7/CAJ192 C9 did not display cross-resistance/hypersusceptibility to other agents, suggesting that TBBQ has a novel MOA. Eukaryotic toxicity of the compound was investigated using a human skin equivalent. Exposure to TBBQ for 24 hours did not irritate or damage skin. Therefore, TBBQ appears to interact specifically with bacterial membranes and could potentially be used as a topical treatment. In view of the potent bactericidal and antibiofilm activity of TBBQ, and absence of development of high-level resistance or damage to skin, this compound warrants further investigation as a candidate antistaphylococcal agent.

4.2 Introduction

4.2.1 MOA studies

Characterisation of the MOA of antibacterial agents is an important phase of preclinical evaluation, as it can guide further development of potentially useful agents (O'Neill & Chopra, 2004). There are a large number of tools available to investigate MOA, and the experiments descried in the following section have been successfully used in previous studies (O'Neill & Chopra, 2004). Initial MOA studies may include measurements of effects of compounds on macromolecular synthesis (MMS) through incorporation of

radiolabeled precursors into DNA, RNA and protein (Wilson *et al.*, 1995). Compounds may also be screened against whole-cell *B. subtilis* biosensors (Mariner *et al.*, 2011). Expression profiling was used to identify promoters that are induced following exposure to inhibitors of known biosynthetic pathways (DNA, RNA, protein, cell-envelope or fatty acid synthesis). Promoter-luciferase reporter constructs were then used to generate bacterial biosensors (Urban *et al.*, 2007), which can be employed in high-throughput screening of inhibitors to identify agents that affect specific pathways.

Compounds that damage the bacterial membrane simultaneously inhibit all MMS pathways and may not induce any of the biosensors (Ooi *et al.*, 2009). Membrane perturbation can be confirmed by measuring ingress/egress of fluorescent dyes and leakage of intracellular components (Ooi *et al.*, 2009). Until relatively recently, membrane-damaging activity had been considered an undesirable property of a novel inhibitor, as some compounds may have promiscuous interactions with both prokaryotic and eukaryotic membranes (Hurdle *et al.*, 2011). Daptomycin is a membrane-damaging antibiotic that disrupts bacterial cytoplasmic membranes specifically by inserting into the phospholipid bilayer in the presence of calcium and oligomerises to form a pore through which intracellular components are lost (Randall *et al.*, 2013; Hobbs *et al.*, 2008). The successful introduction of daptomycin into clinical practice suggests that selective toxicity of membrane-damaging agents may be achieved by optimisation of the dosing regimen (Dvorchik *et al.*, 2003). Therefore, compounds that disrupt the bacterial membrane are receiving increasing interest, particularly due to the emerging concept that membrane-active agents may be usefully employed against non-growing and persistent bacterial populations (Hu *et al.*, 2010; Hurdle *et al.*, 2011).

Agents with novel targets or those that affect essential bacterial components are desirable as resistance to these agents is less likely to arise by minor alterations to either existing resistance mechanisms or conserved structures. The development of resistance should be assessed during preclinical evaluation, as resistant mutants can provide insight into

the cellular target of the compound (O'Neill & Chopra, 2004). Artificial under- or over-expression of the inhibitor's target should cause hypersusceptibility or resistance to the compound respectively, and can be used to confirm that the gene of interest is indeed the target of the inhibitor (O'Neill & Chopra, 2004).

4.2.2 Toxicity studies

Upon characterisation of an antibacterial agent's MOA, the compound should be tested for selective toxicity. Since the primary concern with membrane-damaging agent agents is human toxicity, any promising inhibitor that displays this MOA must undergo rigorous toxicity testing. There is an array of experiments that can be used to investigate detrimental effects of compounds on eukaryotic cells, including those that measure leakage of haemoglobin from erythrocytes (Oliva *et al.*, 2003); metabolic activity as an indicator of cell viability via reduction of dyes (Fotakis & Timbrell, 2006); release of inflammatory cytokines indicating irritation (Bernhofer *et al.*, 1999); physical damage to the structure of human skin equivalents (Rasmussen *et al.*, 2010); or *in vivo* LD_{50} determinations with test animals (Lorke, 1983). Compounds that are active through a non-specific MOA are likely to display host toxicity *in vivo*, and may not be taken further in antibacterial drug discovery programmes (Silver, 2011).

4.2.3 Putative MOA of TBBQ

The rapid bactericidal and antibiofilm activity of TBBQ against staphylococci suggest that the compound has potential to be used clinically as an antibacterial agent. The MOA of TBBQ has not been determined, however attempts have been made to assess the impact that the precursor TBHQ has on bacteria (Malone *et al.*, 2008). In Chapter 3 it was shown that TBHQ is rapidly converted to TBBQ and that the antibacterial activity attributed to TBHQ is due to TBBQ. Therefore, previous MOA studies of TBHQ may in

fact have described the MOA of TBBQ. TBHQ was reported to induce single- and double-strand breakage of supercoiled double stranded plasmid DNA at concentration ≥ 0.1 mM ($\sim$16 μg/mL) (Okubo *et al.*, 1997). DNA damage was enhanced in the presence of transition metals, and the addition of ROS scavengers or metal chelators inhibited DNA cleavage. This suggests that TBHQ may undergo metal-assisted redox reactions causing lesions in DNA (Okubo *et al.*, 1997; Li *et al.*, 2002).

The contribution of ROS generation to antibiotic MOA was investigated by Kohanski *et al.* (2007), who suggested that bactericidal but not bacteriostatic antibiotics induce lethal generation of ROS. It was proposed that fluoroquinolones, β-lactams and aminoglycosides lead to hyperactivation of the electron transport chain, increasing NADH consumption and inducing superoxide generation. This reactive species destabilises iron-sulphur cluster-containing enzymes, causing leaching of iron. Subsequently, iron-assisted Fenton reactions generate hydroxyl radicals, which contribute to cell death through damage to bacterial macromolecules (Kohanski *et al.*, 2007). Numerous research papers investigating this hypothesis have appeared in the literature, some in support of Kohanski's theory (Wang & Zhao, 2009) and some in opposition (Ricci *et al.*, 2012). Although the subject has divided the research community (Wright *et al.*, 2013), two recent papers provide compelling evidence that refute the hypothesis (Liu & Imlay, 2013; Keren *et al.*, 2013). The following observations were of particular significance: bactericidal antibiotics caused an equivalent loss of cell viability anaerobically as aerobically; antibiotics did not induce significant increases in hydrogen peroxide levels; strains lacking hydrogen peroxide detoxification enzymes were not more sensitive to all bactericidal antibiotics; and DNA repair mutant strains did not display increased susceptibility to kanamycin or ampicillin, (Liu & Imlay, 2013; Keren *et al.*, 2013). Therefore, under certain conditions some bacteria may generate ROS following exposure to some bactericidal antibiotics, but the extent to which this contributes to cell death is debatable.

Investigations into the contribution of ROS in antibiotic MOA highlight the importance of identifying the primary lethal interaction as opposed to the response of stressed or dying cells. Whilst TBHQ may be capable of inducing ROS production that damages DNA, these studies serve as a reminder that attempts should be made to distinguish between primary and secondary effects following antibacterial challenge. Furthermore, TBHQ-induced ROS damage to DNA has not been measured in whole-cell assays with bacteria. Experiments carried out on cellular components *in vitro* must be confirmed under culture conditions, as identified effects may not contribute to MOA in living organisms.

No further research has been carried out into the MOA of TBHQ. However the antibacterial activity of another antioxidant that is related to TBBQ has been studied. The food preservative 3-*tert*-butyl-4-hydroxyanisole (BHA) interacts with *E. coli* membrane phospholipids, and solubilises membrane proteins, affecting the molecular organisation of membranes (Okubo *et al.*, 1997; Simonetti *et al.*, 2002). TBBQ, like structurally related BHA, could affect bacterial membranes. Furthermore, compounds that are rapidly bactericidal displaying activity against bacterial biofilms may be expected to be active through disruption of the cytoplasmic membrane (Ooi *et al.*, 2009, 2010; Hurdle *et al.*, 2011).

4.2.4 Aims and Objectives

Work described in this chapter was intended to determine the mechanism by which TBBQ exerts it antibacterial activity, and to identify any detrimental effects of the compound on eukaryotic cells. To assess the potential for the compound to be used clinically, synergistic interactions with existing antibiotics and the rate of resistance development will be assessed. A staphylococcal transposon library will be screened with the intention of isolating mutants that are hypersusceptible to TBBQ, and these strains, along with TBBQ-resistant mutants, will be characterised in order to provide further information about the cellular target of TBBQ.

4.3 Results and discussion

4.3.1 Effects of TBBQ on biosynthetic pathways

As part of the preclinical evaluation of TBBQ, the MOA of the compound was investigated at the cellular level. Antibacterial agents commonly exert their activity through inhibition of macromolecular biosynthesis pathways (O'Neill & Chopra, 2004). Therefore, preliminary experiments examined the effects of TBBQ on bacteria using a suite of *B. subtilis* biosensors responsive to inhibition of specific biosynthetic pathways (Mariner *et al.*, 2011; Urban *et al.*, 2007). The DNA, RNA, protein, cell wall and fatty acid biosynthesis biosensors were induced in response to the positive control agents ciprofloxacin, rifampicin, tetracycline, vancomycin, and triclosan respectively. Positive controls caused a maximum increase in luminescence of 80.5-, 12.3-, 5.2-, 2.6- and 8.6-fold respectively in comparison with the drug-free control. No induction was observed with any of the biosensors for TBBQ in the concentration range 0.005 - 20 μg/mL (Figure 4.1). Therefore, exposure to TBBQ does not induce promoters that are responsive to these pathways in *B. subtilis*.

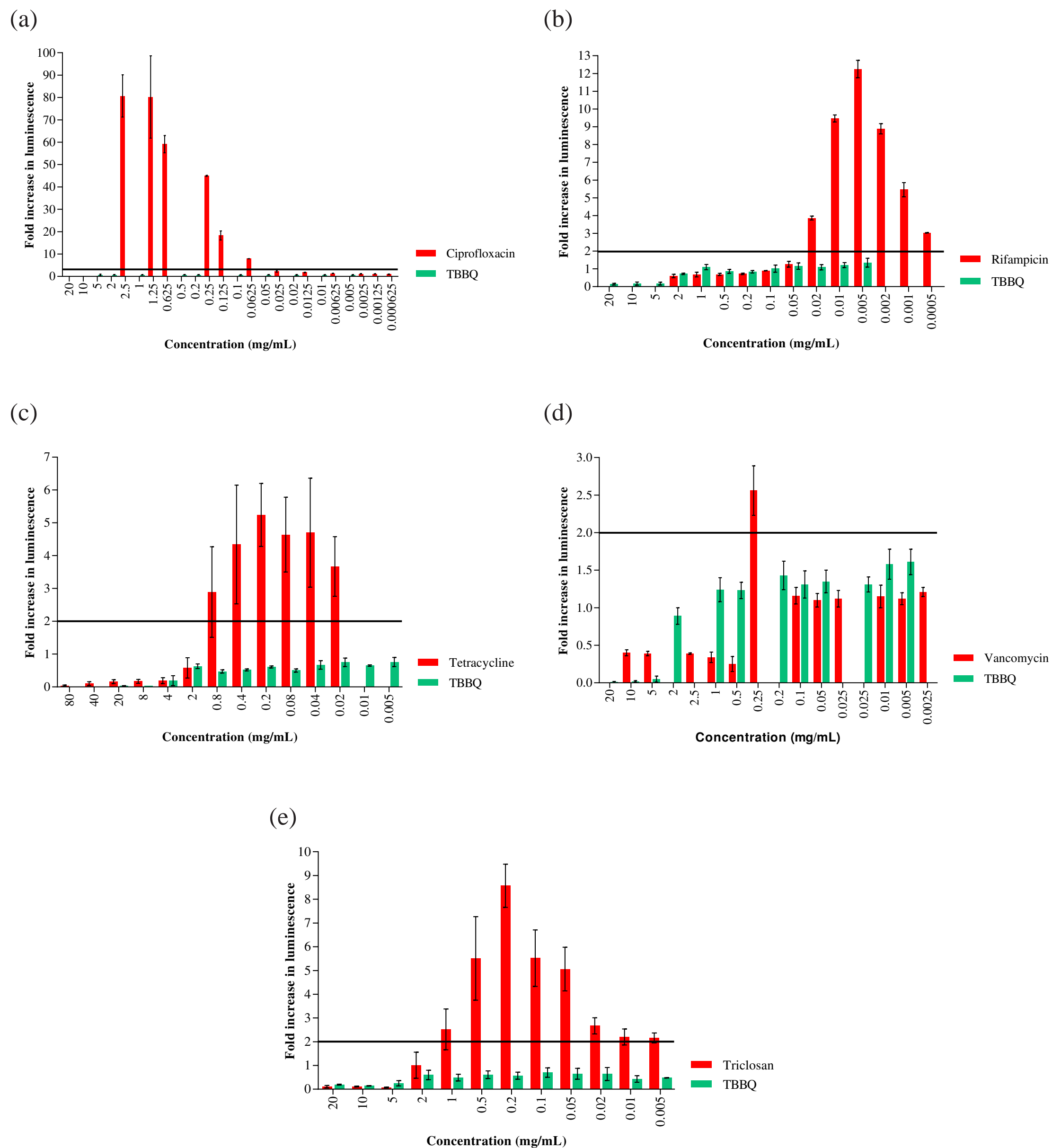

Figure 4.1: **Effects of TBBQ and pathway specific inducers on *B. subtilis* pathway specific biosensors.** Panel (a): DNA biosensor (*yorB* promoter-luciferase reporter fusion strain); Panel (b): RNA biosensor (*yvgS* promoter-luciferase reporter fusion strain); Panel (c): protein biosensor (*yheI* promoter-luciferase reporter fusion strain); Panel (d): cell wall biosensor (*ypuA* promoter-luciferase reporter fusion strain); Panel (e): fatty acid biosensor (*fabHB* promoter-luciferase reporter fusion strain). Horizontal line indicates 2-fold increase in relative luminescence. Mean of at least three independent replicates; error bars show standard errors.

To investigate whether TBBQ inhibits synthesis of specific macromolecular biosynthetic pathways in staphylococci, incorporation of radiolabeled precursors into macromolecules was compared with known inhibitors of DNA, RNA and protein synthesis (ciprofloxacin, rifampicin and tetracycline respectively). At 4x MIC TBBQ inhibited all three macromolecular synthetic pathways within 10 minutes, with no evidence for preferential inhibition of a single biosynthetic process (Figure 4.2).

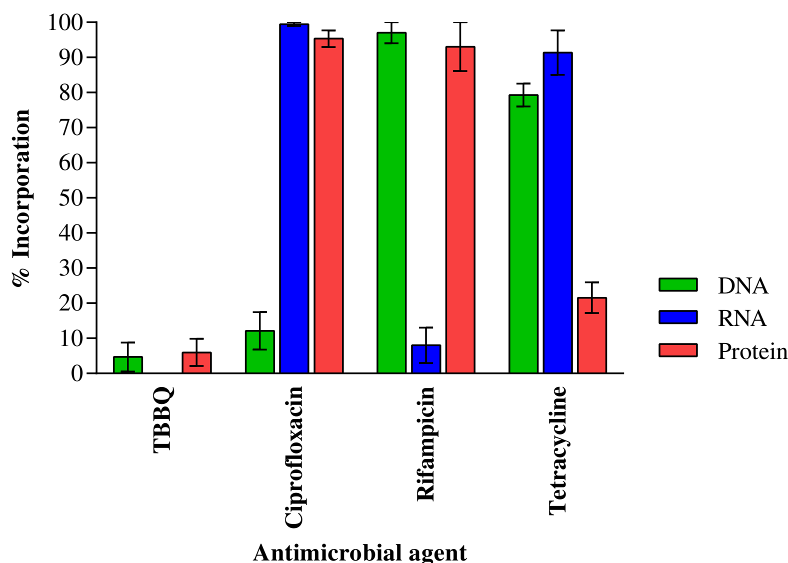

Figure 4.2: **Effects of TBBQ and comparator agents on major macromolecular biosynthesis pathways in *S. aureus*.** Percentage incorporation of ^{3}H thymidine, uridine and glutamine into SH1000 DNA, RNA and protein respectively (mean of at least three independent replicates; error bars show standard errors).

4.3.2 Membrane damaging assays

Rapid bactericidal activity, failure to induce pathway-specific biosensors, and non-specific inhibition of macromolecular synthesis are features common to antibacterial agents that act on bacteria by compromising the integrity of the cytoplasmic membrane and/or

inhibiting energy metabolism (Ooi *et al.*, 2009; Shapiro & Baneyx, 2002; O'Neill & Chopra, 2004). Therefore, the BacLight™ assay was used to examine whether TBBQ acts on staphylococcal membranes. This method involves exposing bacteria to antimicrobial agents before staining with two fluorescent nucleic acid dyes: SYTO® 9 and propidium iodide. The bacterial cell membrane is permeable to SYTO® 9, which can stain all cells, but propidium iodide only stains cells with a compromised membrane. Membrane damage allows increased propidium iodide staining and reduces SYTO® 9. Therefore, the ratio of the dyes is a direct measure of compound-induced membrane damage (Hilliard *et al.*, 1999). A number of antibiotics and known membrane-damaging agents (nisin, CTAB and valinomycin) were used as comparator agents. Nisin is a lantibiotic that generates pores in the membrane, which dissipates the proton motive force and promotes leakage of cellular components (McAuliffe *et al.*, 2001). Similarly, the biocide CTAB forms micelles that interact with the lipid component of membranes causing depolarisation and lysis (Simões *et al.*, 2005; Vieira & Carmona-Ribeiro, 2006). The MOA of valinomycin is distinct from the other agents, acting as an ionophore that stimulates passive permeabilisation of the membrane to potassium ions (Bhattacharyya *et al.*, 1971).

S. aureus cells exposed to ampicillin, rifampicin, vancomycin, tetracycline, and daptomycin for 10 minutes retained between 90 and 100% membrane integrity, while TBBQ and the known membrane disruptors nisin, CTAB and valinomycin reduced membrane integrity over the same time period (Table 4.1).

Antimicrobial compound	% membrane integrity ($\pm$ SE)
None	100 ± 0
Ampicillin	99.9 ± 0.1
CTAB	0.5 ± 0.3
Daptomycin	94.7 ± 5.3
Nisin	4.6 ± 1.5
Rifampicin	94.7 ± 5.3
5% SDS	0 ± 0
TBBQ	66.1 ± 4.3
Tetracycline	90.9 ± 9.1
Valinomycin	27.4 ± 3.8
Vancomycin	100 ± 0

Table 4.1: **Effect of TBBQ and comparator agents at 4x MIC on *S. aureus* SH1000 membranes.** Results are the means of at least three independent determinations, SE - standard error.

