Evaluation of the mode of action of novel metal-based antimicrobial compounds

A thesis submitted to Maynooth University for the degree of Doctor of Philosophy

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Declaration

This thesis has not been submitted whole or in part to this or any other university for any other degree and is the original work of the author except where otherwise stated.

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Characterisation of the antimicrobial mode of action of gallium maltolate. **M. Piatek**, D. Griffith and Kevin Kavanagh. The 3rd Symposium of the Irish Biological Inorganic Chemistry Society (IBICS-3), RCSI, Dublin, November 2019.

Characterisation of the proteomic response of *Candida parapsilosis* to the silver(I) compound SBC3. **M. Piatek**, C. O'Beirne, Z. Beato, M. Tacke and Kevin Kavanagh. Department of Biology Research Day, Maynooth University, May 2022.

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Exposure of *Candida parapsilosis* to the silver(I) compound SBC3 induces alterations in the proteome inhibiting protein synthesis and reducing virulence. **M. Piatek**, C. O'Beirne, Z. Beato, M. Tacke and K. Kavanagh. The 5th Symposium of the Irish Biological Inorganic Chemistry Society (IBICS-5), virtual event, November 2021.

Exposure of *Candida parapsilosis* to the silver(I) compound SBC3 induces alterations in the proteome and reduces virulence. **M. Piatek**, C. O'Beirne, Z. Beato, M. Tacke and K. Kavanagh. Microbiology Society Annual Conference, ICC Belfast, April 2022.

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Abbreviations

°C	Degrees Celsius
ABC	ATP-binding cassette
ABPA	Allergic bronchopulmonary aspergillosis
ALS	Agglutinin-like-sequence
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BEC	Buccal epithelial cell
CDC	Centers for Disease Control and Prevention
CDR1 and 2	*Candida* drug resistance 1 and 2
CF	Cystic fibrosis
CFEM	Common in fungal extracellular membranes
CFU	Colony forming units
COX	Cytochrome *c* oxidase
CPA	Chronic bronchopulmonary aspergillosis
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
FDA	U.S. Food and Drug Administration
FDR	False discovery rate
FsC	Fusarinine C
g	G-force
g	Grams
Ga	Gallium
GaC	Gallium citrate
GaM	Gallium maltolate
GaN	Gallium nitrate
GaPPIX	Ga(III)-protoporphyrin IX
GCS	Glycine cleavage system
GO	Gene ontology
GOBP	Gene ontology biological process
GOCC	Gene ontology cellular component
GOMF	Gene ontology molecular function
h	Hours
HCl	Hydrochloride
IA	Invasive aspergillosis
IAA	Iodoacetamide
Ig	Immunoglobulin

Kg	Kilogram
L	Litre
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LFQ	Label-free quantitative
M	Molar
MATE	Multi-drug and toxic compound extrusion
MDR	Multidrug resistance
MDR1	Multidrug resistance 1
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
mg	Milligram
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
mM	Millimolar
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSCRAMM	Microbial surface component recognising adhesive matrix molecules
MSSA	Methicillin-sensitive *S. aureus*
NADH	Nicotinamide adenine dinucleotide (NAD) and hydrogen (H)
ng	Nanogram
NHC	*N*-heterocyclic carbene
nl	Nanolitre
nm	Nanometre
ns	Not significant
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
Papain-like protease	PL^{pro}
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCA	Principal component analysis
PMSF	Phenylmethylsulfonyl fluoride
PQS	*Pseudomonas* quinolone signal
PRIDE	PRoteomics IDEntifications Database
QS	Quorum-sensing
RIA	Reductive iron assimilation
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
ROS	Reactive oxygen species

rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
S.E.	Standard error
SBC3	1,3-dibenzyl-4,5-diphenylimidazol-2-ylidene silver(I) acetate
SD	Standard deviation
Sec	Seconds
SEM	Scanning electron microscopy
SIT	Siderophore-Iron-Transporter
SMR	Small multidrug resistance
SSDA	Statistically significant and differentially abundant
STRING	Search Tool for the Retrieval of INteracting Genes
T1SS	Type 1 secretion system
T2SS	Type 2 secretion system
T3SS	Type 3 secretion system
T6SS	Type 6 secretion system
TAFC	Triacetylfusarinine C
TCA	Tricarboxylic acid
TFA	Trifluoroacetic acid
TLCK	Tosyl-L-lysine chloromethyl ketone
tRNA	Transfer ribonucleic acid
TTC	Triphenyltetrazolium chloride
USP	Universal stress protein
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization
YEPD	Yeast extract peptone dextrose
β	Beta
µg	Microgram
µl	Microlitre
µM	Micromolar

Abstract

The global threat of antimicrobial resistance (AMR) is steadily on the rise jeopardising the efficacy of conventional antimicrobial agents. This crisis is driven by the inappropriate use of antimicrobials and decline in novel drug discovery. The most threatening pathogens have developed resistance to multiple drug classes and demand particular attention. Part of the ESKAPE pathogen list, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are major causes of severe nosocomial infection and frequent colonisers of cystic fibrosis patient lungs. Moreover, *Candida* and *Aspergillus* spp. contribute to the vast majority of fungal infections and exacerbate morbidity and mortality in the immunocompromised.

Metal ions have a long history of antimicrobial use however, the advent of AMR and the desire for more innovative strategies have revived interest. Metal ions possess unique (and multiple) modes of action. In addition, the diverse physiochemical properties of metal-based complexes (e.g. coordination number, geometry, reactivity and type and number of ligands) enables the production of vast libraries of agents with differing properties. Indeed, the design, synthesis, characterisation and microbiological assessment relies upon an interdisciplinary approach in the fields of inorganic chemistry and biology. This project conducted microbiological assessment of novel gallium(III)- and silver(I)-based agents whereby drug candidates were screened against a range of bacterial and fungal pathogens. The most susceptible of which were subjected to label-free quantitative proteomic analysis to uncover the likely mechanistic roles.

The iron-mimicking capabilities of gallium and resulting disruption in iron ion homeostasis has led to the generation of therapeutic formulations including gallium maltolate (GaM). Anti-*Pseudomonal* assessment *in vitro* translated *in vivo* using *Galleria mellonella* as an insect model whilst subsequent proteomic studies revealed a number of affected pathways which would contribute to the attenuated growth and virulence of the pathogen. The poor bioavailability of gallium compounds prompted the development of water-soluble heteroleptic Ga(III) polypyridyl compounds ($[Ga(bipy)_2(2,3\text{-}DHBA_{-2H})](NO_3)$ (**1**), $[Ga(bipy)_2(3,4\text{-}DHBA_{-2H})](NO_3)$ (**2**) and $[Ga(bipy)_2(2,3,4\text{-}THBA_{-2H})](NO_3)$ (**3**)) bearing a catecholate moiety. These siderophore conjugates, which aimed to enhance gallium uptake, were evaluated

against *Aspergillus fumigatus*. Proteomic and biochemical analyses revealed the superior activity of these novel agents to existing gallium nitrate and effect on mitochondria.

Subsequent work analysed the antimicrobial activity of the silver(I) *N*-heterocyclic carbene (NHC) complex, 1,3-dibenzyl-4,5-diphenylimidazol-2-ylidene (NHC*), termed SBC3. Alterations in the *Candida parapsilosis* proteome revealed a significant decrease in protein synthesis and directed further studies on the anti-virulence capacities of SBC3 in terms of cell adhesion, biofilm formation and cell morphology. Possessing broad-spectrum activity, SBC3 was also examined against two structurally different bacteria: *P. aeruginosa* (Gram-negative) and *S. aureus* (Gram-positive). Both were susceptible to SBC3 treatment however, proteomic findings identified distinct variations in affected proteins and associated pathways.

The results presented in this thesis offer novel insights on the activity of these selected metal complexes for the treatment of resistant microbial pathogens. Proteomic analysis is a useful tool in drug discovery and development, which is key in addressing escalating rates of AMR.

Chapter 1

Introduction

1.1 General introduction

Prior to the 20th century, infectious diseases such as cholera, diphtheria, small pox, tuberculosis, typhoid fever etc., were among the leading causes of mortality among humans, with life expectancy averaged at 47 years of age (Adedeji, 2016). The discovery of penicillin in 1928 revolutionised modern medicine, significantly reducing morbidity and mortality and increasing life expectancy by about 23 years (Hutchings, Truman & Wilkinson, 2019). Sir Alexander Fleming pioneered the antibiotic era and triggered the discovery and development of more potent antibiotics for the treatment of bacterial infections (Lobanovska & Pilla, 2017). The period between 1950-1970 marked the golden era of antibiotic discovery with the introduction of approximately 20 novel antibiotic classes. The vast majority served the public for 60 years (Dhingra et al., 2020). The rampant use and misuse has since encouraged the development of drug-resistant bacterial isolates and jeopardised the efficacy of these agents (Lobanovska & Pilla, 2017; Llor & Bjerrum, 2014). The fight against microbial pathogens has worsened with increasing rates of immunosuppressed individuals suffering from HIV/AIDS, cancers, and those undergoing immunosuppressive therapies such as chemotherapy and organ transplants. In this regard, microbes which do not typically pose a threat to healthy individuals become opportunistic and life-threatening to vulnerable individuals (Kauffman, 2001; Yusuf, Sampath & Umar, 2023; Riccardi, Rotulo & Castagnola, 2019).

Furthermore, the broad-spectrum use of antibiotics in combination with medical advances have promoted increased incidence of fungal infections since the 1940s (Homei & Worboys, 2013). The elimination of bacterial competitors creates opportunity for fungal pathogens to reproduce and thrive. For example, it is believed that the disruption of the host microbiota and resulting increased resource availability can prompt invasive *Candida* growth leading to candidiasis (Homei & Worboys, 2013; Tan et al., 2021). The surge in fungal infections also coincided with the onset of HIV/AIDS, chemotherapy and organ transplantation in the 1980s (Fisher-Hoch & Hutwagner, 1995).

Griseofulvin was first isolated in 1939 and commercially produced for dermatophyte infections in 1959. This was followed by the introduction of the polyene amphotericin B, and flucytosine in the 1960s (Sheehan, Hitchcock & Sibley, 1999; Bennett, 2009).

The latter was developed for cryptococcal infections however, it was mainly used in combination with polyenes due to high levels of monotherapy resistance. In the 1970s, azoles became a first-line therapy for invasive infections and remain a fundamental option for broad-spectrum use. The echinocandins were the last group of antifungal agents developed for clinical use and were introduced in the early 2000s. The low levels of toxicity of echinocandins make them a desirable choice for fist line-therapy for invasive candidiasis (Perfect, 2017).

The management of fungal, and indeed bacterial, infections is threatened by the growing rates of antimicrobial resistance (AMR). It is estimated that over 1.2 million deaths as a result of bacterial AMR occurred globally in 2019, exceeding HIV/AIDS and malaria deaths, and AMR-related deaths are predicted to increase to about 10 million by 2050 (Tang, Millar & Moore, 2023; Thompson, 2022). Moreover, this has significant financial ramifications with an estimated $55 billion already being spent on healthcare and loss of productivity associated with AMR in the United States alone (Dadgostar, 2019). There is therefore a critical need to replenish the antimicrobial pipeline with novel and/or repurposed therapies.

1.2 Multidrug resistant bacterial pathogens

The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) employ a range of resistance mechanisms broadly categorised as (1) drug target site modification, (2) enhanced drug detoxification/efflux, (3) alterations in membrane permeability and (4) enzymatic drug degradation (Santajit & Indrawattana, 2016). The diminished efficacy of first-line and last resort therapies pose great challenges in the treatment of infections. These pathogens can contribute to high morbidity and mortality rates in nosocomial settings and community-acquired infections from environmental reservoirs such as wastewater, surface water, soils, and contaminated food (Denissen et al., 2022; Masoud et al., 2022). The World Health Organization (WHO) has listed the ESKAPE pathogens among 12 priority bacteria for which new antibiotics are critically needed (**Table 1.1**) (Asokan et al., 2019).

Table 1.1. List of bacterial priority pathogens adapted from the WHO catalogue of pathogens (2017) that pose the greatest risks to human health. The objectives are to raise awareness on AMR and promote the research and development of novel antimicrobials (Organization, 2017).

Priority 1: Critical	Priority 2: High	Priority 3: Medium
Acinetobacter baumannii	*Enterococcus faecium*	*Streptococcus pneumoniae*
Pseudomonas aeruginosa	*Staphylococcus aureus*	*Haemophilus influenzae*
Enterobacteriaceae	*Helicobacter pylori*	*Shigella* spp.
	Campylobacter spp.	
	Salmonellae	
	Neisseria gonorrhoeae	

1.2.1 *Pseudomonas aeruginosa*

Over 140 species belong to the *Pseudomonas* genus with most being saprophytic living in soil, vegetation and aquatic environments. About 25 species are linked to human colonisation although the majority of infections are opportunistic. The Gram-negative, rod-shaped bacterium *P. aeruginosa* is one of the most clinically relevant and frequently isolated pseudomonads recovered from clinical samples (about 80% in combination with *Pseudomonas maltophilia*) (Iglewski, 1996). *P. aeruginosa* has the capacity to infect plants and animals (Ambreetha & Balachandar, 2022). Susceptibility to infection often coincides with the use of invasive medical devices (catheter-associated urinary tract infections and ventilator-associated pneumonia) and/or predisposing conditions (cystic fibrosis (CF), HIV/AIDS and skin wounds) (Bassetti et al., 2018). The moisture, nutrients and structural damage within the CF lung provide a conducive environment for bacteria to thrive and therefore, *P. aeruginosa* is one of the most prevalent and serious pathogens isolated from patient lungs (Bhagirath et al., 2016). Environmental adaptation and the production of virulence factors contribute to chronic infection thus accelerating the deterioration of pulmonary function that leads to poor or fatal patient outcomes (Jurado-Martín, Sainz-Mejías & McClean, 2021).

1.2.2 *P. aeruginosa* virulence factors and resistance

The large *P. aeruginosa* genome (5.5 – 7 Mbp) comprises of an invariable "core genome" and a highly variable "accessory genome" that makes up approximately 10% of the former and is strain specific (Klockgether et al., 2011; Ozer, Allen & Hauser, 2014). Elements within the accessory genome encode resistance and virulence traits that enable rapid adaptation to varied and hostile ecological niches such as those with limited nutrient availability and drug-induced toxicity (Ozer, Allen & Hauser, 2014). This allows *P. aeruginosa* to outcompete and occupy environments inhospitable to other microorganisms. For instance, *P. aeruginosa* can survive in temperatures ranging from 4° - 42°C, distinguishing it from other pseudomonads and it is also a facultative anaerobe, capable of growing in oxygen-depleted environments and utilising nitrates or other oxidised forms of nitrogen (Iglewski, 1996).

P. aeruginosa is a model organism for virulence and cell communication studies (Diggle & Whiteley, 2020). Virulence factors can be divided into three categories based on: (1) cell structure, (2) secreted factors and (3) cell-to-cell communication systems (quorum-sensing (QS)) (Liao et al., 2022). The latter coordinates the expression of hundreds of virulence-encoding genes by sensing changing environmental conditions such as pH, osmolarity, nutrient supply and cell density (Smith & Iglewski, 2003). QS in *P. aeruginosa* relies on two systems that use acyl-homoserine lactone signals (*las* and *rhl*) and a third system (*pqs*) that uses quinolone signals (Smith & Iglewski, 2003; Wilder, Diggle & Schuster, 2011).

P. aeruginosa is currently known to employ six secretion systems (Type 1 secretion system (T1SS) to Type 6 secretion system (T6SS)) for the transport of bacterial molecules to external environments or delivery into host cells (Qin et al., 2022). The Type 3 secretion system (T3SS) is one of the most complex systems which facilitates host tissue invasion and damage by utilising a syringe-like apparatus to inject effector toxins into host cells to subvert the immune response and disrupt host cell signalling processes (Coburn, Sekirov & Finlay, 2007). These four effector proteins - ExoU, ExoY, and ExoT and ExoS possess specific virulence functions which include induction of necrotic cytotoxicity, disruption of barrier integrity, inhibition of cytokinesis, and induction of apoptosis of eukaryotic cells, respectively (Kaminski et al., 2018). The Type 2 secretion system (T2SS) secreted exotoxin A, is a potent toxin

and inhibitor of protein synthesis and immune system interference, along with proteases (Protease IV), elastases (LasA and LasB), lipases (LipA and LipC), phospholipases (phospholipase C) and pyocyanin for host tissue injury (Pollack, 1983; Qin et al., 2022).

A wide variety of microbes must acquire iron to sustain growth and survival (Cherayil, 2011). *P. aeruginosa* uses a number of iron acquisition mechanisms including the production of two virulent siderophores: pyochelin and pyoverdine for iron uptake in limiting conditions. These iron-chelating molecules permit iron retrieval from host-sequestered sources like lactoferrin or transferrin. Alternative modes involve the uptake and use of xenosiderophores produced by other organisms, the uptake of heme from host hemoproteins and lastly, under anaerobic (or microaerobic conditions), ferric iron (3^+ oxidation state) is reduced extracellularly to ferrous iron (2^+ oxidation state) via phenazine compounds as part of the Feo system. Soluble ferrous iron is transported into the cell cytoplasm by the FeOABC transport system that includes the FeoB permease and FeoA and FeoC proteins (Cornelis & Dingemans, 2013).

The motility of *P. aeruginosa* permits dissemination from the infection site and adaptation to diverse environments (Arora et al., 2005; Sultan, Arya & Kim, 2021). It is driven by its appendages – a single flagellum and multiple pili. Fibre-like motorised type IV pili permit swarming and twitching motilities and adhesion to surfaces. These are key aspects for biofilm formation and movement towards favourable substrates (Feldman et al., 1998). Moreover, *P. aeruginosa* can grow as planktonic or sessile cells. The latter consists of cellular aggregates encapsulated in a ‘sticky’ matrix defined as biofilm which protects against hostile environments and aids with nutrient acquisition and exchange of genetic material (Tuon et al., 2022). Various components comprise the biofilm matrix including extracellular DNA, proteins and lipids and exopolysaccharides such as Psl, Pel, which are involved with initial surface attachment of non-mucoid phenotypes, and alginate which contributes to the mucoid phenotype, biofilm maturation and antibiotic resistance (Thi, Wibowo & Rehm, 2020).

Biofilms are a form of adaptive resistance whereby gene expression is modified in response to an external stimulus. The biofilm matrix acts as an impenetrable barrier to antibiotics however, this form of resistance is transient whereby it becomes deactivated upon stress removal (Langendonk, Neill & Fothergill, 2021; Donlan, 2000). Intrinsic

resistance attributes however, are encoded within the core genome and include low outer membrane permeability, expression of efflux pumps and the production of antibiotic-inactivating enzymes such as β-lactamases and aminoglycoside-modifying enzymes (Pang et al., 2019). Acquired resistance occurs through mutations or the horizontal acquisition of resistance genes via plasmids, transposons integrons and prophages (Pang et al., 2019).

1.2.3 Conventional treatment of *P. aeruginosa*

Aminoglycosides (e.g. amikacin, gentamicin and tobramycin) bind to the amino-acyl tRNAsite of the 30S ribosomal subunit inhibiting protein synthesis (O'Sullivan et al., 2018). Part of the β-lactam class, carbapenems (imipenem, meropenem, and doripenem) gain access via porins and bind to the peptidase domain of penicillin binding proteins (PBPs) preventing transpeptidation and thus damaging the structural integrity of the cell wall (Armstrong, Fenn & Hardie, 2021). Cephalosporins (ceftazidime, cefepime) and monobactams (aztreonam) work in a similar manner (Klein & Cunha, 1995). Fluoroquinolones (ciprofloxacin, levofloxacin) target two type II topoisomerases required for DNA replication (Fàbrega et al., 2009). The intrinsic and acquired resistance mechanisms in *P. aeruginosa* can overcome antibiotic efficacy, including to last-resort carbapenem treatment. Understanding the underlying molecular mechanisms of resistance is imperative for the development of novel therapeutic alternatives, and are summarised in section **1.2.7**. Clinical applications often favour synergistic combination therapy, particularly antibiotics of differing classes and modes of action, to maximise infection control and patient clinical outcome (Yadav et al., 2017; Torella, Chait & Kishony, 2010).

1.2.4 *Staphylococcus aureus*

S. aureus is a Gram-positive bacterium and commensal of the skin and mucosal membranes of healthy individuals (Jenkins et al., 2015). It is estimated to colonise the anterior snares of approximately 20 – 80% of the population (Brown et al., 2013). Its opportunistic nature enables immune system evasion, entry into the bloodstream and invasion of internal tissues to cause life-threatening bacteraemia and endocarditis, along with osteomyelitis and skin and soft tissue infections (abscesses, cellulitis, folliculitis, impetigo etc) (Tong et al., 2015). Production of a multitude of virulence

factors, immune evasion factors and resistance have hindered treatment. Methicillin-resistance (methicillin-resistant *S. aureus* (MRSA)) is a community- and hospital-based infection that has rendered an entire class of antibiotics (β-lactams) ineffective (Katayama, Zhang & Chambers, 2004). MRSA first emerged in the 1960s but rapidly spread in the 1990s to become one of the leading nosocomial pathogens increasing patient morbidity and mortality and duration of hospitalisation. Osteomyelitis, meningitis, pneumonia and empyema are all associated invasive infections (Turner et al., 2019).

1.2.5 *S. aureus* virulence factors

S. aureus pathogenesis is facilitated by a range of toxin and immune evasion virulence factors. The production of factors such as hemolysins, leukocidins and protein A are controlled by the *S. aureus* QS *agr* system (Le & Otto, 2015).

Microbial surface component recognising adhesive matrix molecules (MSCRAMM) are the largest class of surface proteins that function in adhesion, biofilm formation, immune evasion and, host cell and tissue invasion. Among them is the IgG-binding Staphylococcal protein A (SpA) that inhibits phagocytosis and anti-staphylococcal immunity whilst extracellular adherence protein (Eap) prevents neutrophil recruitment to the target site (de Jong, van Kessel & van Strijp, 2019). Other proteins include Clumping Factor A and B (ClfA and ClfB) for fibrinogen adhesion, Collagen-binding protein (Cna) for collagen-rich tissue binding, Serine aspartate repeat protein (SdrC) and Fibronectin binding proteins A and B for biofilm formation. Secreted coagulase, proteases (such as aureolysin, staphopain A and B, and V8 protease, for example), staphylokinase enzymes along with cytolytic toxins (hemolysins, leukotoxin, enterotoxin, toxic-shock syndrome toxin-1 etc) further aid in host cell/tissue damage and invasion and immune defence. Toxic-shock syndrome toxin-1 can bind to antigen-presenting cells leading to T-cell activation and uncontrolled cytokine production. Susceptible hosts can therefore be at risk of toxic shock syndrome and death (Foster, 2019a; Martí et al., 2010; Ohbayashi et al., 2011). Moreover, cell lysis enables iron retrieval to aid bacterial growth (Kong, Neoh & Nathan, 2016). *S. aureus* produces an extensive number of these disease-promoting virulence factors which have previously been studied in detail (de Jong, van Kessel & van Strijp, 2019; Foster, 2019a).

1.2.6 Conventional treatment of *S. aureus*

β-lactam antibiotics covalently bind to PBPs as their target site and prevent these enzymes from carrying out the terminal steps of peptidoglycan cross-linking in Gram-positive and -negative species (Mora-Ochomogo & Lohans, 2021). β-lactams remain a key option for the treatment of methicillin-sensitive *S. aureus* (MSSA) infections, although use has declined because of rapid MRSA spread (Foster, 2019b). The destruction of β-lactam antibiotics is induced by β-lactamase enzymes produced by *S. aureus*. Co-administration with β-lactamase inhibitors such as clavulanic acid, tazobactam or sulbactam however, has restored the efficacy of β-lactams to an extent (Castle, 2007). For more severe MRSA bacteraemia, vancomycin is a gold-standard therapy and also works by inhibiting cell wall biosynthesis (McGuinness, Malachowa & DeLeo, 2017). Teicoplanin is another alternative with similar efficacy for bacteraemia and bone and joint infections and has greater tolerability (Ferry et al., 2016). Linezolid adopts a different mode of action through ribosomal protein synthesis disruption. Pneumonia and skin and soft tissue infections are often treated with linezolid which demonstrates high oral bioavailability and suitability for intravenous use (Rasmussen et al., 2011). Daptomycin is a lipopeptide antibiotic with a unique mode of action for disrupting cell membrane potential and is also suitable for complicated skin infections and endocarditis caused by MSSA and MRSA (Eisenstein, 2007; Steenbergen et al., 2005).

1.2.7 Bacterial resistance mechanisms

AMR is driven by repeated exposure to antimicrobials whereby bacteria have developed highly specialised resistance mechanisms to counteract noxious environments. The three fundamental mechanisms of resistance involve (1) bacterial enzymes that chemically alter or inactive an antibiotic, (2) mutations of antibiotic binding sites and (3) cell membrane interactions that regulate the passage of substances in and out of the cell, or a combination of these to achieve high levels of protection (**Figure 1.1**). Resistance mechanisms however, can be specific to a drug and bacterial type based on cellular structure (i.e. Gram-positive or Gram-negative).

Enzymatic degradation or alteration of antibiotics sterically hinder the drug and weaken its affinity to the target binding site. This can occur through hydrolysis, a

functional group transfer within the antibiotic molecule or redox mechanisms (Wright, 2005). The β-lactam ring, consisting of an amide bond, is fundamental to the activity of β-lactam drugs. β-lactamase enzymes hydrolyse this functional site to confer resistance to penicillin and cephalosporins (Tooke et al., 2019). A similar approach is used against macrolide antibiotics such as erythromycin whereby esterase enzymes hydrolyse the macrolactone ring (Morar et al., 2012). Transferases, on the other hand, modify the antibiotic by a group transfer that consequently restricts antibiotic target site binding (Egorov, Ulyashova & Rubtsova, 2018). The chemical approaches to modify structure include glycosylation, acylation, phosphorylation and thiol group transfer (Wright, 2005).

High-level fluoroquinolone resistance is often linked with mutations of genes that encode primary and secondary drug targets (Redgrave et al., 2014). Broad-spectrum quinolone drugs target topoisomerase IV and DNA gyrase enzymes crucial for DNA replication, transcription, recombination and repair (Jacoby, 2005). *GyrA* and *gyrB* encode two A the subunits of DNA gyrase and *parC* and *parE* encode the B subunits of topoisomerase IV. Mutations of *gyrA* or *parC* contribute to fluoroquinolone resistance while mutations of *gyrB* and *parE* seldom occur (Piddock, 1998). Double DNA gyrase and topoisomerase IV mutations give rise to highly resistant isolates (Hooper & Jacoby, 2016).

A distinct characteristic of Gram-negative bacteria is the presence of an outer membrane which provides added protection against antibiotics without impeding exchange of nutrients (Delcour, 2009). Porins are transmembrane proteins with a β-barrel structure that form water-filled open channels to permit the diffusion of hydrophilic antibiotics (Choi & Lee, 2019). Mutations that result in porin loss, reduced abundance or alteration alter membrane permeability, impede entry of β-lactams and fluoroquinolone (Fernández & Hancock, 2012). Acquired resistance to these drug classes in *P. aeruginosa* is due to low outer membrane permeability (Kapoor, Saigal & Elongavan, 2017).

Efflux systems found in Gram-positive and -negative bacteria selectively expel toxic substrates from the cytoplasm. Protein channels may be specific to one or several drug types that vary structurally thus conferring multidrug resistance (Handzlik, Matys & Kieć-Kononowicz, 2013). Mutations lead to the overexpression of efflux pumps and

the selection of which is categorised based on their structure and energy source (Anes et al., 2015). Resistance-nodulation-division (RND) superfamily members transport metal ions, xenobiotics and drugs among others through active transport. The ability to associate with multiple drug classes is carried out by small multidrug resistance (SMR) pumps. In conjunction with major facilitator superfamily (MFS) and multi-drug and toxic compound extrusion (MATE) pumps, they all require a proton gradient to expel toxins. Alternatively, ATP-binding cassette (ABC) proteins acquire energy from ATP hydrolysis (Barabote et al., 2010; Delmar, Su & Yu, 2015)

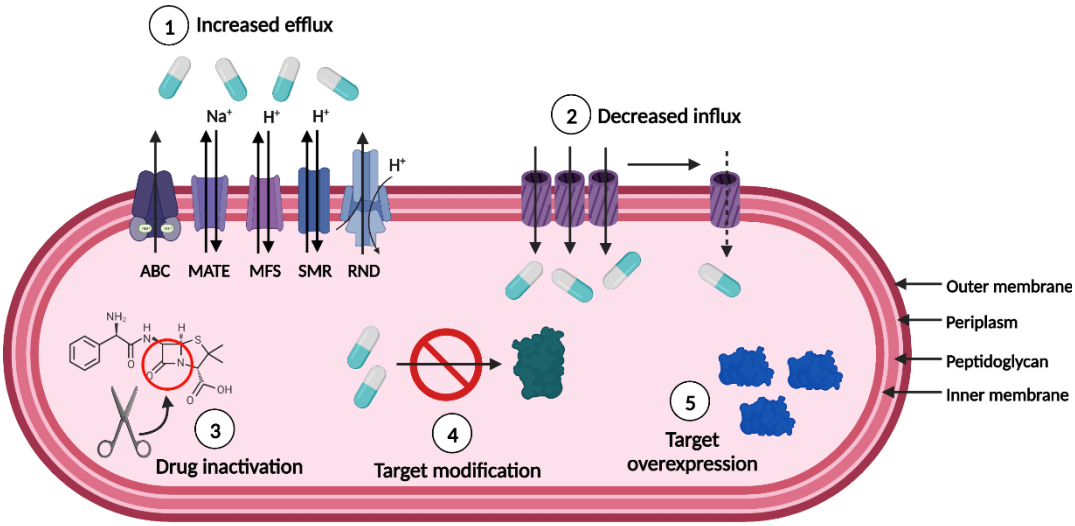

Figure 1.1. Antibiotic resistance mechanisms in bacteria. Multidrug resistant bacteria can employ one or more of the following mechanisms: (1) efflux pumps to expel solutes out of the cell. (2) Porins provide entry of small hydrophilic antibiotics via the outer membrane into the periplasm in Gram-negative bacteria. (3) Inactivation of antibiotics is carried out by enzymes that degrade or modify the antibiotic structure. (4) Mutations of genes that encode the target protein or enzymatic alterations of the target site inhibit antibiotic binding and function. (5) Overexpression of genes encoding a drug target elevates antibiotic MIC, thereby diminishing drug efficacy. Adapted image from (Darby et al., 2023; Piddock, 2006).

1.3 Multidrug resistant fungal pathogens

Fungi colonise many diverse habitats and therefore must compete against their microbial competitors in the form of chemical defence (Hyde et al., 2019). The resulting production of secondary metabolites has led to the isolation of therapeutic agents with anticancer, immunosuppressive, hypercholesterolemic and antimicrobial properties (Künzler, 2018; Hyde et al., 2019; Manzoni & Rollini, 2002). The fungal

primary metabolism has also been exploited for the production of an abundance of agricultural, cosmetic and industrial commodities (Dowd & Kelley, 2011; Hyde et al., 2019). As eukaryotes, fungi are attractive model organisms for the study of cellular processes in mammals. Almost a quarter of yeast genes are homologous to humans and so have been employed for genetic, developmental and pathological studies with easier, faster and cheaper cultivation and generation of results (Liu et al., 2017b). On the other hand, fungi can be serious pathogens of humans and are responsible for an estimated 1.6 million deaths due to infections annually, a number likely to increase with continued use of antibiotics and immunosuppressive therapies, and the emergence of drug-resistant isolates (Fausto, Rodrigues & Coelho, 2019; Low & Rotstein, 2011). **Table 1.2** lists the most critically important fungal pathogens as deemed by the WHO in 2022 (Parums, 2022). Immunocompromised individuals are particularly susceptible to infections that can range from (recalcitrant) superficial infections to life-threatening systemic infections (Odom, 1994). Although *Candida* species are more frequently isolated in ICU patients, mortality rates are exacerbated by the filamentous fungus, *Aspergillus fumigatus*, which according to the Centers for Disease Control and Prevention (CDC), mortality exceeds 50% in invasive aspergillosis patients (Turner & Butler, 2014; Beer et al., 2018). Similarities between fungi and host cells have hindered antifungal drug development due to limitations in drug selectivity (Mazu et al., 2016). Consequently, there are limited treatment options to combat the increasing demand for novel and/or repurposed therapies. Additionally, phytopathogenic fungi pose a serious threat to crop productivity resulting in an estimated global loss of 200 billion dollars yearly (Villamizar-Gallardo, Osma & Ortíz-Rodriguez, 2019).

Table 1.2. The WHO list of priority fungal pathogens (2022) (Parums, 2022).

Priority 1: Critical	Priority 2: High	Priority 3: Medium
Cryptococcus neoformans	*Candida glabrata*	*Scedosporium* spp.
Candida auris	*Histoplasma* spp.	*Lomentospora prolificans*
Aspergillus fumigatus	*Eumycetoma* causative agents	*Coccidioides* spp.
Candida albicans	*Mucorales*	*Candida krusei*
	Fusarium spp.	*Cryptococcus gattii*
	Candida tropicalis	*Talaromyces marneffei*
	Candida parapsilosis	*Pneumocystis jirovecii*
		Paracoccidioides spp.

1.3.1 *Candida* species

Fifteen *Candida* species contribute to invasive candidiasis, however, *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* account for over 90% of infections and are all included in the WHO's list of priority fungal pathogens (Parslow & Thornton, 2022; Fisher & Denning, 2023) (**Table 1.2**). *C. albicans* accounts for 65% of candidiasis cases and although it remains the most common cause, infections are declining in comparison with the incidence of infections due to non-*albicans* species. This coincides with the rapid emergence of *Candida auris*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis* as significant pathogens in recent decades (Turner & Butler, 2014; Egger et al., 2022).

1.3.2 *Candida* and candidiasis

Candidiasis broadly refers to mycoses caused by *Candida* species and can arise in three forms: cutaneous, mucosal and systemic (Nurdin et al., 2021; Raska et al., 2008). *Candida* yeasts are commensals of the human mycobiome however, dysbiosis induced by antibiotics and lifestyle habits such as diet (e.g. poor nutrition or high sugar intake) and hygiene can trigger superficial infections like intertrigo, balanitis, paronychia and oropharyngeal, vulvovaginal and interdigital candidiasis (Hrncir, 2022; Metin, Dilek & Bilgili, 2018). Vulvovaginal candidiasis is a common pathology affecting about

70% of women at least once in their lifetime and 8% experiencing recurrent infection. *C. albicans* is responsible for about 90% of infections with non-*albicans* species such as *C. glabrata*, *C. krusei* and *C. parapsilosis* also identified (Nyirjesy, Alexander & Weitz, 2005; Jeanmonod & Jeanmonod, 2019; Güzel et al., 2013). Superficial infections are easily managed with topical antifungals (nystatin, clotrimazole, amphotericin B or miconazole) in immunocompetent hosts but can become recurrent or progress to severe systemic infection in the immunocompromised. Candidemia is an invasive form of infection present in the blood that can lead to deep-seated tissue infections in vital organs. Symptoms can present as sepsis and septic shock and end-organ damage (Mora Carpio & Climaco, 2020).

1.3.3 *Candida parapsilosis*

C. parapsilosis was once deemed non-pathogenic despite a fatal case of endocarditis in 1940 (Trofa, Gácser & Nosanchuk, 2008). Incidences of infections due to this pathogen have surged in the past three decades and in some cases, surpassing nosocomial infections of *C. albicans* (Chow, Linden & Bliss, 2012). *C. parapsilosis* is a commensal of human skin and the gastrointestinal tract, and disease most predominantly occurs in immunocompromised individuals particularly critically-ill and premature neonates, along with AIDS, cancer and catheter-related healthcare. The latter is a typical route of transmission along with the hands of healthcare workers (Trofa, Gácser & Nosanchuk, 2008).

Candida yeasts grow in either yeast (round cells) or filamentous forms (elongated and attached cells). Hyphae and pseudohyphae are filamentous morphologies however, unlike *C. albicans* and *Candida dubliniensis*, *C. parapsilosis* cannot produce true hyphal forms. Biofilms therefore comprise a basal layer of yeast cells and pseudohyphae encapsulated by an extracellular matrix (ECM) (Holland et al., 2014). Yeast forms are presented as a smooth and/or crater colony phenotypes whilst pseudohyphal forms are described as crepe and/or concentric (Branco, Miranda & Rodrigues, 2023). *C. parapsilosis* is incapable of undergoing opaque-white switching. Shifts between these two cell types alter the expression of genes related to adhesion, metabolism and mating behaviours to suit different environments. *C. parapsilosis* does not undergo mating and reproduces asexually (West et al., 2021; Bennett, 2010).

1.3.4 *C. parapsilosis* virulence factors

Virulence factors facilitate initial colonisation and the spread of *Candida* infection. They vary depending on the type of species, infection type and site (Saiprom et al., 2023). *C. parapsilosis* has an excellent capacity to adhere to biotic (host cells/tissues especially mucosal) and abiotic (medical and prosthetic devices) surfaces. The fungal cell wall offers strength and protection from environmental stressors and also functions in host-pathogen interactions including adhesion, biofilm formation, host immune recognition and evasion (Gong et al., 2023). The agglutinin-like-sequence (ALS) genes encode a family of adhesin proteins for adhesion to surfaces or other fungal cells. These glycosylphosphatidylinositol-linked glycoproteins bind to human fibronectin, vitronectin, laminin, plasminogen and kininogen along with N-cadherin to facilitate endocytosis by endothelial cells. The ALS proteins are best characterised in *C. albicans* although the roles of CpALS7 in *C. parapsilosis* have been investigated (Neale et al., 2021; Kozik et al., 2015).

Biofilm formation succeeds successful adhesion and is governed by the hydrophobicity, texture, quality and quantity of available nutrients and the expression of adhesins. Attached yeast cells develop into microcolonies and undergo dimorphic transition from round yeast cells to pseudohyphal forms. Mature biofilms produce an ECM made of polysaccharides, lipids, proteins, minerals and nucleic acids and further aid microbial adhesion and cohesion. Treatment of biofilms is problematic due to reduced antifungal penetration and host immune susceptibility (Al-Fattani & Douglas, 2004).