Experiments with the fluorescent dye $DiSC_3(5)$ are a more sensitive measure of membrane perturbation. $DiSC_3(5)$ accumulates in polarised cells and is released following loss of membrane potential (Wu & Hancock, 1999). TBBQ, like other membrane disrupting agents, caused rapid dissipation of membrane potential in staphylococcal cells suspended in HEPES and glucose buffer (Figure 4.3). Within 5 minutes $\sim$70% membrane potential was lost, an effect comparable with that of the known membrane-damaging agent, nisin.

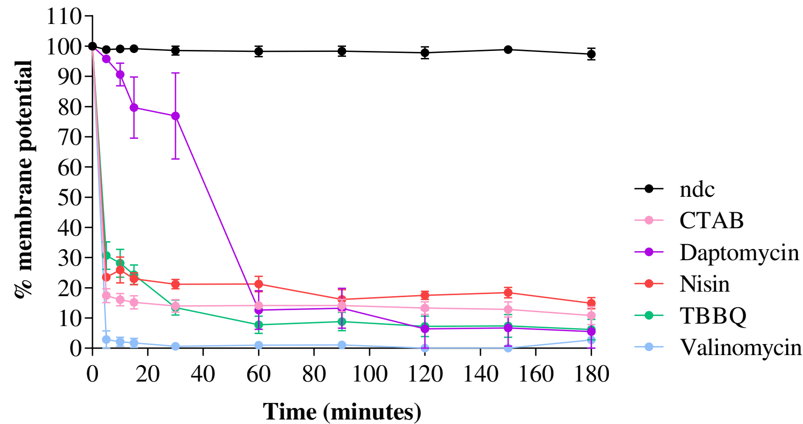

Figure 4.3: **Effect of TBBQ and comparator agents at 4x MIC on staphylococcal membrane potential.** Mean of at least three independent replicates; error bars show standard errors.

In order to determine whether membrane perturbation following exposure to TBBQ was extensive enough to allow leakage of intracellular ions, release of potassium and magnesium was measured. TBBQ induced leakage of potassium from cells exposed to compound at 4x MIC in HEPES and glucose buffer, but did not cause release of magnesium (Figure 4.4). Therefore, the interaction of TBBQ with the membrane could either specifically promote active potassium export, or enhance passive diffusion of K^+ through potassium specific transporters. Since loss of membrane potential precedes gradual efflux of potassium ions, it is likely that membrane depolarisation triggers K^+ export in an attempt to recover membrane potential (Shapiro, 2000). Therefore, in contrast with other membrane-damaging agents, such as daptomycin (Hobbs *et al.*, 2008), TBBQ may have relatively subtle effects on the membrane and not simply form pores allowing non-specific diffusion of intracellular components.

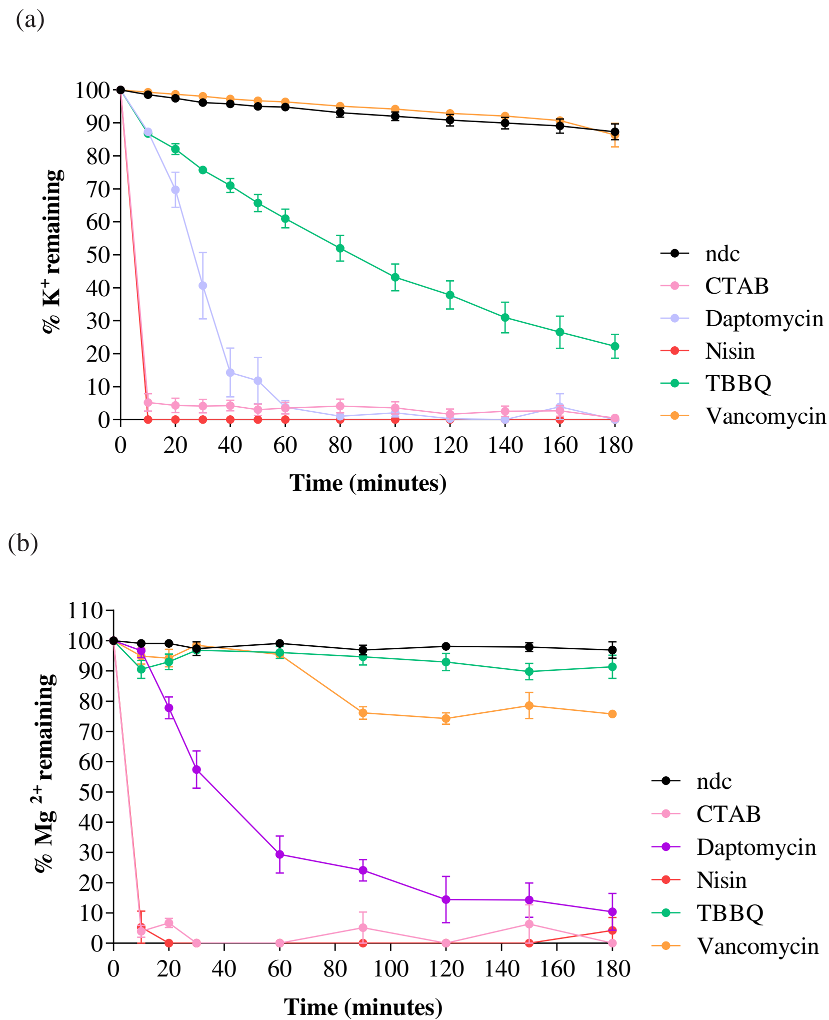

Figure 4.4: **Release of intracellular ions following exposure of *S. aureus* SH1000 to TBBQ and comparator agents at 4x MIC.** Panel (a): potassium leakage; Panel (b): magnesium leakage (mean of at least three independent replicates; error bars show standard errors).

To establish whether TBBQ interacts with the phospholipid bilayer or other components of the membrane, staphylococcal liposomes were exposed to compounds at 4x MIC for 3 hours (Table 4.2). TBBQ did not cause leakage of dye contained within liposomes, suggesting that the compound does not directly disrupt the staphylococcal lipid bilayer. Since TBBQ does not cause bacterial lysis, generalised leakage of intracellular components or destructure liposomes, the interaction with the membrane is not surfactant-like. Instead, damage may be through disruption of components embedded within the lipid bilayer, such as the proteins of the electron transport chain.

Compound	Liposome integrity $\pm$ SE
ndc	100 ± 0
CTAB	98.0 ± 0.0
Daptomycin	23.5 ± 1.2
Nisin	111.5 ± 4.3
TBBQ	92.0 ± 0.5
Tetracycline	87.9 ± 1.6
Vancomycin	100.6 ± 0.4

Table 4.2: **Effect of TBBQ on staphylococcal liposomes.** Experiments were carried out by C. Randall, University of Leeds (mean of at least three independent replicates; SE - standard errors).

Since antibacterial compounds that compromise the bacterial membrane may show comparable effects on mammalian membranes, it is important to establish the bacterial selectivity of agents with this MOA (Payne *et al.*, 2007). Therefore, TBBQ was tested for its ability to cause haemolysis of mammalian erythrocytes. Agents that do not damage membranes caused <5% loss in erythrocyte integrity, whilst the known membrane disruptors nisin, valinomycin and CTAB induced haemolysis (Table 4.3). TBBQ had little effect on erythrocyte integrity (Table 4.3), which indicates that the interaction with cell membranes is bacteria-specific.

Antimicrobial compound	% Erythrocyte Integrity (± SE)
ndc	100 ± 0
Ampicillin	97.7 ± 1.1
CTAB	0 ± 0
Daptomycin	97.9 ± 0.7
Nisin	54.9 ± 4.6
5 % SDS	0 ± 0
TBBQ	93.5 ± 2.0
Tetracycline	99.2 ± 0.4
Valinomycin	49.1 ± 3.4
Vancomycin	96.3 ± 3.7

Table 4.3: **Effect of TBBQ and comparator agents at 4x MIC on erythrocytes.** Results are the means of at least three independent determinations, SE - standard error.

4.3.3 Effect of TBBQ on staphylococcal metabolism

Rapid bactericidal activity and cessation of macromolecular synthesis may be induced by altered energy metabolism in addition to membrane disruption. Since TBBQ does not appear to disrupt the phospholipid bilayer of staphylococci, the interaction with the membrane may be through disruption of other components, such as membrane-embedded proteins that comprise the electron transport chain. Measurement of ATP leakage from cells is a standard gauge of membrane disruption (Hobbs *et al.*, 2008; Ooi *et al.*, 2009). However, experimentation revealed that TBBQ did not cause leakage of ATP, measured by decreases in intracellular and concurrent increases in extracellular ATP levels. Instead, total ATP levels were depleted in cells exposed to TBBQ, indicating that the compound induced hydrolysis of intracellular ATP stores (Figure 4.5). The ability of TBBQ to diminish ATP levels was unusual, as this property was not observed with other antibacterial agents.

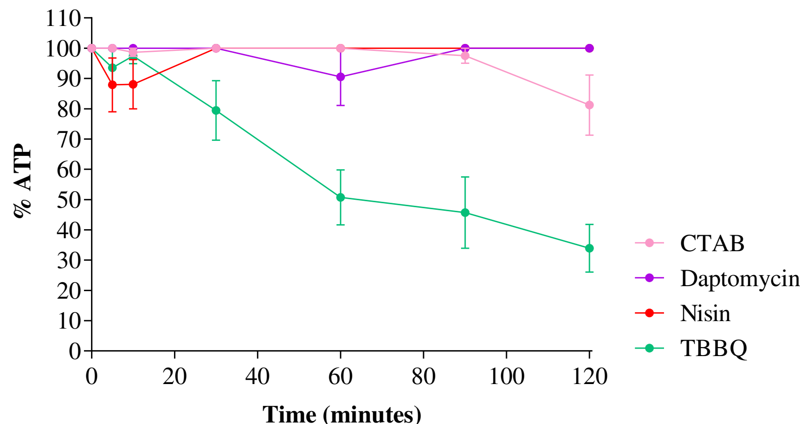

Figure 4.5: **Cellular ATP levels following exposure to TBBQ and comparator agents at 4x MIC.** Values were calculated as a percentage of an untreated control. Mean of at least three independent replicates; error bars show standard errors.

The effect of supplementation with the ATP synthase/ATPase inhibitor DCCD and uncoupling agent CCCP on TBBQ activity was tested (Figure 4.6). This experiment aimed to identify whether active ATP generation and/or ATP hydrolysis is required for TBBQ to effect antibacterial activity. Neither of the agents reduced TBBQ activity, suggesting that the primary MOA is not a consequence of disruption to ATP/ADP cycling. Since the addition of respiratory inhibitors does not abolish TBBQ activity, it can be assumed that an active electron transport chain coupled with an active ATP synthase is not required for bactericidal activity. Thus, ATP depletion is likely to be a secondary effect of TBBQ's interaction with its target, and may be due to upregulation of ATPase activity in an attempt to recover membrane potential (Figure 4.6). Since energy production genes are involved in persister formation (Mah & O'Toole, 2001), ATP depletion may contribute to TBBQ's ability to sterilise cultures. Fewer persisters are formed from populations of mutants with reduced energy production, suggesting that maintenance of ATP pools is implicated in persister formation (Mah & O'Toole, 2001). Hydrolysis of ATP following exposure to TBBQ may impede the generation of persisters and contribute

to the compound's ability to sterilise bacterial cultures.

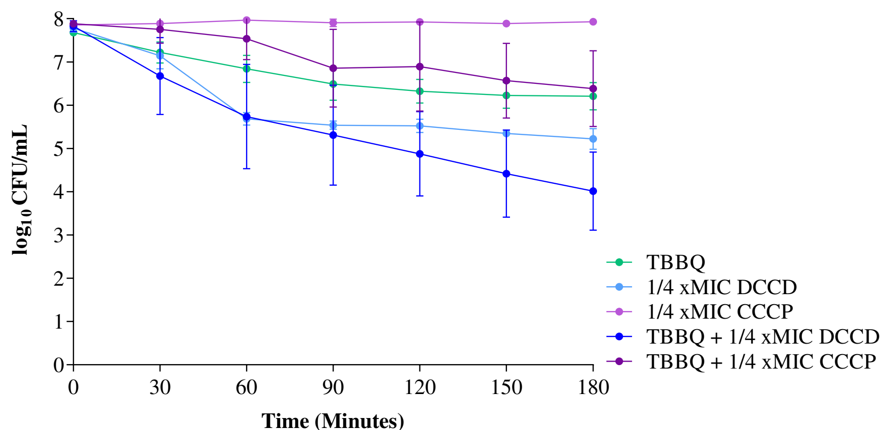

Figure 4.6: **Effects of respiratory uncouplers on activity of TBBQ against *S. aureus* SH1000.** Cells were exposed to TBBQ in HEPES and glucose buffer at 4x MIC supplemented with 0.25x MIC DCCD or CCCP (mean of at least three independent replicates; error bars show standard deviations).

Although TBBQ does not inhibit phospholipid-bound ATP synthase/ATPase, it may affect other components of metabolic pathways. Small colony variants (SCVs) are slow-growing cells that arise spontaneously in staphylococcal populations (Singh *et al.*, 2009). SCVs have reduced membrane potential, restricted energy metabolism, and are less susceptible to antibiotics (Von Eiff *et al.*, 2006). To investigate whether TBBQ is active against bacteria with a defective electron transport chain, time-kill experiments were carried out in MHB with SH1000 *hemB*::*ermB* and SH1000 *menD*::*ermC*. SCVs were resistant to the activity of the aminoglycoside gentamicin (Figure 4.7b), as has been described previously (Von Eiff *et al.*, 2006). TBBQ retained some activity against SH1000 *hemB*::*ermB*, but was not active against the *menD* knockout, which has a more restricted energy metabolism (Figure 4.7c) (Von Eiff *et al.*, 2006).

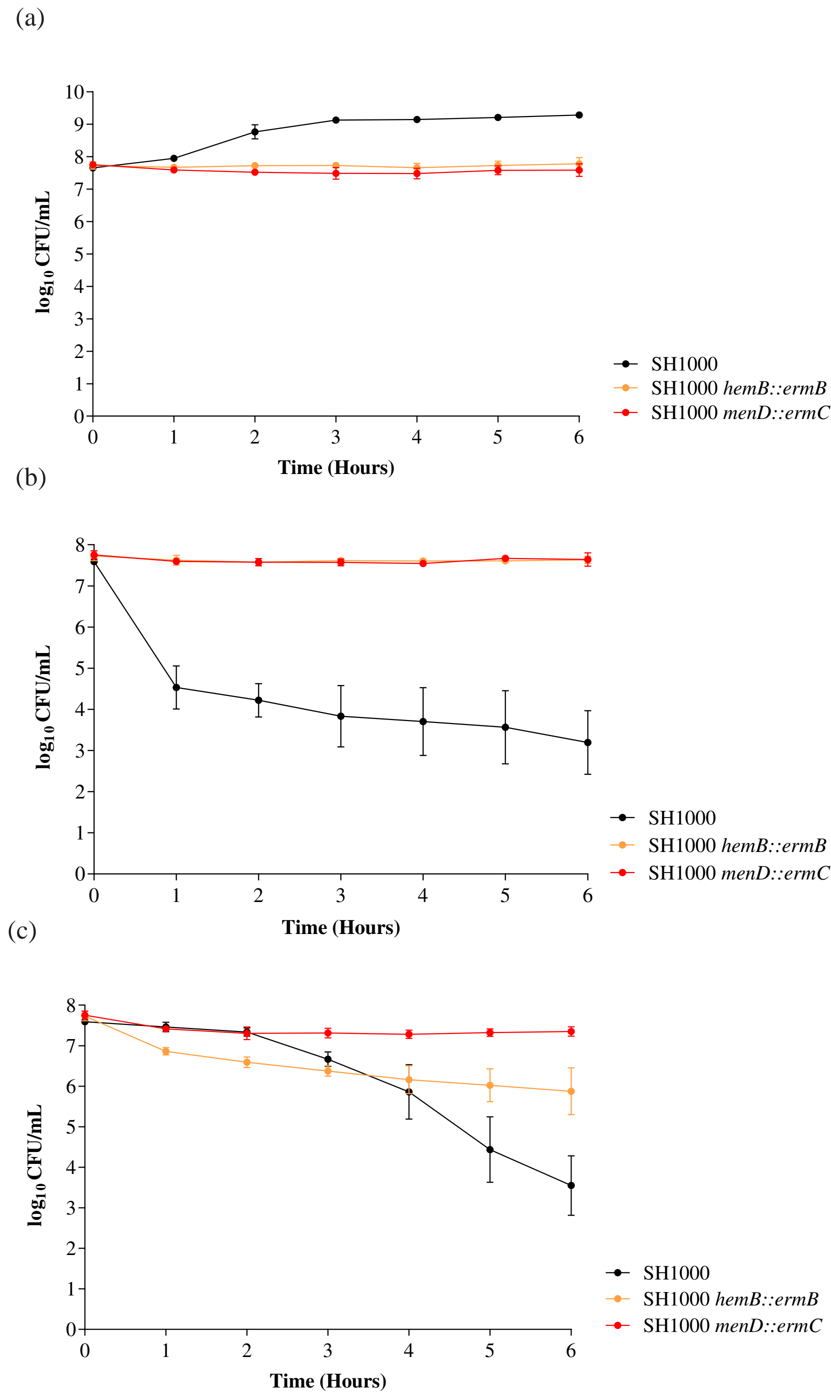

Figure 4.7: **Antibacterial activity of compounds at 4x MIC against small colony variants.** Panel (a): drug-free control; Panel (b): gentamicin; Panel (c): TBBQ. Mean of at least three independent replicates; error bars show standard deviations.

The limited activity of TBBQ against SCVs, may be due to their altered membrane potential ($\Delta\psi$). SH1000 had a $\Delta\psi$ of -72.9 $\pm$ 2.0 mV, and exhibited a 4.5 $\log_{10}$ reduction in CFU/mL following exposure to TBBQ for 6 hours (Figure 4.7c). The $\Delta\psi$ of SH1000 *menD*::*ermC* was $\sim$15% of that of SH1000 (-12.4 $\pm$ 4.7 mV), and TBBQ induced little cell death in time-kill experiments with the strain (Figure 4.7c). SH1000 *hemB*::*ermB* had an intermediate effect on membrane potential ($\Delta\psi$ = -26.3 $\pm$ 5.7) and TBBQ antibacterial activity, displaying $\sim$1.5 $\log_{10}$ reduction in CFU/mL over 6 hours. Since TBBQ retained activity against stationary phase and persister cells (Figure 3.9 and Figure 3.10), reduced activity against SCVs cannot be attributed to inability to kill slow-growing cells. Therefore, TBBQ activity may be dependent upon the ability to depolarise the membrane and/or disrupt components of normal metabolic pathways that are bypassed in SCVs.

Menadione is a precursor of menaquinone, an element of the staphylococcal electron transport chain that is required for normal growth of staphylococci (Goldenbaum *et al.*, 1975; Von Eiff *et al.*, 2006). Since TBBQ has reduced activity against the menadione-deficient SH1000 *menD*::*ermC*, the compound may inhibit the normal function of this precursor or a downstream product. In order to test this hypothesis, SH1000 *menD*::*ermC* cultures were exposed to TBBQ supplemented with menadione at 1/4x MIC (4 μg/mL). TBBQ has little activity against SH1000 *menD*::*ermC* at 4x MIC (see Figure 4.7c). Therefore, to identify alterations in killing activity, TBBQ concentration was increased to 8x MIC.

The addition of menadione to cultures of SH1000 *menD*::*ermC* caused resumption of active growth, with cultures showing increases in viable cell numbers over 6 hours (Figure 4.8a). This suggests that menadione can penetrate cells and be used as a precursor for menaquinone. Since active growth was resumed in SH1000 *menD*::*ermC* supplemented with menadione, susceptibility to TBBQ was expected to be restored. However, menadione abrogated the bactericidal activity of TBBQ against SH1000 *menD*::*ermC*

(Figure 4.8a). This suggests that TBBQ does not target menadione or a downstream product. As menadione could not suppress TBBQ activity against SH1000 (Figure 4.8b), the supplement cannot simply degrade TBBQ leading to reduced activity against SH1000 *menD*::*ermC*. Therefore, exogenous menadione may interfere with binding of TBBQ to a target that has altered expression in SH1000 *menD*::*ermC* due to its restricted energy metabolism.

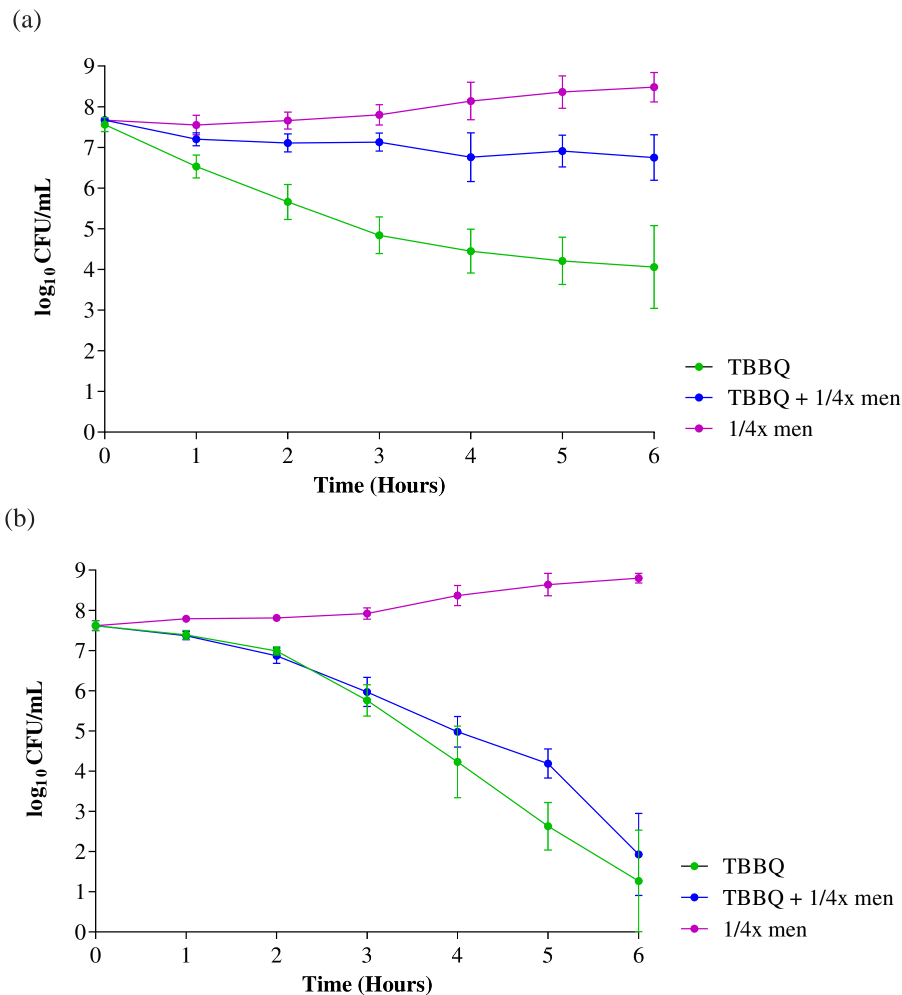

Figure 4.8: **Time-kill studies with staphylococci exposed to 8x MIC TBBQ in MHB supplemented with menadione.** Menadione (men) was added at 4 μg/mL (1/4x MIC). Panel (a): menadione deficient SH1000 *menD*::*ermC*; Panel (b): SH1000 (mean of at least three independent replicates; error bars show standard deviations).

4.3.4 Effect of TBBQ on ROS generation

It has been suggested that antibiotics stimulate the formation of hydroxyl radicals, which contribute to cell death through damage to bacterial macromolecules and membranes (Kohanski *et al.*, 2007). TBBQ is capable of participating in redox reactions and is therefore likely to produce ROS (Okubo *et al.*, 1997) Therefore, the effect of the antioxidant on ROS protection defective mutants was investigated to examine whether ROS generation could contribute to its MOA. In *S. aureus*, *sodA* codes for the major Mn-SOD that dismutates endogenous superoxide, and *sodM*, is produced in response to extracellular superoxide (Karavolos *et al.*, 2003). Hydrogen peroxide induces expression of catalase encoded by *katA*, and alkyl hydroperoxide reductase coded by *ahpC*, which are responsible for degrading this ROS (Clements & Foster, 1999). The MICs of TBBQ, antibiotics and membrane-damaging agents were determined against SH1000, the superoxide dismutase double knockout (MHK11AM), and the catalase and peroxidase knockout (KC043) (Table 4.4). A number of compounds appeared to inhibit growth of MHK11AM and KC034 at lower concentrations than SH1000. Since this was not the case for all compounds the strains are not simply unfit, and the differences in MICs may be due to differences in ROS generation by different agents.

	S. aureus MIC (μg/mL)		
Antibacterial agent	SH1000	MHK11AM	KC043
Ampicillin	0.5	0.125	0.25
Chloramphenicol	4	4	4
Ciprofloxacin	4	1	1
Clofazamine	4	0.5	0.5
Daptomycin	2	2	1
Fosfomycin	16	8	8
Fusidic acid	0.125	0.0625	0.0625
Gentamicin	0.125	0.125	0.125
Kanamycin	2	1	1
Linezolid	4	2	2
Norfloxacin	8	4	4
Rifampicin	0.016	0.016	0.016
Spectinomycin	64	32	32
Vancomycin	2	2	2
CCCP	2	1	2
Chlorhexidine	1	1	1
CTAB	2	2	2
Nisin	8	8	8
SDS	256	256	256
TBBQ	8	2	4
Triclosan	0.25	0.25	0.125
Valinomycin	2	1	1

Table 4.4: **MIC determinations of TBBQ and comparator agents against *S. aureus* SH1000 and ROS protection defective mutants.** MHK11AM is superoxide dismutase deficient and KC043 is catalase and peroxidase deficient. Values in green denote hypersusceptibilty to compounds relative to SH1000.

MHK11AM and KC043 were exposed to TBBQ and comparator antibiotics to identify whether ROS generation is a significant contributing factor to cell death. Bacteria were exposed to compounds at 4x MIC of SH1000 in order to identify differences in susceptibility of the strains. TBBQ exhibited enhanced activity against the strain lacking superoxide dismutase (MHK11AM) (Figure 4.9b), suggesting that the MOA of TBBQ could involve generation of electron transport chain-derived superoxide. In MHK11AM TBBQ is likely to induce production of superoxide and subsequent formation of hydroxyl radicals that damage DNA, lipid and protein, leading to cell death Wang & Zhao (2009); Kohanski *et al.* (2010). This is not necessarily the case in SH1000 which has a functioning ROS defense mechanism; however, this experiment serves to confirm that TBBQ has the ability to induce oxidative stress. Similarly, Schlievert *et al.* (2013) concluded that ROS generation may contribute to the MOA of the antioxidant menadione, but that production of ROS was not the sole mechanism by which bacteria were killed. It is perhaps not surprising that antioxidants disrupt the bacterial metabolism since the compounds are capable of redox cycling (Kim & Pratt, 1990).

In contrast with TBBQ, bacteria lacking the ability to eradicate superoxide and hydrogen peroxide were less susceptible to ciprofloxacin (Figure 4.9c), an antibiotic that inhibits DNA synthesis in actively growing cells (Ng *et al.*, 1996). Since these strains are more susceptible to ROS damage of DNA and are likely to be undergoing DNA repair (Gaupp *et al.*, 2012), the potency of ciprofloxacin may be reduced as DNA synthesis rates are diminished. The difference in susceptibility of strains challenged with ampicillin was minor, and by 24 hours there was no difference between the strains (Figure 4.9d). In support of a number of studies (Ricci *et al.*, 2012; Liu & Imlay, 2013; Keren *et al.*, 2013) that dispute the claims initially proposed by Kohanski *et al.* (2007), this study found that strains unable to detoxify ROS were no more susceptible to bactericidal antibiotics. Whilst ROS may have been generated upon exposure to antibiotics, their production is most likely a secondary effect following antibiotic stress, and ROS are unlikely to contribute to cell death in *S. aureus*.

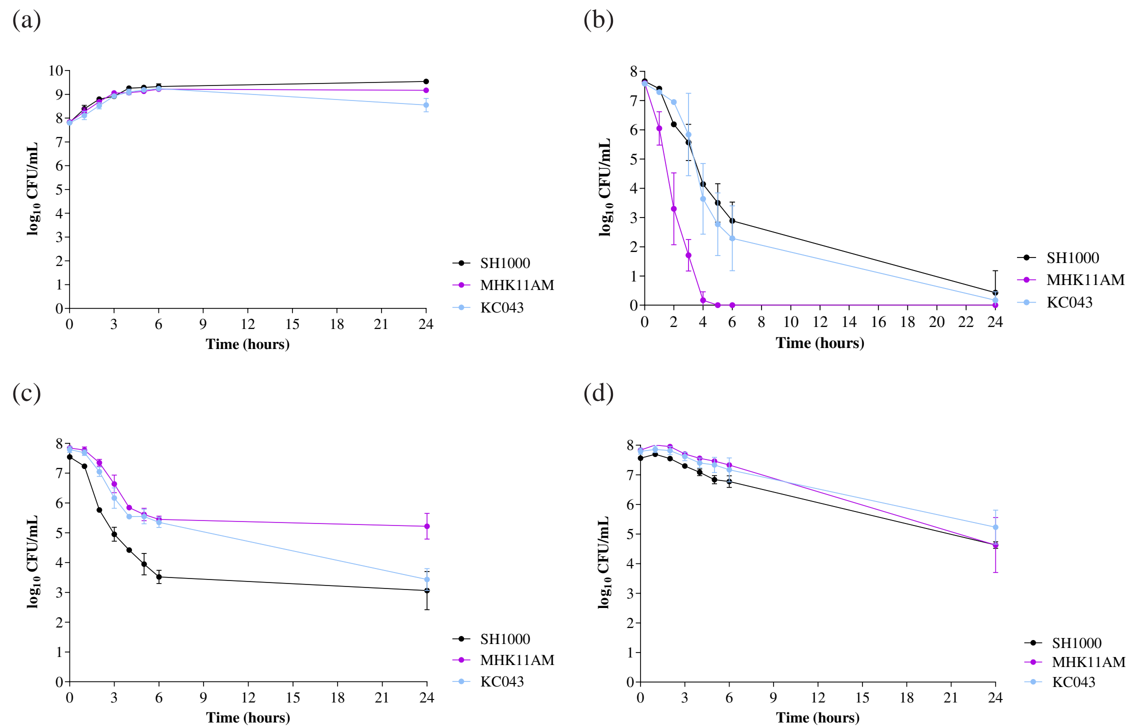

Figure 4.9: **Time-kill experiments with *S. aureus* SH1000 and ROS protection deficient mutants exposed to TBBQ and comparator agents at 4x MIC in MHB.** MHK11AM is superoxide dismutase deficient and KC043 is catalase and peroxidase deficient. Panel (a): drug-free control; Panel (b): TBBQ; Panel (c): ciprofloxacin; Panel (d): ampicillin (mean of at least three independent replicates; error bars show standard deviations).

To investigate the contribution of ROS generation to the MOA of TBBQ further, time-kill experiments were carried out with SH1000 exposed to TBBQ supplemented with superoxide and hydroxyl radical scavengers. The scavengers tiron and thiourea displayed MICs of >32.8 and 32.8 mg/mL, respectively. Therefore, cultures were exposed to TBBQ at 4x MIC in MHB supplemented with sub-inhibitory concentrations of scavengers (8 mg/mL) (Figure 4.10). Neither tiron nor thiourea significantly reduced the bactericidal activity of TBBQ, suggesting that although TBBQ may enhance ROS production, innate

protection mechanisms in *S. aureus* are sufficient to overcome oxidative stress.

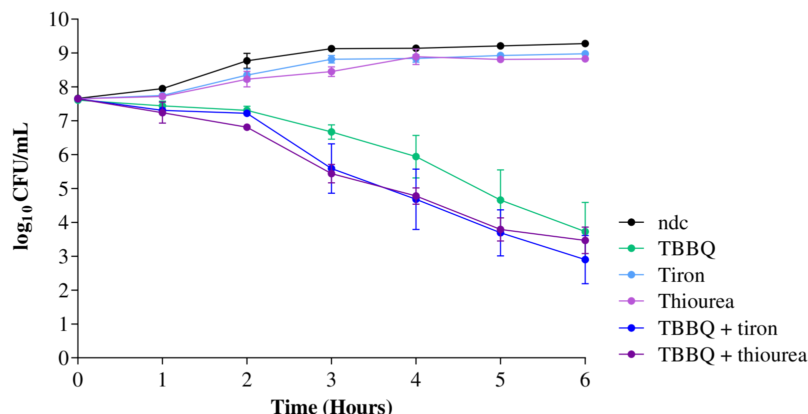

Figure 4.10: **Time-kill studies with *S. aureus* SH1000 exposed to TBBQ at 4x MIC in MHB supplemented with ROS scavengers at 8 mg/mL.** Mean of at least three independent replicates; error bars show standard deviations.

4.3.5 Identification of TBBQ-resistant and hypersusceptible mutants

4.3.5.1 Isolation of a TBBQ-resistant strain

Bacteria exposed to topical antibacterial agents may be exposed to a steep concentration gradient and experience a strong selection pressure to develop resistance to the compound (Hermsen *et al.*, 2012). As such, the ideal topical antibacterial would be both strongly bactericidal and would not readily select resistance. The membrane-perturbing MOA of TBBQ may be advantageous in this regard, as there is evidence to suggest that bacteria do not readily acquire resistance to membrane-active agents (Ooi *et al.*, 2009; Hurdle *et al.*, 2011). Attempts were made to isolate mutants resistant to TBBQ using standard mutation frequency determination methodology (Ryder *et al.*, 2012). However, no TBBQ-resistant mutants were identified (mutation frequency $<5.0 \times 10^{-9}$), suggesting that TBBQ may

not have a single target site and that resistance to TBBQ may not arise readily (O'Neill & Chopra, 2004). Therefore attempts were made to select mutants using an extended concentration range over prolonged periods. The extended spectrum MIC method was employed with daptomycin as a positive control (Friedman *et al.*, 2006). Following 40 passages the most resistant strain selected with daptomycin had an MIC of 32 μg/mL: a 16-fold increase in daptomycin MIC. Following 40 passages the most resistant strain selected with TBBQ had an MIC of 32 μg/mL: a 4-fold increase in TBBQ MIC (strain B7). This level of resistance was identified following 20 passages, but no further increase in resistance was observed. Reduced susceptibility to TBBQ was not lost over 4 drug-free passages, suggesting that stable resistance can arise, but only low level resistance was observed. TBBQ may disrupt the staphylococcal membrane in such a way that the effects of the compound may only be overcome by gross alterations in the architecture of the membrane, which is likely to perturb a number of essential cellular processes. Therefore, only mutants with relatively minor modifications may be viable, explaining why only low level TBBQ resistance develops.

Experiments were carried out to assess whether resistant mutants that arise under TBBQ selection would be more or less likely to survive in a clinical setting. Resistant mutant fitness was assessed by comparing the doubling time of the parental strain, SH1000 (doubling time of 31.3 $\pm$ 2.4 minutes) to that of B7 (doubling time of 64.1 $\pm$ 2.4). B7 was growth attenuated, suggesting that TBBQ-resistant mutants may have reduced survival or ability to cause disease.

4.3.5.2 Identification of a TBBQ hypersusceptible strain

A near-saturation SH1000 transposon library generated and maintained at the University of Leeds was screened for TBBQ hypersusceptibility. The library comprises $\sim$20500 mutants containing the transposon InsTet^{G+}2^{CM} (Blake & O'Neill, 2012; Bertram *et al.*, 2005). The transposon carries a strong outward facing promoter, thus Tn mutants

can display either overexpression or knock-down of gene expression depending upon the location and orientation of transposon insertion. Mutants in which the transposon is inserted with the promoter in the sense direction upstream of the gene display overexpression. Downstream antisense insertion of the promoter leads to the formation of antisense mRNA or collision of transcription forks, causing knock-down of gene expression. Transcription of antisense mRNA that displays reverse complementarity is hypothesised to form of antisense-gene mRNA duplexes that sterically hinder translation or are targeted for degradation by the cell. This may reduce the quantity of mRNA available for translation and concentration of target protein (O'Neill & Chopra, 2004). Approximately 12500 mutants were screened and one strain, CAJ192 C9, displayed hypersusceptibility to TBBQ, with an MIC of 2 μg/mL: a 4-fold reduction in MIC.