Candida species secrete an array of hydrolytic enzymes to enable destruction of physical barriers and invade a host (Branco, Miranda & Rodrigues, 2023). The host epithelial or endothelial cell layer is strengthened with ECM and must be impaired (Tóth et al., 2019). Aspartic proteinases disrupt mucosal membranes and destroy structural and immunological proteins like collagen and fibronectin and immunoglobulin G and C3 protein. Phospholipases cleave ester linkages in glycerophospholipids and are believed to impact cell membranes whilst lipases are employed in lipid digestion for nutrient uptake, host tissue damage and destruction of competing microbes. Two lipase genes have been identified in *C. parapsilsosis* – *CpLIP1* and *CpLIP2* (Trofa, Gácser & Nosanchuk, 2008).

1.3.5 *Aspergillus fumigatus*

Aspergillus fumigatus is a filamentous fungus that occupies a number of ecological niches such as soil, air and decaying debris. As a saprophyte it has an important role in carbon and nitrogen cycling in nature (Latgé, 1999). Asexual sporulation permits efficient dispersal of conidia. The presence of melanin in the cell wall yields a distinctive blue-green colour to conidia/hyphae and provides UV irradiation protection (Upadhyay, Torres & Lin, 2013). Not only can conidia tolerate freezing and dehydration, but have excellent dispersal capabilities due to their hydrophobicity (Kwon-Chung & Sugui, 2013). Humans are estimated to inhale several hundred conidia daily and the small size of conidia (2 – 3 μm in diameter) enables mucociliary clearance evasion and access to lung alveoli (Kwon-Chung & Sugui, 2013; Latgé, 1999). The majority of infections are therefore respiratory. Severe infections are rare in immunocompetent individuals where alveolar macrophages eliminate conidia as the first-line innate defence (Beisswenger, Hess & Bals, 2012). *A. fumigatus* is an opportunistic pathogen and the increasing numbers of immunocompromised patients such as CF, organ transplant, chemotherapy and corticosteroid users have increased the prevalence and severity of infection (Latgé, 1999). When left unchecked, conidia can germinate into hyphae and invade and damage host tissues. *A. fumigatus* is one of the most serious respiratory fungal pathogens and systemic infections that contributes to mortality rates of up to 90% in vulnerable patient groups (Dagenais & Keller, 2009).

1.3.6 *A. fumigatus* and aspergillosis

Bronchopulmonary aspergillosis is an umbrella term for a range of *A. fumigatus* infections with the three main being allergic, chronic and invasive aspergillosis (IA), each differing in severity (Patterson et al., 2016). Allergic bronchopulmonary aspergillosis (ABPA), chronic bronchopulmonary aspergillosis (CPA) and aspergilloma generally occur in patients with pre-existing lung conditions (Bongomin et al., 2020; Agarwal et al., 2020). Airway colonisation can induce a hypersensitivity reaction to *A. fumigatus* antigens presented as bronchospasm, bronchiectasis, eosinophilia, mucous plugging and pulmonary infiltrates in ABPA (Ueki et al., 2018; Zeng et al., 2022; Janahi, Rehman & Al-Naimi, 2017) which occurs mainly in asthmatics and CF patients. CPA is a progressive, debilitating disease characterised by the presence of one or more lung cavities, with or without an aspergilloma, or lung

nodules. An aspergilloma is a fungus ball (mycetoma) comprised of hyphae and cellular debris and mucous (Moodley, Pillay & Dheda, 2014). Severely immunocompromised patients are most at risk of IA, having a mortality rate of up to 90% (Deigendesch, Costa Nunez & Stenzel, 2018) Originating in the bronchopulmonary system, IA disseminates via the bloodstream to other organs such as the brain (infarction), heart (endocarditis), kidneys and liver (Kousha, Tadi & Soubani, 2011). *A. fumigatus* species account for about 90% of IA cases (Abad et al., 2010).

1.3.7 *A. fumigatus* virulence factors

Soil and decaying vegetation are the primary habitats of *A. fumigatus*. Morphological and stress-tolerance characteristics enable the fungus to occupy diverse ecological niches that can make it equipped for human colonisation (Latgé & Chamilos, 2020). Moreover, the immune status of a patient and a repertoire of fungal virulence factors govern the shift of *A. fumigatus* from a saprophytic to pathogenic form (Latgé, 1999; Shankar et al., 2018). *A fumigatus* virulence is a multifaceted interaction that fine-tunes metabolism, morphology and stress resistance to withstand hostile environments. Virulence molecules and genes in *A. fumigatus* enable thermotolerance, cell wall maintenance, stress and host immune resistance, the production of toxins, nutrient uptake, metabolism regulation and the production of allergens (Abad et al., 2010).

Optimal growth of *A. fumigatus* occurs at 37°C and pH of 3.7 – 7.6, although it is capable of growing between 12° and 65°C and over the pH range of 2.1 – 8.8. This ability favours the growth of *A. fumigatus* at fever temperature (38°C) in the host and makes *A. fumigatus* a remarkable human pathogen (Latgé & Chamilos, 2020). Successful adherence and penetration of epithelia in the respiratory system however, require adhesins (complement receptor, laminin receptor, hydrophobins) and enzymes (hydrolases, peptidases, phospholipases, and proteases). Antioxidant catalases and superoxide dismutases combating oxidative stress-inducing macrophages during phagocytosis (Latgé & Chamilos, 2020).

A. fumigatus produces a host of secondary metabolites including toxins such as gliotoxin and fumagillin (Gayathri, Akbarsha & Ruckmani, 2020). Mycotoxins are a means of protection against competitors and predators in ecological niches however,

production in a human host can impact DNA, RNA and protein syntheses, and damage the cell membrane, to the detriment of host cell survival (Awuchi et al., 2022). Gliotoxin is one of the most well recognised *A. fumigatus* toxins and displays immunosuppressive properties such as inhibition of B- and T-cell activation, phagocytosis and the respiratory burst and induces leukocyte apoptosis (Coméra et al., 2007). It can also induce oxidative damage via redox cycling and generation of reactive oxygen species (ROS) (Ye et al., 2021). Other toxic molecules are hemolysins and RNase ribotoxin for erythrocyte apoptosis and rRNA gene cleavage and protein biosynthesis inhibition, respectively (Lacadena et al., 2007; Nayak, Green & Beezhold, 2013).

The *A. fumigatus* cell wall is composed of about 90% polysaccharides (glucans, chitins, and galactomannans) and proteins and offers physical protection against osmotic and environmental stressors and a means of host cell adhesion by recognition of extracellular matrix components such as laminin and fibronectin (Abad et al., 2010). The pigment melanin enhances cell strength and rigidity, can resist various toxins and damaging enzymes and also shields against host immunity by scavenging ROS or physically concealing pathogen-associated molecular patterns (PAMPs) from immune recognition. *A. fumigatus* produces two melanin types: dihydroxynaphthalene and pyomelanin (Cordero & Casadevall, 2017).

Adequate nutrient supplies are essential to virulence. Iron is an essential micronutrient for growth, development and antioxidative stress defence. Efficient uptake and storage strategies are employed to meet demands and maintain non-toxic levels (Misslinger et al., 2021). Siderophore-mediated and reductive iron assimilation (RIA) are high-affinity uptake systems. Extra- and intracellular siderophores are small molecule, ferric iron chelators for uptake and storage, respectively. Fusarinine C and triacetylfusarinine scavenge iron and hyphal siderophore ferricrocin and condidial hydroxyferricrocin are responsible for iron storage, with the latter having added roles in germination and oxidative stress resistance (Schrettl et al., 2010). RIA is a three-step process of ferric to ferrous iron reduction using metalloreductases (e.g. FreB), reoxidation of ferrous to ferric iron via the FetC oxidoreductase and finally, cellular uptake of ferric iron (Iron(III))by the FtrA permease (Isidor et al., 2023).

Air-borne conidia and hyphal fragments released by *A. fumigatus* can trigger an IgE-mediated allergic response in the form of *A. fumigatus*-sensitised asthma and ABPA which can be life-threatening (Luo et al., 2020). Asthmatics and CF patients are susceptible to this antigen hypersensitivity, manifested as dyspnea and coughing with expectoration, bronchospasm along with pulmonary infiltrates and eosinophilia (Knutsen & Slavin, 2011; Shah & Panjabi, 2014; Thia & Balfour Lynn, 2009; Matsumoto et al., 2019). Asp f1 – 4 and f6 are major *A. fumigatus* allergens with Aspf1 being the most important. It is secreted during spore germination and early fungal invasion (Luo et al., 2020).

A. fumigatus is equipped with powerful virulence attributes that enable it to persist and enhance morbidity and mortality in infected patients (Nierman et al., 2005).

1.3.8 Conventional treatment of fungal pathogens

Current antifungal drug classes predominately target structural components of the cell i.e. the cell membrane and cell wall. Polyenes bind directly to the cell membrane, azoles inhibit the synthesis of the cell membrane and the most recently developed echinocandins target cell wall synthesis (Hasim & Coleman, 2019). Azoles (e.g. miconazole, clotrimazole, econazole, fluconazole, itraconazole, voriconazole and isavuconazole) target Cyp51 (Erg11), lanosterol 14α-sterol demethylase, which converts lanosterol to ergosterol (Hossain et al., 2022). Depletion of ergosterol production and aggregation of 14-α sterols disrupts both the structure of the plasma membrane and associated functions such as nutrient transport and chitin synthesis. Cell growth is consequently inhibited (Sheehan, Hitchcock & Sibley, 1999). Polyenes (e.g. nystatin and amphotericin B) bind to ergosterol, create aqueous pores causing cellular leakage of ions and ultimate death (Carolus et al., 2020). Polyenes also have some affinity for cholesterol in human cells and so exert nephro- and hepatotoxicities (Birch & Sibley, 2017). Newer lipid-based delivery systems such as AmBisome and amphotericin B lipid complex, developed in the 1990s greatly reduced toxicity (Carolus et al., 2020; Perfect, 2017). These spherical vesicle formulations comprise of an aqueous centre encapsulated by a phospholipid bilayer (Brüggemann, Jensen & Lass-Flörl, 2022). Echinocandins (e.g. caspofungin, micafungin, rezafungin etc) disrupt β-(1,3)-D-glucan synthase thus interfering with cell wall synthesis and inducing cell death (Szymański et al., 2022). The lower toxicity of this drug class

compared to other agents is attributed to its fungal-specific enzyme target which is absent in human cells (Perlin, 2015). Whilst griseofulvin remains in use for tinea capitis and onychomycosis treatment, use has been largely replaced with itraconazole and terbinafine (Bennett et al., 2000). The latter is a squalene epoxidase inhibitor whereby the conversion of squalene to sterols in the ergosterol biosynthetic pathway is impeded (Garaiová et al., 2014). Griseofulvin on the other hand, functions as a mitosis inhibitor by binding to fungal microtubules and affecting the mitotic spindle assembly. It has been repurposed in recent decades for its anti-cancer and antiviral properties (Aris et al., 2022; Vishkautsan, Sykes & Papich, 2021).

The eukaryotic nature of fungi creates challenges in the discovery and development of antifungals with specificity and low toxicity, which is exacerbated with escalating rates of AMR.

1.3.9 Antifungal resistance mechanisms

Antifungal therapy is confined to three major classes of drugs, all of which are associated with host toxicity, limited spectrum of action and pharmacokinetic issues. The advent of AMR has magnified this treatment crisis. Resistance mechanisms fall into five categories: (1) drug target alteration, (2) drug transport alteration, (3) induction of a cellular stress response and (4) the presence of complex multicellular structures (biofilms; **Figure 1.2**) (Lee et al., 2021; Sanglard, Coste & Ferrari, 2009).

Target alterations occur for two drug classes: azoles and echinocandins and is conferred by mutations in the ERG11/CYP51 and FKS1 genes, respectively. Point mutations in the ERG11 gene (which encodes 14α-sterol demethylase) consist of amino acid substitutions that structurally alter the target enzyme and hence, reduce the affinity for an azole (Xiang et al., 2013). Upregulation of ERG11 can also occur, where saturation overshadows azole activity (Sanglard, Coste & Ferrari, 2009). The FKS1 gene encodes a subunit of the β-(1,3)-D-glucan synthase in *C. albicans*. Mutations, which are located in two "hotspot" regions, HS1 and HS2, significantly increase MIC values and reduce glucan synthase sensitivity by 3000-fold in *C. albicans*. Resistance to these agents is most often seen in *Candida*, *Cryptococcus* and *Aspergillus* species (Sah, Hayes & Rustchenko, 2021; Lee et al., 2021).

Upregulation of plasma efflux pumps is frequently responsible for azole resistance and the decrease in effective drug concentration is mediated by several drug transporters. Overexpression of ABC transporters, *Candida* drug resistance 1 (CDR1) and 2 (CDR2) confer fluconazole resistance in *C. albicans* along with multidrug resistance 1 (MDR1) which encodes a membrane protein of the MFS (Wirsching et al., 2000; Jin et al., 2018). *A. fumigatus* ABC transporter genes, *atrF* and *AfuMDR4* are implicated in itraconazole resistance (Sanglard, Coste & Ferrari, 2009).

Biofilms not only provide a protective barrier against physical and chemical stressors in the form of an ECM, but are also linked with community-coordinated gene expression and metabolic cooperation (Ramage et al., 2012). In addition, cells within biofilms are intrinsically drug resistant due to increased drug efflux systems and the presence of highly resistant and metabolically dormant persister cells that are believed to be 1000-fold more resistant than planktonic cells (Ramage et al., 2012; Kaur & Nobile, 2023). Both filamentous and yeast fungi form biofilms however, most microbes exist as part of polymicrobial biofilm communities. The synergies of which contribute to more severe and recalcitrant infection (Costa-Orlandi et al., 2017).

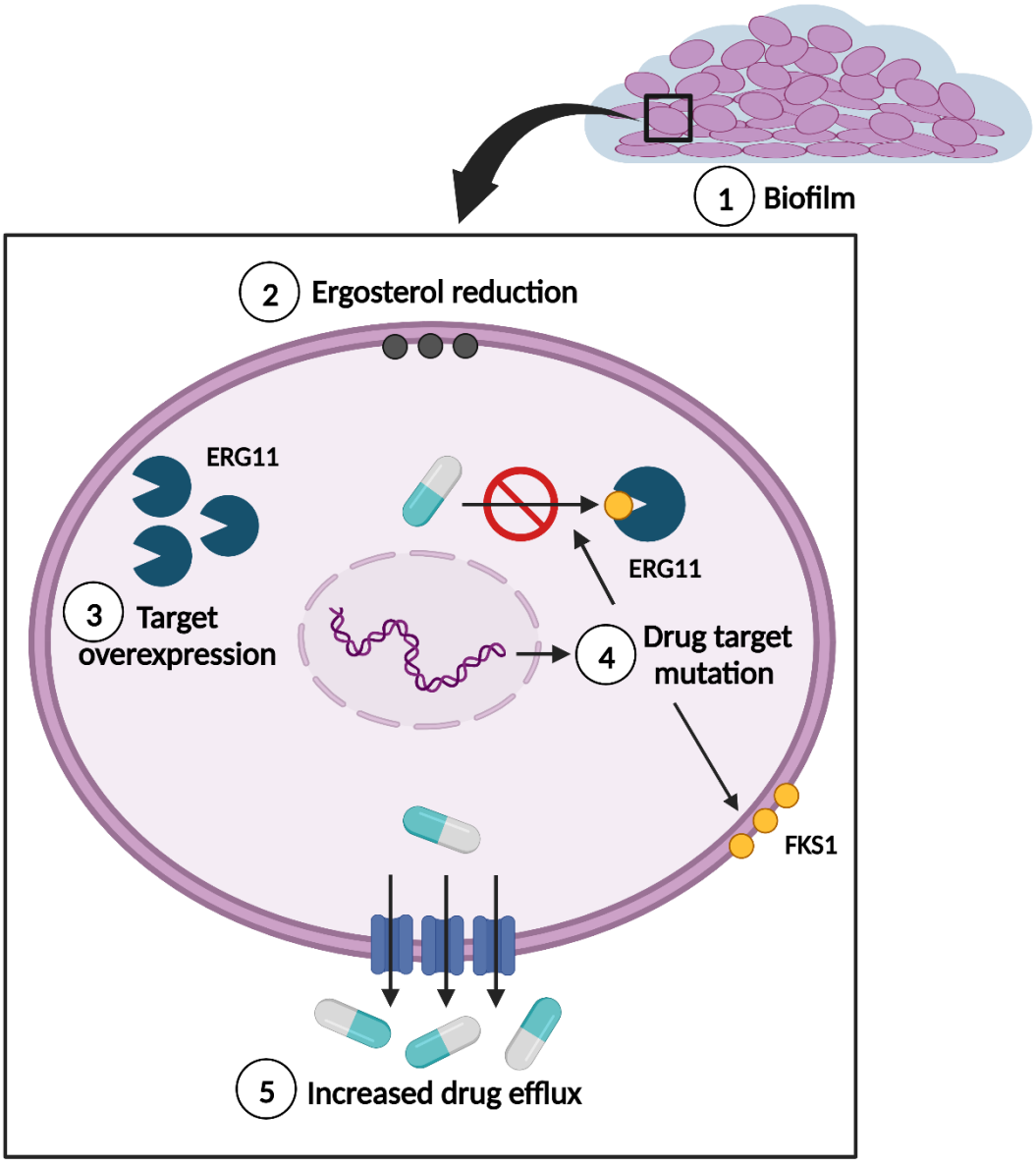

Figure 1.2. Antifungal drug resistance mechanisms. Biofilms can resist antifungal agents and host immune clearance. Persister cells within confer high levels of resistance (1). Polyene resistance is linked with altered expression or mutation resulting in reduced ergosterol expression (2). Azole resistance arises from the overexpression or point mutation of the drug target site (e.g. ERG11) to increase ergosterol biosynthesis overwhelming drug activity (3 and 4) (Berkow et al., 2015; Urbanek et al., 2022). Mutations in the FKS1/FKS2 genes confer resistance to echinocandins (5). Upregulation of efflux pumps reduce intracellular drug concentration (6). Image adapted from (Stevenson et al., 2022).

1.4 Metal-based antimicrobial agents

The antimicrobial properties of metals have been recognised since ancient times with the first recorded use of copper documented in Egypt, 2600 BC for water sanitisation

and the treatment of chest wounds, scalds and itching (Arendsen, Thakar & Sultan, 2019). Use of more structurally-defined metals for the treatment of diseases continued into the 20th century (Claudel, Schwarte & Fromm, 2020). In 1910, Paul Ehrlich introduced arsenic-containing Salvarsan as an effective organo-metallic treatment for syphilis (Williams, 2009). Despite its replacement with penicillin in 1940, interest in the use of metal-based compounds resurfaced following the emergence of AMR and advancements in inorganic chemistry (Turner, 2017).

The future of antimicrobial therapies demands innovative strategies to minimise the reappearance of resistance. Ehrlich conceptualised the "magic bullet" design of chemicals to selectively kill microbes but not human cells (Strebhardt & Ullrich, 2008). Conventional antimicrobials emulate this concept having a singular target (e.g. cell membrane/wall (synthesis), DNA replication and protein synthesis), which in turn can expedite resistance (Turner, 2017). Conversely, metals affect a number of cellular pathways and have a pleiotropic effect on microbial cells (Turner, 2017).

Transition metals such as copper, iron, manganese and zinc are required in trace amounts for the growth and development of almost all living organisms (Szabo, Bodolea & Mocan, 2021; Juttukonda & Skaar, 2015). In certain forms, or excess amounts, metals can exert toxicity deleterious to some biological processes (Lemire, Harrison & Turner, 2013). Acting as enzyme co-factors, metal ions catalyse many biochemical reactions (Valls & De Lorenzo, 2002; Moustakas, 2021). While some comprise structural constituents of enzymes, others are involved in electron transfer in redox reactions or mediate the binding of molecules to corresponding receptors to control biological processes (Zhang & Zheng, 2020). Optimal growth of bacteria demands approximately 10^{-7} to 10^{-5} M iron, for example (Loutet et al., 2016). Microbes face the challenge of acquiring and/or competing for essential micronutrients in scarce environments (Kramer, Özkaya & Kümmerli, 2020). In the context of infection, mammalian hosts sequester iron via metal-binding proteins (e.g. lactoferrin) as part of "nutritional immunity" to starve microbial pathogens (Lu et al., 2020). Successful bacterial and fungal pathogens employ efficient uptake strategies (i.e. the production and uptake of iron-chelating siderophores, acquisition from heme and host iron-containing proteins, and ferric to ferrous iron reduction and transport mechanisms) to circumvent this (Kronstad & Caza, 2013).

The chemical affinity of metals for thiol groups can disrupt enzyme specificity (Yazdankhah, Skjerve & Wasteson, 2018). High concentrations of metals, both essential and non-essential, have the capacity to disrupt the correct functioning of enzymes, catalyse redox cycling and the production of ROS, damage ion regulation and DNA and protein formation (Igiri et al., 2018; Yazdankhah, Skjerve & Wasteson, 2018). In yeasts, this can damage ergosterol biosynthesis and cell wall integrity and increase cellular leakage whilst in filamentous fungi it can increase chitin deposition and hence inhibit hyphal extension, and interfere with conidial and conidiophore development (Robinson, Isikhuemhen & Anike, 2021). Microbes must therefore selectively manage metal transport systems to maintain homeostasis (da Silva Neto, Staats & Pontes, 2023).

Antimicrobial application of metals can adopt one of two approaches: (1) exert toxicity to inhibit/kill microbial pathogens and (2) exploit nutritional vulnerabilities to inhibit the growth, development and virulence of pathogens. Transition metals of the d-block elements (e.g. cobalt, copper, gold, iron, manganese, ruthenium, silver, zinc etc.) are increasingly incorporated into metal-based formulations in addition to metals and metalloids from groups 13 – 16 (bismuth, gallium, germanium, selenium etc.) (Truong et al., 2021; Piatek, Griffith & Kavanagh, 2020; Alharbi et al., 2012; Ji et al., 2023; Abass et al., 2021; O'Shaughnessy et al., 2022; Piatek et al., 2022; Li et al., 2012a; Zhang et al., 2021; Zhou et al., 2012).

1.4.1 Antimicrobial applications of silver

Silver is a soft and lustrous transition metal with an abundance of applications in electronics, tableware, jewellery and medicine (Drake & Hazelwood, 2005). Therapeutic use of silver was first recorded approximately 1500 BC by the Han dynasty in China whilst Hippocrates documented the wound healing and food and water preservation properties of silver in 400 BC (RubenMorones-Ramirez et al., 2013; Sim et al., 2018). Use of silver was common since ancient civilisations and eventually gained regulatory approval as an antimicrobial agent in the early 20th century (Chopra, 2007). Modern day uses of silver involve wound care, bone prostheses, cardiac devices, catheters, surgical appliances and dentistry (Polívková et al., 2017; Talapko et al., 2020).

Silver surface coatings have been an effective strategy to limit microbial adhesion and infection. Silver nanoparticles are either deposited directly onto the surface or contained within a polymeric surface coating (Knetsch & Koole, 2011). Implanted medical devices can harbour bacterial and fungal pathogens that are presented as obstinate biofilms impeding antimicrobial and host immune clearance (Dhaliwal et al., 2021; Lafuente-Ibáñez de Mendoza et al., 2021). The most commonly isolated pathogens are *E. coli*, *Enterococcus faecalis*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *Staphylococcus epidermidis* and *Streptococcus viridans* (Khatoon et al., 2018). A number of studies detail improved clinical outcomes using silver-coated central venous catheters, urinary catheters, and ventilator endotracheal tubes (Rupp et al., 2005; Davenport & Keeley, 2005; Kollef et al., 2008). Orthopaedic hardware is associated with pin tract infection in external fixations and silver-coating showed reduction in bacterial growth (Massè et al., 2000). In the case of orthopaedic megaprosthetic implantations, Agluna® (Accentus Medical), MUTARS® (Implantcast) and PorAg® (Waldemar Link) are three examples of commercially available silver coatings. Studies have demonstrated their efficacy for prosthetic joint infections compared to untreated devices (Fiore et al., 2021).

Silver has a variety of applications in dentistry. Silver nitrate was initially applied for the prevention of dental caries (tooth decay) before later incorporation with fluoride in the 1960s. Silver nanotechnologies became a more preferable option as antimicrobial agents to tackle the growth, adhesion and biofilm formation of *Streptococcus mutans*. Moreover, silver has been employed in dental implantology and denture production (Talapko et al., 2020; Butrón Téllez Girón et al., 2020).

Burn wound application of silver originated in the 1960s with the use of 0.5% silver nitrate solution and silver sulfadiazine cream. These therapies demanded re-hydration every few hours which led to the development of nanoparticle- and silver salt-based gels (Sim et al., 2018). Silver Acticoat™ is a multi-layered wound dressing impregnated with silver nanoparticles that has demonstrated superior activity to standard wound dressings. Acticoat7 has a prolonged seven-day efficacy without the need of reapplication and Acticoat Absorbant™ comprises of an alginate dressing with an absorbent property to form a gel with wound exudate and release nanocrystalline silver cations into the wound (Fong & Wood, 2006).

1.4.2 The antimicrobial mechanisms of action of silver

Silver possess antibacterial, antifungal and antiviral properties and has shown complete killing (bactericidal/fungicidal) and growth reducing (bacteriostatic/fungistatic) potential (Panáček et al., 2009; Ribeiro et al., 2023; Anees Ahmad et al., 2020; Ratan et al., 2021). Silver exerts its biological activity when it is soluble in aqueous environments, as either Ag^{+}, or Ag^{0} clusters. The ionic form, Ag^{+}, is released from silver compounds like silver nitrate and silver sulfadiazine, among other ionic silver compounds. Nanocrystalline silver contains the uncharged Ag^{0} metallic form (Fong & Wood, 2006). Various mechanisms of action have been proposed. For instance, released Ag^{+} cations have a high affinity for negatively charged cell membrane surfaces due to the carboxyl, phosphate and amino groups thus allowing silver to penetrate the membrane and alter permeability (Morozova, 2021). Consequently, the dissipation of the proton motive force damages the cell membrane. Other reports have stated that silver ions have a high affinity for thiol groups and thiol-containing proteins in the cell membrane to form protein complexes with electron donors containing oxygen, phosphorous, nitrogen, or sulfur atoms. Membrane-bound enzymes and proteins are therefore inactivated via disulfide bond and active site interaction (Mikhailova, 2020). Moreover, silver nanoparticles may affect membrane fluidity and the lipid bilayer by altering the cis/trans ratio of unsaturated fatty acids. Interference with membrane structure can result in the loss of membrane integrity (Mikhailova, 2020). Shrinkage of the cytoplasm and detachment from the membrane ultimately cause cell wall rupture (Dakal et al., 2016). Structural modifications of the cell can dysregulate transport activity via the cell membrane and cause loss of crucial cellular components such as proteins, ions, sugars and energy stores in the form ATP. Sufficient uptake of vital components such as micronutrients (e.g. potassium) can also be impeded (Dakal et al., 2016). Furthermore, cell adhesion to substrates initiates biofilm formation and the interaction of silver on the cell surface can impede these processes (Chaw, Manimaran & Tay, 2005).

The penetration of silver into the cell impairs the function of intracellular structures (mitochondria and ribosomes) and biomolecules (DNA, lipids and proteins) (Dakal et al., 2016). Interaction with disulphide bonds in respiratory chain enzymes (such as NADH-succinate dehydrogenase) may inactivate them and impact the electron

transport chain (Naik & Kowshik, 2017). This can lead to increased production of ROS and other deleterious free radicals (hydrogen peroxide (H_2O_2), superoxide anion ($O2^-$), hydroxyl radical (•OH), hypochlorous acid (HOCl) and singlet oxygen) which in turn damage the mitochondrial membrane and cause necrosis and cell death (Morozova, 2021; Dayem et al., 2017).

Mitochondrial, endoplasmic reticulum and antioxidant enzyme dysfunctions leading to elevated ROS (and other free radical) levels can promote genotoxicity in the form of chromosomal mutations, DNA strand breaks and DNA base damage (Dakal et al., 2016). Mikhailova (2020) revealed that Ag^+ complex formation with nucleic acids breaks the hydrogen bonds between antiparallel base pairs. In addition, silver treatment can promote DNA condensation and reduce replication (Mikhailova, 2020).

The binding of silver ions to thiol groups is linked to functional defects of various proteins. Phosphomannose isomerase, for example, is a target of silver nanoparticles and disruption prevents the formation of glycolysis cycle intermediates in sugar metabolism (Bhattacharya & Mukherjee, 2008). Similarly, silver can attach to subunits and denature ribosomal activity to prevent protein translation and synthesis (Kędziora et al., 2018).

Gram-negative bacteria have shown to be more susceptible to silver treatment due to a thinner cell wall and the presence of a lipopolysaccharide cell membrane (More et al., 2023). The negatively charged cell membrane attracts silver adhesion and deposition onto the cell surface. The presence of thick, negatively charged peptidoglycan in Gram-positive can trap silver and prevent its activity (Dakal et al., 2016). Despite this, numerous studies have outlined the potent effects of silver for the treatment of Gram-positive pathogens (Domínguez et al., 2020; Adibhesami et al., 2017; Piatek et al., 2023; O'Beirne et al., 2021). Inhibition of these metabolic processes important for energy generation (respiration), protein function and synthesis, DNA structure and replication, antioxidant mechanisms along with nutrient uptake/release can have detrimental effects on microbial growth and survival (**Figure 1.3**). The modes of action of silver are largely universal among bacterial and fungal pathogens (Gibała et al., 2021; Li et al., 2022b). This shows great potential in the fight against mono- or dual microbial infections.

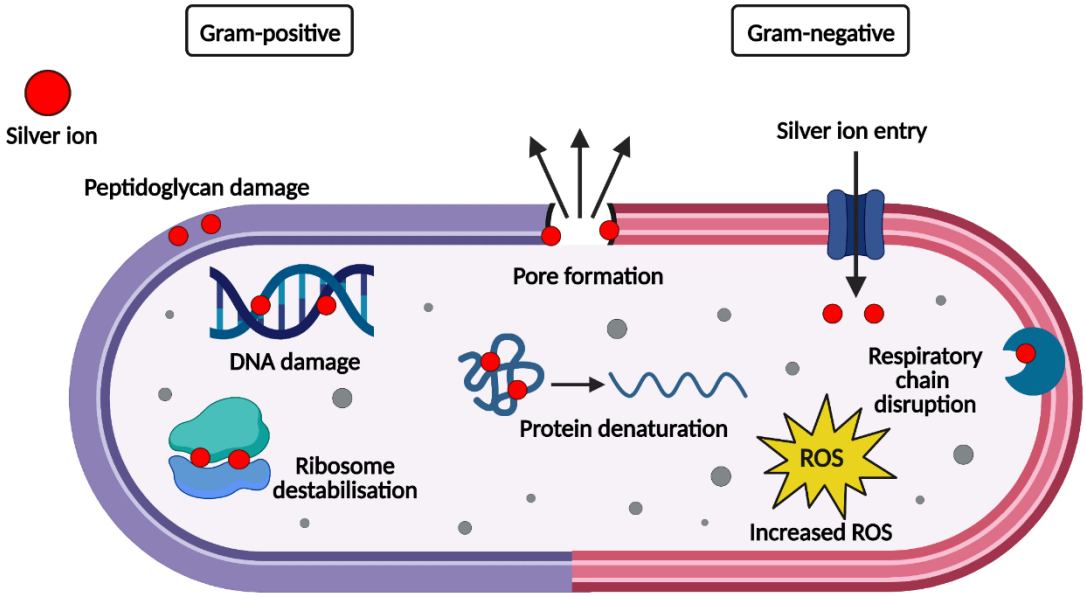

Figure 1.3. The general antibacterial mechanisms of silver ions in Gram-positive and Gram-negative bacteria. Silver ions gain entry into cells via porin proteins and/or adhere to the cell wall/membrane. Pore formation/membrane damage induces ion and metabolite leakage. Inactivation of respiratory chain enzymes can lead to increased ROS generation and oxidative stress which can denature proteins/inactivate enzymes (Kędziora et al., 2018). Binding to the 30S ribosomal subunit can deactivate the ribosomal complex and inhibit protein synthesis (Mikhailova, 2020). Silver ions can induce DNA condensation and disruption in replication (McNeilly et al., 2021; Dakal et al., 2016). Image adapted from (Kędziora et al., 2018; Sportelli et al., 2018; Saha & Kim, 2022).

1.4.3 *N*-heterocyclic carbene complexes and SBC3

N-heterocyclic carbene (NHC) ligands have been incorporated into novel organometallic complexes with anticancer and/or antimicrobial properties due to their efficient synthesis and strong σ-donor capability to form stable complexes with transition metals (Ott, 2017). Carbenes are molecules with a neutral, divalent carbon atom and two unshared valence electrons. Their electrophilic or nucleophilic reactivity is based on whether the unshared electrons are unpaired (triplet carbene) or paired (singlet carbene) (Grubbs, Trnka & Sanford, 2003). NHCs can be defined as any chemical with a carbene centre and at least one nitrogen atom within a heterocyclic ring (**Figure 1.4**) (Wang et al., 2015).

In the early 1960s, Wanzlick postulated the strong hydrogen bond-accepting properties of NHCs and later substantiated this with theoretical calculations and experiments (Hollóczki, 2020). Applications of the first NHC metal complex by Wanzlick and

Öfele, were recorded in 1968. Attempts at NHC isolation remained unsuccessful until 1991, when Arduengo and co-workers isolated the first stable crystalline NHC. Arduengo's contributions prompted a wave of research in the field of organometallic chemistry including transition metal and main group element chemistry.

Current NHCs consist of imidazol-2-ylidene and imidazolin-2-ylidene scaffolds derived from the deprotonation of imidazolium or imidazolinium salts with a strong base. The productivity of synthesis is continuously evolving. Complexes that are air- and moisture-sensitive demand specialised equipment like the Schlenk line for their synthesis (Opalka et al., 2013). Continuous flow synthesis is an attractive, alternative approach that uses a continuous stream of reactants, reagents and solvents introduced into a reactor to carry out a steady chemical reaction. This offers numerous advantages over conventional batch processing that include improved safety, scalability, heat and mass transfer, yield and reproducibility and reduced waste. Moreover, real-time reaction monitoring allows more accurate control. This technique has therefore become a fundamental procedure in academic and industrial production (Capaldo, Wen & Noël, 2023; O'Beirne et al., 2021).

NHC complexes have become an important component of bioinorganic and medicinal chemistry. Increasing interest in transition metal NHC complexes incorporating gold, silver, copper, platinum and palladium has led to the development of an abundance of NHC complexes for anticancer and/or antimicrobial use (Büssing et al., 2021; O'Beirne et al., 2021; Pan et al., 2021; Wan et al., 2021; Ghdhayeb et al., 2021; Lenis-Rojas et al., 2021). The stability (including in physiological environments) of NHCs such as Ag(I)-NHC complexes permits slow release of silver ions to prolong activity. Chelation to metal ions decreases polarity and hence increases the lipophilicity of the compounds for cellular uptake across cell membranes (Jakob et al., 2021; Zhang et al., 2018).

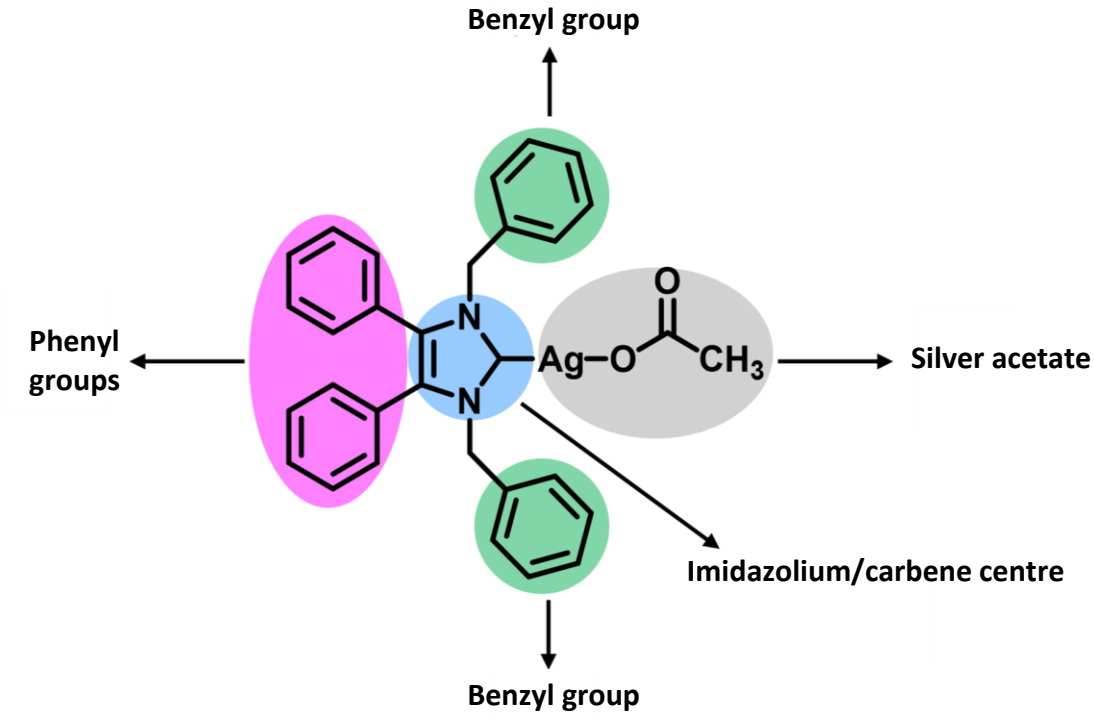

Figure 1.4. The molecular structure of the NHC silver(I) acetate complex, SBC3, derived from 1,3-dibenzyl-4,5-diphenylimidazole-2-ylidine (NHC*). NHC silver(I) acetate contains is complexed with lipophilic benzyl substituents.