4.3.6 Characterisation of TBBQ mutants

Extensive ATP consumption was identified in SH1000 exposed to TBBQ (Figure 4.5). To confirm that this effect is linked to susceptibility to TBBQ, the rate and extent of ATP hydrolysis was compared for mutant strains following exposure to TBBQ. Both mutants displayed a similar doubling time, suggesting that they have similar metabolic activity; as such their ATP consumption would be expected to be similar. However, the TBBQ-resistant strain consumed less ATP following exposure to TBBQ than the parental strain, and the hypersusceptible strain displayed enhanced ATP hydrolysis (Figure 4.11). This confirms that the interaction of TBBQ with staphylococci leads to ATP depletion.

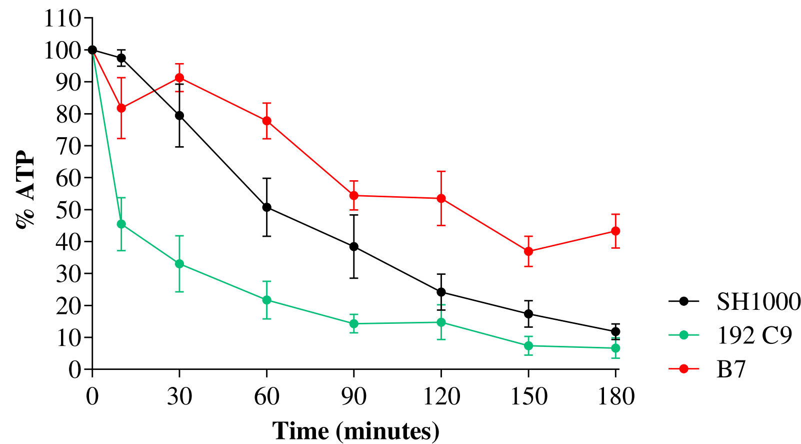

Figure 4.11: **ATP hydrolysis following exposure to TBBQ at 4x MIC in HEPES and glucose buffer.** Values were calculated as a percentage of an untreated control. Mean of at least three independent replicates; error bars show standard errors.

Cross-susceptibility/resistance studies were also carried out with the hypersusceptible and resistant mutants challenged with a number of antibiotics and membrane-damaging agents (Table 4.5). Decreased/increased MIC for all other antimicrobial agents against CAJ192 C9/B7 would suggest that the strains have altered susceptibility to TBBQ due a general decrease/increase in fitness. Alternatively, changes in susceptibility to specific antibacterial agents could identify a shared drug target. Susceptibility of CAJ192 C9 was not altered for any of the antibacterial agents tested. B7 displayed enhanced susceptibility to ciprofloxacin (for discussion see Section 4.3.7), but did not show enhanced resistance to any other agent, suggesting that TBBQ may target a novel component of the bacterial membrane.

	MIC (μg/mL)		
Compound	SH1000	B7	192 C9
Ampicillin	0.5	0.5	0.5
Chlorhexidine	2	2	1
Ciprofloxacin	2	0.5	2
Clofazamine	4	4	2
CTAB	2	2	2
Daptomycin	1	1	1
Erythromycin	0.5	0.5	0.5
Gentamicin	0.5	0.5	0.25
H_2O_2	16	8	8
Nisin	2	2	2
TBBQ	8	32	2
Tetracycline	1	1	1
Valinomycin	2	2	2
Vancomycin	1	1	2
XF-73	1	1	1
XF-70	1	1	1

Table 4.5: **MICs of established antibiotics against TBBQ mutants B7 and CAJ192 C9.** Values in green and red denote hypersusceptibilty and resistance to compounds relative to SH1000.

4.3.7 Genotypic characterisation of TBBQ mutants

To investigate the genetic basis of altered susceptibility to TBBQ in mutants, the site at which the transposon inserted in the genome of CAJ192 C9 was determined, and whole genome sequence determination (WGS) of B7 was carried out to identify mutations. The location of transposon insertion was identified by direct sequence determination using genomic DNA from CAJ192 C9 and the primer Tn_out2. The DNA sequence generated was checked for homology with the *S. aureus* 8325 genome using the BLAST algorithm

available on the National Centre for Biotechnology Information website. Homology with a particular region indicated the approximate insertion site of the transposon, and PCR amplification and sequence determination of that region identified the precise location and confirmed the orientation of transposon insertion. The transposon in CAJ192 C9 was located in the intergenic region directly downstream of SAOUHSC_01723 (coding for a homologue of a recD/traA type DNA helicase or exodeoxyribonuclease) in the antisense orientation (Figure 4.12). This might cause knock-down of expression of genes contained within the operon (containing SAOUHSC_01723 and SAOUHSC_01724). To confirm that alterations in susceptibility to TBBQ are directly attributable to insertion of the transposon at this location, the region was transduced into a clean host (SH1000) using phage ϕ11. Transduction of the transposon insertion site resulted in co-transfer of the TBBQ-hypersusceptible phenotype. Thus, this suggests that disruption of this operon specifically induces hypersusceptibility to TBBQ. The role of the SAOUHSC_01723-containing operon in TBBQ hypersusceptibilty was confirmed by complementation. The operon of interest was amplified from SH1000 by PCR using primers 01723op_XmaI_Fwd and 01723op_XbaI_Rev (Appendix A) and introduced to CAJ192 C9, *in trans*, using the xylose-inducible vector pEPSA5. In the presence of xylose, MIC determinations revealed that complemented CAJ192 C9 displayed wild-type susceptibility to TBBQ (TBBQ MIC of 8 μg/mL).

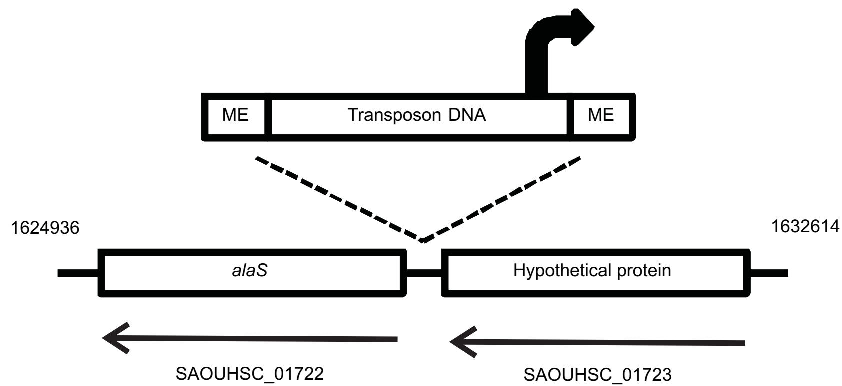

Figure 4.12: **Schematic of the insertion site of the transposon in CAJ192 C9.** Straight black arrows show the direction of gene expression and the curved arrow shows the direction of expression from the outward-facing promoter of the transposon. SAOUHSC_01722 is an alanyl-tRNA synthetase, SAOUHSC_01723 is a putative DNA helicase/exonuclease, and ME represents mosaic ends of the transposon.

Both transduction and complementation suggest that knock-down of expression of the DNA helicase/exonuclease encoded by SAOUHSC_01723 enhances TBBQ susceptibility. It is hypothesised that CAJ192 C9 produces fewer molecules of DNA helicase and may have reduced unwinding and repair of double-stranded DNA. This could enable TBBQ-induced ROS damage to DNA to accumulate and cause oxidative stress, which could act in concert with lethal membrane-damaging activity of TBBQ. In SH1000 ROS damage may play only a minor role in the MOA due to a functional ROS defense mechanism and DNA repair response.

WGS of B7 identified putative mutations in SAOUHSC_00341, SAOUHSC_01866, SAOUHSC_02663 and SAOUHSC_02990. PCR amplification and sequence determination of these genes confirmed that the resistant strain contained a single amino acid substitution ($W_{33}X$) in SAOUHSC_01866. This gene codes for a hypothetical protein with homology to staphylococcal aminoglycoside phosphotransferases, and choline/ethanolamine kinases. Sequence determination of SAOUHSC_01866 in TBBQ

mutants recovered in parallel selections that showed modest increases in TBBQ MIC (2-fold increase) did not share this mutation. To confirm that the single nucleotide polymorphism confers TBBQ resistance, B7 was complemented with SAOUHSC_01866 from SH1000. This resulted in a 2-fold reduction in TBBQ MIC (16 μg/mL), and suggests that the product of SAOUHSC_01866 does indeed contribute to TBBQ resistance.

The function of SAOUHSC_01866 is not yet known, but it shares homology with a number of other genes, including a staphylococcal aminoglycoside phosphotransferase. *S. aureus* are resistant to aminoglycosides through expression of *O*-phosphotransferases and *N*-acetyltransferases, which modify the drug, resulting in poor binding to the ribosome and reduced aminoglycoside activity (Mingeot-Leclercq *et al.*, 1999). However, aminoglycoside susceptibility is not altered in B7 (Table 4.5), therefore SAOUHSC_01866 is unlikely to act as an aminoglycoside modifying enzyme. SAOUHSC_01866 may instead function as a choline or ethanolamine kinase, with which the gene has homology. Since mutation in SAOUHSC_01866 enhances resistance to TBBQ, this suggests that a product of one of these enzymes may be targeted by TBBQ. In *S. aureus* choline is an osmoprotectant that is oxidised to glycine betaine allowing the bacterium to resist osmotic pressure (Graham & Wilkinson, 1992). Phosphorylation of choline by functional SAOUHSC_01866 could reduce intracellular choline pools rendering bacteria susceptible to stressful environmental conditions. Therefore, mutations in choline kinase may ensure maintenance of intracellular choline levels, enabling B7 to survive in increased concentrations of TBBQ. This type of response is seen in daptomycin-resistant strains which upregulate gylcine betaine production to overcome antibiotic-induced stress (Song *et al.*, 2013). However, many of the comparator agents are expected to induce stress responses, and since there is no cross-resistance it is unlikely that B7 is resistant to TBBQ due to enhanced ability to survive under stressful conditions. Alternatively, resistance to TBBQ could be a consequence of altered membrane lipid composition. Ethanolamine is a precursor for the phospholipid phosphatidylethanolamine which is present in small quantities in the staphylococcal cytoplasmic membrane (Short

& White, 1970, 1971). TBBQ may interact with this phospholipid in the membrane of SH1000, but mutation of the ethanolamine altering enzyme of B7 could generate a membrane devoid of phosphatidylethanolamine. Since B7 has only a 4-fold reduction in TBBQ susceptibility, it is unlikely that phosphatidylethanolamine is the primary target causing membrane disruption. However, the absence of a functional lipid may either reduce TBBQ membrane binding efficacy, or cause alterations in membrane structure rendering bacteria less susceptible to stresses generated by TBBQ. Indeed, alterations in membrane structure have been identified in daptomycin-resistant strains, leading to increased membrane fluidity, altered surface charge, and reduced susceptibility to antibiotic induced membrane depolarisation (Jones *et al.*, 2008b). Therefore, further experimentation could be carried out to confirm this hypothesis, and is discussed in Section 6.2.

Hypersusceptibilty of B7 to ciprofloxacin was identified in cross-resistance studies (Table 4.5). Altered susceptibility could be due to enhanced access of ciprofloxacin to the bacterial chromosome through alterations in phospholipid bilayer composition and membrane structure/fluidity. However, hypersusceptibility may not be directly attributable to the mutation in SAOUHSC_01866, but could be a consequence of phenotypic alterations due to transcriptional modifications. Since the topoisomerase-ciprofloxacin-DNA complex behaves as a poison creating lesions in DNA, resistance to the antibiotic is conferred by reduced expression of DNA gyrase (Ince & Hooper, 2003). Therefore, the reverse may be true if DNA gyrase is overexpressed. In CAJ192 C9, reduced DNA helicase activity and DNA repair may render strains more susceptible to TBBQ. Conversely, B7 could alter gene expression, inducing upregulation of DNA synthesis and repair enzymes to counteract the effects of TBBQ. As a consequence, more DNA gyrase may be available for inhibition by ciprofloxacin in B7, causing increased susceptibility to the antibiotic.

The putative DNA-repair attenuated CAJ192 C9 displayed only a 4-fold reduction in

TBBQ MIC. This indicates that the increase in susceptibility to TBBQ due to unrepaired DNA damage is relatively minor, and damage to DNA is unlikely contribute significantly to the antioxidant's MOA. Bacteria may evade the effects of TBBQ through subtle alterations in the structure of the cytoplasmic membrane, as has been suggested in B7. This indicates that TBBQ is active through interactions with the staphylococcal membrane. Thus, sequence determination of hypersusceptible and resistant strains provides further support for the conclusions drawn from experiments with ROS protection knockouts and membrane damaging assays regarding TBBQ MOA.

4.3.8 Synergistic interaction of TBBQ with antibiotics

It has been suggested that compounds which compromise the integrity of the bacterial membrane could be used in combination with existing antibiotics, particularly when treating infections cause by biofilms (Hurdle *et al.*, 2011). Application of permeabilising agents, such as colistin administered with imipenem against a *P. aeruginosa* sepsis model, has had some success and the use of colistin combination therapy has been re-introduced in the clinic (Cirioni *et al.*, 2007; Bassetti *et al.*, 2008). Since TBBQ reduces bacterial membrane integrity, attempts were made to identify synergistic interactions with other antibacterial agents. Synergism of TBBQ with antibiotics (ciprofloxacin, erythromycin, gentamicin, oxacillin and tetracycline) was not observed with planktonic cultures (FIC $\geq$ 0.69). However, TBBQ acted synergistically in combination with gentamicin, an antibiotic that cannot eradicate biofilms $\leq$256 μg/mL, against biofilms of SH1000 grown using the Calgary Biofilm Device (FIC $\leq$ 0.28) (Table 4.6). The combination of 1 μg/mL gentamicin with 16 μg/mL TBBQ could eradicate staphylococcal biofilms. Therefore, TBBQ could potentially be used with gentamicin to eradicate staphylococcal biofilms without application of high concentrations of either compound. Combination therapy may be employed most usefully against biofilms at the skin surface, such as those formed in burn wounds and in the nares, which are currently treated with the use of topical agents

like mupirocin (Coates *et al.*, 2009).

Compounds	Planktonic FIC index	Biofilm eradication FIC index
TBBQ + ciprofloxacin	0.69	>0.5
TBBQ + erythromycin	0.75	>0.5
TBBQ + gentamicin	1.02	**≤0.28**
TBBQ + oxacillin	1.00	>0.5
TBBQ + tetracycline	0.88	>0.5

Table 4.6: **Synergism of TBBQ with established antibiotics against planktonic and biofilm cultures of *S. aureus* SH1000.** FIC index ≤ 0.5 is a synergistic interaction, equal to 1 is an additive interaction and ≥ 2 is an antagonistic interaction.

4.3.9 Effects of TBBQ on a human skin equivalent

For a compound to be developed as a topical antibiofilm agent, it should be able to be applied to the skin without causing damage or irritation. Keratinocytes release primary cytokines, such as IL-1α, in response to the presence of irritants. This induces an inflammation response and the extent of IL-1α release is relative to the degree of skin irritation (Bernhofer *et al.*, 1999). To determine whether TBBQ has detrimental effects on the skin at concentrations above those required to kill bacteria, a living skin equivalent (LabSkin™) was exposed to TBBQ for 24 hours. At 10x MIC TBBQ did not irritate LabSkin™, causing no detectable increase in IL-1α release. The topical agent mupirocin, which is used clinically for nasal decolonisation, was also non-irritant (Liu *et al.*, 2011).

Hematoxylin and eosin staining of tissue sections confirmed that 24 hour exposure to the irritant SDS (5%) was severely damaging to skin, causing shedding of the top layers of skin (stratum corneum and epidermis) and causing injury to the dermis below (Figure 4.13c). Conversely mupirocin and TBBQ (10x MIC) had no visible detrimental effects on skin (Figure 4.13d and 4.13e). Therefore, TBBQ does not physically damage or irritate

fully differentiated skin at higher concentrations than is required to sterilise bacterial cultures. These suggest that TBBQ does not have toxic effects on skin cells and has the potential to be used as a topical agent.

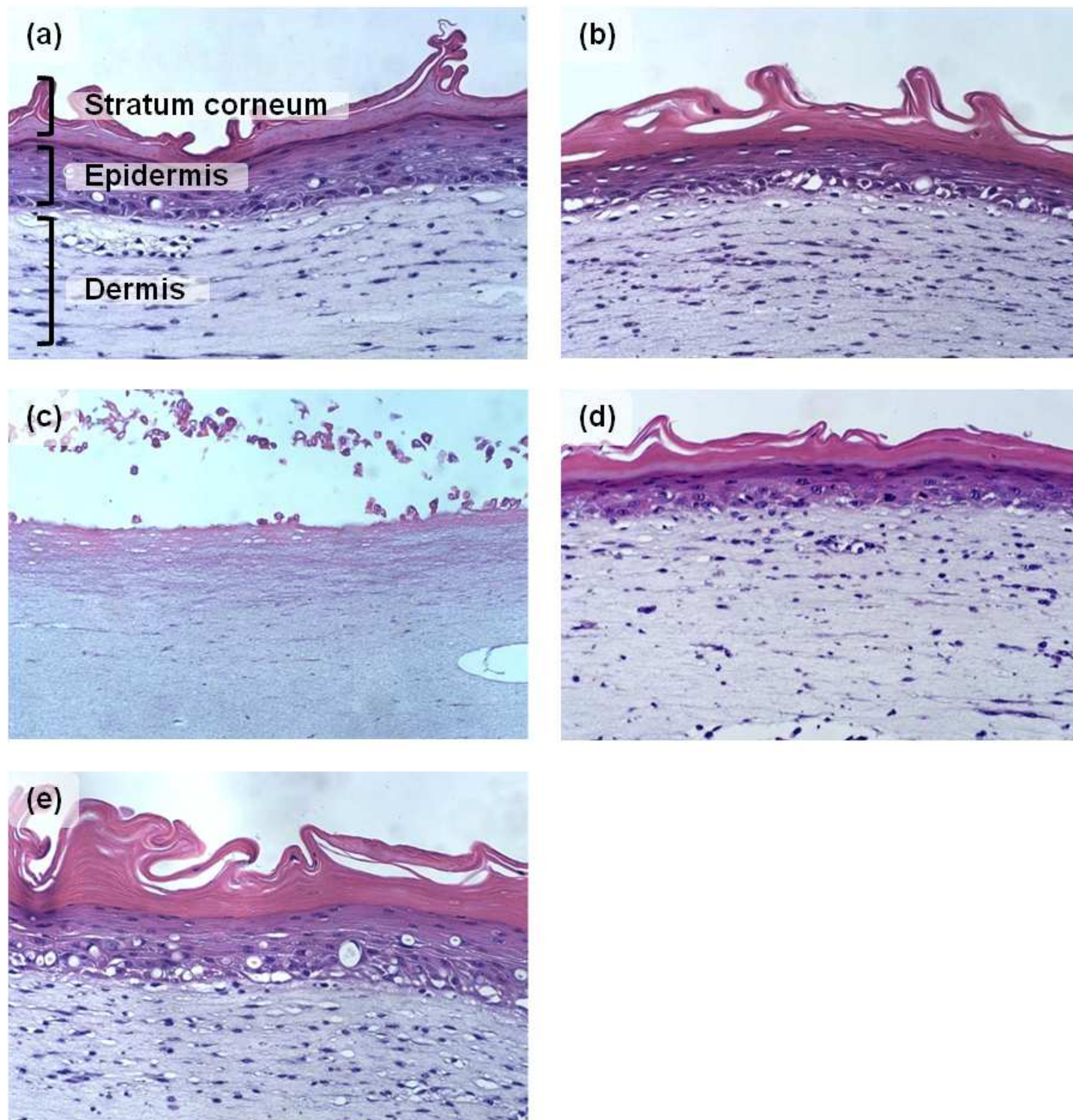

Figure 4.13: **Hematoxylin and eosin stained sections of LabSkin™ exposed to compounds for 24 hours.** Panel (a): drug-free control; Panel (b): ethanol (0.2% v/v); Panel (c): 5% SDS; Panel (d): mupirocin 10x MIC; Panel (e): 10x MIC TBBQ. H&E staining and imaging carried out by J Bradley, Evocutis.