1.4.4 The medicinal properties of gallium

Categorised as a post-transition metal in Group 13 of the periodic table, gallium is an extremely rare element primarily found in the +3 oxidation state (Boronski et al., 2021). Synthetically-produced gallium is silvery-white and liquid at room temperature (Chitambar, 2010). Gallium does not play any physiological roles in the human body but is instead utilised for its similarities to ferric iron and involvement in iron metabolism (Chitambar, 2016). Consequently, gallium has been employed for diagnostic and therapeutic purposes (Chitambar, 2010).

The medicinal value of gallium was first recognised in 1940 following observations that gallium concentrated at sites of osteogenic activity (Hayes, 1978). A gallium scan consisting of intravenous administration of a gallium isotope aimed to generate nuclear medicine images to detect cancer and abscesses in the body. Attempts at diagnosis and therapy of malignant bone lesions using ^{72}Ga in the early 1950s failed. This however, provided valuable insight and along with improved instrumentation led to the

successful use of ^{68}Ga and ^{67}Ga radionuclides (Hayes, 1978). Preliminary reports identified Hodgkin's Lymphoma using radioactive ^{67}Ga citrate which led to use for a broader range of diseases including other cancers and lymphomas, bone infections and inflammatory diseases of the lung (England, Rui & Cai, 2017). The levels of ^{67}Ga uptake, which is primarily transferrin receptor-mediated, are proportional to the tumour proliferation rate and presence of viable malignant cells thus indicating the severity of the tumour and need for therapy (Chitambar, 2010). The high expression of transferrin receptors on the surfaces of many cancer cell types not only permits more targeted delivery but can also minimise side effects on healthy cells (Daniels et al., 2012).

The localisation of ^{67}Ga in tumour cells triggered antineoplastic studies of gallium salts in murine samples. With the strongest efficacy and lowest toxicity, gallium nitrate (GaN) was a strong contender and entered Phase I and II clinical trials. It demonstrated particularly potent activity against non-Hodgkin's Lymphoma and bladder cancer (Chitambar, 2012).

The ability of gallium to disrupt iron metabolism led to antimicrobial investigation with initial reports in 1931 (Li et al., 2022a). This seems logical given the high replication rate of microbes and diagnostic use of gallium for fast-growing cancers. GaN administered intravenously treated syphilis and *Trypanosoma evansi* in rabbits and mice, respectively (Levaditi et al., 1931). GaN was U.S. Food and Drug Administration (FDA)-approved and branded as Ganite® for cancer-associated hypercalcemia however, has since been repurposed for antimicrobial use (Chitambar, 2010; Hijazi et al., 2018). Formulations have developed from first generation GaN $(GaNO_3)_3$, to second generation gallium maltolate (GaM), a Ga(III) coordination complex of maltol, and third generation Ga(III)-protoporphyrin IX (GaPPIX), among others (**Figure 1.5**) (Thompson et al., 2015; Hijazi et al., 2018; Li et al., 2022a; Scaccaglia et al., 2022). The antibacterial activity of gallium is well recognised within an abundance of studies on Gram-positive bacteria: *E. faecalis*, *Mycobacterium tuberculosis*, *S. aureus*, *Staphylococcus epidermidis*, and Gram-negative bacteria: *Acinetobacter baumannii*, *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Stenotrophomonas maltophilia* (Xu et al., 2017; Olakanmi et al., 2013; Piatek, Griffith & Kavanagh, 2020). The antifungal effects of gallium are less

frequently examined although it has shown efficacy against *Candida* species (*C. albicans*, *Candida auris*, *Candida duobushaemulonii*, *Candida haemulonii* and *C. glabrata*) and *Cryptococcus* yeasts (*Cryptococcus gatti* and *Cryptococcus neoformans*) and *A. fumigatus* (Bastos et al., 2019, 2010).

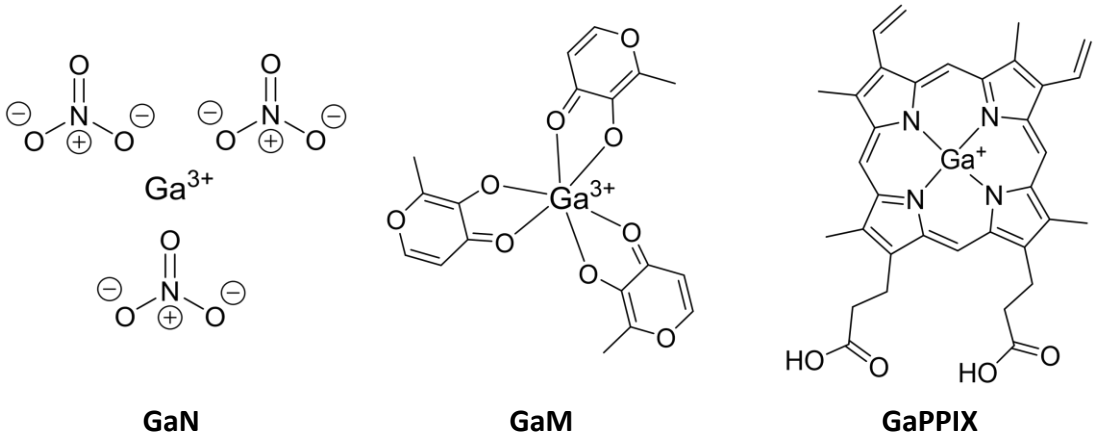

Figure 1.5. Molecular structures gallium-based compounds Ga(III)-nitrate (GaN; first generation), Ga(III)-maltolate (GaM; second generation) and Ga(III)-protoporphyrin IX (GaPPIX; third generation). Image adapted from (Hijazi et al., 2018).

1.4.5 The antimicrobial mechanisms of action of gallium

Gallium(III) shares similar chemical properties with ferric iron, Fe(III), that can make these elements indistinguishable to cells. Similarities include matching charge (3^+), similar ionic radii (0.62 Å for Ga(III) and 0.65 Å for Fe(III)), and preferred coordination numbers of 6 to form similar complexes with other atoms or ions (Vágner et al., 2018; Chitambar, 2016). As a result, cells (including microbes), incorporate gallium in place of iron which is needed for iron-dependent functions vital for cell growth and viability as part of a Trojan Horse strategy (Sun et al., 2023). Crucially, gallium is redox-inactive and incapable of reducing from a trivalent to divalent form in normal physiological conditions whereas iron can exist in two stable oxidation states: 2^+ (ferrous) and 3^+ (ferric) (Li et al., 2022a). Interchangeability (oxidation and reduction) between these states drives essential metabolic processes like the tricarboxylic acid (TCA) cycle and respiratory electron transport for energy generation, and DNA replication since iron is a co-factor and catalytic component of

proteins and enzymes (Kronstad & Caza, 2013). Iron species partake in the Fenton reaction whereby iron(II) reacts with H_2O_2 to generate •OH, a highly reactive and damaging ROS to biomolecules (Touati, 2000). Homeostatic mechanisms must therefore tightly regulate iron levels in order to gain sufficient bioavailability without inducing oxidative stress. This is done via an interplay of iron import, storage and export proteins (Bradley et al., 2020).

Iron is a vital micronutrient for the growth and survival of microorganisms (Frawley & Fang, 2014). Three established iron uptake mechanisms in bacteria are: (1) siderophore- (2) heme/hemeprotein- and (3) transferrin and lactoferrin-based (Krewulak & Vogel, 2008). Direct interaction with iron (and iron sources) and transport into the cell can occur via membrane receptors. Gram-positive bacteria use receptors on the cytoplasmic membrane to detect iron or iron-bound siderophores. Iron(III) is disassociated from the complex and reduced to iron(II) following uptake into the cytoplasm. An outer membrane receptor recognises iron in Gram-negative bacteria before transport to the periplasm and entry into the cell through the plasma membrane (Krewulak & Vogel, 2008). Gallium gains access into cells by using the host's iron acquisition receptors (Li et al., 2022a).

1.5 Proteomics as a tool for drug discovery

High through-put omics technologies have become pivotal tools in the quest for novel drug candidates (Paananen & Fortino, 2020; Aborode et al., 2022). The screening of biological samples at the genomic, transcriptomic, metabolomic and proteomic levels can deeply enhance our understanding of drug targets, efficacy and side-effects (Paananen & Fortino, 2020). The proteome is defined as the entire set of expressed proteins in a cell, tissue or organism and proteomics is the large-scale study of these proteins in terms of composition, function, structure and interaction (Cho, 2007; Al-Amrani et al., 2021). Regarded as a "new" type of omics, proteomics has rapidly evolved due to technological advancements, largely replacing traditional gel-based techniques that lack sensitivity and prevent whole proteome studies (Baggerman et al., 2005; Al-Amrani et al., 2021; Chandramouli & Qian, 2009). Mass spectrometry (MS) is based on the measurement of the mass-to-charge ratio of ions and is the most comprehensive approach for the identification and quantification of peptides, proteins and small molecules within complex mixtures (Rockwood, Kushnir & Clarke, 2018).

The wealth of raw data generated demands robust software for data visualisation and interpretation. Innovations of MS instruments have therefore coincided with the development of intricate bioinformatics tools (Chen et al., 2020a; Bouyssié et al., 2020).

1.5.1 Label-free quantitative proteomics

Quantitative proteomics techniques can be classified as labelled or label-free. The labelling of multiple peptide samples with isobaric tags (e.g., Tandem-mass tag MS) permits in-depth proteome coverage, reduced technical variability, the detection of low-abundance molecules, and the analysis of multiple samples at a time. However, interfering signals can underestimate fold changes. This is not of concern in label-free quantitative (LFQ) approaches which is also a cheaper method and requires less sample preparation (Nakayasu et al., 2021). The general workflow of all label-free methods involves protein extraction, reduction, alkylation and digestion followed by sample separation via liquid chromatography (LC) and analysis by tandem MS (MS/MS) (Duong & Lee, 2023). Each sample undergoes individualised processing from sample preparation to instrument loading to analysis. Protein quantification relies on two measurements: (1) chromatographic peak intensities and (2) spectral counting of identified proteins after MS/MS analysis (Zhu, Smith & Huang, 2010). The intensity of peptide signals is used to quantify peptide abundance and is aided with the use of software tools to analyse mass spectra. In-solution digestion of intact proteins into peptides prior to MS analysis is termed shotgun proteomics. Indirect measurement of proteins occurs via the resulting peptides (Zhang et al., 2013). This ‘bottom-up’ approach differs from ‘top-down’ methods whereby intact proteins are analysed (Toby et al., 2019).

1.5.2 The use of proteomics for studying microbial responses and drug activity

Proteomics provides new insights on host-pathogen interactions and response to antimicrobial agents at a molecular level (Biron et al., 2011; Amiri-Dashatan et al., 2018). Microbes are attractive organisms to study due to less complex proteomes, simple culture conditions and rapid cell turnover for fast generation of results (Stekhoven et al., 2014; Meunier, Cornet & Campos, 2021). This opens the way for the development of novel antimicrobial agents and identifying therapeutic targets

whilst investigation of current antibiotics can better inform bacterial adaptation and resistance mechanisms (Torres-Sangiao et al., 2022).

Similarly, proteomics has enabled better understanding of the clinical consequences of pathogenic fungi as well as their biotechnological potential (Doyle, 2011). The production of fungal secondary metabolites has led to the isolation of therapeutic agents with anticancer, immunosuppressive, hypercholesterolemic and antimicrobial properties whilst the fungal primary metabolism has also been exploited for the production of an abundance of agricultural, cosmetic and industrial commodities (Künzler, 2018; Hyde et al., 2019; Manzoni & Rollini, 2002; Dowd & Kelley, 2011).

Microbial studies are just one of many applications of proteomics – a powerful tool in human health research which has provided invaluable insight into novel biomarkers, disease prognosis and progression (Nayak et al., 2015). Despite this, there is scope for further improvements. Complex organisms and/or physiological processes, vast data sets, intricate and costly instrumentation requiring extensive skills and knowledge, risk of false positivity and hindered detection of low abundance proteins are among some limitations of proteomic studies (Dupree et al., 2020; Gerszten et al., 2008).

1.6 *Galleria mellonella*: a model organism for studying drug toxicity and efficacy *in vivo*

Insects are well established model organisms in the fields of genetics, neuroscience, evolution and developmental and behavioural biology (Tonk-Rügen, Vilcinskas & Wagner, 2022). *Drosophila melanogaster* (fruit fly) is one of the most widely used models along with *Manduca sexta* (tobacco horn moth), *Bombyx mori* (silk worm) and *Galleria mellonella* (greater wax moth). Insects have played a pivotal role in reinforcing the 3R (Replace, Reduce and Refine) policy to reduce the numbers of mammals required in experimentation (Browne & Kavanagh, 2013). The use of insect models is not restricted by ethical or legal legislations. Other advantages include easy handling and inoculation, inexpensive purchase and storage, survival at 37°C for human pathogen studies, rapid generation of results within 24 – 48 h and importantly, they possess structural and functional similarities to mammalian innate immunity. These conserved features enable the study of microbial pathogens and therapeutic

agents with comparable results to mammalian models (Browne, Heelan & Kavanagh, 2013).

Insects occupy ecologically diverse habitats which necessitates a robust immune system to resist pathogen attack. Insects like *G. mellonella* possess a highly active innate immune response that quickly recognises and kills or immobilises pathogens (Sheehan, Farrell & Kavanagh, 2020; Sheehan, Clarke & Kavanagh, 2018). *G. mellonella* innate immunity consists of three components: (1) physical and chemical barriers against the external environment, (2) a cellular response and (3) a humoral response (Trevijano-Contador & Zaragoza, 2018). The hindgut and trachea are composed of chitin whilst the cuticle prevents intruder entry. The presence of enzymes and low pH in the midgut deters the growth of microorganisms (Vertyporokh, Hułas-Stasiak & Wojda, 2020). The cellular and humoral responses work in combination to protect the insect from infection and heal wounds in the cuticle to prevent further invasion ((James & Xu, 2012; Krautz, Arefin & Theopold, 2014). Cellular immunity in *G. mellonella* is mediated by immune cells called hemocytes, which recognise and phagocytose material, largely mimicking human neutrophil activity. Hemocytes circulate freely in hemolymph (insect blood) and are also found attached to the fat body and digestive tract (Pereira et al., 2018). At least six hemocyte types have been identified: prohemocytes, plasmatocytes, granular cells, coagulocytes, spherulocytes enocytoids and are employed in encapsulation, nodulation and phagocytosis (Pereira et al., 2018). The phagocytic activity of hemocytes (more specifically plasmatocytes) is facilitated by the opsonisation of microorganisms using complement-like proteins. The consequent generation of ROS occurs by an NADPH oxidase complex and homologous proteins to human neutrophils (e.g. $gp91^{phox}$, $p67^{phox}$, and $p47^{phox}$) (Bergin et al., 2005). Microbial killing is further aided with the release of enzymes in the process of degranulation (Browne, Heelan & Kavanagh, 2013).

Pathogens, or pathogen derived material (e.g. lipopolysaccharide), is recognised by pattern recognition receptors (e.g. Toll-like receptors) and induce the immune deficiency pathway leading to the activation of the humoral immune response (Asai et al., 2021). This consists of antimicrobial peptide production, coagulation of hemolymph and melanisation (Mowlds, Barron & Kavanagh, 2008). Melanin functions in exoskeleton pigmentation, cuticle sclerotisation, wound healing and in

innate immunity (Sugumaran & Barek, 2016). A hardened exterior prevents dehydration and desiccation and hinders microbial entry. The prophenoloxidase cascade is responsible for the formation of melanin which in turn induces oxidative and toxic intermediate generation for microbial killing (Smith et al., 2022; Eleftherianos & Revenis, 2011). This cascade has been likened to the mammalian complement system (Sheehan et al., 2018).

G. mellonella are widely used in research for evaluating the virulence of microbial pathogens as well as the *in vivo* toxicity and efficacy of antimicrobial agents. some of which are listed in **Table 1.3**.

Table 1.3. Novel and conventional antimicrobial agents tested against a range of bacterial and fungal pathogens in *G. mellonella*.

Bacteria	Antimicrobial agent	Reference
Gram-negative		
Acinetobacter baumannii	Polymyxin B/netropsin	(Chung et al., 2016)
	Daptomycin/colistin	(Yang et al., 2015a)
	Manganese(i) tricarbonyl complexes	(Güntzel et al., 2019)
Burkholderia spp.	Ceftazidime, imipenem, doxycycline, ciprofloxacin	(Thomas et al., 2013)
Enterobacter cloacae	Piperacillin, cefotaxime, imipenem, amikacin, ciprofloxacin	(Yang et al., 2017)
Escherichia coli	Kanamycin	(Leuko & Raivio, 2012)
Klebsiella pneumoniae	K1-ULIP33, KL106-ULIP47, KL106-ULIP54 phages	(Thiry et al., 2019)
Pseudomonas aeruginosa	Cecropin A2/tetracycline	(Zheng et al., 2017)
	Manganese(i) tricarbonyl complexes	(Güntzel et al., 2019)
	1,10-phenanthroline-5,6-dione-based compounds	(Galdino et al., 2019)
Shigella spp.	Geraniol	(Mirza et al., 2018)
Gram-positive		
Clostridium perfringens	Penicillin, bacitracin, tetracycline, neomycin	(Kay et al., 2019)
Enterococcus spp.	Rifampicin, vancomycin, linezolid, tigecycline	(Skinner et al., 2017)
	Ampicillin, gentamycin, streptomycin, photodynamic therapy	(Chibebe Junior et al., 2013)
Listeria monocytogenes	*Cannabis sativa* L. essential oil	(Marini et al., 2018)
Mycobacterium spp.	Amikacin, meropenem, azithromycin	(Meir, Grosfeld & Barkan, 2018)
	Amikacin, ciprofloxacin and isoniazid	(Entwistle & Coote, 2018)
Staphylococcus aureus	Vancomycin/myricetin	(Silva et al., 2017)
	Cinnamaldehyde	(Ferro et al., 2016)

	Triclosan	(Latimer, Forbes & McBain, 2012)
	Cu(II)-steroid complexes	(Barrett et al., 2018)
Fungi		
***Candida* spp.**		
Candida albicans	Caspofungin and DsS3 (1-16)	(MacCallum, Desbois & Coote, 2013)
	$Ag_2(mal)(phen)_3$	(Rowan et al., 2009)
	Triazole-amino acid hybrids	(Aneja et al., 2016)
Candida haemulonii complex	Fluconazole, amphotericin B	(Silva et al., 2018)
	Cu(II), Mn(II), Ag(I) 1,10-phenanthroline chelates	(Gandra et al., 2020)
Candida tropicalis	Amphotericin B, caspofungin, fluconazole, voriconazole	(Mesa-Arango et al., 2013)
Candida glabrata	Fluconazole, amphotericin B	(Ames et al., 2017)
Candida parapsilosis	Fluconazole	(Souza et al., 2015)
***Cryptococcus* spp.**		
Cryptococcus neoformans	Voriconazole	(de Castro Spadari et al., 2020)
	Pedalitin/amphotericin B	(Sangalli-Leite et al., 2016)
***Aspergillus* spp.**		
Aspergillus fumigatus	Itraconazole, voriconazole, posaconazole	(Gomez-Lopez et al., 2014)
Aspergillus terreus	Amphotericin B	(Maurer et al., 2015)
Aspergillus lentulus (mixed infection with *A. fumigatus*)	Voriconazole	(Alcazar-Fuoli et al., 2015)

Several parameters are used to assess infection response and progression which include mortality, melanisation level (**Figure 1.6**) alteration in hemocyte density and/or population composition, changes in microbial load, formation of pupa, movement, alteration in gene expression and variations in the proteome (Jorjão et al., 2018; Sheehan, Clarke & Kavanagh, 2018; Cools et al., 2019; O'Shaughnessy et al., 2022).

Figure 1.6. *G. mellonella* larvae with varying degrees of *S. aureus* infection 24 h post-inoculation. *S. aureus* cell inoculum/larva from left to right: uninfected control larva (PBS only), 2×10^8, 1×10^8, 5×10^7, 2×10^7, 1.5×10^7, 1×10^7, 5×10^6 cells/larva.

The size and ease of handling enable administration of a defined inoculum by force feeding or intra-hemocoel injection to larvae (**Figure 1.7**) (de Jong et al., 2022). Moreover, larvae produce a large volume (60–80µl/larva) of hemolymph that can be analysed by a variety of methods (Thomas et al., 2013; Hill, Veli & Coote, 2014; Astvad et al., 2017; Maurer et al., 2019; Maguire et al., 2017). Hemocytes can be subjected to a range of *ex vivo* cellular assays in response to microbial pathogens and/or to determine the effect of chemical agents (Bergin et al., 2005; Renwick et al., 2007; Banville et al., 2011; Fallon, Reeves & Kavanagh, 2011).

Figure 1.7. Intra-hemocoel injection of *G. mellonella* larvae via the last-left proleg (Kavanagh & Sheehan, 2018).

There are some limitations associated with the use of *G. mellonella* larvae including the lack of a fully sequenced genome and the absence of organs similar to those in humans (e.g. lungs) (Mukherjee et al., 2010; Lange et al., 2018; Tartes, Kuusik & Vanatoa, 1999). The generation of mutant strains is well established in other insect models but has not yet become a possibility in *G. mellonella* and lastly, the short life span of larvae may prohibit the study of some processes associated with chronic infection (Larsson, Zhang & Rasmuson-Lestander, 1996; Brundage et al., 1996; Mukherjee et al., 2010; Kawamura & Maruyama, 2019; Yamamoto et al., 2014; Jorjão et al., 2018).

1.7 Thesis objectives

Increasing rates of AMR demands innovative strategies to treat drug resistant pathogens. Possible approaches are the development of therapies for synergistic use with existing treatments, the repurposing of existing agents and the development of agents with unconventional and/or multiple mode(s) of action. Understanding drug mechanism(s) of action is key in the discovery and development stages for a number of reasons:

1. To provide confidence in drug efficacy

2. Gain insight on the toxicity of an agent, i.e. drug selectivity
3. To better inform drug synthesis and hence improve formulations
4. Identify drug targets which microbes may alter/mutate, and hence, become resistant to in the future

The overall aim of this project was to evaluate the antimicrobial efficacy and mode(s) of action of novel/repurposed metal-based agents for the treatment of bacterial and fungal pathogens. This project heavily focuses on LFQ mass spectrometry to characterise the response of microbes to novel treatments. Alterations in the proteome have established potential drug targets and reveal insight on the mode(s) of action. LFQ proteomics, in conjunction with cellular and biochemical techniques, was therefore selected to assess the activity of silver- and gallium-based agents with the following specific aims:

1. Investigate the activity of GaM against *P. aeruginosa in vitro* and *in vivo* using *G. mellonella* larvae to measure the efficacy and toxicity of the complex. Apply LFQ proteomics to gain a deeper understanding of the effects of the agent on the *P. aeruginosa* proteome to reveal altered/inhibited protein pathways.
2. As an extension of previous work on gallium, study the effects of novel siderophore-conjugated gallium complexes against a range of bacterial pathogens and *A. fumigatus* and compare to other existing gallium formulations. Perform proteomics on gallium-treated *A. fumigatus* to gain insight into the mechanistic roles and contrasts with the pre-reported gallium formulation, GaN. This is part of a collaborative study involving the synthesis, characterisation and formulation of aqueous and powder gallium solutions intended for inhaler and nebuliser pulmonary administration.
3. To assess the antibacterial and antifungal efficacies of the silver-based NHC complex termed SBC3 and identify the most susceptible pathogens. Conduct subsequent proteomic evaluations on selected pathogens of interest to deduce target proteins and pathways.
4. Perform additional assays to validate previous proteomics findings and further assess the anti-virulence capabilities of SBC3 on the fungal pathogen, *C. parapsilosis*.

Chapter 2

Quantitative proteomics reveals gallium maltolate induces an iron-limited stress response and reduced quorum-sensing in *Pseudomonas aeruginosa*

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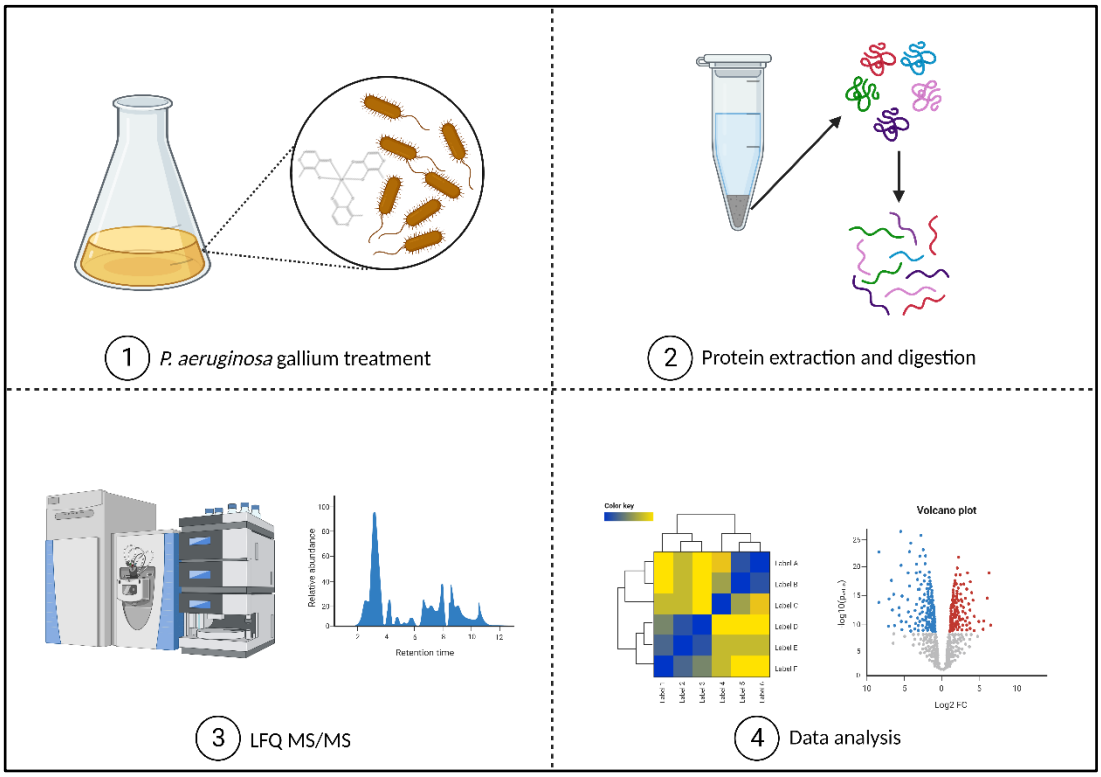
Author Contributions

All experiments, data analysis and manuscript writing were performed by MP. Compound synthesis and characterisation, experimental design and manuscript editing were performed by DG. Funding, experimental design and manuscript editing were performed by KK.

Abstract

Gallium-based drugs have been repurposed as antibacterial therapeutic candidates and have shown significant potential as an alternative treatment option against drug resistant pathogens. The activity of gallium (Ga^{3+}) is a result of its chemical similarity to ferric iron (Fe^{3+}) and substitution into iron-dependent pathways. Ga^{3+} is redox inactive in typical physiological environments and therefore perturbs iron metabolism vital for bacterial growth. Gallium maltolate (GaM) is a well-known water-soluble formulation of gallium, consisting of a central gallium cation coordinated to three maltolate ligands, [$Ga(Maltol_{-1H})_3$]. This study implemented a label-free quantitative proteomic approach to observe the effect of GaM on the bacterial pathogen, *Pseudomonas aeruginosa*. The replacement of iron for gallium mimics an iron-limitation response, as shown by increased abundance of proteins associated with iron acquisition and storage. A decreased abundance of proteins associated with quorum-sensing and swarming motility was also identified. These processes are a fundamental component of bacterial virulence and dissemination and hence suggest a potential role for GaM in the treatment of *P. aeruginosa* infection.

Graphical abstract



2.1 Introduction

Antimicrobial resistance (AMR) is a growing concern globally and has become problematic for the treatment of Gram-negative infections (Exner et al., 2017). The presence of an outer membrane in the bacterial envelope distinguishes Gram-negative from Gram-positive bacteria, and can confer resistance by inhibiting the entry and/or retention of antimicrobial agents (Ghai & Ghai, 2018; Jubeh, Breijyeh & Karaman, 2020). A significant contributor to increased AMR incidences is the Gram-negative bacterium *Pseudomonas aeruginosa* - a pathogen among immunocompromised individuals that can cause urinary tract infections and bacteraemia in nosocomial and community-based settings (El Zowalaty et al., 2015; Wang, Hou & Wang, 2019; Bassetti et al., 2018). The presence of the pathogen is a particular threat for cystic fibrosis patients and often indicates poor clinical outcome due to the ability to persist for long periods of time and drastically affect lung function (Faure, Kwong & Nguyen, 2018; Sordé, Pahissa & Rello, 2011). The adaptability and versatility of the pathogen is accounted for by a relatively large genome (5-7 Mb) that allows the expression of many virulence genes and regulatory enzymes involved in metabolism, efflux of organic compounds and resistance and enables the pathogen to withstand hostile environments (Moradali, Ghods & Rehm, 2017; Frimmersdorf et al., 2010). Moreover, Gram-negative bacteria can increase expression of efflux systems and modify outer membrane proteins and/or drug targets (e.g. penicillin binding proteins) to render many broad-spectrum antibiotics ineffective; frequent use of which can accelerate the development of these mechanisms (Melander, Zurawski & Melander, 2018; Pachori, Gothalwal & Gandhi, 2019; Beceiro, Tomás & Bou, 2013).

The advent of AMR necessitates the production of novel treatments with unique modes of action. Interference with bacterial iron metabolism has proven effective as an alternative method to eliminate infection since iron is a vital nutrient for the growth, survival and virulence of many bacteria (Goss et al., 2018; Chitambar, 2017; Kaneko et al., 2007; Cherayil, 2011). A fully functioning immune system and iron sequestration (for redox homeostasis, respiration and DNA synthesis and repair) are protective strategies of the human body to limit access to pathogens (Ganz & Nemeth, 2006; Puig et al., 2017; Kronstad & Caza, 2013). Successful pathogens such as *P. aeruginosa* can overcome this through multiple iron uptake systems. Pyochelin and

pyoverdine are among two siderophores that act as iron scavengers whilst the Feo system utilises phenazines to reduce insoluble ferric iron (Fe^{3+}) to soluble ferrous iron (Fe^{2+}) that can readily diffuse into the cells (Cornelis & Dingemans, 2013; Lau, Krewulak & Vogel, 2016; Dumas, Ross-Gillespie & Kümmerli, 2013).

The semi-metallic element, gallium, shares chemical properties with iron that allow it to act as an iron mimetic in biological environments (Chitambar, 2017). Transport of gallium in the blood closely resembles that of iron, whereby gallium forms a complex with the iron transporter transferrin, and with the aid of transferrin receptor 1, enters cells via endocytosis (Chitambar & Zivkovic-Gilgenbach, 1990; Chikh et al., 2007). Approximately 2/3 of transferrin is unbound in physiological conditions, leaving it vacant for the attachment of gallium (and other metals) (Chitambar, 2016; Harris & Pecoraro, 1983).

The medicinal application of gallium commenced with the use of gallium-based radiopharmaceuticals to detect and monitor cancerous tissue. The concentration of gallium incorporated into cells is directly proportional to the metabolic and proliferative activity, hence these scans can reveal the severity of some cancers such as lymphomas (Van Amsterdam et al., 1996; Chitambar, 2012). Use later expanded to the development of gallium-based complexes as potential anti-cancer drugs (Chitambar, 2012; Hayes, 1978; Timerbaev, 2009). Gallium limits the availability of iron to malignant cell lines and compromises mitochondrial function through the stimulation of calcium efflux and consequent initiation of apoptosis (Hara, 1974; Sephton & Harris, 1975; Chitambar & Seligman, 1986; Chitambar, Wereley & Matsuyama, 2006). Significantly, the gallium-based complex, tris(8-quinolinolato)gallium(III), KP46, (**Figure 2.1**), has recently reached a phase I/II clinical trial as a potential anticancer agent (Kubista et al., 2017).

The simple gallium salt, gallium nitrate, showed promising antineoplastic and antimicrobial activity as a first-generation compound (Hart et al., 1971). Gallium maltolate (GaM), a Ga(III) coordination complex of maltol, $[Ga(Maltol_{-1H})_3]$, first reported by Finnegan *et al.* (1987) as a neutral water-soluble complex of "medical interest" is a second-generation gallium-based compound which has potentially enhanced bioavailability as an oral and topical treatment (Chitambar, 2016; Giacani et al., 2019; Finnegan et al., 1987; Bernstein et al., 2000).

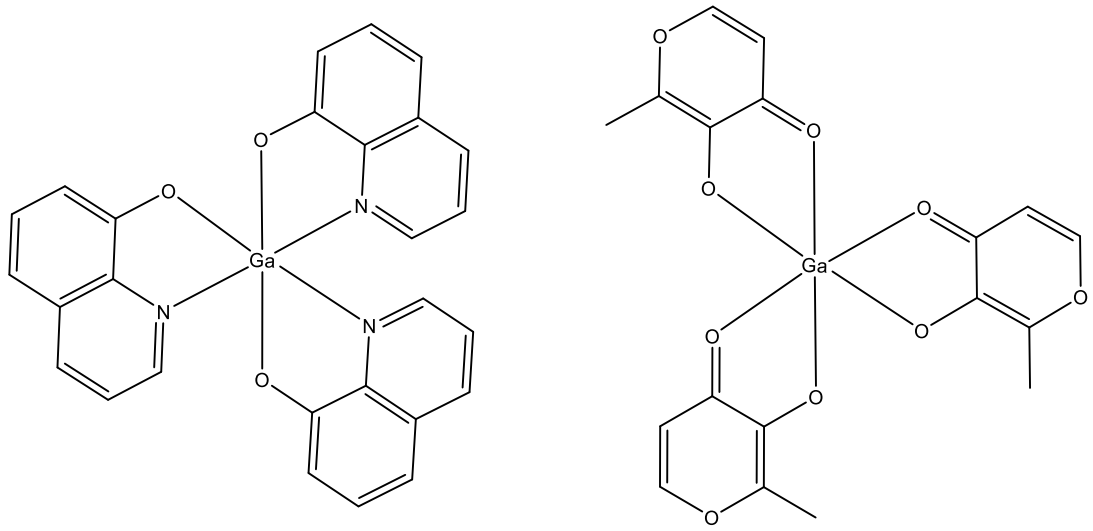


Figure 2.1. Chemical structures of KP46 (left) and GaM (right).

More recently, the physiological and pathological requirements of iron in bacteria have sparked interest in the repurposing of gallium as an antimicrobial agent. The inability of bacterial cells to differentiate between iron and gallium results in the detrimental incorporation of gallium into the cell (DeLeon et al., 2009). Gallium is unable to alternate from a trivalent to divalent form under normal physiological conditions and therefore disrupts bacterial iron metabolism and inhibits cell growth (Giacani et al., 2019).

Studies on antineoplastic and antimicrobial applications of gallium have described similar modes of action whereby gallium interferes with iron-dependent ribonucleotide reductase enzymes to prevent DNA synthesis (Kaneko et al., 2007; Myette, Elford & Chitambar, 1998; Olakanmi et al., 2013; Bernstein, van der Hoeven & Boer, 2012; Jakupec & Keppler, 2005; Merli et al., 2018). Myette *et al*., explored the synergistic effect of gallium nitrate with ribonucleotide reductase inhibitors gemcitabine and hydroxyurea on leukemic cells and with the help of previous findings, revealed the role of gallium in replacing iron in the M2 subunit of ribonucleotide reductase to cease DNA synthesis and cell proliferation (Myette, Elford & Chitambar, 1998; Chitambar et al., 1988; Narasimhan, Antholine & Chitambar, 1992). CCRF-CEM cells were highly susceptible to a combination of gallium nitrate and gemcitabine due to the suspected cytotoxic effect and incorporation of gallium into DNA, preventing strand elongation (Myette, Elford & Chitambar, 1998).

Treatment of *Mycobacterium tuberculosis* with gallium nitrate reduced ribonucleotide activity by a maximum of 60%. The Ib class of ribonucleotide reductases in *M. tuberculosis* is vital for cell growth and a likely target of gallium (Olakanmi et al., 2013; Mowa et al., 2009). These findings were highly comparable with those shown in *P. aeruginosa*, where gallium was presumably targeted by one class of ribonucleotide reductase enzyme (Goss et al., 2018).

Unsurprisingly, rapidly proliferating cancer cells demand more iron and therefore exposure to gallium increases concentration at these target sites (Pfeifhofer-Obermair et al., 2018; Wang et al., 2019c; Chitambar, 2010, 2004a). This is further enhanced in the cases of lymphomas and bladder cancer due to increased transferrin receptor 1 expression (Başar et al., 1991; Habeshaw et al., 1983).

Chitambar (2012) summarised the progress of human clinical trials on a range of gallium compounds (Chitambar, 2012). FDA-approved gallium nitrate has been used for the treatment of Non-Hodgkin's lymphoma and bladder cancer, with minimal safety issues when administered with caution. The optimum dosing regimen involves intravenous infusion of the drug over a number of days (Chitambar, 2004b). Doses ranging between 100 and 500 mg showed no adverse effects on healthy individuals while additional studies revealed reductions in tumor mass and symptoms in a hepatocellular carcinoma patient (Bernstein et al., 2000; Bernstein, van der Hoeven & Boer, 2012). Gallium-containing compounds have generally been well tolerated in human subjects although some side-effects including nausea, vomiting and anaemia have been reported (Malfetano et al., 1991). Alternatively, the use of gallium as an antimicrobial has shown promising results with no adverse toxicity as demonstrated in phase 1 clinical trials on CF patients with *P. aeruginosa* infection. Intravenous therapy improved lung function and was comparable with existing antibiotics (Goss et al., 2018).

The importance of iron and limited host supply opens a window of opportunity to target the nutritional demands of bacteria. This novel approach along with an abundance of literature documenting the therapeutic capability of gallium (and GaM), have encouraged these studies to obtain additional insight on the mode of action (Kaneko et al., 2007; Hijazi et al., 2018; Rzhepishevska et al., 2011; Wang et al., 2019b; Arnold et al., 2012; Fecteau et al., 2014).

The aim of the work presented here was to assess the ability of GaM to inhibit the growth of *P. aeruginosa in vivo* and *in vitro* and using label free mass spectrometry to uncover the response of this bacterium to this metal.

2.2 Materials and methods

2.2.1 *P. aeruginosa* culture conditions

P. aeruginosa PAO1 was cultured for 24 h in nutrient broth (Oxoid, UK) at 37°C in an orbital shaker (200 rpm). Stocks were kept on nutrient agar (Oxoid).

2.2.2 Gallium maltolate synthesis

GaM was synthesised as previously reported by (Finnegan et al., 1987).

2.2.3 Bacterial toxicity assays

P. aeruginosa PAO1 was cultured for 24 h in nutrient broth at 37°C to the stationary phase and samples were diluted to 1/100 in nutrient broth (overnight growth produced cultures with an optical density of approximately 1.0 at 600 nm (OD_{600}) representing ~3 x 10^8 colony forming units (CFU)/ml of bacteria). Aliquots (100 µl) were added to serially diluted GaM (0.997-250 µg/ml) and nutrient broth in a 96-well plate (Sarstedt, Germany). Plates were incubated at 37°C for 24 h and growth was measured at 600 nm.

2.2.4 *Galleria mellonella* viability assays

Sixth instar larvae of the greater wax moth, *Galleria mellonella*, (Livefoods Direct Ltd., Sheffield, UK) were stored at 15°C prior to inoculation. Ten healthy larvae (i.e. no appearance of melanisation), weighing 250 ± 50 mg were selected and stored in 9 cm petri dishes containing wood shavings.

Bacterial cells were diluted in phosphate buffered saline (PBS) to give concentrations of 3 x 10^0, 3 x 10^1, 3 x 10^2, 3 x 10^3 CFUs/ml and 20 µl aliquots of bacterial suspension were injected into larvae via the last left pro-leg using U-100 insulin syringe (Terumo Europe, N.V., Belgium). Aliquots (20 µl) of 500 or 1000 µg/ml GaM treatment and PBS as a control were injected into the last right pro-leg 30 min post bacterial infection. Larvae were incubated at 37°C for all studies. Larval survival was based on the level of melanisation and/or response to touch.

2.2.5 Determination of hemocyte density

G. mellonella larvae were inoculated with 20 µl aliquots of 500 and 1000 µg/ml GaM solutions or PBS and incubated at 37°C. Larvae, (n = 3, per sample), were bled to extract a total of 90 µl of hemolymph. Hemolymph was diluted in 100 µl sterile PBS and *N*-phenylthiourea to prevent melanisation in pre-chilled microcentrifuge tubes. Hemocyte density was calculated using a hemocytometer and determined as number of cells per ml.

2.2.6 *P. aeruginosa* protein extraction and purification

P. aeruginosa cultures grown overnight to the stationary phase were split (50/50) and re-grown in fresh media supplemented with GaM (500 µg/ml and 1000 µg/ml) at 37°C for an additional 6 h (at which stage early stationary phase was reached (**Appendix 8.1**). Proteins were extracted using 6 M urea, 2 M thiourea and a selection of protease inhibitors (phenylmethylsulfonyl fluoride (PMSF; 50mM), aprotinin, leupeptin, pepstatin A, and Tosyl-L-lysine chloromethyl ketone (TLCK; 1 mg/ml), Sigma). Cell debris was pelleted by centrifugation at 9000 x *g* for 5 min. Proteins were quantified via the Bradford protein assay and acetone precipitated (100 µg) overnight at −20°C. The acetone was removed and proteins were re-suspended in 25 µl of resuspension buffer (6 M Urea, 2 M Thiourea, 0.1 M Tris-hydrochloride (HCl; pH 8.0) dissolved in deionized water). The Qubit^{TM} protein quantification system (Invitrogen) was used to quantify 2 µl aliquots of protein samples. Ammonium bicarbonate (50 mM) was added to the remaining samples and proteins were reduced with 0.5 M dithiothreitol (DTT) (Sigma-Aldrich) at 56°C for 20 min and alkylated with 0.5 M iodoacetamide (IAA) (Sigma-Aldrich) in the dark at room temperature for 15 min. Proteins were digested with Sequence Grade Trypsin (0.5 µg/µl) (Promega) and incubated overnight at 37°C. Trifluoroacetic acid (TFA;1 µl; Sigma-Aldrich) was added to inhibit tryptic digestion. Following 5 min incubation at room temperature, samples were centrifuged at 13,000 x *g* for 10 min. Peptides were purified using C-18 spin columns (Pierce) to yield a total of approximately 30 µg of protein and dried in a SpeedVac concentrator (Thermo Scientific Savant DNA120) at 39°C for 2 h. Samples were resuspended in acetonitrile (2% v/v) and TFA (0.05% v/v) and sonicated in a water bath for 5 min followed by centrifugation at 15,500 x *g* for 5 min. The supernatant was extracted and used for mass spectrometry.

2.2.7 Mass spectrometry

Digested *P. aeruginosa* protein samples (0.75 µg) were loaded onto a QExactive Mass Spectrometer (ThermoFisher Scientific) connected to a Dionex Ultimate™ 3000 (RSLCnano) chromatography system. Purified hemolymph proteins were loaded in the same manner as described by (Sheehan & Kavanagh, 2018). An acetonitrile gradient was used to separate peptides in a BioBasic™ C18 PicoFrit™ column (100 mm in length, 75 mm inner diameter) using a 65 min reverse phase gradient at a flow rate of 250 nl/min. The mass spectrometer was operating in an automatic dependent switching mode to acquire all data. A high resolution MS scan (300-2000 Dalton) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS.

Protein identification and LFQ normalisation of MS/MS data was carried out using MaxQuant version 1.6.6.0 (http://maxquant.org/) following established procedures outlined previously (Sheehan & Kavanagh, 2018). The Andromeda search engine in MaxQuant matched MS/MS data against a UniProt-SWISS-PROT database for *P. aeruginosa* PAO1 (Bateman, 2019).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD019265 (Perez-Riverol et al., 2019).

2.2.8 Data analysis

General procedures for data processing and graphic generation were performed on Perseus v.1.6.6.0 (http://maxquant.org/) as described by (Deslyper et al., 2016). Proteins not identified in at least 2 out of the 3 replicates were removed. Imputation of the data replaced missing values with values that mimic low abundance proteins randomly selected from a distribution specified by a downshift of 1.8 times the mean standard deviation (SD) of all measured values and a width of 0.3. Two sample *t*-tests were carried out with a cut-off of $p < 0.05$. For additional analysis, protein names and functions were obtained from searching the Uniprot ID from the Uniprot Knowledgebase (www.uniprot.org).

2.3 Results

2.3.1 Analysis of the *in vitro* and *in vivo* effect of GaM against *P. aeruginosa*

The growth of *P. aeruginosa* PAO1 when exposed to a range of GaM concentrations *in vitro*, was measured after 24 h at 37°C (**Figure 2.2**). A concentration of 62.5 µg/ml inhibited growth by approximately 50% and the maximum concentration used (250 µg/ml) inhibited growth by 70%.

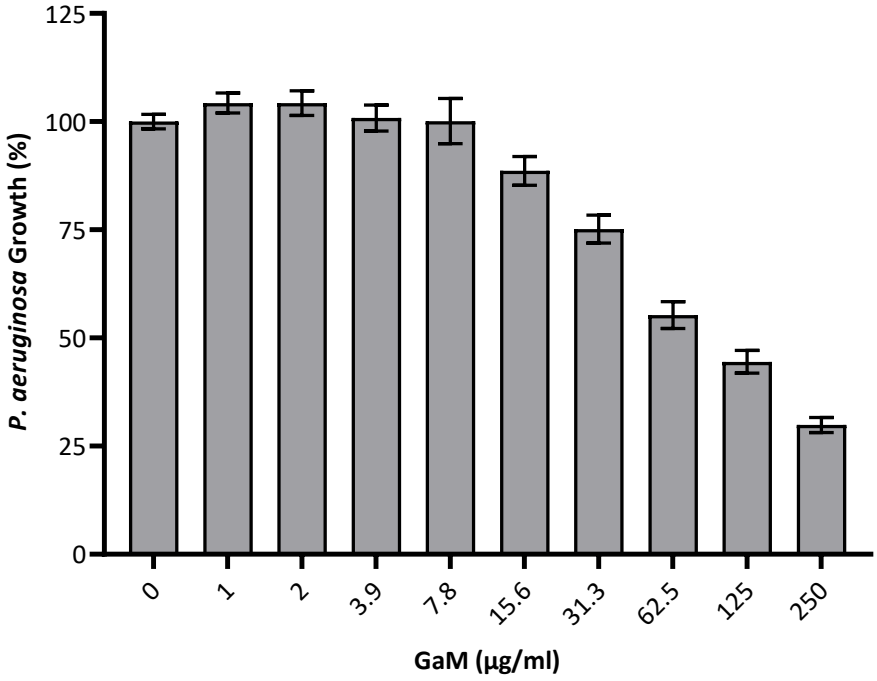


Figure 2.2. Growth of *P. aeruginosa* treated with GaM *in vitro*. Cultures grown overnight were subjected to GaM and incubated at 37°C and evaluated after 24 h. All values are the mean ± standard error (S.E.) of eight samples.

Larvae of *G. mellonella* were administered GaM doses of 125, 250, 500 and 1000 µg/ml in order to measure toxicity in the host. Larvae administered GaM showed no reduction in viability (**Appendix 8.2**) and there was an increase in the hemocyte (immune cell) density at 24 h in those larvae that received a dose of 500 or 1000 µg/ml (**Appendix 8.3**). The ability of GaM to act in the host and prolong survival of larvae infected with *P. aeruginosa* was assessed. Treatment of larvae infected with 3×10^2 and 3×10^3 CFUs of *P. aeruginosa* (doses that result in 100% mortality at 24 h) with doses of 500 and 1000 µg/ml GaM 30 min post-infection significantly increased larval survival at 24 h. Treatment of larvae infected with 3×10^2 CFUs *P. aeruginosa* with a

dose of 1000 µg/ml GaM post infection gave 95 ± 5 % survival at 24 h. Furthermore larvae infected with 3 x 10^3 CFUs of *P. aeruginosa* and administered a dose of 1000 µg/ml post-infection showed 90 ± 10 % survival at 24 h, (**Figure 2.3**).

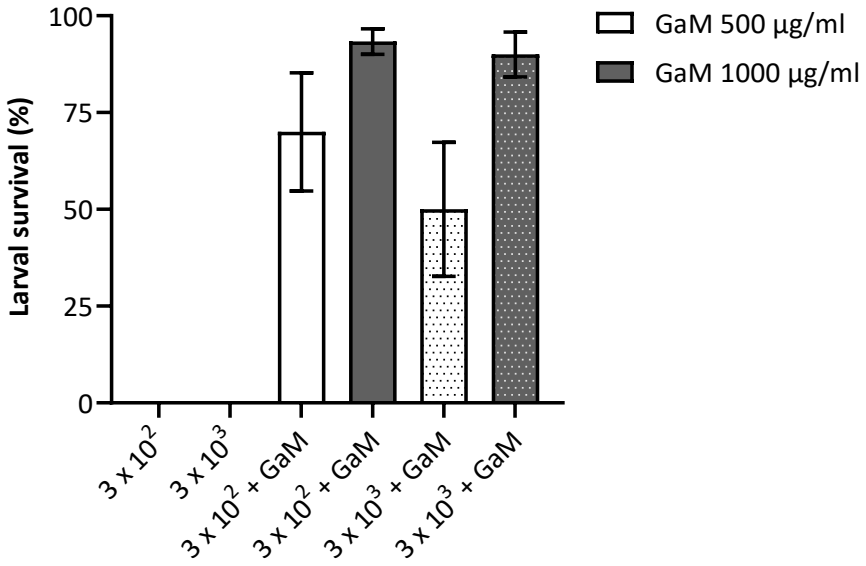


Figure 2.3. Survival of *G. mellonella* larvae inoculated with *P. aeruginosa* and GaM (500 µg/ml and 1000 µg/ml. Larvae (n =10, per group) were inoculated with 3 x 10^2 and 3 x 10^3 CFUs of *P. aeruginosa* prior to GaM administration (20 µl single dose). Control groups include infected larvae injected with PBS. Larvae were observed after 24 h at 37°C. Mean values ± S.E. were obtained from three independent experiments.

2.3.2 Proteomic analysis of the response of *P. aeruginosa* following exposure to GaM

Proteomic analysis allows a comparison of alterations in the whole proteome of an organism in response to a stress or treatment and can be used as a tool to identify potential modes of action of compounds on cells or whole organisms. The effect of GaM on *P. aeruginosa* was analysed via label-free quantitative mass spectrometry and visual representation of acquired data allowed for the identification of proteins and their associated pathways affected by GaM.

To investigate the proteomic response of *P. aeruginosa* to GaM *in vitro*, LFQ proteomics was performed on whole cell lysates. *P. aeruginosa* was grown in the presence of GaM (500 and 1000 µg/ml) for 6 h to the early stationary phase. An

exposure time of 6 h was chosen to ensure adequate incorporation of the compound into the cell in order to elicit a pronounced proteomic change.

A total of 2,606 proteins were initially identified and 1,748 remained after filtering out contaminants and peptides identified by site from the initial data matrix. A total of 1,673 proteins were present in all samples, 56 of which were exclusive to both treatment samples and six were exclusive to control samples (**Supp Dataset 2.3**). Post-imputation, 389 were deemed statistically significant and differentially abundant (SSDA), analysis of variance (ANOVA) $p < 0.05$) with a fold change of >1.5 (**Supp Dataset 2.1**).

A distinct difference between the proteomes of treated and control samples can be observed in the principal component analysis (PCA; **Figure 2.4**) of filtered proteins. The overlap between 500 µg/ml and 1000 µg/ml treatment samples indicates the limited effect of increasing concentrations of GaM on the *P. aeruginosa* proteome. Three replicates of treatment and control samples were resolved through hierarchal clustering of *z*-score normalised intensity values for all SSDA proteins, (n = 389), and visualised via a heatmap (**Appendix 8.4**). The heatmap generated on Perseus statistical software grouped proteins based on similar median expression trends and identified two major protein clusters, A and B, representing three replicates in each sample group (**Supp Dataset 2.2**).

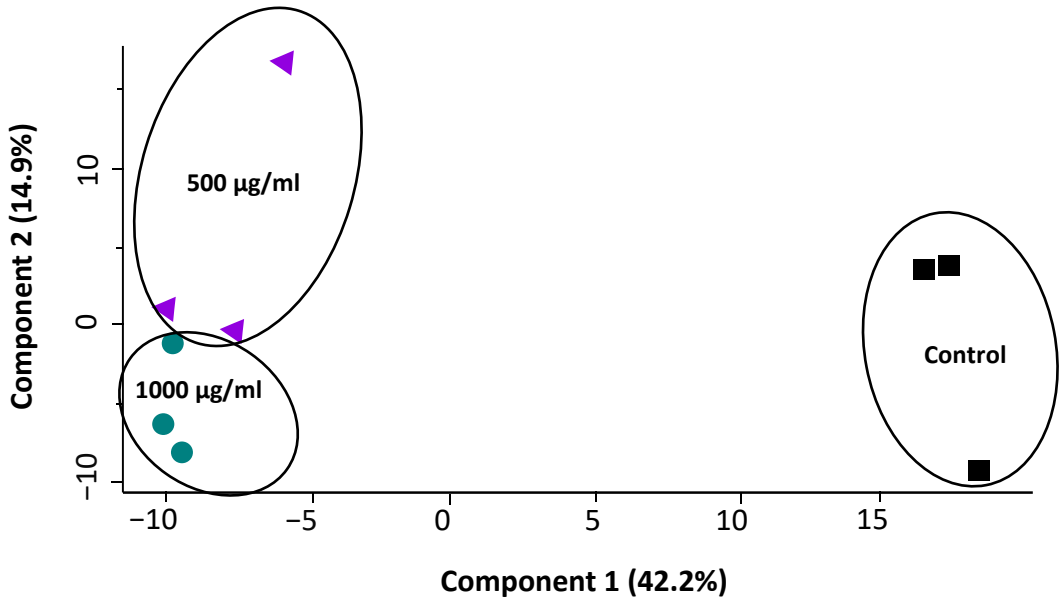


Figure 2.4. Principal component analysis (PCA) of untreated *P. aeruginosa* (black), *P. aeruginosa* treated with 500 µg/ml (green) and 1000 µg/ml (blue) GaM. A contrast is shown between both treated groups and untreated controls.

The volcano plot displays 1,748 filtered proteins with altered abundance levels of treated samples relative to controls. Among the top SSDA proteins increased in abundance in *P. aeruginosa* treated with 1000 µg/ml GaM in comparison to non-treated control samples include *arsC*-encoded protein-tyrosine-phosphatase (37.99-fold), arsenic-responsive ArsR protein (15.13-fold) FMN_red domain-containing protein associated with oxidation-reduction (8.98-fold), methylated-DNA-protein-cysteine methyltransferase associated with DNA-repair (8.5-fold), heme oxygenase HemO (7.6-fold), biotin synthase (7.14-fold), co-chaperone protein HscB homolog for maturation of iron-sulfur cluster-containing proteins (4.83-fold) and hemin degrading factor (4.19-fold; **Appendix 8.5**). SSDA proteins decreased in abundance in GaM-treated samples relative to the control group include elastase (−16.35-fold) and protease A (−11.83-fold) quorum-sensing (QS) enzymes, anaerobic and virulence activator-modulator AnvM (−9.75-fold), dihydrolipoyl dehydrogenase involved in pathogenesis and cell redox (−7.07-fold) and Type 4 fimbrial biogenesis protein PilY2 (−4.6-fold), (**Figure 2.5**; **Appendix 8.5**).

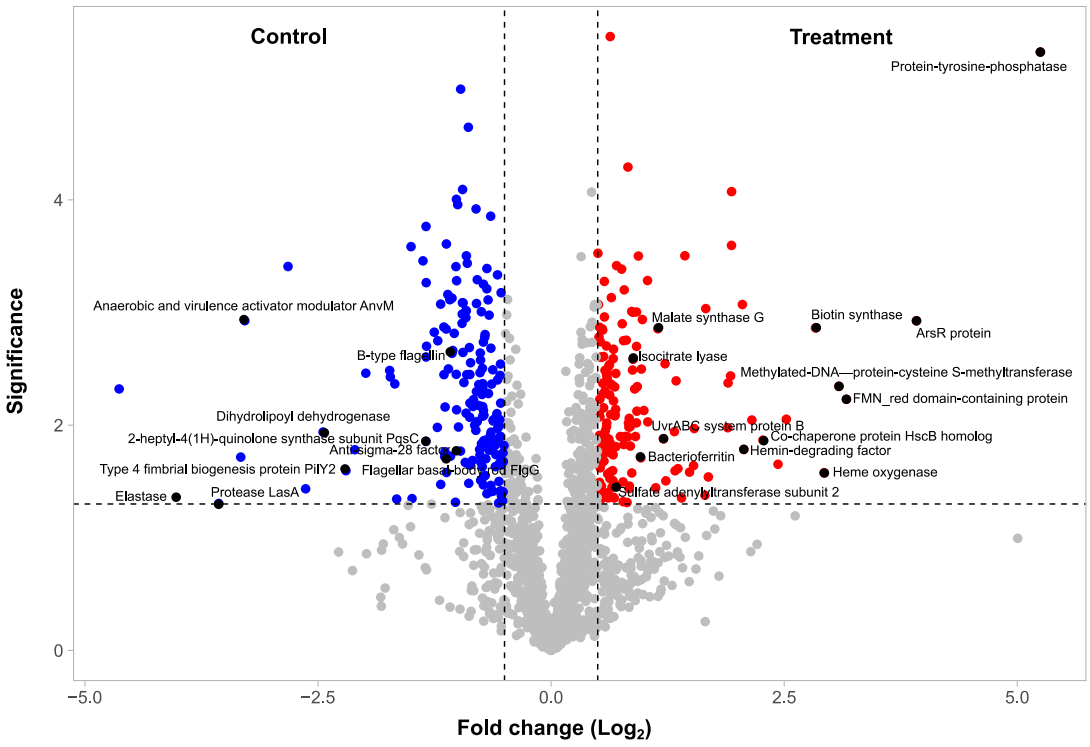


Figure 2.5. Volcano plot representing differentially abundant proteins in *P. aeruginosa* treated with 1000 µg/ml GaM for 6 h. The distribution of quantified proteins is based on significance ($-\log_{10}$ *p*-value) versus the fold change (Log_2 LFQ intensity difference). Statistically significant (*p*-value < 0.05) proteins are located above the horizontal line. Expression transcripts with relative fold changes of >1.5 are shown with increased expression to the right (red) and decreased expression to the left (blue) of the vertical lines. SSDA proteins are annotated (black).

The Search Tool for the Retrieval of INteracting Genes (STRING) database was used to identify biological pathways and protein networks between SSDA proteins increased and decreased in abundance versus control samples. Pathways associated with stress response, DNA damage/repair, iron-sulfur clusters and heme storage were upregulated in GaM-treated P. aeruginosa (**Figure 2.6A**). QS, flagellar motility and cell-redox pathways were downregulated (**Figure 2.6B**).

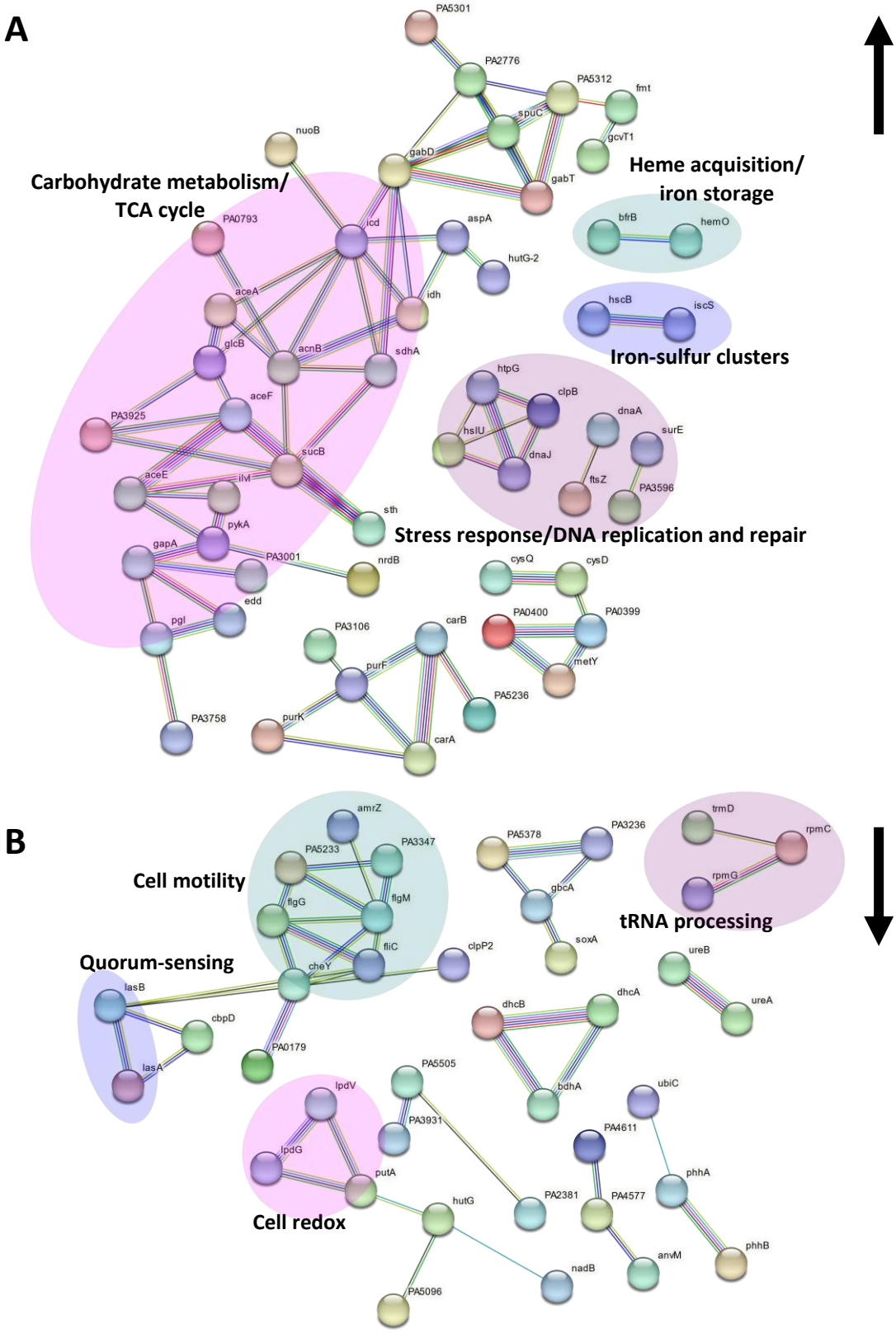


Figure 2.6. Network analysis of proteins increased and decreased in abundance in *P. aeruginosa* treated with 1000 µg/ml GaM. Data obtained from the STRING database using gene lists from SSDA proteins from pair wise *t*-tests ($p < 0.05$) shows interactions among individual proteins and associated pathways (highlighted in colour). (A) Protein pathways upregulated in GaM-treated *P. aeruginosa*. (B) Protein pathways downregulated in GaM-treated *P. aeruginosa*.

2.4 Discussion

The survival of virtually all bacterial pathogens is heavily dependent upon the presence of iron within their host and an ability to access this metal (Skaar, 2010). GaM targets this weakness to disrupt iron metabolism. Although numerous studies have described mechanisms of action, little is known about the proteomic response of bacteria to GaM (Chitambar, 2016; DeLeon et al., 2009; Hijazi et al., 2018; Arnold et al., 2012; Cohen et al., 2015). Analysis of protein expression changes in *P. aeruginosa* in response to GaM can further inform potential modes of action.

P. aeruginosa was susceptible to GaM *in vitro* and 70% growth inhibition was achieved at a concentration of 250 µg/ml. This study utilised *G. mellonella* larvae as an efficient *in vivo* model to further validate the efficacy of GaM within a host. The insect immune system is similar to the mammalian innate immune response: the cuticle and skin are physical barriers against pathogens; blood-like hemolymph circulates hemocytes (immune cells) for phagocytosis and superoxide production, much like mammalian neutrophils (Sheehan & Kavanagh, 2018; Browne & Kavanagh, 2013). Despite lacking an adaptive immune response, *G. mellonella* larvae have been widely used in toxicity assays to elucidate the efficacy of both conventional and novel drugs and have provided comparable results to those from mammalian studies (Browne & Kavanagh, 2013; Kavanagh & Sheehan, 2018). The assessment of *G. mellonella* larvae viability revealed that GaM has growth-inhibiting activity *in vivo* and maintains larval survival for up to 24 h, thereafter survival rates declined. Larvae challenged with lethal concentrations of *P. aeruginosa* prior to GaM administration showed significantly increased survival in comparison to controls at 24 h. Furthermore, GaM demonstrated no toxic effects *in vivo* as larvae inoculated with varying concentrations of GaM showed 100% survival with no signs of melanisation for up to 24 h. To identify whether GaM induced an immunomodulatory effect, larval immune cells (hemocytes) were extracted and enumerated. Administration of GaM increased production of hemocytes, which may enhance antibacterial activity within the larvae. Determination of hemocyte density is a common criterion assessed in many *G. mellonella* studies and inoculation of antimicrobial agents has been shown to induce immune priming responses (Taszłow, Vertyporokh & Wojda, 2017; Sheehan, Clarke & Kavanagh, 2018; Gandra et al., 2020; Fuchs et al., 2016).

Quantitative mass spectrometry identified a distinct difference between the proteomes of GaM-treated *P. aeruginosa* cells versus control cells. Protein-tyrosine-phosphatase was one of the most highly upregulated proteins (37.99-fold), and although the consequence of this is uncertain, the roles of this protein in the cell stress response/resistance, secretion of polysaccharides and biofilm formation have been proposed (Kiley & Stanley-Wall, 2010; Klein, Dartigalongue & Raina, 2003; Standish & Morona, 2014; Musumeci et al., 2005). Indication of a cell stress response was seen through increases in oxidation-reduction via the FMN_red domain-containing protein (8.98-fold) and DNA repair protein methylated-DNA—protein-cysteine S-methyltransferase (8.5-fold), which catalyses cysteine methylation (Yao et al., 2014). The methylation process signals for the repair of mutations in the genome induced by drug toxicity and stress (Schlagman, Hattman & Marinus, 1986; Cohen et al., 2016; Ghosh et al., 2020). Additional counteraction of GaM-induced DNA damage occurred as part of an SOS response via upregulation of UvrABC system protein B (2.3-fold). This component of the UvrABC repair system scans and cleaves abnormalities within DNA (Truglio et al., 2006; Burby & Simmons, 2019; Crowley & Hanawalt, 1998). Furthermore, upregulation of the heme oxygenase (HemO) component of the heme acquisition system (7.6-fold), biotin synthase (that catalyses biotin production through sulfur insertion; 7.14-fold) and co-chaperone HscB protein homolog associated with maturation of iron-sulfur clusters (4.83-fold) are possibly indicative of cellular mechanisms employed to counteract inadequate iron supplies in the cell (Cornelis & Dingemans, 2013; Nguyen et al., 2014; O'Neill et al., 2012; Reyda, Fugate & Jarrett, 2009; Romsang et al., 2014; Miller & Auerbuch, 2015). Interestingly, proteomic studies on the response of *P. aeruginosa* to iron-limited conditions also showed upregulation of heme acquisition components (such as HemO), whereas a previous study utilised gallium porphyrins to disrupt heme uptake pathways and inhibit growth in *P. aeruginosa* (Nelson et al., 2019; Choi, Britigan & Narayanasamy, 2019). Hemoproteins like haemoglobin provide an additional source of iron and can be degraded and liberated via HemO in the heme acquisition pathway (Choi, Britigan & Narayanasamy, 2019; Wegele et al., 2004). With the exception of IscU, iron-limitation also resulted in upregulation of iron-sulfur cluster proteins HscAB, IscR and IscX. In contrast, GaM induced expression of the iron storage protein bacterioferritin B (1.95-fold) and TCA cycle component malate synthase GlcB (2.21-fold), both of which were downregulated in iron-limited conditions (Nelson et al., 2019). Upregulation of

isocitrate lyase AceE (1.71-fold) and cysteine biosynthesis protein sulfate adenylyltransferase subunit 2 (1.63-fold; encoded by *cysD*) following GaM exposure coincided with the effects of iron-limitation (Nelson et al., 2019).

Exposure of *P. aeruginosa* to GaM downregulated QS, an important cell-to-cell communication system that relies upon a range of signalling molecules that enable bacteria to coordinate the expression of virulence genes in response to cell density (Rutherford & Bassler, 2012). The *las* and *rhl* systems and their respective transcriptional activators, LasR and RhlR, are regulated via *N*-acylhomoserine lactone signalling molecules (Glessner et al., 1999). These systems mediate a multitude of virulence factors responsible for tissue damage, nutrient acquisition, evasion of host immunity and enhanced dissemination of infection (Turkina & Vikström, 2019). These include *lasA* (protease A) and *lasB* (elastase), both of which showed significant decreases in abundance (−11.83-fold and −16.35-fold, respectively) in GaM-treated *P. aeruginosa*. The alternative QS system, *Pseudomonas* quinolone signal (PQS) system, employs alkylquinolone signals mediated by *pqsB* and *pqsC* genes (Drees et al., 2016). The latter gene, which encodes the 2-heptyl-4(1H)-quinolone synthase subunit PqsC protein and is responsible for the synthesis of quinolone signalling molecules 2-heptyl-4(1H)-quinolone and 2-heptyl-3(1H)-quinolone, was decreased in abundance by 2.54-fold (Liu et al., 2018). Inhibition of PQS genes has been linked to reduced biofilm, elastase, pyocyanin and siderophore virulence factor synthesis (Diggle et al., 2003; Heeb et al., 2011). Repression of the transcriptional regulator *pvdS* and hence biosynthesis of pyoverdine has been suggested as one mode of action adopted by GaM in disrupting iron metabolism (Goss et al., 2018). Direct inhibition of pyoverdine synthesis was not explicitly identified in this study, however, the association between QS and pyoverdine regulation has previously been outlined (Whiteley, Lee & Greenberg, 1999; Stintzi et al., 1998). LasR plays roles in the regulation of *lasA* and *lasB* genes and has been shown to mediate pyoverdine biosynthesis (Stintzi et al., 1998; Coin et al., 1997). It has been proposed that cell density can play a role in siderophore regulation in the Gram-negative pathogen, *Vibrio vulnificus*. This idea has also been explored in *Burkholderia cepacia*, *Vibro harveyi* and *P. aeruginosa* and may provide a link between QS inhibition and the suppression of iron uptake via GaM therapy (Wen et al., 2012).

The invasive nature and success of *P. aeruginosa* in colonising diverse environments is accounted for by twitching/swarming motilities, that are modulated by QS pathways with the aid of flagellar movement, to sense and move towards favourable environments (Zolfaghar, Evans & Fleiszig, 2003; Murray, Ledizet & Kazmierczak, 2010; Chuang et al., 2019; Lakshmanan et al., 2020). A number of regulatory genes are required in flagellar biosynthesis including the anti-sigma-28 factor protein (encoded by *flgM*) that regulates expression of the B-type flagellin (encoded by *fliC*), and *flgG*-encoded flagellar basal-body rod protein FlgG which mediates flagellar rod assembly (Frisk et al., 2002; Burrage, Vanderpool & Kearns, 2018; Zhu et al., 2019). Downregulation of flagellar basal-body rod protein FlgG, the anti-sigma-28 factor protein and B-type flagellin occurred following GaM treated with fold decreases of −2.1, −1.96 and −2.09, respectively, and are also presented on the STRING network analysis (**Figure 2.6**). In addition, the type 4 fimbrial biogenesis protein PilY2, is a surface sensor and showed a −4.6-fold decrease in abundance in GaM-treated *P. aeruginosa*. Comparisons of iron-limitation and GaM treatment have shown inconsistencies in twitching motility in *Pseudomonas* strains. Studies by (Nelson et al., 2019) correlate with research showing iron-rich environments promote biofilm-forming phenotypes, whereas iron deficiency upregulates twitching motility in *P. aeruginosa* (Nelson et al., 2019; Patriquin et al., 2008). Iron limitation in *Pseudomonas fluorescens* reduces expression of flagellar motor proteins FliN (fold change (log_2) -1.96) and FliG (fold change (log_2) -1.75) and flagellar M-ring protein FliF (fold change (log_2) -1.74) as examples (Lim et al., 2012).

While the anti-*Pseudomonal* activity of GaM is well characterised, the potency of the complex in terms of its minimum inhibitory concentration that inhibited 80% growth (MIC_{80}), (>250 μg/ml), is rather low when compared to some existing antibacterial drugs (Sader et al., 2017; Ekkelenkamp et al., 2020; Mustafa et al., 2016; Pathmanathan, Samat & Mohamed, 2009). Synergistic combination therapy with GaM may therefore be more beneficial, particularly for the treatment of resistant pathogens. A novel proteomic approach was utilised here to shed light on an alternative mechanism of action- the disruption of iron metabolism and attenuation of virulence through reduced QS and swarming ability. There is ample evidence of the physiological links between iron and QS pathways in bacteria and the exploitation of QS as a drug target is well established (Oglesby et al., 2008; Kaur, Lansky & Wilks,

2009; Bollinger et al., 2001; Juhas et al., 2004; Jiang et al., 2019; Asif & Acharya, 2012; Suga & Smith, 2003; Imperi et al., 2013). The development of novel or re-purposed compounds are imperative to combat AMR in clinically relevant bacteria and the results presented here indicate that GaM is active *in vitro* and *in vivo* for the treatment of recalcitrant *P. aeruginosa* infection.

Chapter 3

Characterisation of the proteomic response of *Aspergillus fumigatus* to novel Ga(III) polypyridyl catecholate complexes

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Author Contributions

Experiments, data analysis, and manuscript writing were performed by MP. Experiments, data analysis and manuscript editing were performed by BG. Compound synthesis and characterisation were performed by LMO'F. Funding, experimental design and manuscript editing were performed by AMP, GB, SE, CO'C, DG, AMH and KK.

Abstract

Infections caused by the airborne fungal pathogen, *Aspergillus fumigatus,* are increasing in severity due to growing numbers of immunocompromised individuals and increasing incidence of antifungal drug resistance, exacerbating treatment challenges. Gallium has proven to be a strong candidate in the fight against microbial pathogens due to its iron-mimicking capability and substitution of Ga(III) in place of ferric iron (Fe(III)) disrupting iron-dependent pathways. Iron is a critical nutrient for cell growth and viability, and iron-chelating siderophores employed for iron uptake are hypothesised to incorporate gallium in the same manner. We report the *in vitro* activity and proteomic effects of gallium nitrate (GaN) and a novel Ga(III) polypyridyl catecholate compound against *A. fumigatus*. The siderophore-conjugated compound has demonstrated enhanced growth-inhibition and impact on the *A. fumigatus* proteome compared to GaN. DNA replication and repair mechanisms along with lipid metabolism and the oxidative stress responses are elevated. Crucially, a large number of mitochondrial proteins are reduced in abundance. Respiration is an important source of energy to fuel metabolic processes required for growth, survival and virulence, the disruption of which may be a viable strategy for treatment of microbial infections.

3.1 Introduction

Fungi of the *Aspergillus* genus are responsible for a broad range of diseases termed aspergillosis and include allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) and invasive aspergillosis (IA) (Kanj, Abdallah & Soubani, 2018). *Aspergillus fumigatus* is widely distributed in the environment and is one of the main species to cause fungal disease, particularly among immunocompromised individuals (Sugui et al., 2015). The fungus disseminates via air-borne conidia, several hundred of which are inhaled daily. A healthy pulmonary immune system rapidly identifies and clears conidia, however, in susceptible patients conidia germinate and produce hyphae that can grow in the lung or penetrate the airway epithelial barrier and enter the bloodstream to cause life-threatening invasive infection (Latgé, 1999). *Aspergillus* infections have a high mortality rate among immunocompromised transplant, HIV+, chemotherapy and cystic fibrosis (CF) patients, and the ongoing rise in resistant infections has deemed *A. fumigatus* a critical priority pathogen (Dagenais & Keller, 2009; Parums, 2022). Advances in immunosuppressive therapies and more recently, the COVID-19 pandemic have increased treatment challenges (Kauffman, 2001). The capacity of this pathogen to occupy diverse and hostile environments is due to virulence factors that include metabolism and growth regulation, thermotolerance, intra- and extra-cellular siderophore secretion for iron uptake and homeostasis, secretion of hydrolytic enzymes and the production of secondary metabolites (i.e. mycotoxins) (Irmer et al., 2015; Chang et al., 2004; Robson et al., 2005; Kamei & Watanabe, 2005; Haas, 2012).