4.4 Conclusions

TBBQ exerts its antibacterial MOA by causing selective disruption of the staphylococcal cytoplasmic membrane. Since the antioxidant did not disrupt staphylococcal liposomes, dissipation of membrane potential and associated membrane disruption are unlikely to be dependent upon binding to specific lipids in the bilayer. Downstream effects of membrane perturbation include loss of intracellular potassium ions, extensive hydrolysis of cellular ATP and probable generation of ROS that are detoxified by innate ROS protection mechanisms. That TBBQ acts to compromise the integrity of the bacterial membrane may explain why this compound has the ability to eradicate staphylococcal biofilms; potent antibiofilm activity is a property that is often associated with membrane-perturbing agents (Hurdle *et al.*, 2011; O'Neill, 2011). *S. aureus* does not readily develop resistance to TBBQ, and strains displaying reduced susceptibility suffer fitness costs as measured by reduced growth rate. Since TBBQ potentiates the activity of gentamicin against staphylococcal biofilms, combination therapy could be employed to reduce the dose and thus toxic effects of gentamicin treatment. Furthermore, TBBQ appears to be non-toxic to erythrocytes or a human living skin equivalent. Therefore, at concentrations that could be used to eradicate biofilms on the skin surface, membrane effects are bacteria-specific, and the inability of *S. aureus* to develop high level resistance would allow topical treatment of infections for prolonged periods.

Chapter 5

Antioxidants that target the cell membrane can eradicate staphylococcal biofilms

5.1 Abstract

Biofilms are involved in ∼80% of bacterial infections in humans, and are refractory to the action of most antibacterial agents in clinical use. In the search for novel antibiofilm agents, a selection of antioxidants was assessed for biofilm-eradicating activity. The 15 antioxidants tested all displayed antibacterial activity against planktonic staphylococcal cultures, and seven also eradicated staphylococcal biofilms. Biofilm-eradicating antioxidants were all bactericidal and caused membrane perturbation. Eradication of preformed biofilms following exposure to antioxidants involved dissociation of both cells and matrix, which occurred before reductions in bacterial viability were detected. This suggests that antioxidants degrade the matrix and induce release of biofilm cells; the latter are then subject to the membrane damaging effects of the compounds. The two most promising agents in respect of antibacterial potency and selective toxicity towards

bacterial over mammalian membranes (celastrol and NDGA) did not damage LabSkin™ following topical application. These compounds acted synergistically with gentamicin against both planktonic and biofilm cultures. Whilst NDGA-resistant mutants could not be recovered, a strain was isolated that displayed reduced susceptibility to celastrol. The strain displayed increased mutability and had altered susceptibility to a number of antibacterial agents. Continuous selection over several days was required to generate the strain, which displays a celastrol MIC of 8 μg/mL; 4-fold lower than the minimum biofilm eradication concentration. Therefore, both NDGA and celastrol could potentially be useful in antibiofilm strategies.

5.2 Introduction

5.2.1 The clinical significance of biofilm infections

Biofilms are found in approximately 80% of bacterial infections in humans (Davies, 2003). *S. aureus* and *S. epidermidis* are biofilm-forming organisms implicated in numerous nosocomial infections, with staphylococcal biofilms being prevalent in chronic wounds and the most common cause of infections involving indwelling medical devices (Hall-Stoodley *et al.*, 2004; James *et al.*, 2008; Otto, 2008). Chronic wounds include pressure ulcers and ulcers on the feet of people with diabetes, which frequently necessitate limb amputation following treatment failure (James *et al.*, 2008). The presence of biofilms in burn wounds and ulcers can contribute to persistent inflammation (Wolcott *et al.*, 2010), and the rate of wound healing is believed to be reduced at sites colonised with *S. aureus* (James *et al.*, 2008). Currently, the most effective wound care routine involves regular debridement and treatment with systemic antibiotics, in addition to topically-applied antibacterial agents (Wolcott *et al.*, 2010). Therefore, to address the current difficulties in treating chronic wounds, it is important to discover novel antistaphylococcal agents

that possess antibiofilm activity in addition to activity against planktonic cells. Previous research has indicated that phenylic antioxidants may display the ability to eradicate biofilms, which is rare amongst antibacterial agents (Section 1.4).

5.2.2 Aims and Objectives

Work described in this chapter aims to characterise the antibacterial activity of a number of phenylic antioxidants, with an emphasis on assessing their activity against biofilms, and elucidating their antibacterial MOA/the mechanism by which they eradicate biofilms. Synergistic interactions with existing antibiotics will be identified, and a living skin equivalent will be exposed to agents that display selective toxicity and antibiofilm activity in order to assess tolerability. Finally, resistant mutant generation and subsequent sequence determination will be used to investigate the cellular target of promising compounds.

5.3 Results and discussion

5.3.1 Antibacterial properties of phenylic antioxidants

Although it has been reported previously that all of the antioxidants examined in this study display some antibacterial activity (Toama *et al.*, 1974; Moujir *et al.*, 1990; Eady *et al.*, 1994; Nicholas *et al.*, 1999; Kubo *et al.*, 2002; Zampini *et al.*, 2005; Inatsu *et al.*, 2006; Moreno *et al.*, 2006; Seville & Wilkinson, 2008; Çolak *et al.*, 2009; Chaudhuri *et al.*, 2010; Reddy *et al.*, 2010; Chaieb *et al.*, 2011; Miranda *et al.*, 2011; Martins *et al.*, 2013), most of them have not been evaluated using standardised procedures for susceptibility testing. Using CLSI methodology for susceptibility testing, the 15 antioxidants exhibited MICs ranging from 0.25-128 μg/mL against planktonic cultures of

S. aureus and *S. epidermidis* (Table 5.1). Approximately half of these compounds (seven of 15) also demonstrated complete eradication of established staphylococcal biofilms at concentrations $\leq$256 μg/mL. By contrast, none of the established antibacterial agents tested were able to eradicate SH1000 biofilms at $\leq$256 μg/mL (or, in the case of nisin, at the maximum achievable concentration of 16 μg/ mL) (Table 3.2).

The ability of compounds to eradicate biofilms is suggestive of bactericidal activity. To assess this, antioxidant-mediated killing of SH1000 in planktonic culture was examined by viable counting. Since the antioxidants TBHQ and TBBQ were found to degrade (Section 3.3.1), it was reasoned that other antioxidants may also degrade over 24 hours. Therefore, bacterial viability was assessed 6 and 24 hours post antioxidant exposure to identify compounds that may initially be bactericidal, but that subsequently allow grow-back. Some antioxidants, such as idebenone, were bacteriostatic, whilst others, such as NDGA, were bactericidal at 4x MIC (Figure 5.1a). At the MBEC, benzoyl peroxide, carnosic acid, celastrol and NDGA essentially sterilised planktonic cultures (viable cells $\leq$0.8 $\log_{10}$ CFU/mL) (Figure 5.1b), whilst AO 2246, bakuchiol and totarol killed bacteria initially, but allowed grow-back over a 24 hour period (Figure 5.1c). Therefore, none of the agents with antibiofilm activity (compounds with an SH1000 MBEC of $\leq$256 μg/mL) displayed bacteriostatic properties.

Antioxidant	MIC (μg/mL)			bMIC (μg/mL)			MBEC (μg/mL)		
	SH1000	UAMS-1	RP62A	SH1000	UAMS-1	RP62A	SH1000	UAMS-1	RP62A
AO 2246	4	4	4	16	4	4	16	16	128
Bakuchiol	4	4	4	8	4	4	16	8	8
Benzoyl peroxide	64	32	32	256	32	64	256	32	64
Carnosic acid	32	32	8	64	32	32	128	64	64
Celastrol	1	0.5	0.25	1	0.25	0.25	32	4	4
Dihydroxychalcone	128	128	128	64	64	64	>256	>256	>256
8-hydroxyquinoline	4	2	1	4	1	1	>256	32	>256
Idebenone	64	64	32	64	16	>256	>256	256	>256
Lauryl gallate	64	64	16	32	8	16	>256	256	>256
Menadione	16	8	8	32	8	32	>256	128	256
Nordihydroguaiaretic acid	64	64	64	64	16	32	128	16	256
Thymohydroquinone	16	8	16	32	4	64	>256	16	64
Thymoquinone	16	8	8	32	2	8	>256	4	32
Totarol	4	2	2	4	2	4	16	8	16
Vitamin K5 hydrochloride	32	16	16	32	8	16	>256	64	128

Table 5.1: **MICs, biofilm MICs (bMICs) and minimum biofilm eradication concentrations (MBECs) of antioxidants against staphylococci.**

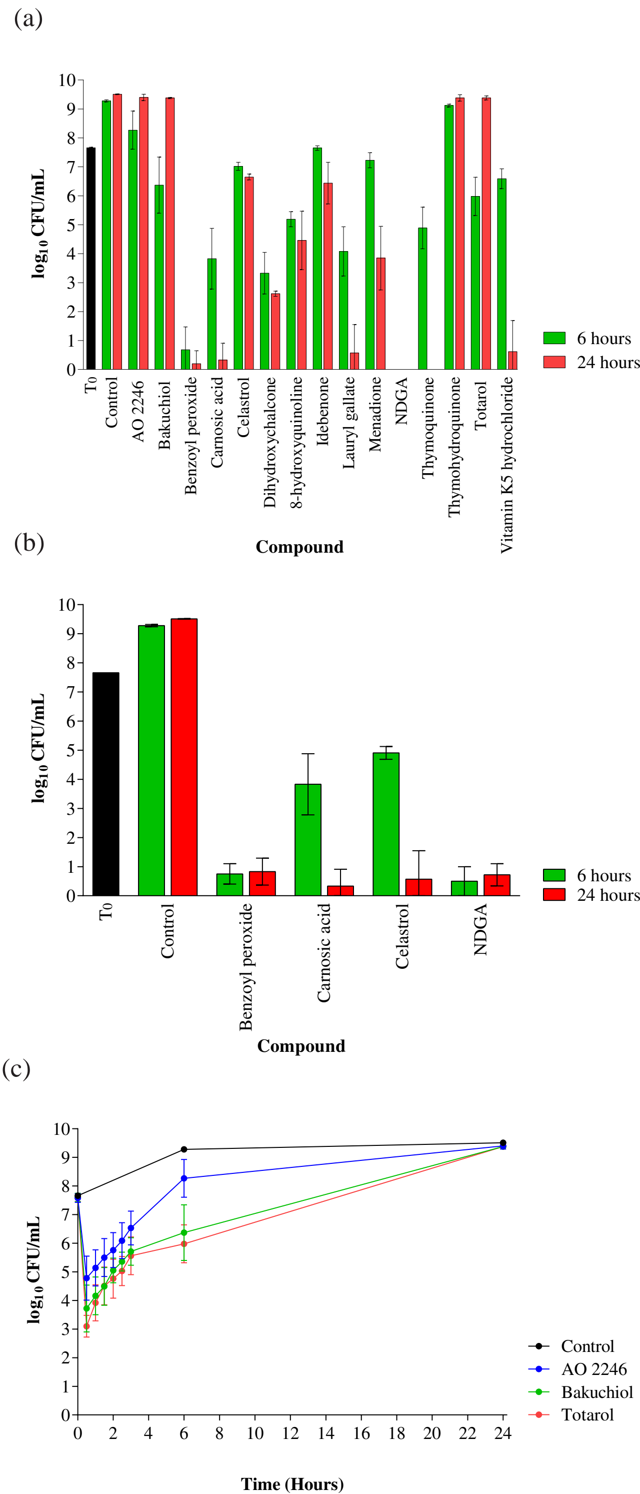

Figure 5.1: **Bactericidal activity of antioxidants against *S. aureus* SH1000.** Panel (a): bacterial viability following exposure to antioxidants at 4x MIC for 6 or 24 hours; Panel (b): bacterial viability following exposure to biofilm-eradicating antioxidants at the MBEC for 6 or 24 hours; Panel (c): killing kinetics of biofilm-eradicating antioxidants that display grow-back at the MBEC. Mean of at least three independent replicates; error bars show standard deviations.

5.3.2 Antibacterial MOA of antioxidants

This study identified membrane damage and disruption of the electron transport chain following exposure of bacteria to the phenylic antioxidant TBBQ. Similarly, a number of the antioxidants under investigation here have previously been reported to disrupt membranes or inhibit the respiratory chain (Reddy *et al.*, 2010; Valacchi *et al.*, 2001; Zhou *et al.*, 2011; Sabzevari *et al.*, 2004; Kubo *et al.*, 2002; Dumont *et al.*, 1999; Bhuvaneswaran & Dakshinamurti, 1972; Foss *et al.*, 2013). Therefore, it was hypothesised that all phenylic antioxidants may have detrimental effects on the bacterial membrane, and that this effect may contribute to the MOA of the compounds.

At 4x MIC, 10 of 15 antioxidants compromised the integrity of the SH1000 membrane within 10 minutes, as measured by the *Bac*Light^{TM} assay (Table 5.2). Since this assay does not reliably detect subtle membrane disruption (*e.g.* loss of membrane potential), or perturbation occurring over longer than a 10 minute window, additional assays were carried out to assess whether the other five antioxidants also mediate membrane perturbation. All five compounds caused loss of membrane potential within 3 hours at 4x MIC, and some compounds caused reductions in intracellular potassium levels over this duration and at this concentration (Figure 5.2b and 5.2a). Therefore, the remaining antioxidants exerted more subtle effects on the membrane, in most instances prompting depolarisation without leakage of intracellular components.

Antimicrobial compound	% Membrane Integrity (± SE)
No drug	100 ± 0
Vancomycin	100 ± 0
Thymohydroquinone	100 ± 0
Thymoquinone	100 ± 0
Celastrol	100 ± 0
Daptomycin	94.7 ± 5.3
Tetracycline	90.9 ± 9.1
8-Hydroxyquinoline	86.3 ± 3.0
Menadione	80.0 ± 10.7
Idebenone	59.0 ± 4.7
Vitamin K5 hydrochloride	23.0 ± 7.0
Dihdroxychalcone	16.7 ± 5.7
AO 2246	10.8 ± 5.2
Totarol	8.8 ± 1.4
Bakuchiol	7.3 ± 1.1
NDGA	5.2 ± 0.7
Lauryl gallate	4.7 ± 3.7
Nisin	4.6 ± 1.5
Carnosic acid	3.6 ± 1.1
Benzoyl peroxide	2.7 ± 1.4
CTAB	0.5 ± 0.3
5% SDS	0 ± 0

Table 5.2: **The *Bac*Light^{TM} assay to determine effects of compounds at 4x MIC on *S. aureus* SH1000 membrane integrity.** Mean of at least three independent replicates, SE - standard error.

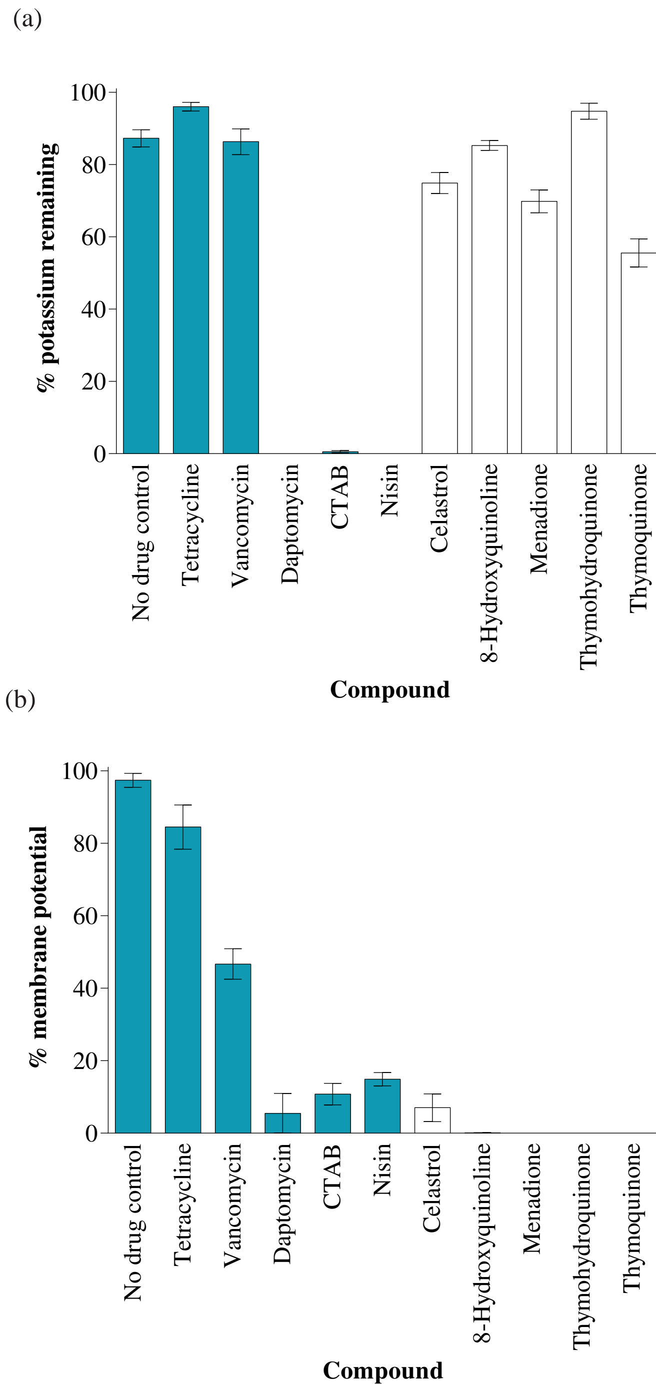

Figure 5.2: **Effect of antibacterial agents on *S. aureus* potassium leakage and membrane potential (4x MIC for 3 hours).** Panel (a): release of intracellular potassium ions; Panel (b): bacterial membrane potential measured by leakage of $DiSC_{(3)}5$. Filled bars show comparator agents (mean of at least three independent replicates; error bars show standard errors).