The primary *Aspergillus* treatment options involve the use of the azole antifungals (namely voriconazole, isavuconazole, posaconazole) and the polyene, amphotericin B in liposomal formulation (Buil et al., 2019). The latter is administered in cases of failed azole treatment due to resistance but its use is associated with increased nephro- and hepato-toxicities (Patel, Crank & Leikin, 2011). Azole resistance in *Aspergillus* has emerged globally and whilst there are low levels of resistance to amphotericin B, resistance to polyenes is predicted to increase with limited alternatives (Fan, Korfanty & Xu, 2021).

With an estimated 1.7 million deaths annually due to fungal infections and dwindling treatment options, the development of novel therapeutics is critical (Kainz et al., 2020).

The repurposing of metals as novel antimicrobial agents has garnered much interest in recent decades. Radiopharmaceutical applications of gallium date back to the 1940s and gallium's use has since expanded to anticancer and antimicrobial treatment (Hayes, 1978; Chitambar, 2012; Li et al., 2022a). Gallium compounds such as gallium nitrate, gallium citrate and gallium maltolate, have shown potent activity against multi-drug resistant (MDR) bacteria *in vitro* and *in vivo* using insect and mammalian models (Antunes et al., 2012; Olakanmi et al., 2013; Piatek, Griffith & Kavanagh, 2020; DeLeon et al., 2009). The antifungal effects are less well characterised.

Gallium is a Group IIIA metal with a similar ionic radius to iron (III), and iron is a fundamental nutrient for microbial growth. Unlike iron, gallium cannot be reduced from the trivalent to divalent form in normal physiological conditions, thereby disrupting iron-dependent proteins required for DNA synthesis, amino acid metabolism, energy generation via respiration and virulence in bacterial and fungal pathogens (Goss et al., 2018; Bastos et al., 2019). Host iron is sequestered and restricted from invading microbes to limit infection. Despite this, bacterial and fungal pathogens have developed powerful counteractive strategies (Parrow, Fleming & Minnick, 2013).

Bacteria and fungi secrete iron-chelating molecules called siderophores in iron-deficient environments. In bacteria, siderophores are categorised based on the type of functional group: hydroxamate, catecholate, carboxylate, phenolate or a mixed combination (Kramer, Özkaya & Kümmerli, 2020). Antibiotic-siderophore conjugates are a promising approach to enhance drug uptake and efficacy (Kong et al., 2019). Sideromycins are naturally occurring siderophores covalently bound to an antibiotic and include albomycin for streptococcal and *Yersinia* infections (Pramanik et al., 2007). Furthermore, the FDA-approved cephalosporin-catecholate conjugate, Cefiderocol, is a potent Gram-negative antibacterial (Ong'Uti et al., 2022). Iron acquisition in fungi such as *A. fumigatus* relies upon four siderophores (fusarinine C (FsC), triacetylfusarinine C (TAFC), ferricrocin and hydroxyferricrocin) but can also employ non-native siderophores (Aguiar et al., 2022; Haas et al., 2003). Khan *et al.* (2021) proved that exposure to *E. coli* catecholate siderophores increases iron accumulation in *Aspergillus nidulans* (Khan et al., 2021).

The work presented here detailed the synthesis and antibacterial activity of five novel water soluble heteroleptic Ga(III) polypyridyl catecholate compounds which also demonstrated low toxicity and some antibacterial efficacy *in vivo* using *Galleria mellonella* larvae (More O'Ferrall et al., 2023). Herein, we assessed the growth inhibitory and proteomic effects of three of these compounds: $[Ga(bipy)_2(2,3\text{-}DHBA_{-2H})](NO_3)$ (**1**), $[Ga(bipy)_2(3,4\text{-}DHBA_{-2H})](NO_3)$ (**2**) and $[Ga(bipy)_2(2,3,4\text{-}THBA_{-2H})](NO_3)$ (**3**) (**Figure 3.1**) on *A. fumigatus*. Proteomic analysis allows the characterisation of the changes in the cell's proteome following a treatment or stress and can be used to identify the likely mode(s) of action of a novel antimicrobial treatment (Tsakou et al., 2020; Senges et al., 2021). Label-free quantitative proteomics was performed to reveal protein abundance changes in *A. fumigatus* in response to the most potent compound, **1**, and commercially available gallium nitrate as a standard, to gain insight into the mechanistic roles of these novel antifungal therapeutics.

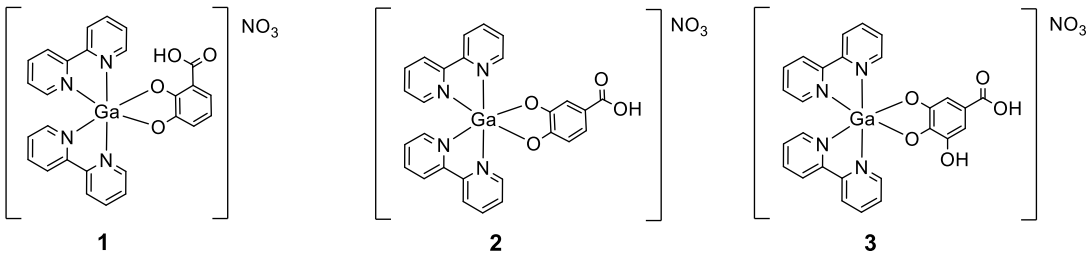


Figure 3.1. Chemical structures of $[Ga(bipy)_2(2,3\text{-}DHBA_{-2H})](NO_3)$ (**1**), $[Ga(bipy)_2(3,4\text{-}DHBA_{-2H})](NO_3)$ (**2**) and $[Ga(bipy)_2(2,3,4\text{-}THBA_{-2H})](NO_3)$ (**3**).

3.2 Materials and methods

3.2.1 *A. fumigatus* culture conditions

A. fumigatus (ATCC 26933) was grown on Sabouraud dextrose agar plates (Oxoid) at 37°C. Conidia were harvested with PBS and 0.01% (v/v) Tween-20 and washed twice in sterile PBS by centrifugation at 2000 x *g*. Conidia were enumerated using a haemocytometer and were added to Sabouraud dextrose medium to give a final concentration of 5 x10^5 conidia/ml and incubated at 37°C in an orbital shaker at 200 rpm.

3.2.2 Compound 1 – 3 preparation

Compounds **1** – **3** were synthesised as previously described and GaC and GaM were synthesised as previously reported (Finnegan et al., 1987; Matzapetakis et al., 2001; More O'Ferrall et al., 2023). Stock solutions of all compounds were prepared in sterile, deionised water up to 2 mg/ml. Dissolution was aided by sonication. Stock solutions were stored at 4°C.

3.2.3 *A. fumigatus in vitro* susceptibility assays

Galllium solutions were serially diluted in 96-well plates (Corning) containing Sabouraud dextrose media to final concentrations of 4 - 500 μg/ml. Aliquots (100 μl) of *A. fumigatus* conidia suspensions were added to all wells to obtain a final concentration of 5 x 10^5 conidia/ml. Plates were incubated at 37°C for 24 h and 48 h in a static incubator. Growth was quantified in a plate reader (Bio-Tek Synergy HT) at 600 nm.

3.2.4 Label-free quantitative mass spectrometry sample preparation

A. fumigatus cultures (n = 4, per sample group) were grown for 24 h at 37°C in an orbital incubator at 200 rpm by inoculating 25 ml Sabouraud dextrose medium with 5 x 10^5 conidia/ml. Compound **1** and GaN were applied in 5 ml aliquots to achieve final concentrations of 250 μg/ml and cultures were grown for a further 24 h. *A. fumigatus* hyphae were isolated by passing through Mira cloth and the wet weights were recorded to ensure standardised growth across all sample groups (**Appendix 8.6**). The hyphal mass was crushed into a fine powder using a pestle and mortar with liquid nitrogen.

Four ml/g hyphae of lysis buffer (8 M urea, 2 M thiourea and 0.1 M Tris-HCl, pH 8.0) supplemented with protease inhibitors (aprotinin, leupeptin, pepstatin A and TLCK (10 μg/ml) and PMSF (1 mM/ml) was added to powdered hyphae and sonicated with a sonication probe (Bandelin Sonopuls, Bandelin electronic, Berlin) at 50% power for 10 sec three times. The cell lysates were centrifuged at 14500 x *g* for 10 min to pellet the cell debris. The protein concentrations in supernatants were quantified using the Bradford method. Supernatants containing 100 μg of protein were combined with ice-cold acetone in a 1:3 ratio, supernatant:acetone, and proteins were left to precipitate at −20°C overnight.

The protein was pelleted via centrifugation at 14500 x *g* for 10 min. The acetone was discarded, pellets were air dried and resuspended in 25 μl resuspension buffer (same as lysis buffer minus the protease inhibitors). Two μl aliquots were used for protein quantification using the Qubit™ quantification kit (Invitrogen). To the remaining sample, 125 μl of ammonium bicarbonate (50 mM) was added followed by 1 μl DTT (0.5 M). Samples were reduced for 20 min at 56°C and alkylated with 2.7 μl IAA (0.55 M) in the dark at room temperature. Five hundred ng of 1% (w/v) ProteaseMAX Surfactant Trypsin Enhancer (Promega) was added to each sample followed by 500 ng of Sequence Grade Trypsin (Promega). Proteins were digested for 19 h at 37°C.

One μl TFA was added to inhibit digestion and samples were incubated at room temperature for 5 min. Samples were centrifuged for 10 min at 14500 x *g* and the supernatants were purified using C-18 spin columns (Pierce). Purified peptides were dried at 38°C for 2-3 h in a SpeedVac concentrator (Thermo Scientific Savant DNA 120) and resuspended in acetonitrile (2% v/v) and TFA (0.05% v/v). Samples (2 μl containing 750 ng protein) were loaded on to a QExactive mass spectrometer (ThermoFisher Scientific) connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Mass spectrometer and MaxQuant software (for protein quantification and normalisation of MS/MS data) parameters were set as described by Margalit *et al*. (2020) (Margalit et al., 2020).

3.2.5 2,3,5-Triphenyltetrazolium chloride assay

A. fumigatus was grown for 24 h and treated with 250 μg/ml **1** and GaN for 24 h as per previous methods for proteomic analysis. A 1.25 mg/ml stock solution of 2,3,5-

triphenyltetrazolium chloride (TTC) was prepared in Sabouraud dextrose medium and filter sterilised using a 0.22 μm pore filter. Twenty ml of TTC stock solution was added to each treated culture to obtain a final TTC concentration of 0.5 mg/ml. Culture flasks were incubated for a further 24 h at 37°C, 200 rpm. Hyphae were isolated and the wet weights were recorded. The hyphal mass was resuspended in 18 ml of acidified isopropanol (0.04 M HCl isopropanol) and incubated for 1 h at 30°C in an orbital shaker at 200 rpm to solubilise the formazan. The mixture was centrifuged for 5 min at 2000 x *g* to separate hyphae and supernatant. The supernatant (200 μl) was applied to wells in a 96-well plate and formazan was quantified using a microplate reader at 570 nm.

3.2.6 Data analysis

Raw MS data were processed via MaxQuant v 1.6.3.4 and Perseus v 1.6.6.0 against a *Neosartorya fumigata* (strain ATCC MYA-4609/Af293/CBS 101355/FGSC A1100) Uniprot-SWISS-PROT database (downloaded August 2022 ; 9647 entries). Pairwise students *t*-tests were performed using a cut-off of $p < 0.05$ on post-imputated data to observe differences between proteomic sample groups. Log_2 fold-changes and $-\log p$ values were plotted on the *x* and *y* axes, respectively, to generate volcano plots for Pairwise comparisons.

GraphPad Prism software v 9.5.1 was used to generate growth and TTC graphs. One-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$) was conducted to identify statistical significance between sample groups.

3.3 Results

3.3.1 The effect of gallium compounds on the growth of *A. fumigatus*

The susceptibility of *A. fumigatus* to compounds **1**, **2** and **3** was measured in conjunction with three existing and widely-reported gallium compounds – gallium citrate (GaC), gallium nitrate (GaN) and gallium maltolate (GaM). The latter compounds revealed weaker potency, achieving growth inhibitions of 19% (GaM), 47% (GaN) and 77% (GaC) at the highest tested dose (250 µg/ml). Compounds **1**, **2** and **3** reduced growths by 92.5 ± 0.5% at this concentration (**Figure 3.2**).

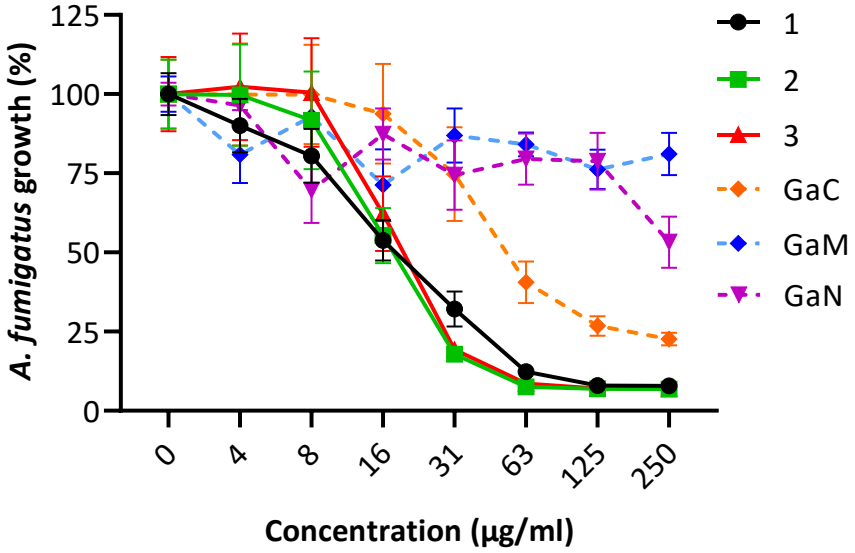


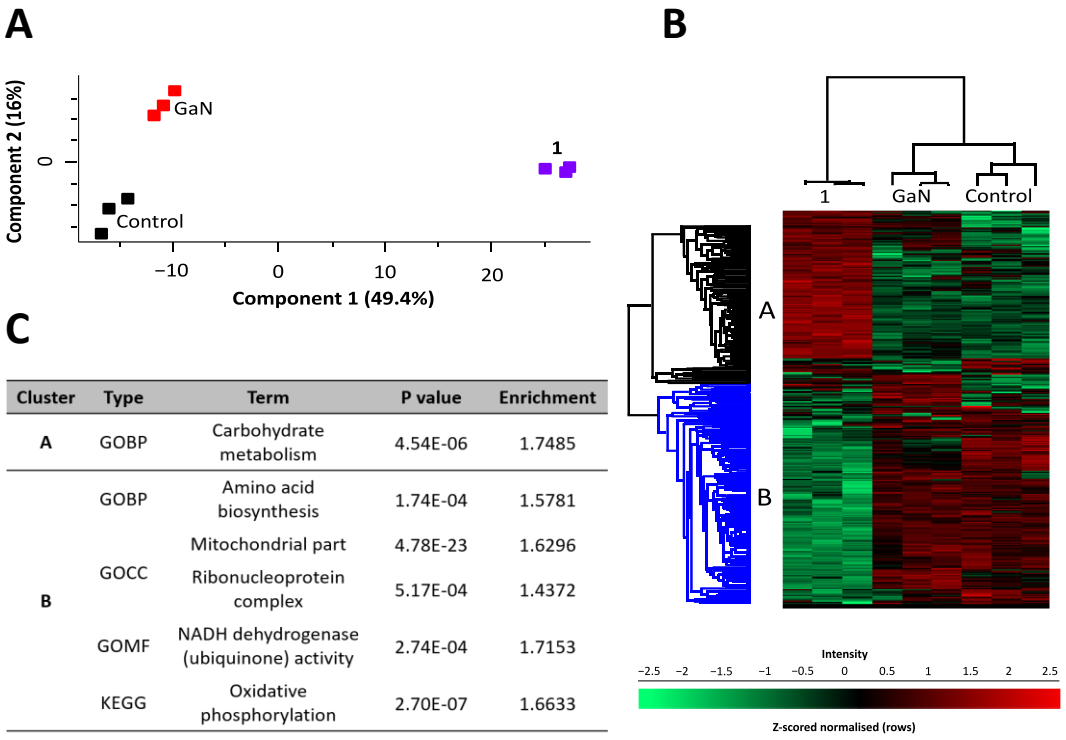
Figure 3.2. The growth of *A. fumigatus* after 24 hours in response to novel Ga(III) polypyridyl compounds **1**, **2** and **3** and commercially available gallium compounds gallium citrate (GaC), gallium maltolate (GaM) and gallium nitrate (GaN).

3.3.2 The proteomic response of *A. fumigatus* to [Ga(bipy)$_2$(2,3-DHBA$_{-2H}$)](NO$_3$) (1) and GaN

The proteomic responses of *A. fumigatus* exposed to **1** and GaN was assessed. *A. fumigatus* cultures were grown for 24 h as described above and then supplemented with doses of 250 µg/ml for a further 24 hours prior to protein extraction and analysis by LFQ proteomics. Analysis of hyphal wet weights showed no significant difference between treatments and control (**Appendix 8.6**). Post-filtration of contaminants and imputation steps, 2331 proteins were identified (**Supp Dataset 3.1**). All filtered

proteins were subjected to a principal component analysis (PCA) with a combined variance (Component 1 and Component 2) of 65.4%. All replicates were resolved into their corresponding sample group. *A. fumigatus* proteomes exposed to **1** displayed a clear distinction in comparison to GaN and control samples (**Figure 3.3A**).

Hierarchal clustering of *z*-score normalised LFQ intensity values for 705 statistically significant ($p < 0.05$) and differentially abundant (SSDA) proteins (fold change ≥ 1.5) was derived from pairwise *t*-tests (**Supp Dataset 3.2**). Columns comprise sample groups (n = 3 biological replicates in each) and individual proteins in rows. Proteins with corresponding expression profiles are grouped together (**Figure 3.3B**). Gene ontology enrichment analysis performed on two resolved clusters, A and B, revealed the main processes altered in response to treatment as listed in **Figure 3.3C**. The proteomes of GaN-treated and untreated *A. fumigatus* show clear distinctions to treatment with **1**.



C

Cluster	Type	Term	P value	Enrichment
A	GOBP	Carbohydrate metabolism	4.54E-06	1.7485
B	GOBP	Amino acid biosynthesis	1.74E-04	1.5781
	GOCC	Mitochondrial part	4.78E-23	1.6296
		Ribonucleoprotein complex	5.17E-04	1.4372
	GOMF	NADH dehydrogenase (ubiquinone) activity	2.74E-04	1.7153
	KEGG	Oxidative phosphorylation	2.70E-07	1.6633

Figure 3.3. (A) Principal component analysis (PCA) of *A. fumigatus* treated with **1**, GaN and untreated control (n = 3, per sample group). The proteomes of *A. fumigatus* challenged with **1** show distinct differences to GaN and control groups, which appear more closely related. (B) Hierarchal clustering of statistically significant proteins derived from *t*-tests ($p < 0.05$) and exclusively expressed proteins between sample groups. Proteins with similar expression profiles are clustered together in clusters A and B. (C) The main statistically enriched gene ontology terms in Clusters A and B are shown in Table C.

3.3.3 The proteomic response of *A. fumigatus* to GaN

Volcano plots derived from pairwise *t*-tests present the differential abundance of all filtered proteins and among them 101 SSDA proteins from GaN versus control comparisons, with $\log_2$ fold changes ranging from −50 to 52 (**Figure 3.4**). The top 10 most differentially abundant proteins are highlighted and annotated with the corresponding gene names, the functions of which are summarised in **Appendix 8.7**. There is an increased abundance in proteins such as nitrosative stress response protein nitric oxide dioxygenase (+52-fold), siderophore biosynthesis proteins N(5)-hydroxyornithine:cis-anhydromevalonyl coenzyme A-N(5)-transacylase sidF (+36-fold), L-ornithine N(5)-monooxygenase (+21-fold), mevalonyl-coenzyme A hydratase sidH (+19-fold) and acyl-CoA ligase sidI (+7-fold), and antioxidant superoxide dismutase [Cu-Zn] (+12-fold). Furthermore, there is a decreased abundance of dual-

functional monooxygenase/methyltransferase psoF (−5-fold) for fumagillin biosynthesis, pseurotin A biosynthesis mediator methyltransferase psoC (−4-fold), ubiquinone biosynthesis protein, putative (−3-fold) for lipid homeostasis, and sterol biosynthesis cytochrome b5, putative (−3-fold). These protein abundance changes indicate an increased stress response and compensatory strategies for iron acquisition. Disruption of lipid homeostasis and secondary metabolism may play roles in growth impairment and attenuation of virulence.

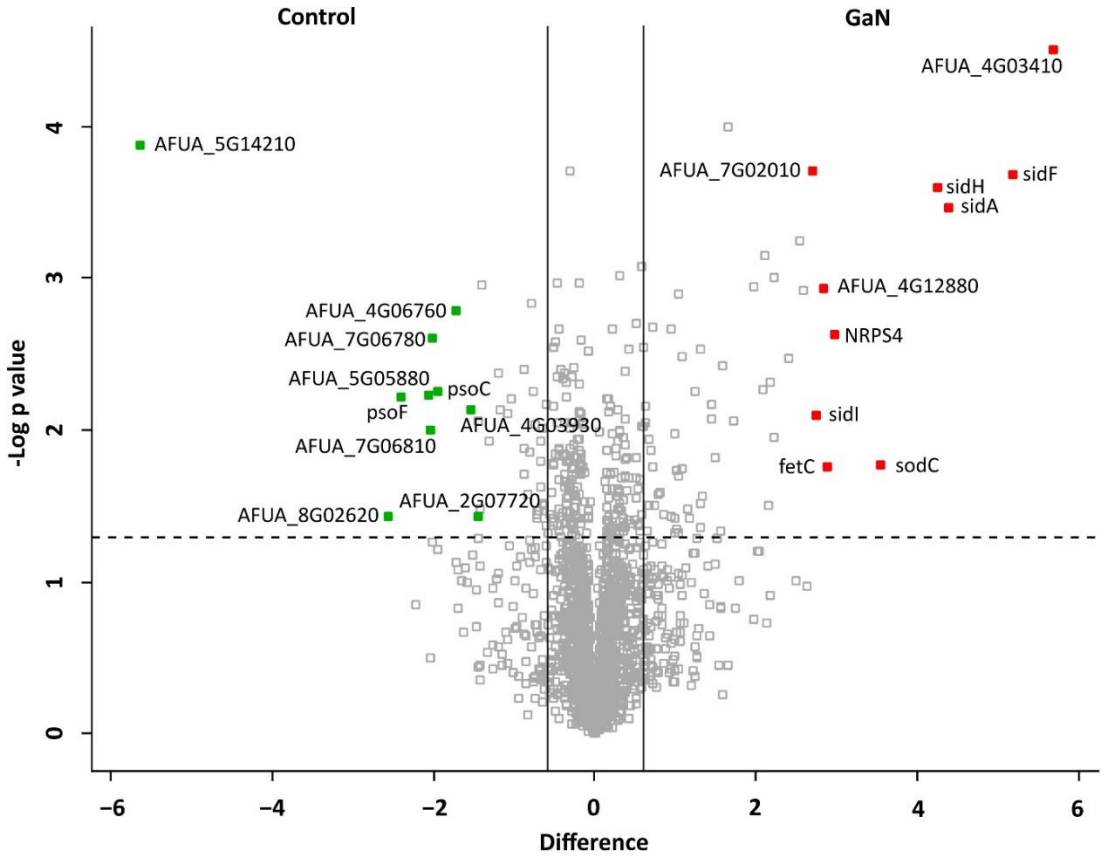


Figure 3.4. Volcano plot of *A. fumigatus* proteome treated with GaN (250 μg/ml) versus controls. The *x* axis denotes the $\log_2$ mean intensity difference, with cut-off of ± 0.59 used to represent a fold change of ± 1.5, indicated with vertical lines. The statistical significance (−log10 *p* value) is located on the *y* axis. Proteins above the dashed horizontal line are statistically significant, $p < 0.05$, and to the right and left of the vertical lines are differentially abundant, fold change ≥ 1.5. The top 10 most differentially abundant proteins increased and decreased are highlighted in red and green, respectively, and annotated with their corresponding gene names. Their protein products and associated functions are listed in **Appendix 8.7**.

3.3.4 The proteomic response of *A. fumigatus* to 1

Volcano plots derived from pairwise *t*-tests present the differential abundance of all filtered proteins and among them 501 SSDA proteins from **1** versus control comparisons with $\log_2$ fold changes ranging from −58 to 398 (**Figure 3.5**). The top 10 most differentially abundant proteins are highlighted and annotated with the corresponding gene names, the functions of which are summarised in **Appendix 8.8**. Increased abundance in proteins such as cupin domain protein (+398-fold) with suspected roles in pathogenesis, superoxide dismutase [Cu-Zn] (+69-fold), N(5)-hydroxyornithine:cis-anhydromevalonyl coenzyme A-N(5)-transacylase sidF (+28-fold), antioxidant DUF323 domain protein (+17-fold) and ergosterol biosynthesis protein methylsterol monooxygenase erg25A (+16-fold), all show evidence of stress-induced and compensatory responses to withstand the perturbation of iron homeostasis caused by **1**. Furthermore, the decreased abundance of cysteine synthase B, putative (−27-fold) and 3-isopropylmalate dehydratase (−7-fold) for amino acid biosynthesis, respiratory chain cytochrome oxidase c oxidase assembly factor 6 (−14-fold) and isocyanide synthase-NRPS hybrid crmA employed in secondary metabolism (−11-fold) all indicate a reduction in energy and protein synthesis which is required for functional growth and development.

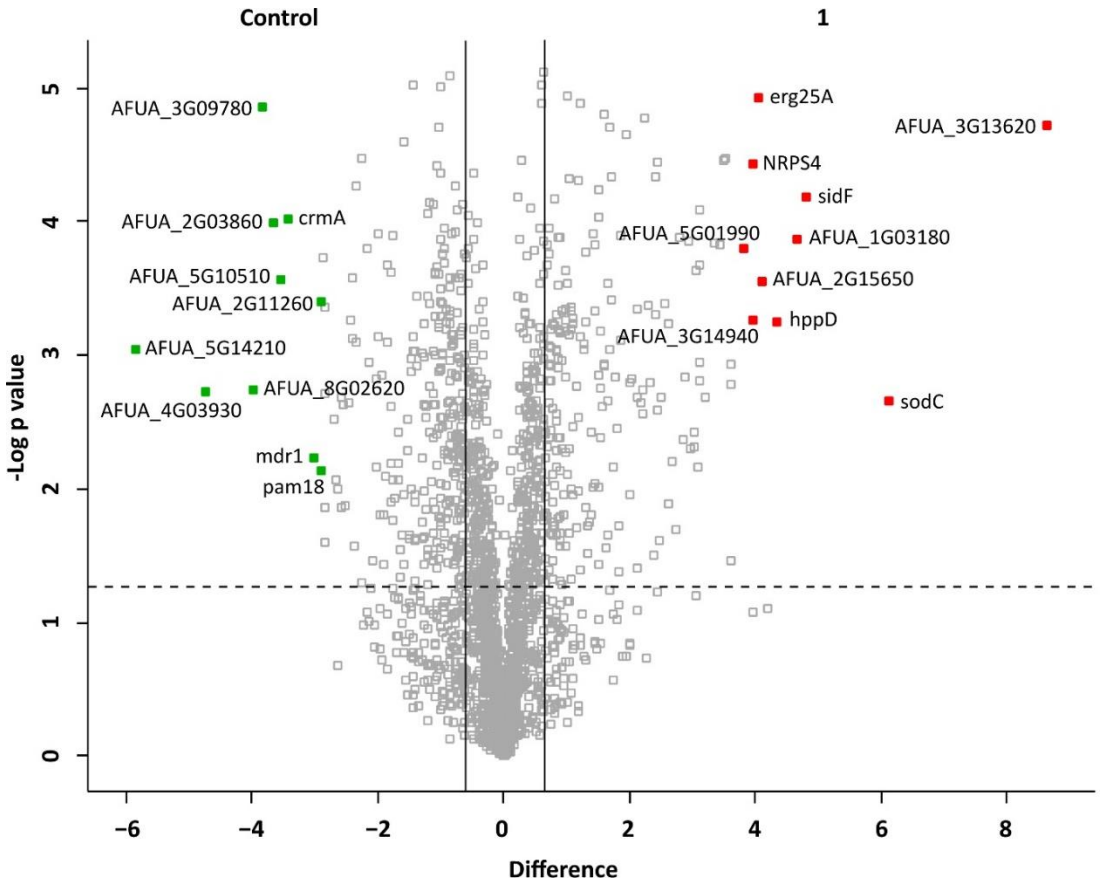


Figure 3.5. Volcano plot of *A. fumigatus* proteome treated with **1** (250 μg/ml) versus controls. The *x* axis denotes the $\log_2$ mean intensity difference, with cut-off of ± 0.59 used to represent a fold change of ± 1.5 indicated with vertical lines. The statistical significance (−log10 *p* value) is located on the *y* axis. Proteins above the dashed horizontal line are statistically significant, $p < 0.05$, and to the right and left of the vertical lines are differentially abundant, fold change ≥ 1.5. The top 10 most differentially abundant proteins increased and decreased are highlighted in red and green, respectively, and annotated with their corresponding gene names. Their protein products and associated functions are listed in **Appendix 8.8**.

STRING enrichment analysis revealed a number of pathways that were altered in response to **1,** such as DNA repair, iron acquisition and lipid biosynthesis, for example. A large abundance of mitochondrial proteins were affected with specific pathways highlighted in **Figure 3.6**. Mitochondrial translation, organisation and transport along with components of the respiration chain (TCA cycle, oxidative phosphorylation and complex IV assembly) are shown. Similar to GaN treatment, amino acid biosynthesis is reduced.

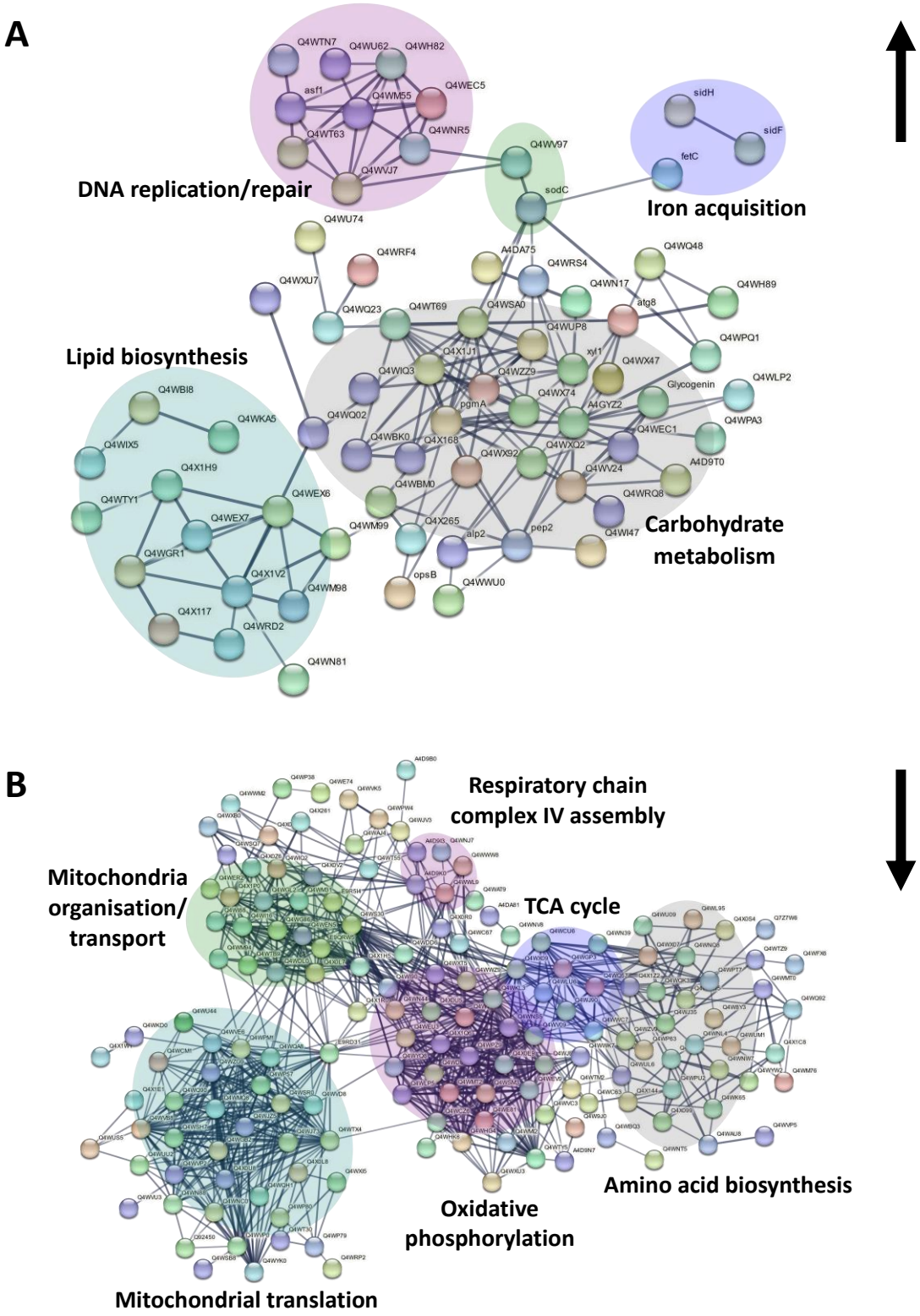


Figure 3.6. STRING network analysis of proteins and pathways altered (A: increased, B: decreased) in response to **1** (250 µg/ml) versus controls. Protein ID search and parameters are as previously described.

3.3.5 The differential response of *A. fumigatus* to 1 and GaN

Proteomic analysis was employed to ascertain differences in the response of *A. fumigatus* to GaN versus the novel gallium compound (**1**). A volcano plot was generated as previously described showing the increased abundance of proteins in response to **1** (right) and decreased abundance proteins in response to GaN (left) (**Figure 3.7**). The top 10 increased and decreased proteins are listed in **Appendix 8.9** and include cupin domain protein (+230-fold), hydroxyphenylpyruvate dioxygenase (+23-fold) for melanin biosynthesis, ribonucleotide reductase small subunit RnrA, putative for DNA synthesis (+17-fold), nitrosative stress response protein nitric oxide dioxygenase (−57-fold), cytochrome c oxidase assembly factor 6 (−14-fold) and glutathione S-transferase, putative for oxidative stress resistance (−9-fold). The results indicate a more toxic environment induced by **1,** as shown by the elevated abundances of oxidative stress proteins in response to this treatment. Moreover, the relative fold changes of SSDA proteins identified in both **1** and GaN groups versus controls are predominantly more abundant in the former, as shown in **Supp Dataset 3.3**.