To establish whether the antioxidants displayed specificity for the bacterial membrane, erythrocytes were challenged with all compounds at 4x MIC, and haemoglobin leakage monitored. Of the 15 antioxidants tested, only six (celastrol, 8-hydroxyquinoline, menadione, NDGA, thymohydroquinone, and thymoquinone) did not induce haemolysis (Figure 5.3), suggesting that antioxidant-membrane interactions were bacteria specific. Of the antioxidants capable of eradicating staphylococcal biofilms, only celastrol and NDGA were non-haemolytic. Subsequent studies focused on these two antioxidants, since selectivity for bacterial membranes and the ability to eradicate staphylococcal biofilms suggested the greatest potential for clinical utility.

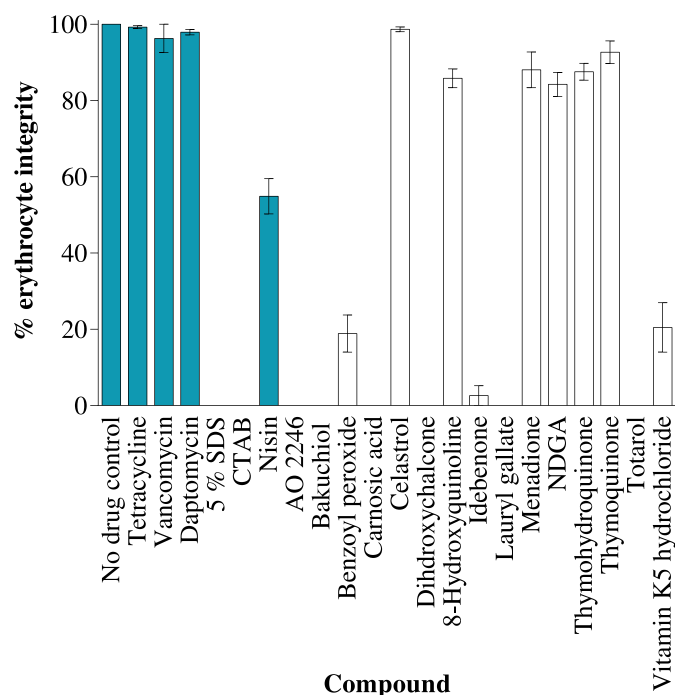

Figure 5.3: **Effect of antioxidants and comparator agents at 4x MIC on mammalian membrane integrity as measured by leakage of haemoglobin from erythrocytes.** Filled bars show comparator agents (mean of at least three independent replicates; error bars show standard errors).

5.3.3 Investigating the mechanistic basis for antibiofilm activity of phenylic antioxidants

Considering that all antioxidants appear to share a similar mechanism of action against planktonic cells, this study sought to determine why some antioxidants have antibiofilm activity whilst others do not. Therefore, a representative antioxidant was studied from each group: 8-hydroxyquinoline, a non-haemolytic antioxidant devoid of antibiofilm activity, and celastrol, a non-haemolytic antioxidant with the ability to eradicate biofilms. Initially, experiments were carried out to identify whether lack of activity was due to a failure of compounds to traverse mature biofilm, as reduced compound penetration has been implicated in the inability of some antibiotics to eradicate biofilms (Del Pozo & Patel, 2007). Both antioxidants and the control agent vancomycin proved able to traverse the biofilm. 8-hydroxyquinoline showed more extensive biofilm penetration (104.6 $\pm$ 2.3%) than vancomycin (56.4 $\pm$ 3.8%) or celastrol (44.0 $\pm$ 3.8%). Therefore, failure of compounds to penetrate the biofilm is unlikely to cause the lack of activity of 8-hydroxyquinoline against biofilms.

Instead, differences in antibiofilm activity may be a result of different effects on slow-growing cells. Biofilms contain bacteria in a variety of metabolic states, including slow-growing, non-growing and persister cells (Davies, 2003; Kubo *et al.*, 2003). Biofilm-eradicating antioxidants could exert their antibiofilm effect by retaining activity against metabolically inactive cells. To examine this, exponential phase and slow-growing cells were challenged with the biofilm eradicator celastrol at the MBEC (32x MIC). Since the comparator agent, 8-hydroxyquinoline, had no measurable MBEC, the compound was used at an equivalent concentration (32x MIC) (Figure 5.4b and 5.4c). Celastrol did not display enhanced activity against stationary phase or biofilm resuspended cells in comparison with 8-hydroxyquinoline over a 6 hour period.

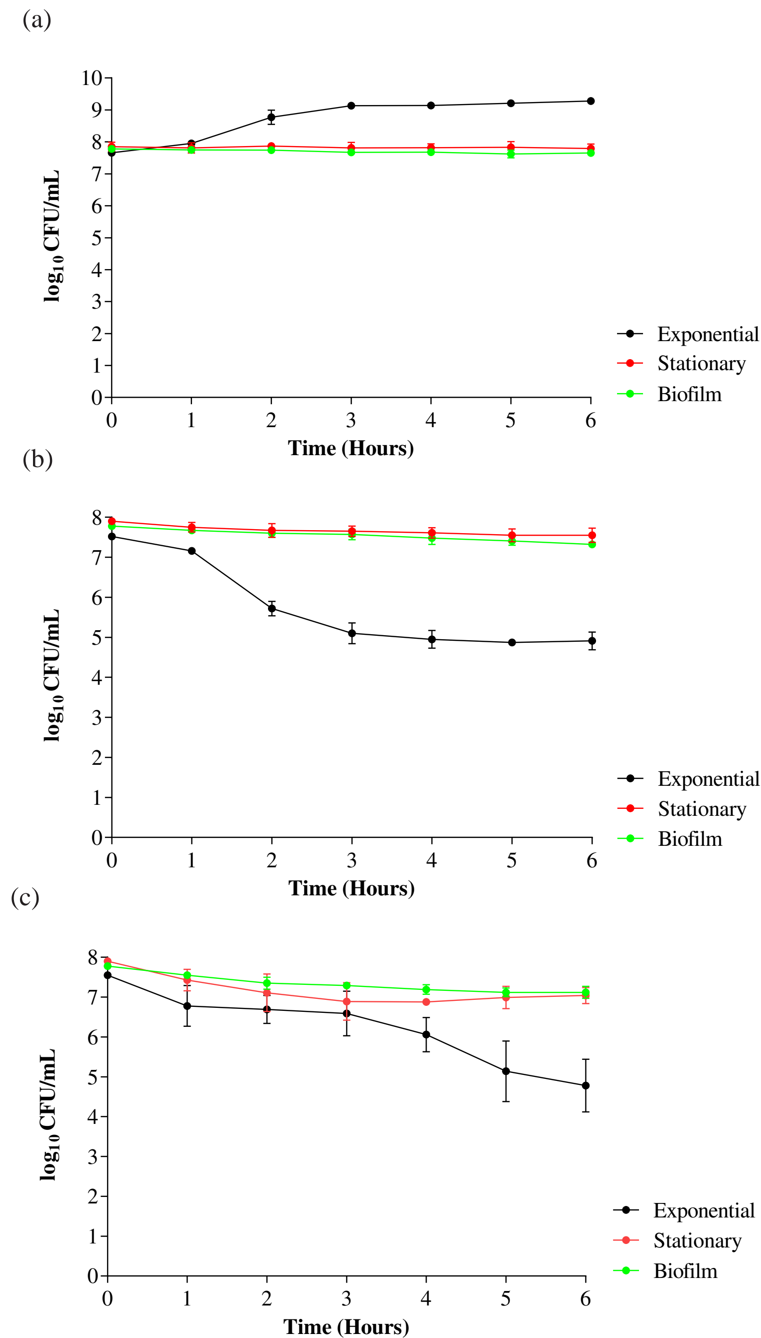

Figure 5.4: **Susceptibility of exponential phase, stationary phase or biofilm-resuspended *S. aureus* SH1000 cells to celastrol or 8-hydroxyquinoline.** Panel (a): cell viability of untreated cells; Panel (b): viability of cells exposed to celastrol at the MBEC; Panel (c): viability of cells exposed to 8-hydroxyquinoline at 32x MIC. Mean of at least three independent replicates; error bars show standard deviations.

Over extended time-periods (24 hours), antioxidants capable of biofilm eradication (celastrol and NDGA) were no more active against stationary phase or persister cells than compounds that are not active against biofilms (Figure 5.5a and Figure 5.5b). Therefore, biofilm eradication by these antioxidants cannot be attributed to activity against the slow- or non-growing cells that have previously been implicated in biofilm resistance to antibiotics (Davies, 2003; Shapiro *et al.*, 2011).

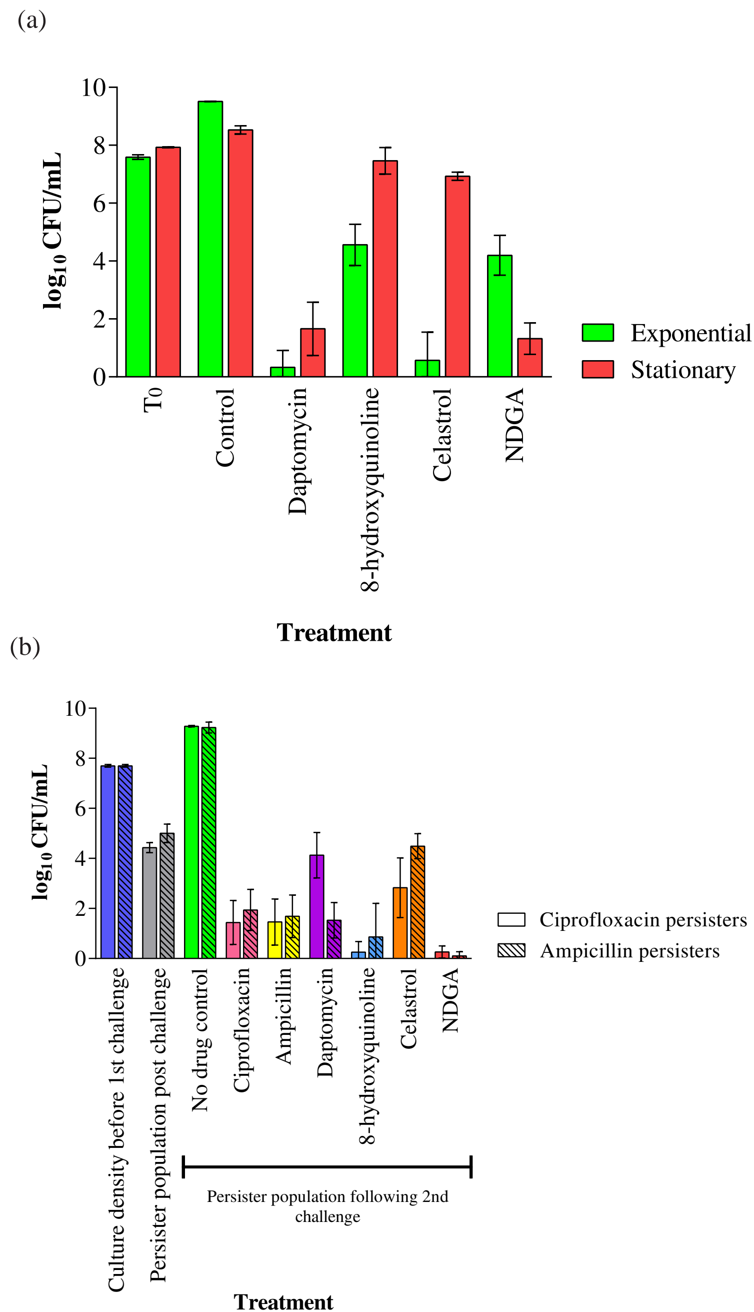

Figure 5.5: **Viability of *S. aureus* SH1000 slow-growing and persister cells following exposure to compounds for 24 hours.** Panel (a): exponential and stationary phase cell viability following exposure to biofilm-active agents at the MBEC, or biofilm-inactive agents at 256 μg/mL. T_0 is the culture density before the addition of compounds; Panel (b): cell viability of ciprofloxacin and ampicillin persisters following exposure to compounds at 10x MIC. Mean of at least three independent replicates; error bars show standard deviations.

Based on the observation that TBBQ triggered extensive hydrolysis of ATP stores, total bacterial ATP levels were measured following exposure to antioxidants; firstly to test the hypothesis that ATP depletion is associated with sterilisation of metabolically inactive cells (Section 4.3.3), and secondly to attempt to distinguish antioxidants with and without antibiofilm activity. Intracellular and extracellular ATP levels were measured following exposure of bacterial populations to celastrol and 8-hydroxyquinolone at 4x MIC in HEPES and glucose buffer (Figure 5.6). Neither compound caused hydrolysis of ATP pools, suggesting that ATP depletion is not a prerequisite for antioxidant-induced biofilm eradication. Furthermore, quantification of ATP levels revealed that the MOA of celastrol and TBBQ are at least partially distinct, despite sharing bacterial membrane-perturbing activity.

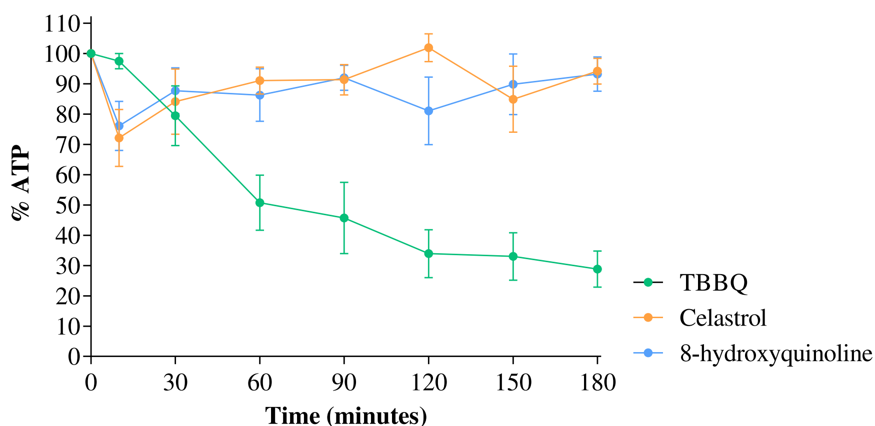

Figure 5.6: **Effects of antioxidants at 4x MIC on total ATP levels in *S. aureus*.** Values were calculated as a percentage of an untreated control. Mean of at least three independent replicates; error bars show standard errors.

Since celastrol and NDGA did not have enhanced activity against heterogeneous populations of cells found within biofilms, they may act by causing physical perturbation of the biofilm. Alterations in biofilm structural components were quantified in an attempt

to distinguish biofilm eradicators from non-eradicators. Following 24 hour incubation with compounds at 256 μg/mL, biofilm eradicators significantly reduced the amount of adherent material (matrix and cells) in comparison with non-eradicating antioxidants (Figure 5.7a and 5.7b).

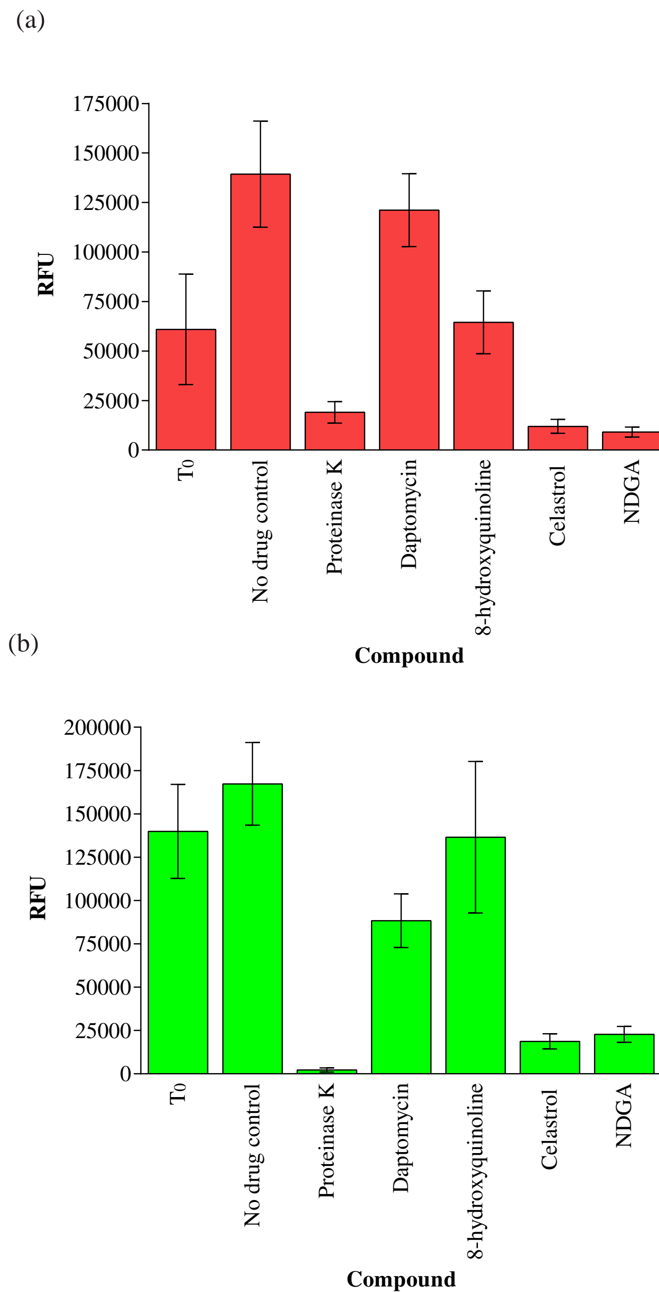

Figure 5.7: **Quantification of matrix material and adherent cells following exposure of biofilms to compounds at 256 μg/mL for 24 hours.** Panel (a): attached matrix; Panel (b): attached cells. The data labeled T_0 shows the quantity of matrix and cells before the addition of compounds. Data are the means of at least three independent replicates, with error bars showing standard deviation.

To investigate whether biofilm dispersion occurs prior to loss of bacterial viability, or is in fact a consequence of cell death, viable cells and adherent material were quantified following exposure to NDGA and celastrol for 1 hour. The antioxidants had little or no effect on total (released and attached cell) viability over this period (Figure 5.8a), while adherent material was significantly reduced within 1 hour (Figure 5.8b and 5.8c). Therefore, short duration experiments revealed that destructuring of the biofilm occurs before initiation of cell killing, implying that biofilm detachment is not simply a consequence of cell death. Thus, eradication of staphylococcal biofilms by celastrol and NDGA involves biofilm disruption, causing release of planktonic cells that are susceptible to the membrane damaging effects of the compounds.

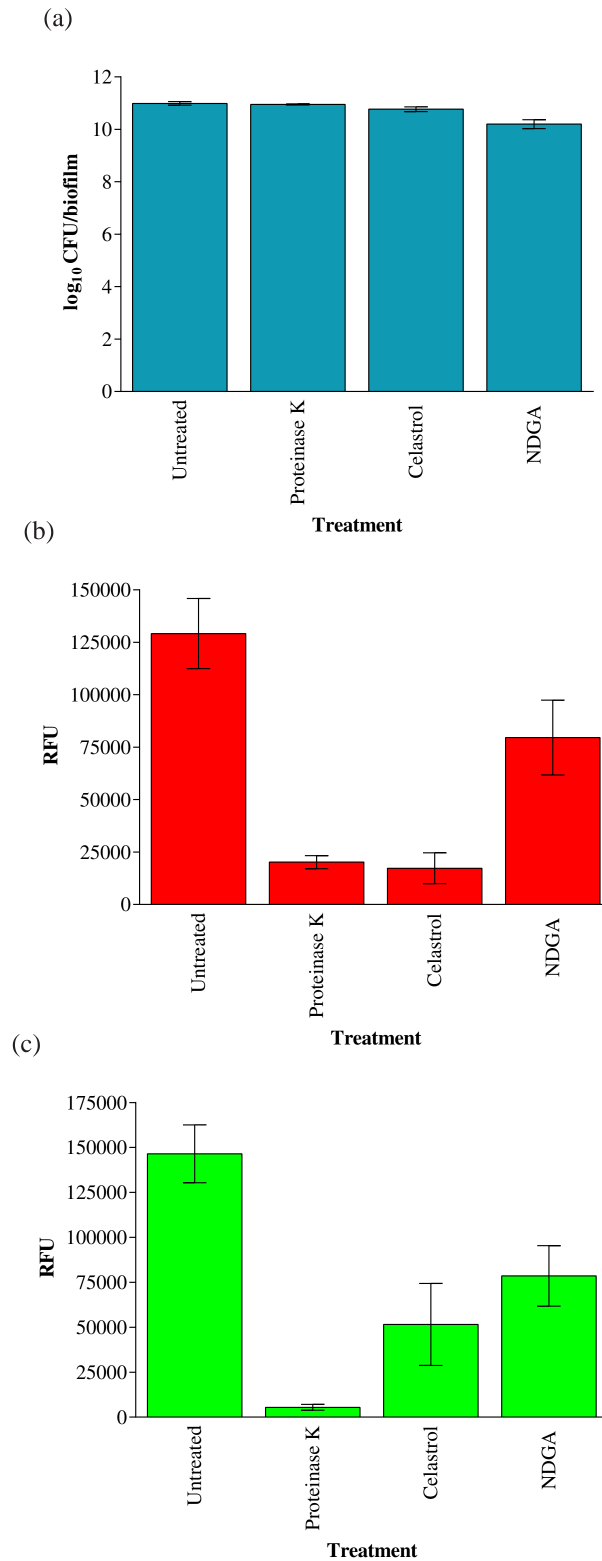

Figure 5.8: **Cell viability and quantification of adherent material following exposure of biofilms to compounds at 256 μg/mL for 1 hour.** Panel (a): total biofilm cell viability; Panel (b): attached matrix; Panel (c): attached cells. Data are the means of at least three independent replicates, with error bars showing standard deviation.

5.3.4 Synergistic interactions of antioxidants with antibiotics

Previous studies with a subset of the compounds under investigation here identified antioxidant-mediated potentiation of antibacterial activity. Carnosic acid enhanced aminoglycoside activity against vancomycin-resistant enterococci (Horiuchi *et al.*, 2007), dihydroxychalcone acted synergistically with doxycycline, gentamicin and ciprofloxacin against *S. aureus* (Tran *et al.*, 2012), and totarol increased carbapenem and β-lactam activity against *S. aureus* (Nicholas *et al.*, 1999). To identify potentially useful synergistic interactions, planktonic cells were exposed to non-haemolytic antioxidants in combination with licensed antibiotics (Table 5.3). Celastrol and NDGA acted synergistically with gentamicin (inhibition of bacterial growth at 0.25 and 16 μg/mL of celastrol and NDGA in combination with 0.03125 or 0.125 μg/mL gentamicin, respectively), whilst all other combinations had additive effects. Therefore, like some other antioxidants and membrane-damaging agents, celastrol and NDGA could be used usefully in combination with existing antibiotics (Horiuchi *et al.*, 2007; Tran *et al.*, 2012; Nicholas *et al.*, 1999; Hu & Coates, 2013).

	Tetracycline	Ciprofloxacin	Erythromycin	Gentamicin	Oxacillin
Celastrol	1.00	0.77	0.88	**0.33**	0.64
NDGA	0.75	0.63	0.75	**0.42**	0.59
Thymoquinone	0.59	0.75	1.06	0.79	0.75
Thymohydroquinone	0.75	0.88	0.69	1.04	0.88
Menadione	0.69	0.53	0.75	1.01	0.69
8-hydroxyquinoline	0.75	0.75	0.75	0.83	0.77

Table 5.3: **Synergism of antioxidants with established antibiotics against planktonic cultures of *S. aureus* SH1000.** FIC index $\leq$0.5 is a synergistic interaction, equal to 1 is an additive interaction and $\geq$2 is an antagonistic interaction.

S. aureus biofilms grown using the CBD were also exposed to combinations of biofilm-eradicating antioxidants and antibiotics, and assessed for synergistic interactions. As was identified in experiments with planktonic cultures, celastrol and NDGA only acted synergistically with gentamicin (Table 5.4). Staphylococcal biofilms were eradicated by the combination of 1 μg/mL gentamicin with 8 μg/mL celastrol or 0.5 μg/mL gentamicin and 16 μg/mL NDGA. Therefore, addition of low concentrations of gentamicin to culture media significantly reduced celastrol and NDGA MBECs (a 4- and 8-fold reduction in MBEC from 32 and 128 μg/mL respectively).

	Tetracycline	Ciprofloxacin	Erythromycin	Gentamicin	Oxacillin
Celastrol	>0.5	>0.5	>0.5	**≤0.25**	>0.5
NDGA	>1	>0.5	>1	**≤0.15**	>0.5

Table 5.4: **Synergism of antioxidants with established antibiotics against *S. aureus* SH1000 biofilms.** FIC index ≤0.5 is a synergistic interaction, equal to 1 is an additive interaction and ≥2 is an antagonistic interaction.

5.3.5 Effects of celastrol and NDGA on human LabSkin™

For a compound to be developed as a topical antibiofilm agent, it should be able to be applied to the skin without causing damage or irritation. NDGA and celastrol had no visible adverse effects on human or mouse skin when investigated as high-concentration topical preparations at 3% (for the treatment of psoriasis) and 5% (for reduction of inflammation) respectively (Newton *et al.*, 1988; Kim *et al.*, 2009). Furthermore, cytotoxicity was not identified in rat basophil leukemia cells treated with ≤10 μM celastrol (∼4.5 μg/mL) (Kim *et al.*, 2009). Exposure to ≤100 μM NDGA (∼30 μg/mL) was not toxic to bovine fibroblasts, though 1 mM NDGA (∼300 μg/mL) induced cytotoxicity (Koob *et al.*, 2001). Therefore some toxicity testing has been carried out

with celastrol and NDGA, but irritation and physical damage to human skin structure has not been assessed at concentrations that are relevant to their use as antibiofilm agents.

Although antioxidants were not haemolytic (Figure 5.3), further studies were carried out to determine whether celastrol and NGDA exhibit sufficient selective toxicity to allow their topical use on human skin. Following a 24 hour exposure of a skin equivalence system (LabSkin^{TM}) to 10x MIC mupirocin and celastrol, no irritation of skin was detected by IL-1α release. The limited solubility of NDGA meant that, in order to keep the solvent load below the level (0.5%) causing damage to LabSkin^{TM}, experiments were carried out with compound at a concentration equivalent to 4x MIC (*S. aureus* SH1000). IL-1α release was below the lowest detectable level for mupirocin, celastrol and NDGA (0 $\pm$ 0% IL-1α release relative to 5% SDS). Hematoxylin and eosin staining of tissue sections confirmed that 24 hour exposure to the irritant SDS (5%) was severely damaging to skin, causing shedding of the top layers (stratum corneum and epidermis) and causing injury to the dermis below (Figure 5.9c). Conversely mupirocin, celastrol and NDGA had no visible detrimental effects on skin (Figure 5.9d, 5.9e and 5.9f). Therefore, celastrol and NDGA did not cause irritation, as measured by IL-1α release, nor did they damage fully differentiated skin at concentrations above those that are able to sterilise bacterial cultures.

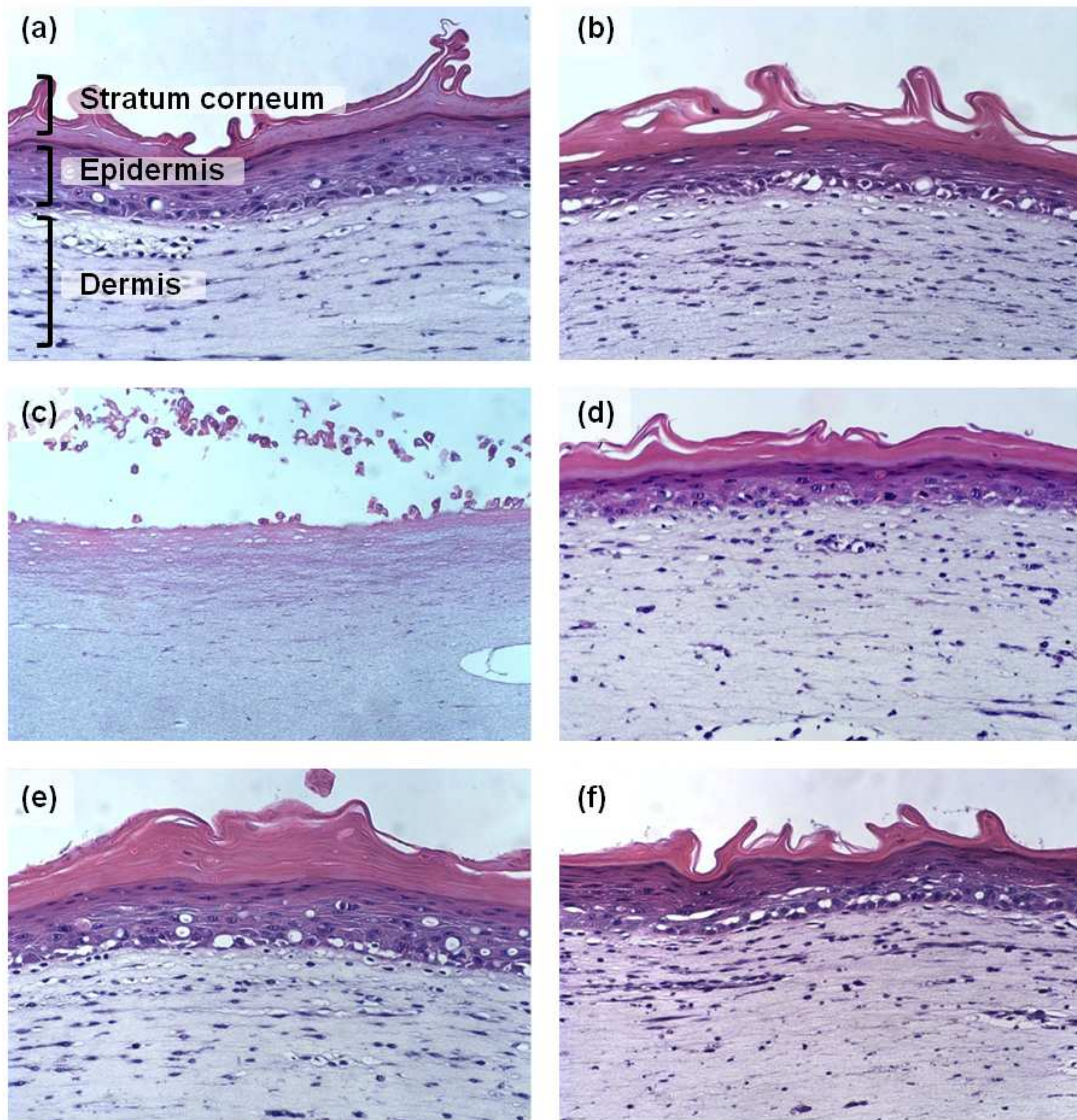

Figure 5.9: **Hematoxylin and eosin stained sections of LabSkin™ exposed to compounds for 24 hours.** Panel (a): untreated control; Panel (b): ethanol (0.2% v/v); Panel (c) SDS (5% w/v); Panel (d): mupirocin 10x MIC; Panel (e) celastrol 10x MIC; Panel (f): NDGA 4x MIC. H&E staining and imaging carried out by J Bradley, Evocutis.

5.3.6 Selection of antioxidant resistant strains

A desirable feature of any potential antibacterial drug is a low propensity to select resistance. The resistance potential of NDGA and celastrol was evaluated initially by plating saturated cultures of SH1000 onto agar containing these compounds at 4x MIC. In neither case were resistant mutants identified (mutation frequency $<5.0 \times 10^{-9}$). This suggests that the antioxidants have low resistance potentials, and that multiple mutations may be required to develop resistance to the agents (O'Neill & Chopra, 2004). Therefore, the extended spectrum MIC method was used to determine whether resistance to these antioxidants would arise in any of the eight separate lineages that were under continuous selection over several days (Friedman *et al.*, 2006). Resistance (defined here as a 4-fold increase in MIC) to the control antibiotic daptomycin was identified in all eight test lineages. The most resistant strain displayed a 16-fold increase in MIC following 40 passages (Figure 5.10). Celastrol resistance was detected in all lineages, and the most resistant strain, C3, had an MIC of 8 μg/mL (Figure 5.10). It is of note that strains displaying reduced susceptibility to celastrol had an MIC that was at least 4-fold lower than the concentration that eradicates staphylococcal biofilms. Resistance to NDGA could not be selected, and strains exposed to the compound over 40 passages retained the same MIC throughout the experiment (64 μg/mL). Therefore, NDGA resistance may not arise readily if the compound were to be used in the clinic. Celastrol resistant strains are more likely to arise, but reduced susceptibility to celastrol may only be selected by continuous exposure of bacteria to the compound over extended periods. Furthermore, this experiment suggests that resistance is unlikely to reach a level that would impact on antibiofilm therapy.

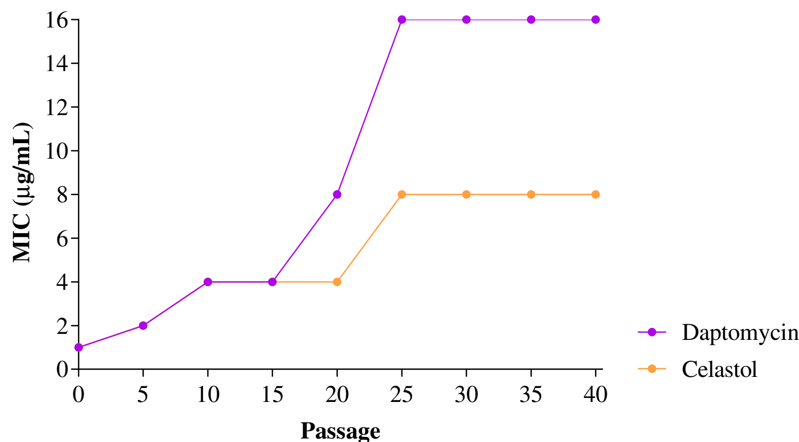

Figure 5.10: **Selection of daptomycin and celastrol resistant mutants.** Experiments were carried out using eight lineages, and results are representative of replicates.

To investigate how alterations in the celastrol resistant strain (C3) affect susceptibility to a range of compounds, cross-resistance/susceptibility studies were carried out with antibiotics, membrane-damaging agents and antioxidants. The MICs of several antioxidants and ciprofloxacin were reduced 4-fold in comparison with the parental strain (Table 5.5). This suggests that a number of antioxidants may interact with similar pathways or components of staphylococci that have been altered in C3 as a response to celastrol exposure. Alternatively, changes in susceptibility could be due to alterations in a generalised resistance mechanism, such as an efflux pump (this will be addressed in the following section).

Compound	MIC (μg/mL)	
	SH1000	C3
Ampicillin	0.5	0.5
AO2246	4	4
Bakuchiol	4	4
Benzoyl peroxide	64	32
Carnosic acid	32	32
Celastrol	1	8
Dihydroxychalcone	128	128
Chlorhexidine	2	1
Ciprofloxacin	2	0.5
CTAB	2	1
Daptomycin	1	1
Erythromycin	0.5	0.5
Gentamicin	0.5	0.5
8-hydroxyquinoline	4	4
Idebenone	64	64
Lauryl gallate	64	64
Menadione	16	8
NGDA	64	64
Nisin	2	4
TBBQ	8	8
Tetracycline	1	0.5
Thymohydroquinone	16	4
Thymoquinone	16	4
Totarol	4	2
Vancomycin	1	1
Vitamin K5 hydrochloride	32	8

Table 5.5: **Susceptibility of the celastrol resistant strain, C3, to antibacterial agents.** Values in green and red denote hypersusceptibilty and resistance to compounds relative to SH1000.

5.3.7 Characterisation of the celastrol resistant strain

WGS of strain C3 was used to provide further information about the target of celastrol. A number of genes and intergenic regions were identified in which there were potential DNA sequence changes (Table 5.6). It should be noted that the role of each individual mutation in celastrol resistance has not yet been confirmed, and further work will be required to do so (see Section 6.2). Broadly the mutations can be grouped into those that potentially affect the cell envelope, those that may impact on DNA repair, and those without a definable effect or function. The majority of mutations appear to be located in genes related to cell envelope function, which may have been anticipated due to the membrane damaging MOA of celastrol (Section 5.3.2). However, due to time restrictions it was not possible to investigate the effect of each individual mutation.