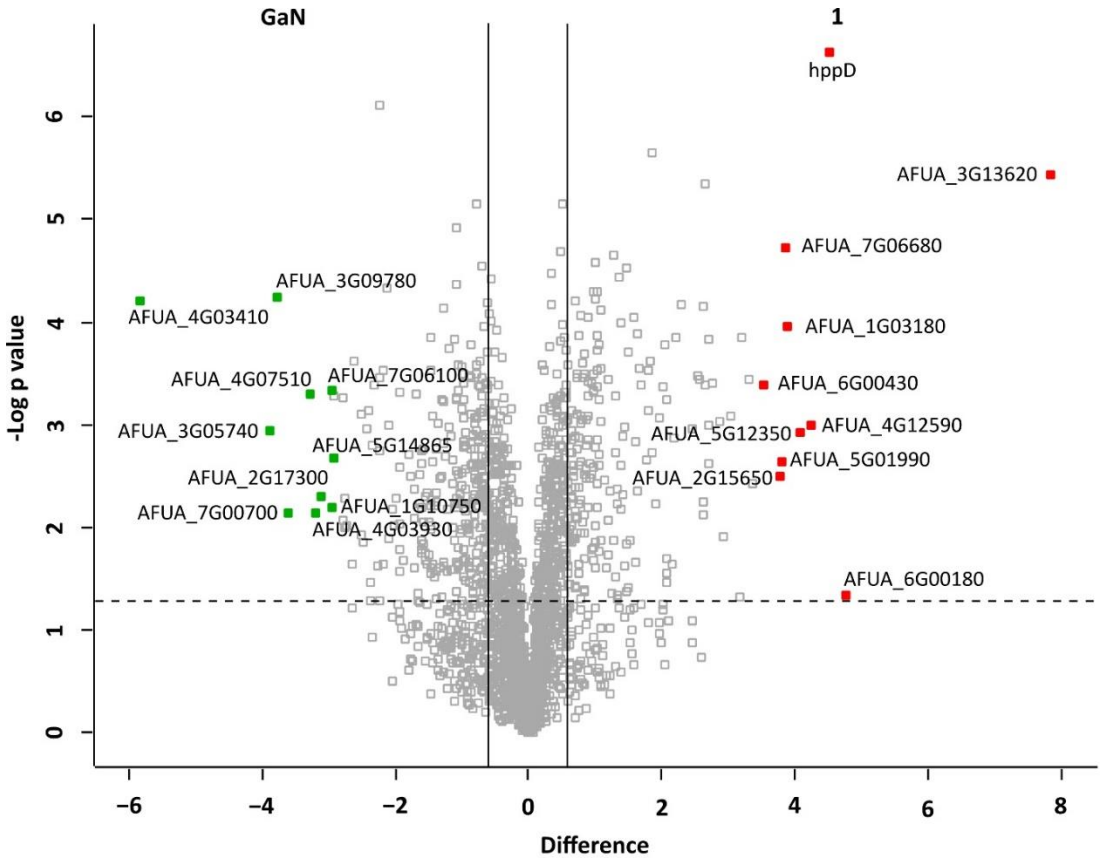
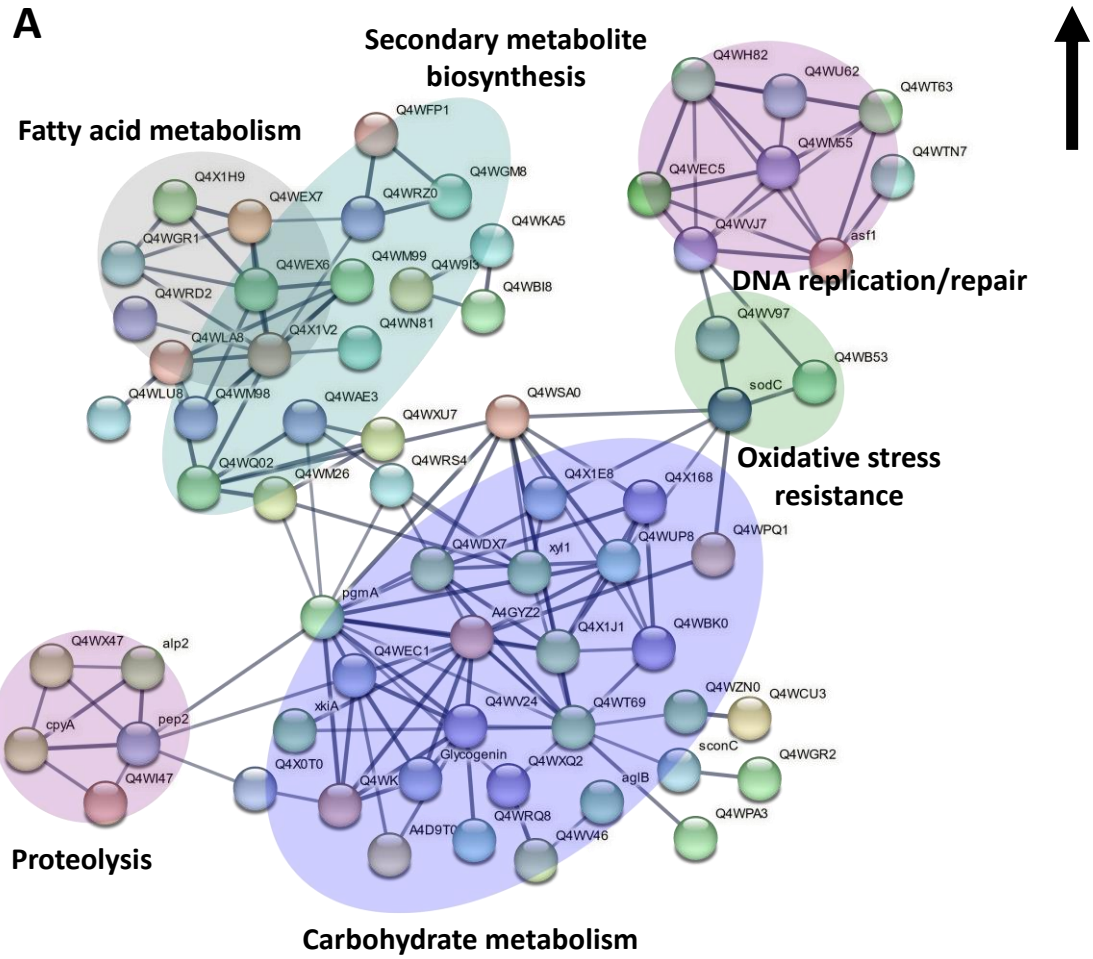


Figure 3.7. Volcano plot of *A. fumigatus* proteome treated with GaN (250 μg/ml) versus **1** (250 μg/ml). The *x* axis *y* axis parameters are as previously described. Proteins above the horizontal dashed line are statistically significant, $p < 0.05$, and to the right and left of the vertical lines are differentially abundant, fold change ≥ 1.5. The top 10 most differentially abundant proteins increased and decreased are highlighted red and green, respectively, and annotated with their corresponding gene names. Their protein products and associated functions are listed in **Appendix 8.9**.

STRING network analysis of SSDA proteins derived from pairwise *t* tests ($p < 0.05$) of **1** versus GaN treatment identified elevated stress resistance, secondary metabolism, and proteolysis pathways in the former treatment. A large proportion of targeted proteins were associated with the mitochondria. Cellular respiration and mitochondrial translation networks in addition to amino acid metabolism were downregulated, and to a greater extent upon exposure to **1** compared to GaN. The mitochondrion provides crucial energy supplies, the disruption of which can have detrimental impacts on growth, virulence and detoxification (**Figure 3.8**) (Calderone, Li & Traven, 2015; Black et al., 2021).


A
Secondary metabolite biosynthesis
Fatty acid metabolism
DNA replication/repair
Oxidative stress resistance
Proteolysis
Carbohydrate metabolism
Q4WFP1
Q4WGM8
Q4WRZ0
Q4X1H9
Q4WEX7
Q4WGR1
Q4WKA5
Q4WEX6
Q4WM99
Q4W9I5
Q4WBI8
Q4WRD2
Q4WN81
Q4X1V2
Q4WLA8
Q4WLU8
Q4WM98
Q4WAE3
Q4WXU7
Q4WSA0
Q4WQ02
Q4WM26
Q4WRS4
Q4X1E8
Q4X168
Q4WDX7
xyl1
Q4WUP8
Q4WPQ1
pgmA
A4GYZ2
Q4X1J1
Q4WBK0
Q4WEC1
Q4WX47
alp2
cpyA
pep2
Q4WI47
xkiA
Q4WV24
Q4WT69
Q4WZN0
Q4WCU3
sconC
Q4WGR2
Q4X0T0
Glycogenin
Q4WXQ2
Q4WK
aglB
Q4WRQ8
Q4WPA3
A4D9T0
Q4WV46
Q4WH82
Q4WU62
Q4WT63
Q4WM55
Q4WTN7
Q4WEC5
Q4WVJ7
asf1
Q4WV97
Q4WB53
sodC

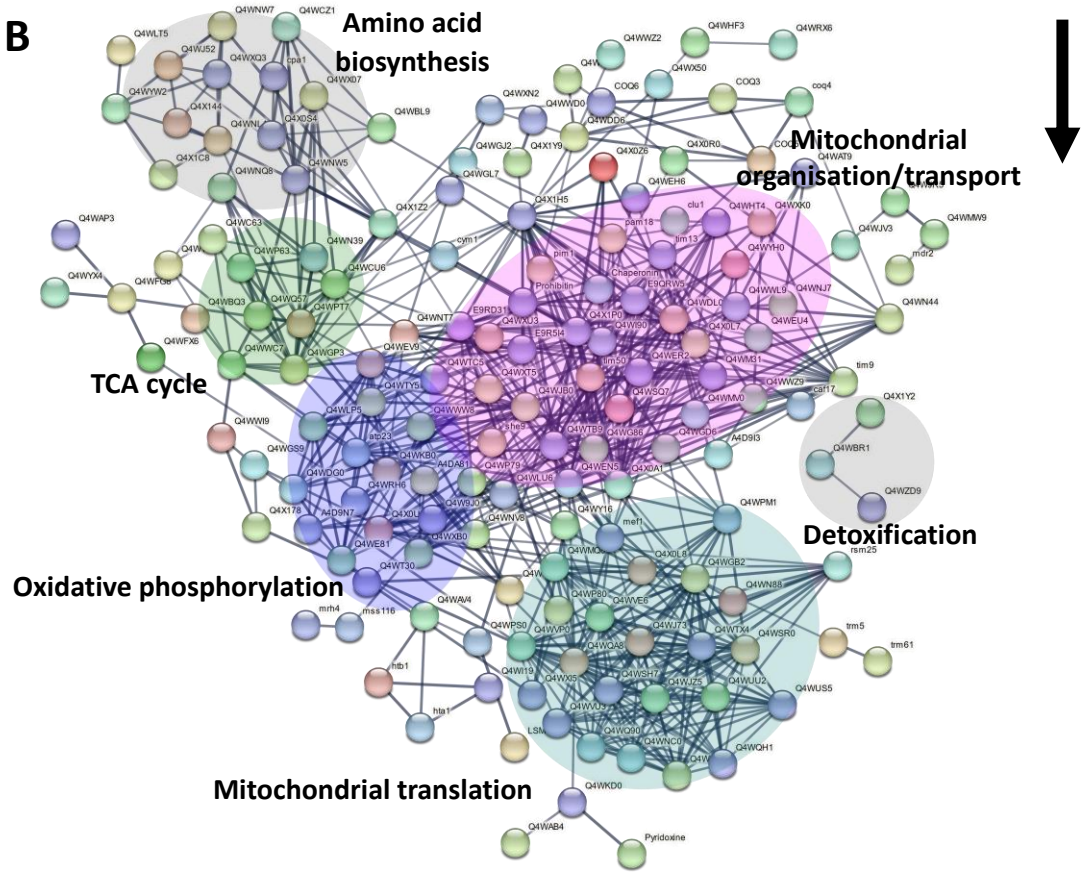


Figure 3.8. STRING network analysis of proteins and pathways altered (A: increased, B: decreased) in response to **1** (250 μg/ml) versus GaN (250 μg/ml). Proteins were searched on the STRING database as previously described.

3.3.6 The effect of gallium treatment on *A. fumigatus* respiration

Proteomics findings prompted further investigation into the effects of gallium on mitochondrial activity. Tetrazolium salts are commonly employed as cell redox indicators for measuring the rate of cellular respiration and viability (Meletiadis et al., 2001). Electron transport chain enzymes (NADH dehydrogenases and cytochrome c oxidases) reduce colourless 2,3,5-triphenyltetrazolium chloride (TTC) to insoluble red 1,3,5-triphenylformazan (formazan) (Tanaka et al., 2021). Formazan crystals accumulate within cells and require a suitable solvent to solubilise and liberate the product into solution (Riss et al., 2016). The calorimetric change (and intensity) is proportional to the rate of cellular respiration and can be assessed qualitatively (**Appendix 8.10**) and quantitatively (**Figure 3.9**). Treatment of *A. fumigatus* with GaN and **1** was carried out as per proteomics methods and revealed an 18% and 37% reduction, respectively, in formazan, and hence respiration rate, relative to untreated

control samples (**Figure 3.9**). Recorded wet weights prior to TTC addition showed no statistically significant difference (**Appendix 8.11**).

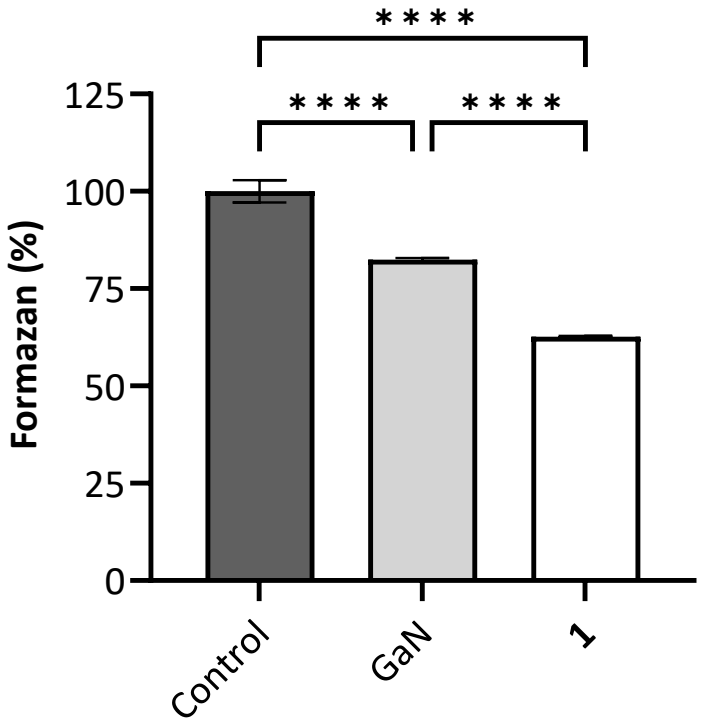


Figure 3.9. Triphenyltetrazolium chloride (TTC) assay measuring cellular respiration in *A. fumigatus*. Statistically significant reductions of formazan products were shown following 24-h GaN and **1** treatment (250 μg/ml) versus untreated control samples. Values are the mean ± S.E. of three independent experiments (n = 3; ****: $p < 0.0001$).

3.4 Discussion

Gallium (Ga) in its +3 oxidation state, Ga(III), is an effective Fe(III) mimic given it has a similar ionic radius and the same charge (Chitambar, 2012). Ga(III) is redox inert under physiological conditions and therefore cannot be reduced. It consequently inhibits critical physiological Fe redox activity and in turn Ga(III)-substituted proteins disrupt important metabolic pathways (Li et al., 2022a). All Ga(III) compounds exhibited good *in vitro* growth inhibition against *A. fumigatus*, in line with previous antibacterial studies. In addition, compound **1** demonstrated good tolerability *in vivo* and therefore was selected for further proteomic evaluation (More O'Ferrall et al., 2023). The data presented here sheds light on the mode of action and shows differences in how it acts when compared to GaN.

3.4.1 Siderophore biosynthesis

The data presented here revealed elevated abundance of siderophore synthesis and uptake proteins following gallium treatment. *SidA* encodes L-ornithine N(5)-monooxygenase, catalysing the first step of biosynthesis of all four siderophores (Khan, Singh & Srivastava, 2018) and was increased by +11- and +21-fold post- **1** and GaN treatment, respectively. *SidA* is a vital element for virulence. *A. fumigatus sidA* knockouts are incapable of retrieving iron from human transferrin and were avirulent in neutropenic mice with invasive aspergillosis (Hissen et al., 2005). Subsequent biosynthetic pathways differ for extra- and intracellular siderophores. For extracellular FsC and TAFC, mevalonate yields anhydromevalonyl via CoA ligation and dehydration by acyl-CoA ligase sidI and mevalonyl-coenzyme A hydratase sidH, respectively. Acyl-CoA ligase sidI (+4- and +7-fold) and mevalonyl-coenzyme A hydratase sidH (+9- and +20-fold) were more abundant after **1** and GaN treatment. Also increased was transacylase SidF, which transfers this moiety onto L-ornithine N(5)-monooxygenase. Intracellular ferrichrome biosynthesis involves acetylation of L-ornithine N(5)-monooxygenase by SidL (Haas, 2014). All siderophores are assembled by non-ribosomal peptide synthetases (NRPS). Assembly of FsC is catalysed by nonribosomal peptide synthase sidD, encoded by *NRPS4*/*sidD* and was +2-fold higher in abundance in **1**- versus GaN-treated *A. fumigatus*.

The broad range and structural diversity of siderophores aims to prevent uptake by non-native species. Despite this, microbes have evolved Siderophore-Iron-Transporters (SITs), capable of recognising and utilizing these siderophores, termed xenosiderophores (Haas, 2012). Compound **1** treatment increased the abundance of some SITs found in *A. fumigatus* - MFS siderochrome iron transporter B (*mirB*) was +7-fold more abundant versus GaN exposure along with MFS siderochrome iron transporter D (*mirD*; +3-fold) and siderochrome iron transporter 2 (*sit2*; +3-fold) for TAFC, FsC and ferrichrome transport, respectively (Aguiar et al., 2022).

Reductive iron assimilation (RIA) by fungi commences with the reduction of ferric to ferrous iron, thus increasing solubility and bioavailability to the cells (Misslinger et al., 2021). Three protein groups are used in this process: (1) ferric reductases, (2) multicopper ferroxidases and (3) high-affinity iron permeases (Stanford et al., 2021). The ferrous iron is subsequently re-oxidised by the cell surface ferroxidase, iron transport multicopper oxidase fetC, encoded by *fetC*, for uptake by *ftrA*-encoded ferric-iron transporter. This ferroxidase was elevated +7-fold in both treatments.

3.4.2 Respiration

Mitochondria are highly iron-dependent and are central to energy metabolism within the cell. Respiratory proteins are complexed with metal ions that act as co factors for electron transfer and catalysis (Black et al., 2021). Approximately 59% of decreased SSDA proteins in **1** versus control comparisons were associated with the mitochondrion. Functional enrichment using the STRING gene ontology database revealed biological processes such as cytochrome complex assembly, mitochondrial transport and structural assembly, respiratory chain complex assembly and the tricarboxylic acid cycle were among those affected. Cytochrome c oxidase assembly factor 6 (*AFUA_3G09780*), cytochrome c oxidase assembly protein (Pet191), putative (*AFUA_5G08965*) and NADH-ubiquinone oxidoreductase B14 subunit, putative (*AFUA_6G04620*) were −14-, −7- and −8-fold less abundant compared to GaN treatment, respectively. In accordance with these results, the TTC assay provided further qualitative and quantitative evaluation of the disruption of mitochondrial activity induced by GaN and **1** (**Appendix 8.10** and **Figure 3.9**).

3.4.3 Mitochondrial translation

Translation is one of the most energy-consuming processes in the cell, the rate of which is governed by oxidative stress and nutrient shortage, including of iron (Haas, 2012). The pathogen must adjust its proteome by reducing global translation and prioritising expression of stress response proteins for survival. Compound **1** reduced small nuclear ribonucleoprotein Sm D3 (*AFUA_4G10240*) abundance by 6-fold compared to GaN. Proteins implemented in the electron transport chain are translated by mitochondrial ribosomes, several of which were also reduced in abundance.

3.4.4 Amino acid metabolism

Ornithine is a non-proteinogenic amino acid and precursor of siderophores. Ornithine and arginine biosynthesis are interconnected, whereby the hydrolysis of arginine generates ornithine and synthesis of arginine from ornithine (Schafferer et al., 2015). Arginine biosynthetic pathways employ several enzymes, some of which were decreased in abundance by **1** 2-fold relative to GaN: arginosuccinase (AFUA_3G07790), acetylornithine transaminase (AFUA_2G12470) and ornithine carbamoyltransferase, mitochondrial (AFUA_4G07190).

Functional enrichment analysis of STRING pathways identified that isoleucine, leucine, valine, alanine, aspartate, glutamate and lysine pathways were downregulated in response to **1**. Moreover, cysteine synthase B, putative (AFUA_4G03930) was the second most decreased protein (−27-fold). Reduction in cysteine biosynthesis has previously been linked with iron-depletion in *P. aeruginosa* (Nelson et al., 2019). Amino acids constitute an important nutritional source and structural component of proteins. Efficient protein synthesis supports growth and yield virulence factors to support morphological changes, biofilm formation, and adaptability in hostile environments (Silao & Ljungdahl, 2022).

3.4.5 Lipid metabolism

Iron starvation is known to decrease ergosterol content and enhance susceptibility to membrane-damaging antifungals (Chung, Haas & Cramer, 2012). A 14-α sterol demethylase-like enzyme was decreased −7-fold in **1** versus GaN groups. Belonging to a P450 cytochrome monooxygenase superfamily, this enzyme is involved in

demethylation on the 14- α position of lanosterol to produce ergosterol (Zhang et al., 2019).

Fatty acids are the main components of lipids, and fungi and have a diverse range of lipids that constitute cell membranes, are used for energy storage and intra- and extra-cellular signalling (DeJarnette et al., 2021). Ergosterol is the most abundant sterol and a target of conventional antifungals to induce cell leakage and death (Herrick & Hashmi, 2020). Delta(7)-sterol 5(6)-desaturase erg3B (*erg3*; +11-fold), delta(14)-sterol reductase erg24A (*erg24A*; +9-fold) and methylsterol monooxygenase erg25A (*erg25A*; +16-fold) ergosterol biosynthesis proteins were all elevated in response to **1**. Lipid metabolism, including ergosterol biosynthesis, were elevated by gallium treatment. Studies have shown links with lipid metabolism and stress resistance. Unfavourable conditions in the presence of gallium are likely to have caused upregulation of this pathway to combat structural damage (Zhu et al., 2022; Rella, Farnoud & Del Poeta, 2016).

3.4.6 Oxidative stress response

Iron is an enzymatic co-factor in oxidative stress defence. Mitochondrial dysfunction due to iron deficiency can impair resistance and conidial growth (Haas, 2012). As described, **1** targets the *A. fumigatus* mitochondria, including a number of stress response enzymes (e.g. NADH-ubiquinone oxidoreductase B14 subunit, putative (*AFUA_6G04620*), Cytochrome c peroxidase, mitochondrial (*ccp1*), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, *AFUA_5G06540*, −2-fold), and Putative aconitate hydratase, mitochondrial (*acoB*)), all of which were less abundant in this treatment (Lambou et al., 2010). Cytoplasmic Fe superoxide dismutase, putative (AFUA_6G07210; −2-fold) is also among these differentially abundant proteins. Conversely, ROS-neutralising thioredoxin (+2-fold) and superoxide dismutase [Cu-Zn] (*sodC*) was elevated +6-fold more with **1** exposure. Superoxide dismutases are employed by the mitochondria to defend against deleterious by-products of respiration. Oberegger *et al*. (2020) reported increased Cu-Zn dismutase in iron-deficient *Aspergilli* (Oberegger et al., 2000).

3.4.7 DNA replication and repair

The DNA-binding properties of metals and gallium-induced inhibition of the iron-dependent enzyme, ribonucleotide reductase, are well-recognised to induce anticancer and antimicrobial effects (Anastassopoulou, 2003; Kircheva & Dudev, 2019; Oyebode et al., 2013; Goss et al., 2018). This enzyme is essential for DNA replication and cell proliferation. Exposure to **1** increased ribonucleotide reductase small subunit RnrA, putative, protein +12-fold compared to controls and +17-fold to GaN-treatment. The DNA-damaging effects of gallium were evident however, through increased DNA repair proteins such as DNA-(apurinic or apyrimidinic site) lyase (AFUA_4G11930), DNA repair protein RAD51 homolog (AFUA_1G10410), DNA replication licensing factor MCM5 (AFUA_5G02520), possible replication factor-a protein (AFUA_6G11130) and replication protein A subunit (AFUA_2G06320) were more abundant following **1** treatment.

3.5 Conclusion

The threat of antimicrobial resistance is rising, and associated deaths are predicted to exceed all cancer-related deaths by 2050 (Adebisi & Ogunkola, 2023). The development of novel antifungal therapies is complex due to the eukaryotic nature of fungi and hence structural similarities with mammalian hosts. Identification of non-toxic and selective targets are urgently required. Gallium has demonstrated potent antimicrobial activity against drug resistant pathogens and ongoing studies aim to provide more efficacious therapeutic options. The disruption of iron homeostasis by gallium has an effect on a number of pathways essential to the microbe's vitality, growth, and virulence, including cellular respiration. Compound **1**, a recently reported novel gallium polypyridyl catecholate compound, induces a greater impact on protein abundance, thus affecting mitochondrial function, cell structure, DNA integrity and the cell stress response to a greater extent when compared to GaN. Having previously exhibited notable activity against *P. aeruginosa*, **1** has now also been shown to exhibit a novel and more potent alternative to existing therapies to limit the spread of *A. fumigatus* infection (More O'Ferrall et al., 2023). The Ga compound, **1**, has potential as a novel therapy for the treatment of *A. fumigatus* and *P. aeruginosa* which frequently co-colonise the lungs of CF patients.

Chapter 4

Exposure of *Candida parapsilosis* to the silver(I) compound SBC3 induces alterations in the proteome and reduced virulence

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Author Contributions

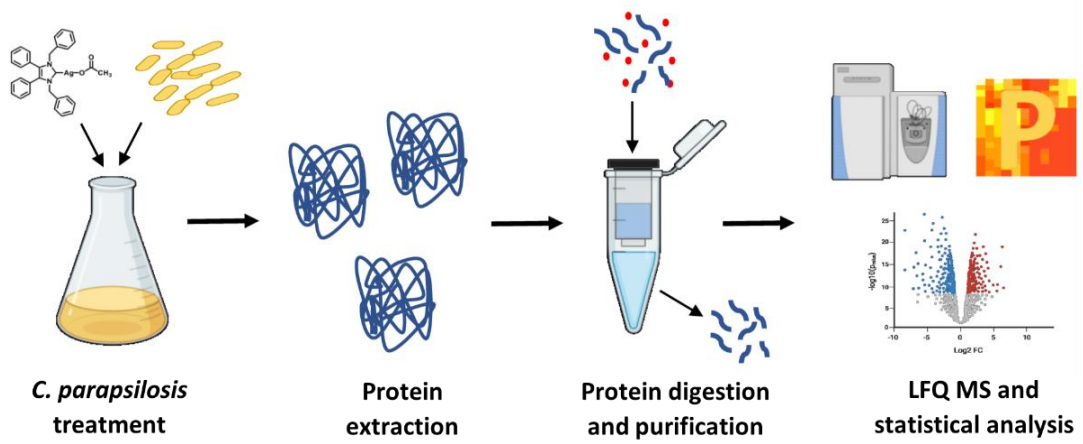
All experiments, data analysis, and manuscript writing were performed by MP. Compound synthesis and characterisation, and manuscript editing were performed by CO'B and ZB. Funding, experimental design and manuscript editing were performed by MT and KK.

We wish to acknowledge Dr. Anatte Margalit for her expertise on mass spectrometry sample preparation and data analysis and Ms. Orla Fenelon and Luke Brennan for their assistance with the scanning electron microscope sample preparation and instrument operation.

Abstract

The antimicrobial properties of silver have been exploited for many centuries and continue to gain interest in the fight against antimicrobial drug resistance. The broad-spectrum activity and low toxicity of silver have led to its incorporation into a wide range of novel antimicrobial agents, including *N*-heterocyclic carbene (NHC) complexes. The antimicrobial activity and *in vivo* efficacy of the NHC silver(I) acetate complex SBC3, derived from 1,3-dibenzyl-4,5-diphenylimidazol-2-ylidene (NHC*), has previously been demonstrated, although the mode(s) of action of SBC3 remains to be fully elucidated. Label-free quantitative (LFQ) proteomics was applied to analyse changes in protein abundance in the pathogenic yeast *Candida parapsilosis* in response to SBC3 treatment. Increased abundance of proteins associated with detoxification and drug efflux were indicative of a cell stress response whilst significant decreases in proteins required for protein and amino acid biosynthesis offer potential insight into the growth-inhibitory mechanisms of SBC3. Guided by the proteomic findings and the prolific biofilm and adherence capabilities of *C. parapsilosis*, our studies have shown the potential of SBC3 in reducing adherence to epithelial cells and biofilm formation and hence decrease fungal virulence.

Graphical abstract



4.1 Introduction

Advances in medicine and technology have improved life quality and reduced mortality rates due to microbial infections over the last 100 years (Fisher, Wicks & Babar, 2016). Despite this, the incidence of fungal infections is increasing, particularly those infections due to *Candida* spp. and *Aspergillus* spp. (Lin et al., 2021). These fungi generally exist as part of the human microbiome, although when left unchecked, have the capacity to establish infections ranging from superficial mycoses of the skin, hair and nails to life-threating systemic infections affecting vital organs (Hay, 2018; Mayer, Wilson & Hube, 2013). Superficial infections are normally easily managed with a fully-functioning immune system but can become recalcitrant in immunocompromised individuals and demand potent antifungal therapy (Odom, 1994). The increasing rates of fungal infections are largely driven by technological advances such as invasive surgeries and medical equipment, the overuse of antibiotic treatments and by environmental factors such as climate change that provide more favourable conditions to thermotolerant species and/or enhance thermotolerance closer to human body temperature (Garcia-Solache & Casadevall, 2010; Azevedo et al., 2015). The survival of infected patients relies upon rapid diagnosis and a suitable treatment regimen, although these factors are compromised by prolonged detection methods (with low sensitivity) and the emergence of resistant isolates to first-line treatments (Riwes & Wingard, 2012; Falci, Stadnik & Pasqualotto, 2017; Antinori, Corbellino & Parravicini, 2018).

The yeast *Candida parapsilosis* is an opportunistic fungal pathogen, and although it is considered less virulent than *Candida albicans*, it has rapidly emerged in recent decades as a serious problem in immunocompromised patients (Trofa, Gácser & Nosanchuk, 2008; Gago et al., 2014). *C. parapsilosis* is a commensal of the gastrointestinal tract and skin and is frequently isolated from human hands – a common source of contamination of medical devices where biofilm growth can occur (Van Asbeck et al., 2007; Kuhn et al., 2002; Trofa, Gácser & Nosanchuk, 2008). Conventional treatment of this pathogen relies upon the use of azole, polyene, or echinocandin antifungal agents, however, the spread of antifungal resistance and limited treatment options have diminished their efficacy (Santos et al., 2018; Spampinato & Leonardi, 2013). The development of novel, non-toxic antifungal

agents with distinct modes of action is urgently required to allow treatment of resistant isolates.

There has been a reawakening of interest in the antimicrobial properties of silver in recent years (Lara et al., 2015; Artunduaga Bonilla et al., 2015). Silver is incorporated into many topical creams, wound dressings and as medical device coatings in nosocomial settings while ongoing research seeks to develop novel silver-based drugs with enhanced efficacy and bioavailability (Sim et al., 2018; Munteanu, Florescu & Nitescu, 2016; Prencipe et al., 2021). Silver exhibits several modes of action including cell wall rupture to induce cell leakage, facilitation of the generation of reactive oxygen species (ROS) and DNA disruption leading to reduced replication (Kim et al., 2009; Hwang et al., 2012; Yang et al., 2009; Abbas et al., 2019). Other mechanisms have been reported including inhibition of respiration and protein synthesis (Fong & Wood, 2006; Park et al., 2019; Holt & Bard, 2005; Guilger-Casagrande & Lima, 2019). The antiviral properties of metallodrugs have also been explored revealing their roles in disrupting the SARS-CoV-2 papain-like protease (PL^{pro}). Gold and silver *N*-heterocyclic carbene (NHC) complexes have shown promising inhibitory activity in disrupting this enzyme required for viral replication (Gil-Moles et al., 2021).

Conjugation of silver to ligands is an effective strategy to improve drug stability and promote more controlled and targeted release of silver (Budagumpi et al., 2013; Jimenez et al., 2017). It is essential to ensure a suitable ligand with an adequate level of water solubility, lipophilicity, and stability in normal physiological environments (Hopkins et al., 2014; Aldabaldetrecu et al., 2018). NHCs constitute a versatile group of organic ligands that readily bind to transition metals (Doddi, Peters & Tamm, 2019). Structural modifications have produced a library of NHCs with distinct properties, however, a divalent carbon atom bound to nitrogen atom(s) within a heterocycle forms the basis (Jahnke & Hahn, 2010; Hopkins et al., 2014). Guided by the work of Wanzlick and Öfele in the 1960s, Arduengo and colleagues successfully isolated the first stable carbene through the deprotonation of azolium salts in 1991 (Arduengo, Harlow & Kline, 1991; Pieczykolan et al., 2020). The ease and efficiency of this new method triggered a wave of NHC research in the development of novel anticancer and antimicrobial agents (Hans et al., 2015; Patil et al., 2015; Eloy et al., 2012). Numerous studies have shown the broad-spectrum antimicrobial activity of NHC silver

complexes for the treatment of Gram-positive bacteria (*Enterococcus faecalis*, *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA)), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) along with several fungal pathogens (*C. albicans*, *Candida tropicalis* and *C. parapsilosis*) among others (Kaloğlu et al., 2016; Kascatan-Nebioglu et al., 2006; O'Beirne et al., 2021; Shahini et al., 2017).

The NHC 1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene silver(I) acetate (SBC3) (**Figure 4.1**) isolated by the Tacke group via Young's synthetic method has shown excellent inhibitory activity against a selection of resistant bacterial and fungal pathogens, both *in vitro* and *in vivo* using the *Galleria mellonella* insect model (O'Beirne et al., 2021; Browne et al., 2014; O'Loughlin et al., 2021). SBC3 promoted *G. mellonella* survival against *S. aureus* and *C. albicans* infections, without displaying any adverse effects at the test concentrations whilst subsequent studies in a murine thigh infection model showed both good tolerability and decreased dissemination of MRSA in response to SBC3 concentrations at the appropriate dosing regimen (Browne et al., 2014; O'Loughlin et al., 2021). The aim of the work presented here was to apply label-free quantitative mass spectrometry (LFQ MS) to *C. parapsilosis* exposed to SBC3 to characterise the proteomic alterations induced by the complex, determine the anti-virulence activity and thus gain an insight into its antifungal mode(s) of action.

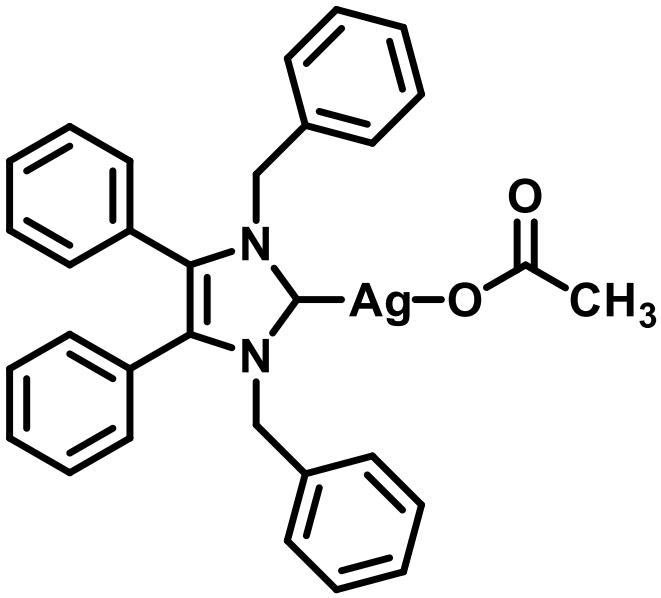


Figure 4.1. Chemical structure of SBC3.

4.2 Materials and methods

4.2.1 *C. parapsilosis* culture conditions

C. parapsilosis was cultured for 24-48 h in yeast extract peptone dextrose (YEPD) medium (2% w/v) glucose (Sigma), 2% (w/v) peptone (Sigma-Aldrich), 1% (w/v) yeast extract (Fisher)) at 30°C in an orbital shaker at 120 rpm. Stocks were kept on YEPD agar (as described with the addition of 2% (w/v) agar (Fisher)).

4.2.2 SBC3 complex synthesis

SBC3 was synthesised as previously described (O'Loughlin et al., 2021).

4.2.3 SBC3 toxicity evaluation

SBC3 was dissolved in YEPD and 5% (v/v) dimethylsulfoxide (DMSO; Merck) and serially diluted in 96-well plates (Corning) to final concentrations of 0.49-125 μg/ml. Aliquots (100 μl) of *C. parapsilosis* cultures grown overnight were diluted in sterile PBS and added to serially diluted complexes to obtain a final optical density (OD) of 0.05 (representing ~ 4.5 x 10^5 cells/ml). Plates were incubated at 30°C for 24 h in a static incubator. Growth was measured in a plate reader (Bio-Tek Synergy HT) at 600 nm.

4.2.4 *C. parapsilosis* protein extraction and purification

Sterile YEPD media supplemented with SBC3 (15 and 25 μg/ml) was inoculated with *C. parapsilosis* and cells were grown for 8 h at 30°C in an orbital shaker at 120 rpm (n = 3, per sample group). Cells were washed three times in sterile PBS, resuspended in lysis buffer (pH 8.0) containing 6 M urea (Sigma), 2 M thiourea (Sigma-Aldrich), 0.1 M Tris-HCl (Sigma) and a range of protease inhibitors (10 μg/ml aprotinin (Caymen Chemical Company), leupeptin (Thermo Scientific), pepstatin A (Sigma), TLCK and 1 mM/ml PMSF (Sigma)) and lysed via sonication. Cell debris was pelleted by centrifugation at 13,000 x *g* for 5 min. Proteins were acetone (Sigma) precipitated in a ratio of 1:3, sample to acetone, overnight at −20°C. The acetone was discarded, and proteins were resuspended in 25 μl of resuspension buffer (same as lysis buffer without protease inhibitors). Aliquots (2 μl) of sample were used to quantify proteins with the Qubit™ quantification system (Invitrogen). To the remaining samples,

105 µl of 50 mM ammonium bicarbonate and 1 µl 0.5 M DTT (Sigma-Aldrich) were added and samples were incubated at 56°C for 20 min. Samples were alkylated with 2.7 µl 0.5 M IAA (Sigma Aldrich) and incubated in the dark at room temperature for 15 min. Proteins were digested with 1 µl 1% (w/v) ProteaseMAX and 1 µl 0.5 µg/µl Sequence Grade Trypsin (Promega) overnight at 37°C. Digestion was inhibited with 1 µl TFA (Sigma Aldrich). Samples were incubated at room temperature for 5 min and centrifuged at 13,000 x *g* for 10 min. Peptides in the resulting supernatant were purified using C-18 spin columns (Pierce) and dried in a SpeedVac concentrator (Thermo Scientific Savant DNA120) at 39°C for approximately 2 h. Samples were resuspended in acetonitrile (2% v/v) and TFA (0.05% v/v), sonicated in a water bath for 5 min and centrifuged at 13,000 x *g* for 5 min. The supernatant was used for mass spectrometry analysis.

4.2.5 Mass spectrometry

Purified samples (2 µl containing 0.75 µg protein) were loaded onto a Q Exactive Mass Spectrometer ThermoFisher Scientific) using a 120 min reverse phase gradient as per previous methods (Kosanovic et al., 2021). Raw MS/MS data were processed through the Andromeda search engine in MaxQuant software v.1.6.3.4 (Cox & Mann, 2008) using a *C. parapsilosis* database obtained from a UniProt-SWISS-PROT database to identify proteins (5777 entries, downloaded February 2021). Search parameters were followed as per previous methods (Margalit et al., 2020).

4.2.6 2,3,5-Triphenyltetrazolium chloride assay

C. parapsilosis was grown in SBC3-supplemented media for 12 h at 30°C in an orbital shaker. Cells were harvested, washed twice with PBS and resuspended accordingly to standardise ODs of all samples to 0.15. A stock solution of 2,3,5-triphenyltetrazolium chloride (TTC) made in PBS was filter sterilised using a 0.22 µm pore filter and added to cell suspensions at a 1:10 dilution. The solutions were added to wells in a 96-well plate in 100 µl aliquots containing 0.5 mg/ml TTC per well and incubated for 8 h at 30°C in a static incubator. The supernatant was removed, and the remaining formazan crystals were solubilised with DMSO for 15 min in an orbital shaker. The absorbance was measured at 550 nm.

4.2.7 Adherence assay

C. parapsilosis was grown in YEPD media containing 15, 25, 50 and 75 μg/ml SBC3 at 30°C for 24 h. Cells were harvested, resuspended in PBS and enumerated. Buccal epithelial cells (BECs) were self-isolated by gently harvesting from the inside cheek of a healthy individual using a tongue depressor. Yeast cells and BECs were combined in a ratio of 50:1, yeast:BEC, in PBS to a final volume of 2 ml. The cell mixture was incubated at 30°C for 120 min at 120 rpm. The solution was passed through a polycarbonate membrane with 30 μm pores and washed with 8 ml of PBS to eliminate non-adhered *C. parapsilosis* cells. The remaining BECs with attached *C. parapsilosis* cells were applied to microscope glass slides that were dried overnight, heat fixed and stained with 0.5% (w/v) crystal violet dye (ACROS Organics) for 1 min. The average number of adhering yeast cells per BEC was determined after counting 100 BEC per treatment.

4.2.8 Biofilm assay

C. parapsilosis was cultured overnight, diluted in YEPD medium and aliquoted (90 μl of OD 0.1 (~9 x 10^5 cells/ml)) into flat-bottom 96-well plates (Corning). A range of SBC3 concentrations were added in 10 μl aliquots. Plates were incubated at 30°C for 48 h in a static incubator. Growth was measured as previously described. Planktonic cells were removed, and plates were washed four times with deionised water. Adhered cells were stained with 0.1% (w/v) crystal violet dye for 15 min. The dye was removed, and plates were washed to remove excess. The remaining stain was air dried overnight and solubilised with 100 μl, 30% (v/v) acetic acid (Fisher). The intensity of staining was measured at 550 nm.

4.2.9 Scanning electron microscopy

C. parapsilosis cell suspensions (OD 0.1) treated with SBC3 were grown in 6-well plates containing glass slides for 48 h at 30°C in a static incubator. SBC3 solutions were made with DMSO and YEPD. Control samples consisted of YEPD and 0.375% DMSO as per treated samples. The supernatants were removed from wells and the remaining adhered cells on glass slides were fixed with 2.5% (v/v) glutaraldehyde (Sigma) for 2 h. Cells were washed with pre-warmed PBS and dehydrated in increasing ethanol solutions (35%, 50%, 70%, 80%, 90% and twice in 100% v/v) for

10 min each. Slides were dried with 100% (v/v) hexamethyldisilazane (Sigma-Aldrich) for 10 min followed by overnight air-drying. Slides were sputtered with gold (6-12 nm) and imaged on a scanning electron microscope (HITACHI S-3200N).

4.2.10 Data analysis

Graphs were constructed and analysed on GraphPad Prism v.9.2.0. Statistical analysis via one-way ANOVA with Tukey's multiple comparisons test was performed on the means of all sample groups.

The resulting label-free quantitative (LFQ) intensity values were processed through Perseus v.1.6.6.0 (www.maxquant.net/) to generate statistical and graphical analysis (Tyanova et al., 2016). Normalised LFQ intensity values were used as protein abundance measurements. Proteins were filtered to remove contaminants and peptides identified by site. LFQ intensities were $\log_2$ transformed and samples were grouped accordingly (Control, 15 μg/ml Treatment and 25 μg/ml Treatment). Proteins not found in three out of the three replicates in at least one group were removed. The data were imputated to replace missing values with values that imitate signals of low abundance proteins randomly selected from a distribution designated by a downshift of 1.8 times the mean standard deviation of all measured values and a width of 0.3 times this standard deviation. Comparisons between two sample groups were conducted via pair-wise Student's *t*-tests with a false discovery rate (FDR) cut-off of 0.05 on post-imputated data. Volcano plots were produced by plotting $\log_2$ fold changes on the *x* axis and $-\log p$ values on the *y* axis for each sample group comparison. SSDA proteins (analysis of variance (ANOVA) $p < 0.05$, fold change ≥ 1.5) were used in subsequent analyses. LFQ intensities were z-score normalised for hierarchal clustering of the median expression values of SSDA proteins using Euclidean distance. A Fisher's Exact test (Benjamini-Hochberg, false discovery rate (FDR) cut-off of 5%) was used to perform gene ontology (GO) term enrichment analysis of major protein clusters for enrichment in gene ontology biological process (GOBP), gene ontology cellular component (GOCC) and gene ontology molecular function (GOMF). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD025693 (Perez-Riverol et al., 2019).

Cytoscape v.3.8.2 bioinformatics software was used to map SSDA proteins retrieved from UniProt gene lists in Perseus. A high confidence score (0.9) was used to generate interaction networks of up- and downregulated protein pathways for treatment versus control groups. Functional enrichment analysis was performed on protein clusters for enrichment in UniProt key words to identify and annotate functions/processes.

4.3 Results

4.3.1 Antifungal evaluation of SBC3 on *C. parapsilosis*

SBC3 treatment resulted in up to 90% growth inhibition of *C. parapsilosis in vitro* at the highest test concentration (**Figure 4.2**). The determined MIC_{50} and MIC_{80} values representing 15 µg/ml and 25 µg/ml, respectively, were selected for subsequent proteomic analysis. The level of growth was also assessed under aerobic conditions (cultures grown in an orbital shaker, **Appendix 8.12**) at these concentrations in accordance with proteomic sample preparation.

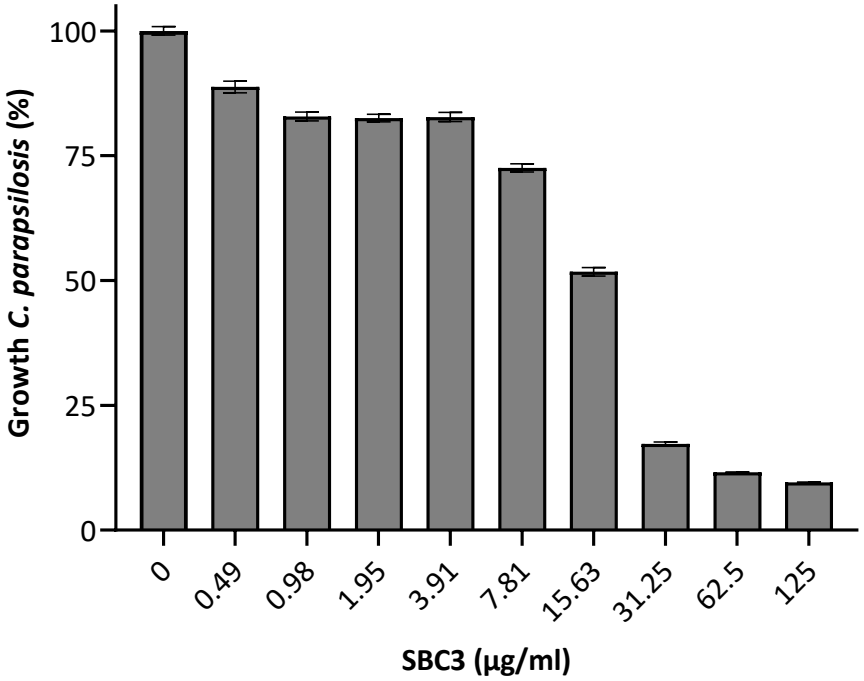


Figure 4.2. Growth inhibition of *C. parapsilosis* in response to SBC3 treatment. Cells were treated for 24 h at 30°C. All values are the mean ± S.E. of three independent experiments, n = 24.

4.3.2 The proteomic response of *C. parapsilosis* to SBC3

C. parapsilosis was exposed to SBC3 (15 and 25 µg/ml) for 8 h and proteins were extracted and purified as described. Purified proteins were identified and quantified via LFQ MS. Statistical analysis confirmed a total of 1281 proteins (post-filtration of

contaminants), 451 of which were considered statistically significant and differentially abundant (SSDA) with fold changes ≥ 1.5 (**Supp Dataset 4.1**).

The entire data set is presented as a principal component analysis (PCA; **Figure 4.3**) whereby replicate samples are reduced into their corresponding sample groups. The positioning of samples depict the variability relative to one another, and here, the combined variance between components 1 and 2 amounts to 63.6%. **Figure 4.3** demonstrates distinct differences in the proteomes of *C. parapsilosis* untreated control samples to those challenged with SBC3.

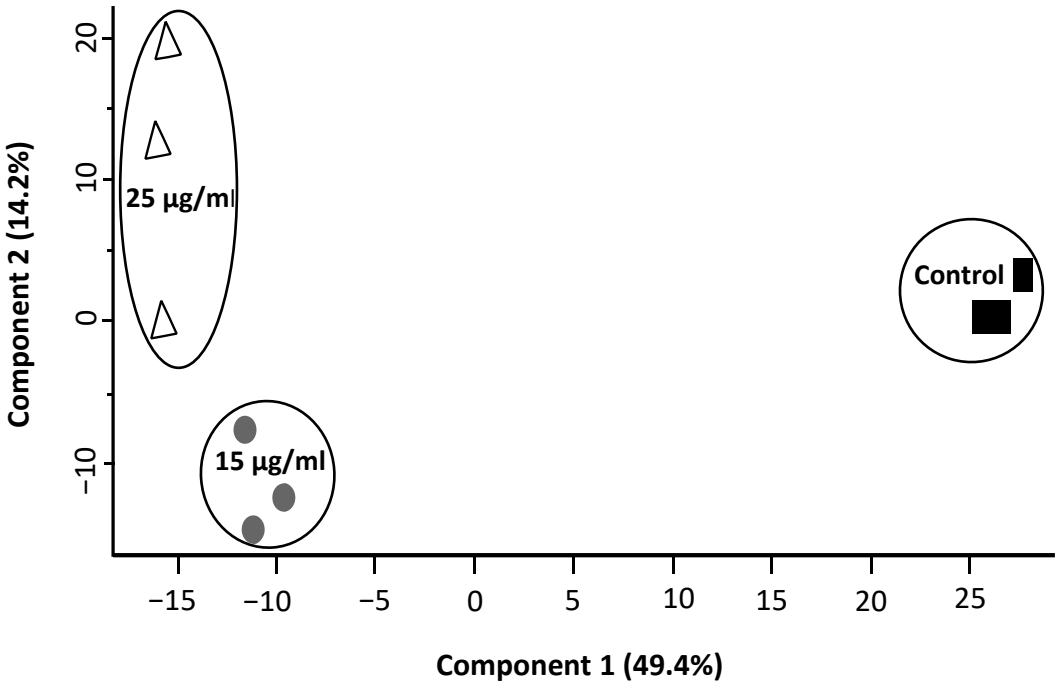
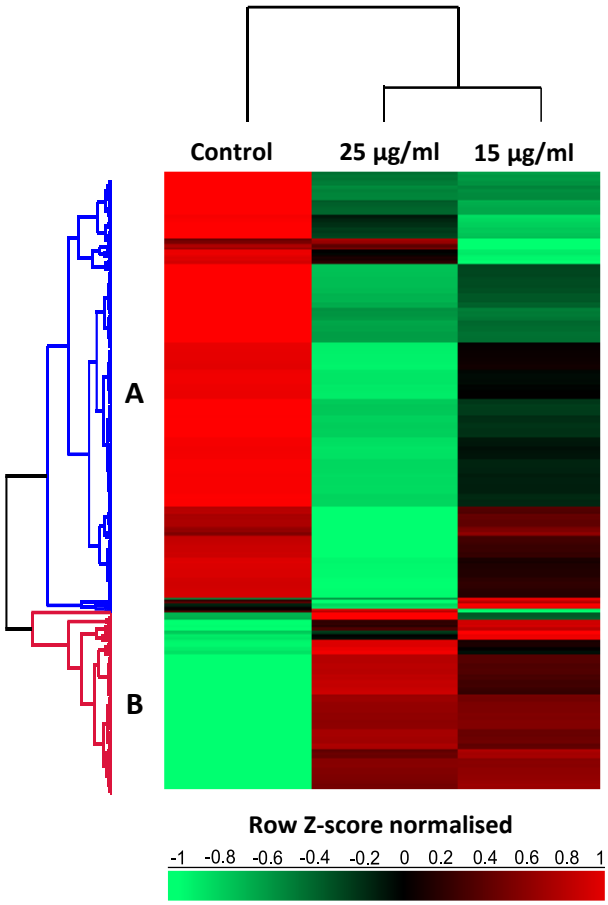


Figure 4.3. Principal component analysis (PCA) of *C. parapsilosis* proteomes treated with SBC3 (15 and 25 µg/ml) versus control samples (n = 3, per sample group). Replicates are resolved into the corresponding sample group and the combined variance between components 1 and 2 amounts to 63.6%.

The distinction between treated and untreated samples can be visualised via hierarchal clustering of *z*-score normalised intensity values for 451 SSDA proteins (ANOVA; Benjamini-Hochberg procedure, FDR cut-off of ≤ 0.05) (**Figure 4.4**). Two row clusters (A and B) were resolved and are based on similar protein abundance trends, with increased and decreased abundance depicted in red and green, respectively. Significant differences in the proteomes of control and SBC3 treated samples are evident.



Cluster	Term	P-value	Enrichment
A	Translation	4.96E-09	1.4182
	Ribonucleoprotein complex	1.54E-11	1.4182
	Ribosome	2.47E-08	1.4182
	RNA binding	3.91E-07	1.4182
B	Glycolysis	3.01E-07	3.391
	Oxidoreductase activity	1.44E-06	1.7824

Figure 4.4. Hierarchal clustering of median expression values of SSDA proteins. Columns comprise of each sample group and rows are clustered (A and B), based on similar protein expression profiles, with increased and decreased proteins identified as red and green, respectively. The table includes a selection of statistically enriched GO terms (Benjamini-Hochberg, FDR ≤ 0.05) identified within clusters A and B.

The main enrichment gene ontology (GO) terms identified within the clusters, which are predominantly linked with protein synthesis, ribosomal components and carbohydrate metabolism are also summarised in **Figure 4.4**. All cluster proteins identified are listed in **Supp Dataset 4.2**.

The distribution of all filtered proteins (1281) is represented on volcano plots (**Figure 4.5**), where statistically significant (ANOVA, $p < 0.05$) and differentially abundant proteins (fold change ≥ 1.5) are located outside of the vertical and horizontal lines. Proteins that increased and decreased in abundance were similar in both treatment groups but different in the extent of their abundance. Among the top 20 proteins most increased in abundance in 15 and 25 μg/ml SBC3 treated *C. parapsilosis*, respectively, were: chitinase (+222- and +139-fold), extracellular membrane proteins linked to iron homeostasis, adherence and virulence CFEM (common in fungal extracellular membranes) 2 (+207- and 179-fold) and CFEM5/6 (+163- and 172-fold), thioredoxin domain-containing protein (+30- and 28-fold) and uncharacterised protein encoded by candida drug resistance 1 (*CDR1*) gene for xenobiotic detoxification (+25- and 33-fold) (**Appendix 8.13** and **Appendix 8.14**).

Treatment of *C. parapsilosis* with 15 μg/ml SBC3 decreased the abundance of several proteins associated with amino acid biosynthesis, with the greatest being 3-isopropylmalate dehydratase (−16-fold). Also decreased in abundance were oxidoreductase glutamine amidotransferase type-2 domain-containing protein (−13-fold), an acyltransferase (−10-fold) and Candida_ALS_N domain-containing protein involved in cell adhesion and pathogenicity (−9-fold). In the cells exposed to 25 μg/ml SBC3, there was decreased abundance of ribosomal protein L37 (−49-fold), proteins associated with respiration and electron transport (−25-fold), uncharacterised protein encoded by CPAR2_407940 associated with cellular stress (−8-fold) and squalene monooxygenase used in sterol biosynthesis (−7-fold; **Appendix 8.14**). These changes are indicative of a high level of cellular stress in response to SBC3. Increased abundance in cell redox and antioxidant proteins is suggestive of coping mechanisms adopted by *C. parapsilosis* to its toxic environment while decreases in amino acid biosynthesis, ribosomal and respiration proteins, among other virulence traits provide an indication of the mechanistic roles of SBC3 in inhibiting cell growth and reducing virulence.

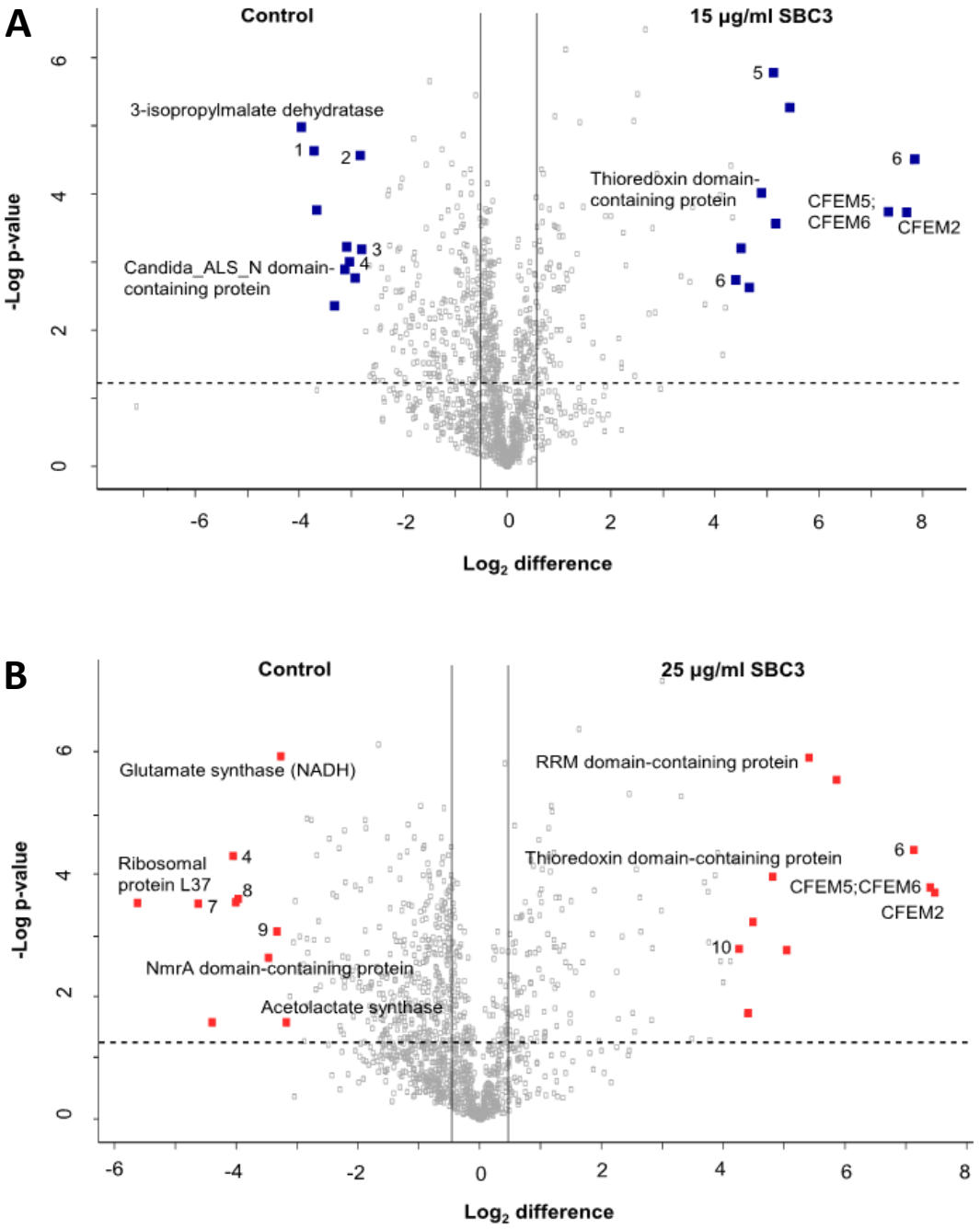


Figure 4.5. Volcano plots of SSDA proteins in *C. parapsilosis* treated with (A) SBC3 15 µg/ml and (B) SBC3 25 µg/ml versus untreated control. Pair-wise Student's *t*-tests (*p* value < 0.05) were used to generate volcano plots with the distribution of quantified proteins according to *p* value ($-\log_{10}$ *p*-value) and fold change ($\log_2$ mean LFQ intensity difference). Statistically significant proteins (*p* value < 0.05) lie above the horizontal dashed line, and differentially abundant proteins (fold change ≥ 1.5) are shown to the right/left of the vertical lines. The top 10 increased/decreased SSDAs are shown in red (unlabelled proteins are uncharacterised; numbered proteins are as follows: 1: Glutamate synthase (NADH); 2: Cytochrome b5 heme-binding domain-containing protein; 3: Oxidored_q6 domain-containing protein; 4: Dihydroxy-acid dehydratase; 5: RRM domain-containing protein; 6: GH18 domain-containing protein; 7: NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial; 8: 3-isopropylmalate dehydratase; 9: zf-CHCC domain-containing protein; 10: Thioredoxin domain-containing protein.

4.3.3 Protein network interaction in response to SBC3

SSDA proteins identified in Perseus were extracted and analysed further using Cytoscape bioinformatics software to map proteins and their associated pathways increased (red) and decreased (blue) in abundance in response to SBC3 treatment. Protein clusters associated with glycolysis/carbohydrate metabolism, ergosterol biosynthesis (also including oxidoreductase activity), components linked with amino acid biosynthesis and the TCA cycle were elevated following treatment (**Appendix 8.15**). A considerable number of ribosomal proteins and others associated with protein synthesis (such as transcription, translation and elongation), were decreased in treated samples relative to controls, in addition to respiration and amino acid biosynthesis proteins. In general, the effects of SBC3 on both treatment groups were comparable with the higher treatment concentration having a more pronounced effect on the differential abundance of proteins.

4.3.4 The effect of SBC3 on cellular respiration

Reduction in respiration in response to SBC3 was ascertained via a 2,3,5-triphenyltetrazolium chloride (TTC) test whereby dehydrogenase enzymes in the mitochondria of viable cells have the capacity to reduce colourless TTC to red formazan crystals in growth media. The calorimetric change is directly proportional to the rate of respiratory activity in cells. The results in **Figure 4.6** substantiate proteomics findings, showing 36-69% reduction in respiration following exposure to SBC3 15-75 μg/ml.

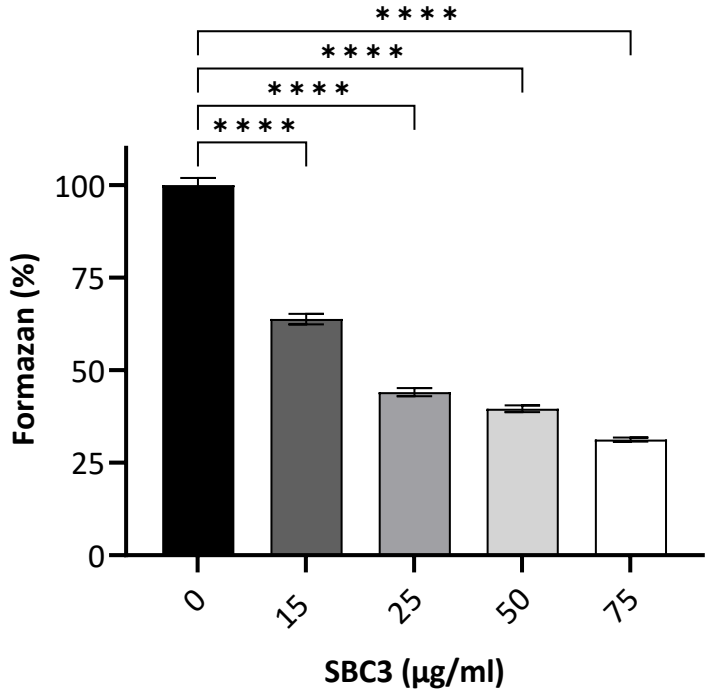


Figure 4.6. Respiratory activity of *C. parapsilosis* treated with SBC3 for 12 h. Statistically significant reductions in respiration are shown by decreased formazan production in SBC3-treated cells. All values are the mean ± S.E. of three independent experiments, n = 48 (**** $p < 0.0001$).

4.3.5 Assessment of the effects of SBC3 on *C. parapsilosis* adherence and biofilm formation

Proteomic analysis of cells exposed to SBC3 indicated alterations in the abundance of proteins associated with cell wall synthesis (chitinases), adherence and biofilm formation (Candida_ALS_N domain-containing protein, CFEM2 and 5/6) (**Figure 4.5, Supp Dataset 4.1**), and ergosterol biosynthesis (encoded by *ERG4*, *ERG11* and *NCP1*) (**Appendix 8.15**). The effects of SBC3 on adherence of *C. parapsilosis* to buccal epithelial cells (BECs) and biofilm formation on an abiotic surface were characterised to ascertain whether the proteomic alterations induced by SBC3 had an impact on the cell's disease-causing potential. *C. parapsilosis* grown with SBC3 for 24 h was isolated and co-incubated with BECs for 120 min to permit the attachment of cells. Adhered yeast cells were enumerated on each BEC for each treatment group revealing pre-treatment with SBC3 (15, 25, 50 and 75 µg/ml) resulted in 76.6 ± 15.5% reduction in adherence (**Figure 4.7A**; **Appendix 8.16**). While the overall trend

indicated a reduction of cell adherence, fluctuations in the response of *C. parapsilosis* were observed and warrant further investigation.

To assess the effect of SBC3 on biofilm formation, *C. parapsilosis* cells were cultured in the presence of SBC3 (10, 15, 20, 25, 50 and 75 µg/ml) for 48 h. The growth of cells was initially measured to verify biofilm reduction was a direct result of SBC3 treatment rather than reduced cell presence. Post-removal of planktonic cells, the remaining adhered cells were stained and measured spectrophotometrically. The results showed a dose-dependent reduction in biofilm formation by 40-76%, while no reduction in cell growth was observed for all test concentrations (**Figure 4.7B**).

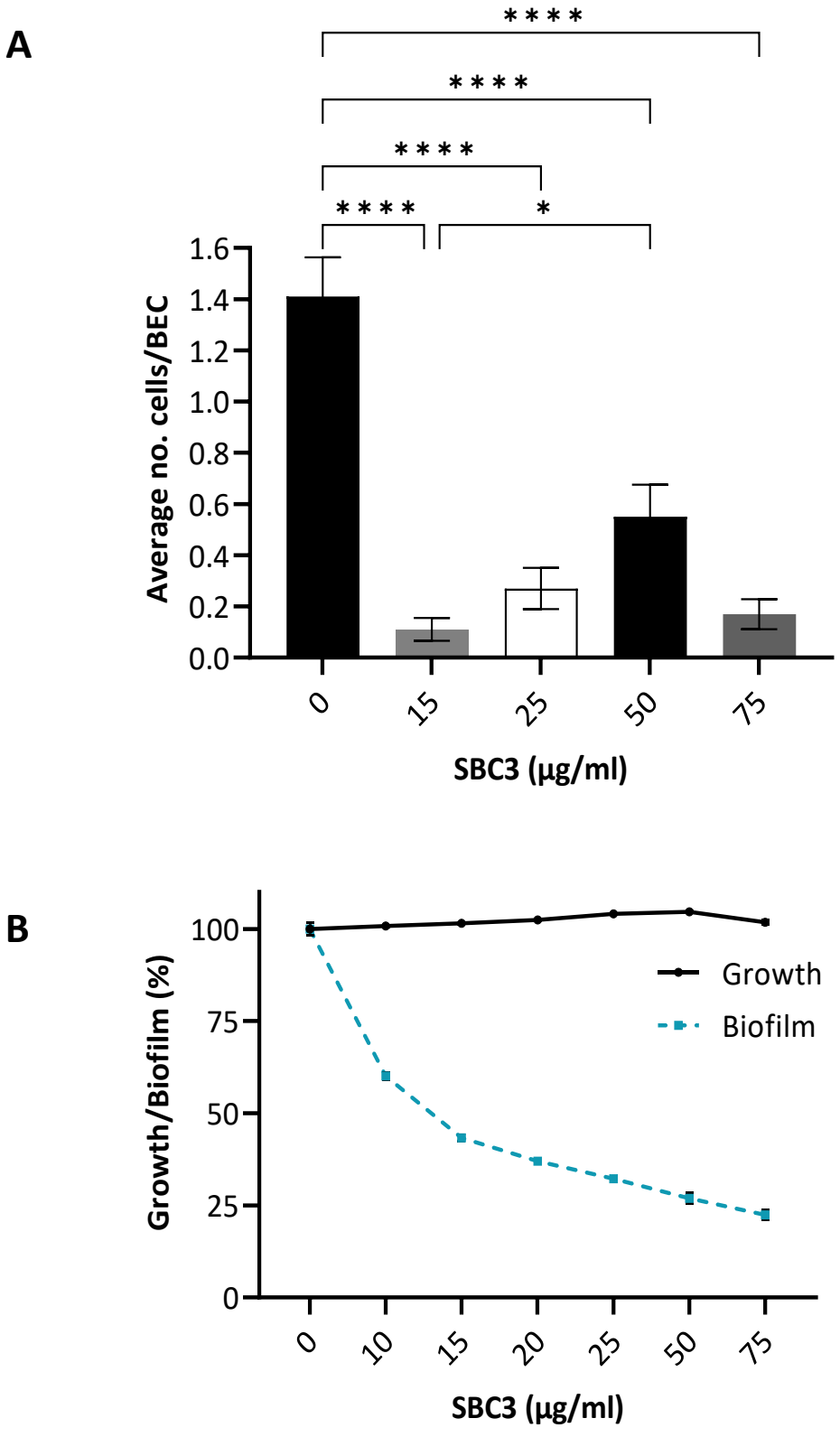


Figure 4.7. The anti-adherence and anti-biofilm effects of SBC3 on *C. parapsilosis*. (A) Pre-treatment of *C. parapsilosis* with SBC3 significantly reduced the number of adhered yeast cells on BECs, compared to control samples. All values are the mean ± S.E., n = 100 (**** $p < 0.0001$). (B) Reduction in biofilm in response to SBC3 after 48 h. Growth remains unaffected for all treatment concentrations. All values are the mean ± S.E., n = 32.

SEM was employed to view structural and morphological changes in *C. parapsilosis*, further supporting the antibiofilm capabilities of SBC3 (**Figure 4.8**). A dense network of cells in both yeast (oval, singular) and pseudo hyphal (elongated, attached) forms was evident in untreated control samples (**Figure 4.8A** and **C**). Filamentous cells layered on top of aggregated cells in the yeast form are adhered to the slide surface, aided by extracellular matrix (ECM) material (**Figure 4.8E**). To observe the full potential of the complex, the highest test dose of SBC3 (75 μg/ml) was selected to treat cells. As a result, cells appeared more dispersed, with reduced presence of biofilm (and ECM material) (**Figure 4.8B**) and were predominantly in the yeast form with a rounded morphology (**Figure 4.8D**) and rough texture characteristic of cell wall damage shown in **Figure 4.8F** (Lara et al., 2015).

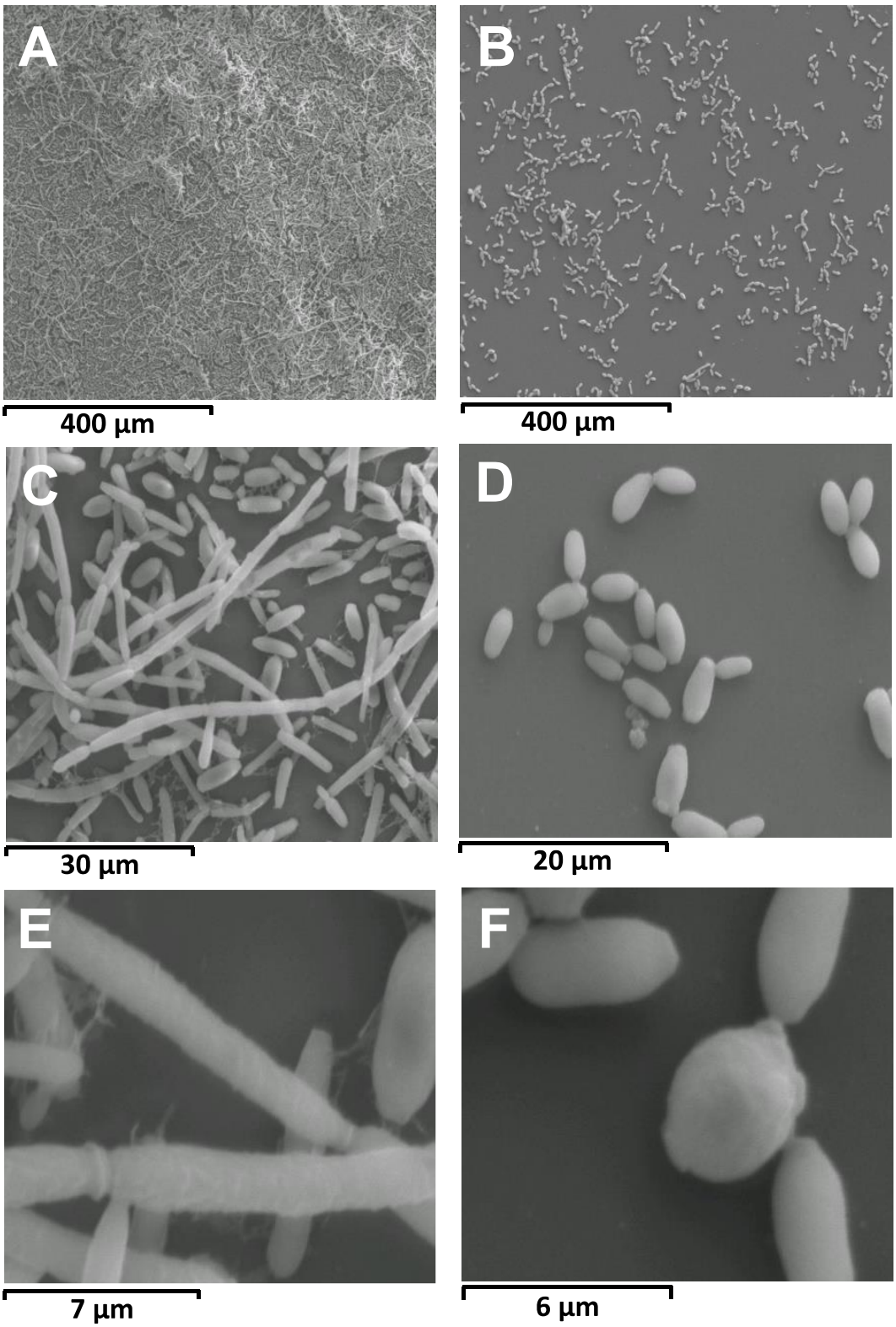


Figure 4.8. SEM images of *C. parapsilosis* grown in the absence (A, C, E) and presence (B, D, F) of SBC3 for 48 h. Various magnification levels exhibit reduction in biofilm and altered morphologies in treated samples.

4.4 Discussion

While *C. albicans* remains a significant fungal pathogen, the epidemiology of *Candida* infections has altered over recent years with increasing reports of *C. parapsilosis* infections (Trofa, Gácser & Nosanchuk, 2008). Incident rates are likely to increase with ongoing use of aggressive immunosuppressive therapies and invasive medical procedures, despite limited treatment options (Kabir & Ahmad, 2013). Much of *C. parapsilosis* virulence is due to its adherence ability and biofilm formation (Modiri et al., 2019).

Silver research has largely focused on the antibacterial activity of silver despite demonstrating promising antifungal properties. The activity of silver against bacteria and fungi appears largely universal having the capacity to induce ROS production. During normal cell activity, viable cells produce low levels of ROS in the mitochondria that are modulated with antioxidants. Silver treatment can perturb this homeostasis via the binding of enzymes in the respiratory chain resulting in oxidative damage to nucleic acids, proteins and lipids. Silver can also contribute to structural damage. Association with cell membranes/walls and formation of pores alters permeability resulting in ion leakage and ultimately, cell death (Rinna et al., 2015; Mussin et al., 2019; Aguirre, Hansberg & Navarro, 2006; Park et al., 2009). Studies have also reported the inhibition of filamentation, and hence biofilm formation, in fungi while Kim *et al*. (2009) demonstrated inhibition of growth via cell cycle arrest and prevention of cell budding in *C. albicans* in response to silver (Lara et al., 2015; Kim et al., 2009).

Quantitative label-free proteomics was employed to assess the effect of SBC3 on the proteome of SBC3-treated *C. parapsilosis* cells and identified considerable increases in the abundance of chitinase. Chitin is a polysaccharide component of the fungal cell wall that provides structural stability to the cell (Lenardon, Munro & Gow, 2010). Cell growth relies on an interplay between biosynthetic chitin enzymes and chitin degrading enzymes (chitinases) for morphological changes and cell turnover (Kuranda & Robbins, 1991; Selvaggini et al., 2004). SBC3 induced increased abundance of chitinase, encoded by *CPAR2_502120*/*CPAR2_502130*. In addition, several CFEM proteins (2, 5-7) were significantly more abundant in both SBC3-treated sample groups. The CFEM domain comprises eight conserved cysteine residues belonging to

a group of proteins specific to fungi (Zhu et al., 2017). CFEM proteins function in adherence, biofilm formation, pathogenicity, with other suspected functions as signal transducers and cell-surface receptors (Kulkarni, Kelkar & Dean, 2003; Pérez et al., 2006). It is believed that CFEM proteins influence biofilm formation in *C. parapsilosis*, although their exact effect has not been fully elucidated (Tóth et al., 2019). We hypothesise the elevated levels of the aforementioned proteins are a compensatory mechanism to withstand the effects of SBC3 in the disruption of cell wall integrity and biofilm formation.