Locus	Function	Affected cellular component	Nucleotide alteration	Amino acid alteration
SAOUHSC_02866	Hypothetical MmpL efflux pump	Cell envelope	$A_{1889}G$	$V_{630}A$
SAOUHSC_02866	Hypothetical MmpL efflux pump	Cell envelope	$A_{1679}G$	$M_{560}T$
SAOUHSC_02866	Hypothetical MmpL efflux pump	Cell envelope	$T_{106}C$	$N_{36}D$
SAOUHSC_02556	Hypothetical acyl-CoA dehydrogenase	Cell membrane	Deleted A_{865}	Frame shift; X_{289}
SAOUHSC_02555*	Hypothetical acyl-CoA dehydrogenase	Cell membrane	Deleted T	-
SAOUHSC_02555*	Hypothetical acyl-CoA dehydrogenase	Cell membrane	Deleted T	-
SAOUHSC_02555*	Hypothetical acyl-CoA dehydrogenase	Cell membrane	Deleted C	-
SAOUHSC_02555*	Hypothetical acyl-CoA dehydrogenase	Cell membrane	Deleted A	-
SAOUHSC_02555*	Hypothetical acyl-CoA dehydrogenase	Cell membrane	Deleted A	-
SAOUHSC_01002	Quinol oxidase AA3 subunit II	Cell membrane	$C_{349}T$	$D_{117}N$

Table 5.6: **Alterations in the genome of the celastrol resistant strain, C3, identified through WGS.** *Mutations in intergenic regions are located upstream of the listed gene.

Locus	Function	Affected cellular component	Nucleotide alteration	Amino acid alteration
SAOUHSC_00020	Two-component response regulator, WalR	Cell wall	$A_{301}G$	$T_{101}A$
SAOUHSC_01287	Glutamine synthetase	Cell wall	$T_{905}C$	$V_{302}A$
SAOUHSC_01664	Hypothetical phosphotransferase	Cell wall	$T_{367}C$	$K_{123}E$
SAOUHSC_01787	Hypothetical lysine-specific permease, LysP	Cell wall	$G_{716}A$	$P_{239}L$
SAOUHSC_02798	Hypothetical cell wall surface anchor protein	Cell wall	Deleted T_{548}	Frame shift;Q_{183}
SAOUHSC_01272	DNA mismatch repair protein, MutS	DNA	$G_{2005}T$	$E_{669}X$
SAOUHSC_01469	Endonuclease III	DNA	$C_{383}T$	$S_{128}N$
SAOUHSC_01403	Cold shock protein (transcriptional regulator)	Unknown	$T_{74}C$	$D_{25}G$
SAOUHSC_01977*	Hypothetical transcriptional regulator	Unknown	A>G	-
SAOUHSC_03022	Hypothetical lipid binding protein of unknown function	Unknown	$C_{359}T$	$P_{120}L$
SAOUHSC_01158*	Hypothetical cell-division initiation protein	Unknown	G>A	-

Table 5.6 (continued): **Alterations in the genome of the celastrol resistant strain, C3, identified through WGS.**

It was of note that there were significantly more mutations identified in WGS of C3 than in the TBBQ-resistant strain, for which only four putative mutations were identified (Section 4.3.7). Hypermutators are found in bacterial populations and arise as a consequence of alterations in the methyl-directed mismatch repair (MMR) system. This DNA repair pathway identifies and repairs mismatches in newly-synthesised DNA duplexes. In *S. aureus* mutations in *mutS* are associated with accelerated rate of antibiotic resistance development (O'Neill & Chopra, 2002). Since one of the mutations in C3 was located in *mutS*, the mutability of the strain was assessed. Mutation frequencies confirmed that C3 is more mutable than the parental strain SH1000, displaying a 7-fold higher frequency of mutation to rifampicin (SH1000 mutation frequency of 1.92x$10^{-7} \pm \leq$ 6.33x10^{-8}, C3 mutation frequency of 1.32x$10^{-6} \pm \leq$ 3.57x10^{-7}). Therefore, the alteration in *mutS* may drive the development of further mutations within C3.

5.4 Conclusions

Since there is an urgent need to develop novel antibiofilm agents, it is of some significance that a number of biofilm-eradicating antioxidants have been identified. Celastrol and NDGA are the most promising of the antioxidants tested, as they display potent and specific activity against staphylococci. These antioxidants eradicate biofilms through disruption of the biofilm structure, causing release of planktonic cells. Subsequent killing involves perturbation of the bacterial membrane, though the precise nature of this interaction varies by compound. Celastrol resistance was identified following continuous selection over several days, whilst NDGA-resistant strains could not be isolated. Resistant bacteria may evade the effects of celastrol by modifying the architecture of the cytoplasmic membrane, which is the putative target of celastrol. Both NDGA and celastrol were applied to skin without measurable detrimental effects. Therefore, these compounds are attractive candidates for development as topical antistaphylococcal

biofilm treatments. Since both antioxidants act synergistically with gentamicin, they could potentially be applied in combination to treat patients with severe skin lesions or burn wounds.

Chapter 6

General conclusions and future work

6.1 General Conclusions

The antioxidants under investigation here were of interest since they are compounds that are already used in the cosmetics industry, as food preservatives or in traditional medicines and have known antibacterial activity (Merrifield & Yang, 1965; Fung *et al.*, 1985; Chaieb *et al.*, 2011; Martins *et al.*, 2013; Chen *et al.*, 2011; Katsura *et al.*, 2001). Therefore, it was hoped that this collection of compounds would display useful antibacterial activity without associated eukaryotic toxicity.

Initial studies focused on the antibacterial activity of the food additive TBHQ. Early experimentation revealed that TBHQ had little innate antibacterial activity, and that antimicrobial properties that had been ascribed to the compound were through conversion to TBBQ under bacterial culture conditions. TBBQ was primarily active against Gram-positive organisms and eradicated staphylococcal biofilms. This unusual property was a consequence of TBBQ's ability to eradicate slow- and non-growing cells. These sub-populations of cells are recalcitrant to the effects of other antibacterial agents, and can repopulate the biofilm following removal of antibacterial challenge. Up to 80% of

bacterial infections are believed to include a biofilm component (National Institute of Health, 2002), which contributes to recurrent infections and treatment failure. Therefore, agents with antibiofilm activity are highly sought after.

The MOA of TBBQ was investigated initially by assessing its effects on biosynthetic pathways using radiolabeled precursors and biosensors that luminesce in response to particular stresses. TBBQ did not preferentially inhibit any single macromolecular synthetic pathway or elicit stress responses in the biosensors, which is suggestive of a compound with membrane-damaging activity (O'Neill & Chopra, 2004). Indeed, TBBQ was found to have lethal interactions with the cytoplasmic membrane, causing depolarisation of cells and loss of intracellular components. A low-level TBBQ-resistant strain was generated under selection, and may have reduced susceptibility due to altered membrane phospholipid composition. However, staphylococcal liposomes were not disrupted by TBBQ, suggesting that the antioxidant is unlikely to bind to and disrupt specific lipids in the membrane. Permeabilisation of the Gram-negative outer membrane rendered *E. coli* susceptible to TBBQ, indicating that the cytoplasmic membrane of Gram-negative bacteria was also susceptible to the antioxidant's action. Since TBBQ did not induce haemolysis at concentrations of antioxidant that damaged bacterial membranes, this suggests that membrane effects were bacteria-specific.

A number of other phenylic antioxidants were screened for antibacterial and antibiofilm activity. Celastrol and NDGA were identified as potentially useful agents, as they eradicated surface-attached communities and displayed specific activity against bacteria. These antioxidants could be distinguished from TBBQ by the mechanism through which they eradicated biofilms. NDGA and celastrol induced dispersal of the biofilm matrix and cells in contrast with TBBQ, which killed metabolically active and inactive cells without significantly destructuring the biofilm. Like TBBQ, celastrol and NDGA disrupted the cytoplasmic membrane, causing death of planktonic cells, though the exact nature of this interaction varied for the different antioxidants.

All three antioxidants acted synergistically with gentamicin against staphylococcal biofilms. Eradication was achieved with a combination of 0.5-1 μg/mL gentamicin and 16 μg/mL TBBQ, 16 μg/mL NDGA, or 8 μg/mL celastrol. At concentrations above those that can eradicate staphylococcal biofilms, antioxidants had no measurable detrimental effects on a human living skin equivalent. Furthermore, TBBQ, celastrol and NDGA all potentiated the antibiofilm activity of gentamicin at concentrations that were tolerated at the skin surface. Amongst other uses, gentamicin is applied as a topical antimicrobial cream (0.1-1% gentamicin [1-10 mg/mL]) for the sterilisation of infected wounds (Castellano *et al.*, 2007; Stojadinovic *et al.*, 2008). The combination of gentamicin and antioxidants could be employed to treat conditions, such as burn wounds and severe skin lesions, where biofilms are likely to arise (Wolcott *et al.*, 2010). Combination therapy could significantly reduce the dose and consequent toxic effects of gentamicin, whilst providing additional antibiofilm activity. Furthermore, an inability of *S. aureus* to develop high-level resistance to these antioxidants would allow topical treatment of infected wounds for prolonged periods.

More generally, the results presented here confirm that compounds with membrane-damaging effects may be a useful source of antibiofilm agents (Hurdle *et al.*, 2011). However, it has been shown that effects on the membrane were varied and it may not be possible to predict which agents will eradicate biofilms based upon the kind of interaction they have with membranes. For example, NDGA caused rapid permeabilisation of the membrane, whilst celastrol compromised the membrane potential without inducing leakage of intracellular ions. Nonetheless, all agents displayed a similar MBEC (4-fold difference between celastrol, TBBQ and NDGA). Even those compounds that caused rapid and extensive membrane damage were not necessarily toxic to eukaryotes, and could be useful as antibacterial agents due to low potential for resistance development.

6.2 Future work

Although it has been established that TBBQ specifically interacts with components of the bacterial membrane, the precise nature of this interaction is not yet known. To provide further insight into the membrane component that TBBQ interacts with, staphylococcal cell ghosts could be generated, comprising a preserved cell envelope in the absence of cytoplasmic material (Langemann *et al.*, 2010; Lubitz, 2010). *E. coli* bacterial ghosts have been produced previously by controlled expression of phage lysis gene E, which forms a transmembrane tunnel through which intracellular components are lost (Langemann *et al.*, 2010). Currently, a chimeric E-L lysis gene that is active in staphylococci is being developed in combination with methodology to re-seal bacterial ghosts using membrane vesicles (Lubitz, 2010). Cell ghosts could be impregnated with fluorescent dye, such as carboxyfluorescein, and exposed to TBBQ. If leakage of dye is not induced, this may indicate that an energised membrane is required for the interaction. This could distinguish between agents that disrupt the function of components embedded within the membrane as opposed to those that simply destructure the membrane.

The contribution of alterations in putative ethanolamine kinase (encoded by SAOUHSC_01866) to TBBQ susceptibility could be confirmed by generating a deletion mutant and testing activity of TBBQ against this strain (O'Neill *et al.*, 2006). In Section 4.3.7 it was hypothesised that the TBBQ-resistant strain (B7) had altered phospholipid production as a consequence of a mutation in ethanolamine kinase. This suggestion could be investigated by determining and comparing the membrane phospholipid composition of *S. aureus* SH1000, B7 and the deletion mutant, in particular quantifying the phosphatidylethanolamine content (Jones 2008). Similarly, membrane fluidity and fatty acid content could be analysed to identify physiological changes in the B7 membrane, which could contribute to the reduction in TBBQ activity (Bayer *et al.*, 2000).

Transcriptional profiling could provide further insight into the target to TBBQ, and

complement the research presented in this thesis. Antibacterial agents disrupt bacterial homeostasis, which induces stress responses that relate to the compound's target. Additionally, bacteria may upregulate the target itself or target-associated genes in order to titrate out the compound and avoid inhibitory effects (O'Neill & Chopra, 2004). Transcriptional profiling would involve extraction of total RNA from untreated planktonic cultures and those that had been exposed to antioxidants. Following mRNA enrichment and fragmentation, complementary DNA (cDNA) would be synthesized for amplification and Next-Generation Sequencing. Reads would be mapped to the reference genome, and expression levels would be assessed based upon read coverage (Pinto *et al.*, 2011). Genes displaying $>$2-fold increase or decrease in expression would be considered to be differentially regulated, and analysis of expression patterns could provide information about the target of TBBQ.

Full analysis of the celastrol resistant strain (C3) would complement MOA studies that have already been carried out with celastrol. WGS has suggested some potential mutations that could contribute to resistance. However, the sequence of each of these genes should be determined individually to confirm the presence of a mutation, then be complemented in C3, as was carried out for the TBBQ-resistant strain. Since a large number of mutations are present and may have compensatory effects, it would be beneficial to delete genes of interest individually in a clean host, for example using site directed mutagenesis (Vickers *et al.*, 2007). Deletion of specific genes causing increased resistance to celastrol would provide further insight into the cellular target of the antioxidant.

Finally, in order to progress TBBQ, celastrol and NDGA as clinical candidates, *in vivo* experimentation with animal models of infection should be carried out. The compounds may be most usefully employed as topical agents for use in the treatment of chronic wounds and surgical site infections, so the experimental design should reflect this situation (Dai *et al.*, 2011). Ideally these experiments would provide information about efficacy,

but also further data regarding tolerability. Collectively, these studies could facilitate development of the antioxidants for human treatments.

Appendices

A Primers used in this study.

Oligonucleotide primer	Sequence
Tnp_out2	5' GCTTGCATGCCTGCAGGTCGACTC 3'
Tn_Fwd	5' CAGGTATAGGTGTTTTGGGAA 3'
Tn_Rev	5' AAAAAGGATTGATTCTAATGA 3'
01722/3inter_Fwd	5' TTTCATCATTTACACATCCTA 3'
01722/3inter_Rev	5' CAAGCGTAAATGTAACTA 3'
01866_Fwd	5' ATTAGGGTGGACACTTGATTC 3'
01866_Rev	5' CCCATGGTTTGTATCGAA 3'
ElitraMCS_Fwd	5' CAGCAGTCTGAGTTATAAAATAG 3'
ElitraMCS_Rev	5' CAGGCAAATTCTGTTTTATCAGAC 3'
01723op_XmaI_Fwd	5' TAACCCGGGGGGGATAACGAATTTA 3'
01723op_XbaI_Rev	5' AAATCTAGAGATGACACAGATAGTCGATTT 3'
01866_SacI_Fwd	5' AAAGAGCTCATTAGGGTGGACACTTGATTC 3'
01866_XmaI_Rev	5' TAACCCGGGCCCATGGTTTGTATCGAA 3'

B MICs of TBHQ and TBBQ against a collection of *S. aureus* clinical isolates.

	Antibiotic	MIC (μg/mL)	
Strain name	susceptibility	TBHQ	TBBQ
G1	MSSA	8	8
G2	MSSA	8	8
G4	MSSA	8	16
G5	MSSA	16	8
G6	MSSA	8	8
G9	MSSA	16	16
G10	MSSA	16	16
G11	MSSA	8	8
G12	MSSA	8	8
G13	MSSA	8	8
G15	MSSA	16	16
G16	MSSA	8	8
G17	MSSA	16	16
G18	MSSA	16	8
G19	MSSA	16	8
G20	MSSA	8	8
G22	MSSA	16	16
G23	MSSA	16	16
G24	MSSA	8	8
G25	MSSA	16	16
G26	MSSA	16	16
G27	MSSA	16	16

	Antibiotic	MIC (μg/mL)	
Strain name	susceptibility	TBHQ	TBBQ
G28	MSSA	16	16
G29	MSSA	8	8
G30	MSSA	8	8
G31	MSSA	8	4
G32	MSSA	8	8
G33	MSSA	16	16
G34	MSSA	16	16
G35	MSSA	16	16
Oxford	MSSA	8	8
R24	MRSA	8	8
R25	MRSA	8	8
R26	MRSA	16	16
R27	MRSA	16	8
R28	MRSA	8	8
R29	MRSA	8	8
R30	MRSA	16	8
R31	MRSA	16	16
R32	MRSA	16	16
R33	MRSA	16	16
R34	MRSA	16	16
R35	MRSA	16	16
R36	MRSA	16	8
R37	MRSA	16	16
R38	MRSA	16	16
R39	MRSA	8	8
R40	MRSA	16	16
R33	MRSA	16	16

Strain name	Antibiotic susceptibility	MIC (μg/mL) TBHQ	TBBQ
12232	MRSA	16	16
12233	MRSA	16	16
W71	MRSA	16	8
W74	MRSA	8	16
W80	MRSA	16	16
W82	MRSA	16	16
W85	MRSA	16	16
W96	MRSA	16	16
W97	MRSA	16	16
W98	MRSA	8	16
W99	MRSA	32	16
EMRSA 15	MRSA	16	16
EMRSA 16	MRSA	16	16
EMRSA17	MRSA	8	8
MW2	MRSA	16	16
VISA 2	VISA	8	8
Mu3	VISA	16	16
New Jersey	VISA	16	16
Mu50	VISA	16	16
V99	VISA	8	8
Michigan	VISA	16	16
VISA 26	VISA	16	16

C Addresses of companies

Alfa Aesar, Heysham, UK

AstraZeneca, Wilmington, USA

Avanti Polar Lipids, Birmingham, USA

Bayer, Leverkusen, Germany

Beckman Coulter Genomics, Takeley, UK

Bioline Reagents, London, UK

Bio-Rad, Hemel Hempstead, UK

BMG LABTECH, Ortenberg, Germany

CP Pharmaceuticals, Wrexham, UK

Cubist Pharmaceuticals, Lexington, USA

Destiny Pharma, Brighton, UK

Duchefa Biochemie, Haarlem, Netherlands

EdgeBio, Gaithersburg, USA

Eurofins MWG Operon, Ebersberg, Germany

Evocutis, Wetherby, UK

Fisher Scientific, Loughborough, UK

GE Healthcare, Little Chalfont, UK

Gene Codes Corporation, Ann Arbor, USA

Greiner Bio-One, Stonehouse, UK

Invitrogen, Paisley, UK

LG Life sciences, Seoul, South Korea

Matrix Biologicals, Hull, UK

Melford, Ipswich, UK

Millipore, Billerica, USA

Molecular Biology Insights Inc., Cascade, USA

MP Biomedicals, Illkirch Cedex, France

Microsynth AG, Balgach, Switzerland

New England Biolabs, Ipswich, USA

Oxoid, Cambridge, UK

Packard Bioscience, Beaconsfield, UK

PerkinElmer, Cambridge, UK

Pfizer, Kalamazoo, USA

Promega, Southampton, UK

Qiagen, Manchester, UK

R&D systems, Abingdon, UK

Sera Laboratories International, Bolney, UK

Sigma-Aldrich, Poole, UK

Sunovion Pharmaceuticals, Marlborough, USA

Theravance, San Francisco, USA

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