ATP-binding cassette (ABC) transporter proteins act as efflux pumps for xenobiotics, drugs and metals, and are encoded by *CDR1* and *CDR2* genes (Holmes et al., 2008; Wasi et al., 2019). Resistance typically coincides with altered expression of multiple genes where *C. parapsilosis* showed reduced susceptibility to fluconazole from increased expression of *CDR1* and *ERG11* (Souza et al., 2015). SBC3 reduced expression of ergosterol synthesis gene *ERG11* while *ERG4* was increased in treated samples in combination with *NCP1*-encoded NADPH cytochrome P450 reductase required for cell detoxification (Kim et al., 2007). The link between increased *ERG4* and azole resistance remains unclear although significantly increased levels of ERG4 mRNA have previously been detected in azole resistant *C. albicans* compared to susceptible isolates (Feng et al., 2017). Ergosterol is a lipid component of biological membranes which maintains structure and permeability of the cell and therefore, these alterations in the cell membrane may possibly reveal a method of cellular detoxification and physical defence against environmental stress (Rodrigues, 2018; Yang et al., 2015b).

Evidence of cellular stress response is further revealed with increased abundance of oxidoreductases. Thioredoxin plays a crucial role in detoxifying peroxides and the flavoenzyme thioredoxin reductase catalyses this process to maintain cell redox homeostasis and protect against oxidative stress (Godoy et al., 2016; Missall & Lodge, 2005). While studies have presented possibilities of thioredoxin reductases as antifungal targets, sub-inhibitory doses of SBC3 significantly elevated levels of thioredoxin domain-containing protein (Abadio et al., 2015; Godoy et al., 2016; Binder et al., 2020).

The most apparent change in SBC3-treated proteomes was the decrease in the abundance of proteins associated with protein synthesis. A large proportion of protein clusters generated on Cytoscape (**Appendix 8.15**) contain translation/elongation factors, tRNA synthetases, ribosomal constituents, along with ribosome biogenesis, RNA binding and rRNA processing proteins. Ribosomes are complex macromolecules that facilitate amino acid linkage to generate peptide chains and assembly of ribosomes is an intricate process of rRNA transcription, folding and coupling of 60S and 40S subunits (Chaker-Margot, 2018; Woolford & Baserga, 2013; Wilson & Cate, 2012). Ribosomal protein L37 contributes to early ribosome assembly and demonstrated the greatest change (−49-fold) among decreased proteins in *C. parapsilosis* exposed to 25 μg/ml SBC3 (Gamalinda et al., 2013). Ribosome assembly, and indeed protein synthesis, are essential to sustain normal cell growth and this vulnerability has been exploited in novel and existing drug targets. Sun *et al*. (2017) suggested a similar mode of action for a repurposed formulation of a thiosemicarbazone via transcriptional analysis (Sun et al., 2017). A novel sordarin derivative has shown specific action against the protein synthesis elongation cycle in some *Candida* spp. and bears no adverse effect on mammalian protein synthesis (Domínguez et al., 1998).

Amino acids are integral components of proteins, and synthesis and metabolism require strict control for adequate protein formation and nutrient acquisition (Boer et al., 2005; Huang et al., 2017). In the yeast *Saccharomyces cerevisiae*, amino acid production relies on feedback regulation of biosynthetic enzymes encoded by *ILV* genes (Boer et al., 2005). The data presented here identified decreased expression of these genes listed as follows with their protein products: *ILV1* (threonine dehydratase), *ILV2* (acetolactate synthase), *ILV3* (dihydroxy-acid dehydratase) and *ILV5* (ketol-acid reductoisomerase). In addition, proteins required for histidine biosynthesis (ATP phosphoribosyltransferase, histidine biosynthesis trifunctional protein and imidazole glycerol phosphate synthase hisHF), leucine biosynthesis (pyruvate carboxyltransferase domain-containing protein), lysine biosynthesis (saccharopine dehydrogenase and alpha-aminoadipate reductase) and tryptophan biosynthesis (anthranilate synthase) were also found within downregulated clusters. Pathogenic fungi employ efficient strategies of nutrient assimilation to support cell growth and various virulence traits (including biofilm formation), and to combat environmental stresses (Ene et al., 2014; Garbe & Vylkova, 2019; Rajendran et al., 2016). Amich and

Bignell (2016) outlined a selection of fungal genes necessary in pathogenesis such as *ILV*, *HIS*, *TRP* and *LEU*, which encode the proteins mentioned above (Amich & Bignell, 2016). Furthermore, the potential of amino acid synthesis/metabolism disruption as a novel antifungal target has been summarised (Jastrzębowska & Gabriel, 2015; K. Mazu et al., 2016; McCarthy & Walsh, 2018). For example, inhibition of the leucine biosynthetic enzyme isopropylmalate dehydrogenase can attenuate virulence in *Cryptococcus neoformans* and phytopathogenic *Magnaporthe* spp. (Do et al., 2015; Jastrzębowska & Gabriel, 2015; Que et al., 2020) and methods of 3-isopropylmalate dehydratase inhibition for use in fungicides have even been patented (Jastrzębowska & Gabriel, 2015). The most significantly decreased protein in response to SBC3 was 3-isopropylmalate dehydratase. These biosynthetic enzymes have proven attractive antifungal targets due to their absence in mammals which must obtain essential amino acids from the diet (Lopez & Mohiuddin, 2020; McCarthy & Walsh, 2018).

The ability of *Candida* spp. to adapt to hostile environments is a complex, multifactorial process dependent upon adequate energy supplies for the maintenance of growth and metabolism (Calderone, Li & Traven, 2015; Duvenage et al., 2019). Components of cytochrome *c* (cytochrome *b-c1* complex subunit 7, cytochrome *b-c1* complex subunit Rieske, cytochrome *c* domain-containing protein encoded by *CYC1*) and a cytochrome *c* oxidase (COX) subunit, encoded by *COX4*, were decreased in abundance. The latter protein catalyses the final step of the electron transport chain and regulates oxidative phosphorylation for ATP synthesis (Coyne et al., 2007; Fontanesi, Soto & Barrientos, 2008; Martins et al., 2011). Furthermore, SBC3 significantly altered the abundance of NADH dehydrogenase [ubiquinone] flavoprotein 1 for NADH electron transfer to the respiratory chain (−25-fold). Other mitochondrial proteins significantly decreased in abundance are listed in **Appendix 8.13** and **Appendix 8.14**.

Mitochondrial function extends beyond energy production to involvement in virulence and resistance mechanisms (Shingu-Vazquez & Traven, 2011; Verma, Shakya & Idnurm, 2018). Defective ATP-generating components of the mitochondria are capable of inhibiting *C. albicans* adherence and biofilm formation (Li et al., 2017) while reduced respiration in phenazine treated *C. albicans* achieved similar results (Morales et al., 2013). SBC3 (15 and 25 μg/ml) decreased Candida_ALS_N domain-

containing protein (encoded by *CPAR2_404800*) by 9- and 5-fold, respectively (**Supp Dataset 4.1**). Agglutinin-like sequence (ALS) proteins are a well-established family of adhesins which initiate microbial colonisation to biotic and abiotic surfaces (Gacser, 2016). Pre-treatment of *C. parapsilosis* with SBC3 reduced yeast adherence to BECs by up to 92%, complementing previous studies by Bertini *et al*. (2016) which showed over 60% reduced adhesion to BECs in *C. parapsilosis CPAR2_404800* null mutants (Bertini et al., 2016). SBC3 exposure can also reduce biofilm formation which is an important virulence factor of *C. parapsilosis* (Borges et al., 2018). Biofilm visualisation by SEM revealed decreased presence of ECM in SBC3-treated cells. Comprised of polysaccharides, lipids, DNA and proteins, ECM aids cell adhesion, offers an impenetrable barrier against antifungal agents and a nutrient source to cells (Nett & Andes, 2020; Mitchell, Zarnowski & Andes, 2016). Furthermore, SBC3 appeared to hinder the transition from yeast to pseudo hyphal forms. A number of genes are expressed during this shift including many cell adhesion proteins and are likely to explain reduced expression of Candida_ALS_N domain-containing protein (Neale et al., 2021). Adherence to host tissue and biofilm formation are essential for the initial stages of infection. The ability of SBC3 to reduce these processes along with morphological shifts would adversely affect the ability of *C. parapsilosis* to colonise and disseminate in the host.

LFQ MS offers a powerful approach to study cell proteomes and permit identification of changes within complex sets of proteins and associated networks/pathways. Therefore, it has been applied to provide insight on the effect(s) of novel and conventional antifungal treatments (Li et al., 2021; Li, Zhao & Zhang, 2021; Guo et al., 2019; Zarnowski et al., 2021). The results presented here indicate that exposure of *C. parapsilosis* to SBC3 results in reductions in proteins associated with protein synthesis, amino acid synthesis and respiration and in virulence as measured by the inhibition of adherence and biofilm formation. These findings provide a novel insight into the mode of action of SBC3 and encourage further research into this interesting antifungal agent.

Chapter 5

Pseudomonas aeruginosa and *Staphylococcus aureus* display differential proteomic responses to the silver(I) compound SBC3

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Author Contributions

All experiments, data analysis, and manuscript writing were performed by MP. Compound synthesis and characterisation, and manuscript editing were performed by CO'B and ZB. Funding, experimental design and manuscript editing were performed by MT and KK.

Abstract

The urgent need to combat antibiotic resistance and develop novel antimicrobial therapies has triggered studies on novel metal-based formulations. *N*-heterocyclic carbene (NHC) complexes coordinate transition metals to generate a broad range of anticancer and/or antimicrobial agents, with ongoing efforts being made to enhance the lipophilicity and drug stability. The lead silver(I) acetate complex, 1,3-dibenzyl-4,5-diphenylimidazol-2-ylidene (NHC*) (SBC3), has previously demonstrated promising growth and biofilm-inhibiting properties. In this work, the responses of two structurally different bacteria to SBC3 using label-free quantitative proteomics were characterised. Multidrug-resistant *Pseudomonas aeruginosa* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) are associated with cystic fibrosis lung colonisation and chronic wound infections, respectively. SBC3 increased the abundance of alginate biosynthesis, the secretion system and drug detoxification proteins in *P. aeruginosa*, whilst a variety of pathways, including anaerobic respiration, twitching motility and ABC transport, were decreased in abundance. This contrasted the affected pathways in *S. aureus*, where increased DNA replication/repair and cell redox homeostasis and decreased protein synthesis, lipoylation and glucose metabolism were observed. Increased abundance of cell wall/membrane proteins was indicative of the structural damage induced by SBC3 in both bacteria. These findings show the potential broad applications of SBC3 in treating Gram-positive and Gram-negative bacteria.

5.1 Introduction

The emergence of drug-resistant pathogens has urged the development of novel antimicrobial agents with alternative modes of action to conventional antimicrobial agents (Kumar et al., 2021). The rise in drug resistance has been caused partly by the incorrect use of treatments and this has led to reduced efficacy against Gram-positive and Gram-negative bacteria (Ventola, 2015). β-lactam derivatives such as cephalosporins, monobactams and carbapenems show enhanced broad-spectrum activity and have tackled growing rates of drug resistance to a certain extent (Worthington & Melander, 2013). β-lactam antibiotics target the synthesis of peptidoglycan – a meshwork of polysaccharide strands and peptides that maintain cell structure and rigidity (Bush & Bradford, 2016). Gram-positive bacteria (such as *Staphylococcus aureus*) possess an inner cytoplasmic membrane and thick peptidoglycan layer functionalised with teichoic acids. These anionic glycopolymers play roles in regulating cell morphology and division, cell adhesion, and defence against temperature, osmotic and toxic stresses (Romaniuk & Cegelski, 2018). Gram-negative bacteria (such as *Pseudomonas aeruginosa*) have a thin peptidoglycan layer sandwiched between an inner cytoplasmic membrane and outer lipopolysaccharide membrane. The latter component offers osmo-protection, regulating the permeability of the cell and the influx and efflux of nutrients and toxins whilst the presence of outer membrane proteins, or porins, create channels that permit passage of molecules in and out of the cell (Delcour, 2009). Decreased production and/or mutations of porins reduce permeability and prevent influx of drugs which can confer resistance. Mutations in expression of the substrate-specific porin, OprD, contribute to carbapenem resistance which is enhanced when combined with increased efflux system expression (Li et al., 2012b). In contrast, Gram-positive bacteria have two main resistance strategies through β-lactamase enzyme production to degrade the β-lactam ring and/or mutations of the drug target site – penicillin binding proteins (PBPs) (Jubeh, Breijyeh & Karaman, 2020).

A decrease in antibiotic discovery since the 1970s has encouraged the search for novel therapeutic candidates (Aminov, 2010). *N*-heterocyclic carbene (NHC) complexes possess broad-spectrum activity against a range of bacterial, fungal and viral pathogens including SARS-CoV-2 (Özdemir et al., 2010; Prencipe et al., 2021). These versatile

ligands offer stability, enhance bioavailability and are compatible with a variety of metals to create agents with antimicrobial and/or anticancer properties (Hindi et al., 2009). The antimicrobial activity of the NHC* metal complex 1,3-dibenzyl-4,5-diphenylimidazol-2-ylidine silver(I) acetate (SBC3) synthesised by the Tacke group has previously been demonstrated *in vitro* and *in vivo* (O'Beirne et al., 2021). *Escherichia coli* thioredoxin reductase is an SBC3 target and contributor in deregulating redox homeostasis (O'Loughlin et al., 2021). The antimicrobial properties of silver are well recognized and have been exploited for centuries (Alexander, 2009). Silver sulfadiazine, silver nitrate and colloidal silver are currently in use as topical skin treatments (Politano et al., 2013).

Proteomic analysis provides enhanced identification of novel biomarkers with improved specificity and sensitivity, increasing our understanding of disease progression and in this context, drug discovery, development and toxicity (Amiri-Dashatan et al., 2018). The identification and quantification of proteins in cells, tissues or whole organisms can provide an insight into the activity of novel therapeutics at a molecular level. Label-free proteomics is an attractive technique with reduced costs, sample preparation time and variability introduced by labelling techniques. An abundance of analytical software tools allows for extensive interpretation of raw MS data. Here, liquid chromatography tandem mass spectrometry (LC MS/MS) was employed to gain an insight into the mode(s) of action of SBC3 and provide a comparison of the differential response of Gram-positive and Gram-negative bacteria to SBC3.

5.2 Materials and methods

5.2.1 Bacterial culture conditions

P. aeruginosa PAO1 and *S. aureus* ATCC 33591 cultures were grown in nutrient broth (Oxoid, Basingstoke, UK) at 37°C in an orbital shaker at 200 rpm. Bacterial stocks were maintained on nutrient agar at 4°C.

5.2.2 Antibacterial susceptibility assays

Bacterial cultures were grown overnight in nutrient broth (Oxoid, Basingstoke, UK) in an orbital shaker at 200 rpm at 37°C. SBC3 (1 mg/ml) was dissolved in sterile nutrient broth with 5% (v/v) DMSO (Honeywell, SLS Scientific Laboratory Supplies, Ireland Ltd). Serial dilutions of the complexes were performed in 96-well plates (Corning®, Somerville, MA, USA) containing nutrient broth (100 µl/well) to give a concentration range of 0.49–125 µg/ml. The optical density (OD) of overnight cultures was measured at 600 nm (OD_{600}) and cell suspensions were adjusted to 0.01 in nutrient broth. Aliquots of cells (100 µl) were added to each SBC3 concentration to obtain a final cell density of approximately 1×10^7 cells/well (*S. aureus*) and 3×10^5 cells/well (*P. aeruginosa*) (DMSO was added to untreated control samples at an equivalent concentration to the highest treatment dose (0.625%)). Plates were incubated in a static incubator at 37°C for 18 h and read at 600 nm in a plate reader (Bio-Tek Synergy HT, Mason Technology, Dublin, Ireland) to measure bacterial growth.

5.2.3 Proteomic analysis of *P. aeruginosa* and *S. aureus* treated with SBC3

Sterile nutrient broth was inoculated with *P. aeruginosa* (~9×10^6 CFUs/ml) and *S. aureus* (~7×10^8 CFUs/ml) and grown in the presence of SBC3 (7.5 µg/ml and 12 µg/ml, respectively) at 37°C in an orbital shaker at 200 rpm for 6 h until the mid-exponential growth phase (**Appendix 8.17**). Cell densities and SBC3 concentrations (approximate $MIC_{50\text{-}80}$ values) were selected based on the microbial response to SBC3 in susceptibility assays. Proteins were extracted, digested and purified as described [64]. Dried samples were resuspended and loaded onto a QExactive Mass Spectrometer (2 µl containing 750 ng of protein) using a 133 min reverse phase gradient.

Protein quantification and label-free quantification (LFQ) normalisation were processed through MaxQuant software version 1.6.3.4 using *a Staphylococcus aureus* NCT8325 (downloaded 12/10/2021; 2889 entries) and *Pseudomonas aeruginosa* PAO1 (downloaded 09/07/2021; 5564 entries) databases following previously described search parameters (Margalit et al., 2020). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD038616.

5.2.4 Data analysis

Statistical and graphical analyses were carried out by processing the resulting LFQ values generated via MaxQuant through the statistical software tool Perseus v.1.6.6.0, with all parameters set in accordance with previous methods [64].

The Search Tool for the Retrieval of INteracting Genes (STRING) v11.5 www.string-db.org/(accessed on 1 November 2022) was used to map statistically significant and differentially abundant (SSDA) proteins using their corresponding gene names retrieved from UniProt gene lists for *P. aeruginosa* PAO1 and *S. aureus* NCTC 8325. A high confidence score (0.7) was used to generate protein/protein interaction networks for treatment versus control sample groups. Disconnected nodes were omitted. Functional enrichment analysis was carried out along with manual searches using the UniProt database to determine individual protein and pathway functions.

5.3 Results

5.3.1 The antimicrobial effect of SBC3

SBC3 demonstrated up to 88.5% and 72.7% growth inhibition at 31.3 and 62.5 µg/ml in *P. aeruginosa* and *S. aureus*, respectively (**Figure 5.1**). Based on the results in **Figure 5.1**, two concentrations of SBC3 that partly inhibited bacterial growth (*P. aeruginosa* (7.5 µg/ml) and *S. aureus* (12 µg/ml)) were identified and these concentrations allowed for sufficient cell density for protein extraction after 6 h, and for cells to demonstrate a proteomic response to the compound.

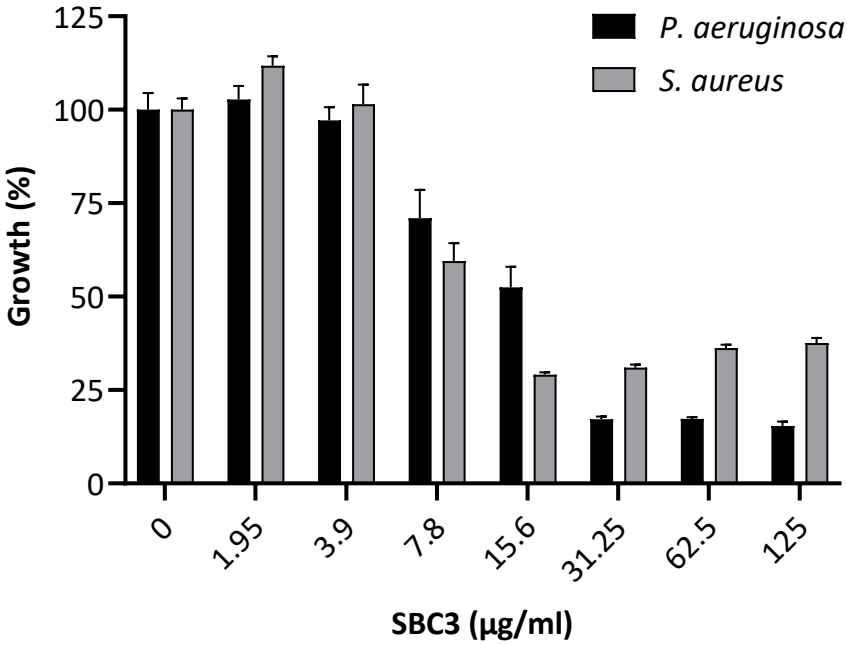

Figure 5.1. Evaluation of the effect of SBC3 on the growth of *P. aeruginosa* and *S. aureus*. All values are the mean ± S.E. of three independent experiments.

5.3.2 Characterisation of the proteomic response of bacteria to SBC3

Label-free quantitative (LFQ) mass spectrometry was employed to characterise changes in the proteomes of SBC3-treated and untreated bacteria. *P. aeruginosa* and *S. aureus* were exposed to the relevant SBC3 concentration for 6 h, after which time proteins were extracted and analysed in order to determine the effect of SBC3 on the whole-cell proteomic response.

5.3.3 Characterisation of the effect of SBC3 on the proteome of *P. aeruginosa*

A total of 2526 *P. aeruginosa* proteins were identified initially, of which 1759 remained following the filtration of contaminants. The entire data set of filtered proteins is shown on the principal component analysis plot (PCA; **Figure 5.2**), whereby all biological replicates (n = 4) are resolved into the corresponding sample groups. Distinct differences are evident between untreated and SBC3-exposed samples, with a combined variance of 79% resulting from Components 1 and 2. Subsequent two-sample *t*-tests ($p < 0.05$) were performed on 1759 proteins post-imputation and identified 362 statistically significant ($p < 0.05$) and differentially abundant (SSDA) proteins with a minimum fold change of 1.5 (**Supp Dataset 5.1**).

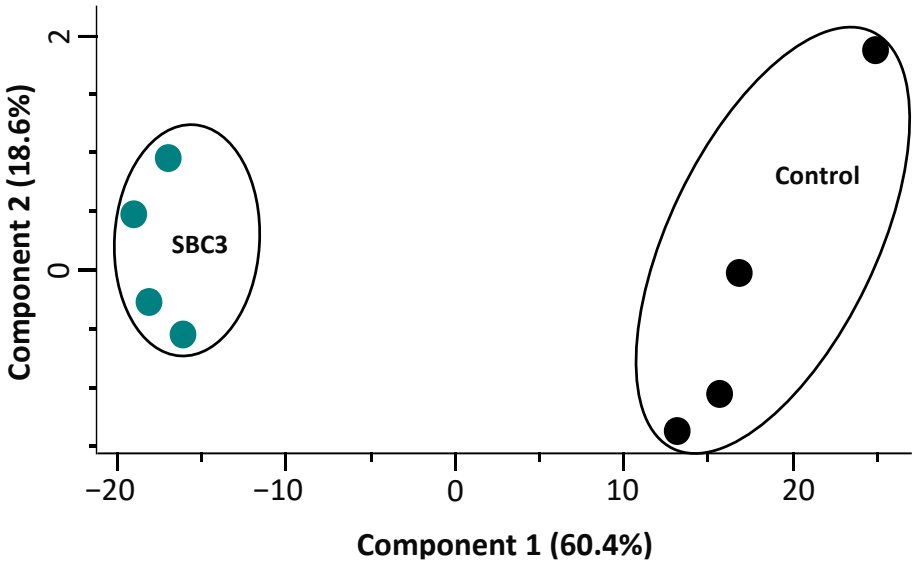

Figure 5.2. Principal component analysis (PCA) of SBC3-treated *P. aeruginosa* versus untreated control samples.

The distribution of all filtered proteins (1631) is represented on a volcano plot (Figure 5.3) conducted via a pairwise Student's *t*-test ($p < 0.05$) and the top ten most differentially abundant proteins (± fold change ≥1.5) are highlighted and annotated with associated gene names. The protein products are listed in **Table 5.1**. Thiol:disulfide interchange protein DsbG (+142-fold), Transcription factor Amrz (+12-fold) required for environmental adaptation, Probable two-component response regulator (+10-fold) for proteolysis and biofilm formation were increased in abundance. Haemagg_act domain-containing protein (−39-fold), ribosome modulation

factor (−37-fold), and probable binding protein component of ATP-binding cassette (ABC) transporter (−11-fold) were decreased in abundance.

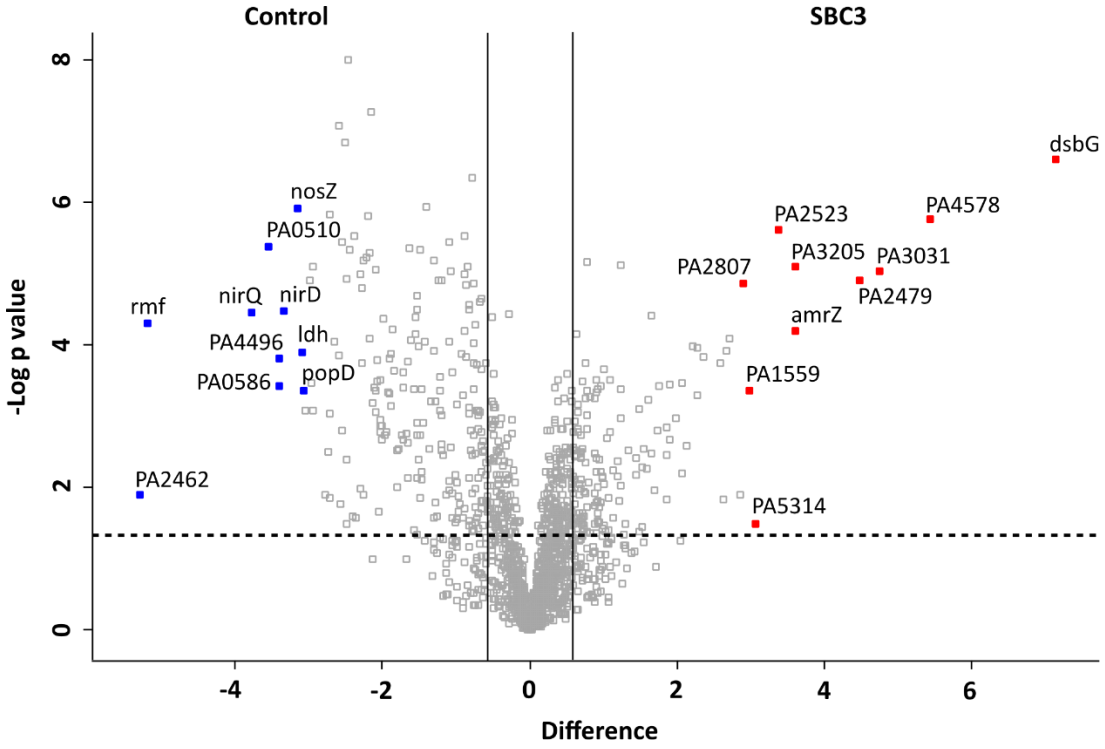

Figure 5.3. Volcano plot generated from a two-sample *t*-test ($p < 0.05$) of *P. aeruginosa* treated with SBC3 (7.5 µg/ml) versus untreated control samples and showing the distribution of all identified proteins (post-filtration of contaminants). The *x*-axis denotes fold change ($\log_2$ LFQ intensity difference), and *y*-axis denotes the P value (−log10 *p* value). Statistically significant proteins ($p < 0.05$) lie above the horizontal, dashed line and proteins with a fold change of ≥ 1.5 are to the right and left of the vertical lines. The top ten most increased and decreased proteins in abundance are coloured red and blue, respectively. The corresponding gene names are annotated, and the protein products and corresponding functions are listed in **Table 5.1**.

Table 5.1. The top 20 differentially abundant proteins identified in SBC3-treated *P. aeruginosa*.

Gene name	Protein name	Protein ID	Function	Fold change
dsbG	Thiol:disulfide interchange protein DsbG	Q9I106	Disulfide bond formation	+142.1
PA4578	Uncharacterised	Q9HVK6	Unknown	+43.7
PA3031	Uncharacterised	G3XCW5	Unknown	+27.1
PA2479	Probable two-component response regulator	Q9I103	DNA binding	+22.5
PA3205	Uncharacterised	Q9HZ35	Unfolded protein binding	+12.2
amrZ	Transcription factor AmrZ	G3XCY4	Transcription; transcription regulation	+12.2
PA2523	Probable two-component response regulator	G3XD16	Positive regulation of proteolysis; positive regulation of single-species biofilm formation on inanimate substrate	+10.4
PA5314	Cupin_3 domain-containing protein	Q9HTP0	Unknown	+8.4
PA1559	NAD_binding_4 domain-containing protein	Q9I3F8	Unknown	+7.9
PA2807	Azurin	Q9I036	Copper binding; electron transfer activity	+7.5
popD	Translocator outer membrane protein PopD	Q9I323	Chaperone binding; Type III secretion system	−8.4
ldh	Leucine dehydrogenase	Q9HYI7	Cellular amino acid metabolic process; oxidoreductase	−8.6
nosZ	Nitrous-oxide reductase	Q9HYL2	Oxidoreductase; denitrification pathway	−8.9
nirD	Siroheme decarboxylase NirD subunit	P95412	Lyase activity; denitrification pathway	−10.1
PA0586	Uncharacterized	Q9I5V1	Unknown	−10.5
PA4496	Probable binding protein component of ABC transporter	Q9HVS5	Peptide transmembrane transporter activity	−10.6
PA0510	Uroporphyrinogen-III C-methyltransferase	G3XD80	Methyltransferase; cobalamin biosynthesis; porphyrin biosynthesis	−11.7
nirQ	Denitrification regulatory protein NirQ	Q51481	Transcription; transcription regulation	−13.8
rmf	Ribosome modulation factor	Q9HZF9	Translation regulation	−36.7
PA2462	Haemagg_act domain-containing protein	Q9I120	Macromolecule metabolic process; primary metabolic process; nitrogen compound metabolic process	−39.4

5.3.4 Characterisation of the effect of SBC3 on the proteome of *S. aureus*

Proteomic analysis of *S. aureus* treated with SBC3 initially identified 1271 proteins of which 1183 were filtered and 251 were SSDA (**Supp Dataset 5.2**). The PCA (**Figure 5.4**) summarises this entire data set, showing the contrast between untreated and SBC3-treated *S. aureus* proteomes. A combined variance of 65.8% arose from Components 1 and 2.

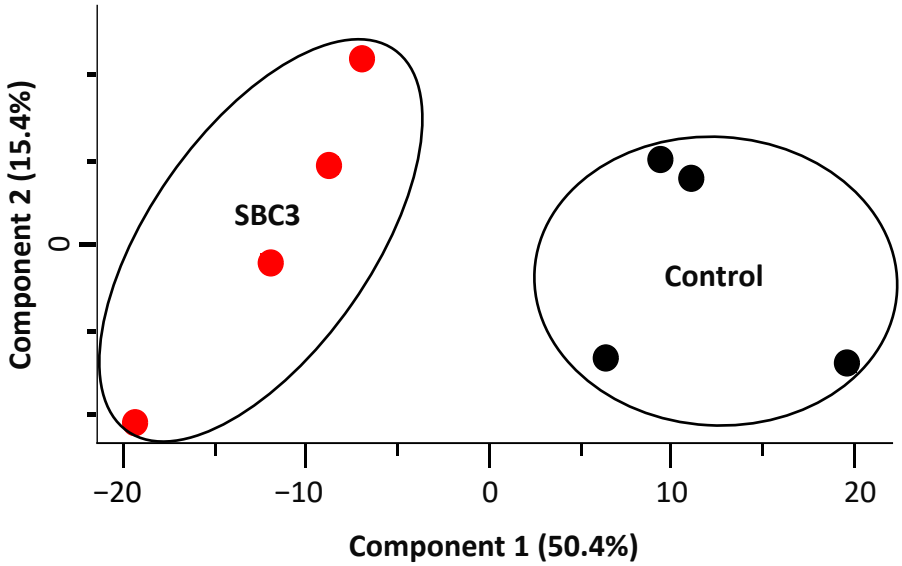

Figure 5.4. Principal component analysis (PCA) of SBC3-treated *S. aureus* versus untreated control samples.

A volcano plot was generated representing the distribution of all filtered proteins (**Figure 5.5**). The top ten most differentially abundant proteins are highlighted and annotated by the corresponding gene names. The protein products and relative fold changes are displayed in **Table 5.2**. A metalloproteinase (+7.4-fold) and proteins associated with cell wall organisation (glycyl-glycine endopeptidase LytM; +6.2-fold, probable autolysin SsaALP; +3.3-fold), DNA replication (DNA topoisomerase 3; +5.2-fold), ABC-type transporter activity (ABC transporter permease; +4-fold) and oxidative stress defence (peroxide-responsive repressor PerR; +3.4) were increased in abundance. Proteins associated with transcriptional regulation of virulence factors (HTH-type transcriptional regulator rot; −8.8-fold), deoxyribonuclease activity (hydrolase TatD; −8.2-fold), and cell redox homeostasis (thioredoxin domain-containing protein; −4.1-fold) were decreased in abundance.

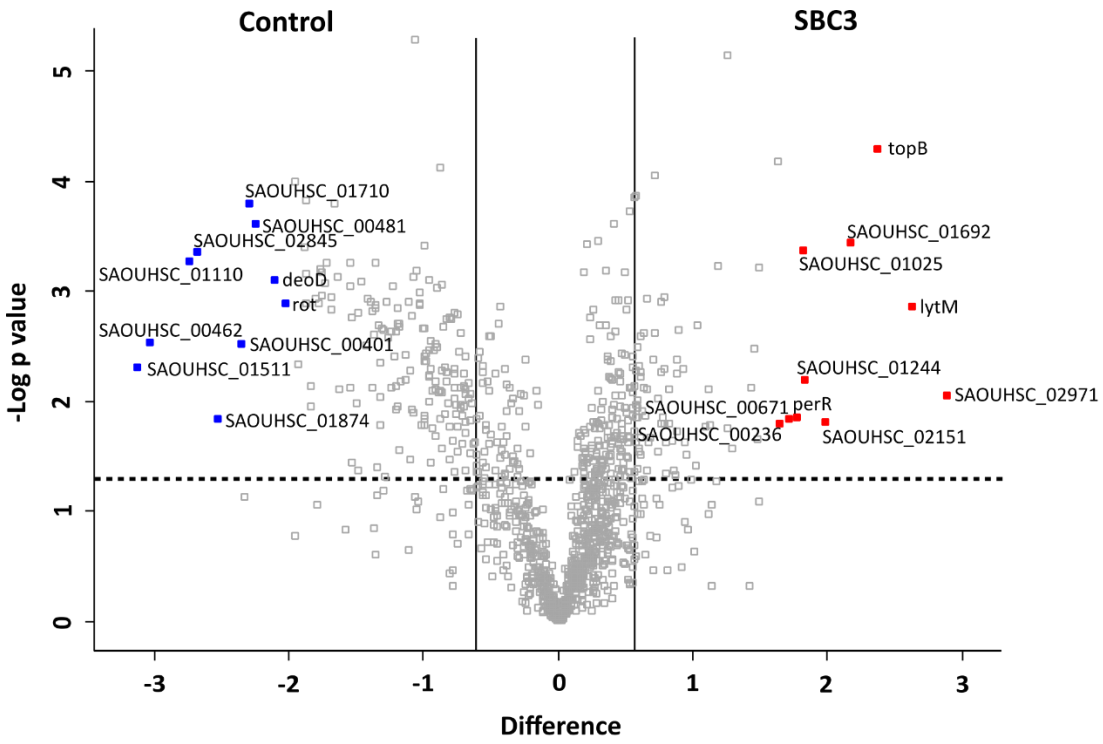

Figure 5.5. Volcano plot generated from a two-sample *t*-test ($p < 0.05$) of *S. aureus* treated with SBC3 (12 µg/ml) versus untreated control samples. All identified and filtered proteins are displayed based on P value (−log10 *p* value) on the *y*-axis and fold change ($\log_2$ mean LFQ intensity difference) on the *x*-axis). Statistically significant ($p < 0.05$) proteins are positioned above the horizontal, dashed line and proteins with a fold change of ≥ 1.5 are to the right and left of the vertical lines. The top ten most differentially abundant proteins are highlighted and annotated with gene names which are listed in **Table 5.2** and including the protein products, functions and fold change.

Table 5.2. The top 20 most differentially abundant proteins identified in *S. aureus* treated with SBC3.

Gene name	Protein name	Protein ID	Function	Fold change
SAOUHSC_02971	Neutral metalloproteinase	Q2FUX4	Metal ion binding; protease	+7.4
lytM	Glycyl-glycine endopeptidase LytM	O33599	Cell wall biogenesis/degradation; virulence	+6.2
topB	DNA topoisomerase 3	Q2FW03	DNA topological change	+5.2
SAOUHSC_01692	CMP/dCMP-type deaminase domain-containing protein	Q2G2A1	Metal binding	+4.5
SAOUHSC_02151	ABC transporter permease	Q2FWX0	ABC-type transporter activity	+4.0
SAOUHSC_01244	YlxR domain-containing protein	Q2G2D1	Unknown	+3.6
SAOUHSC_01025	Membrane spanning protein	Q2FZH9	Unknown	+3.5
perR	Peroxide-responsive repressor PerR	Q2G282	Transcription of antioxidant-encoding proteins (katA, trxB, bcp, ahpCF)	+3.4
SAOUHSC_00671	Probable autolysin SsaALP	Q2G0D4	Cell wall biogenesis/degradation	+3.3
SAOUHSC_00236	6-phospho-beta-glucosidase, putative	Q2G1A9	Carbohydrate catabolic process	+3.1
SAOUHSC_02845	Thioredoxin domain-containing protein	Q2FV89	Cell redox homeostasis	−4.1
deoD	Purine nucleoside phosphorylase DeoD-type	Q2FWB8	Glycosyltransferase	−4.3
SAOUHSC_01874	Rhodanese domain-containing protein	Q2FXH3	Unknown	−4.7
SAOUHSC_01710	Acetyl-CoA carboxylase, biotin carboxyl carrier protein, putative	Q2FXX0	Unknown	−4.9
SAOUHSC_01511	Uncharacterised protein	Q2FY81	Unknown	−5.1
SAOUHSC_01110	Fibrinogen-binding protein	Q2FZC2	Complement binding	−5.8
SAOUHSC_00481	S4 domain-containing protein	Q2G0R5	RNA binding	−6.4
SAOUHSC_00401	Myeloperoxidase inhibitor SPIN	Q2G0X2	Host immune evasion	−6.7
SAOUHSC_00462	Hydrolase TatD	Q2G1S2	Deoxyribonuclease activity	−8.2
rot	HTH-type transcriptional regulator rot	Q9RFJ6	Negatively regulates virulence factors transcription. Positive regulation of cell surface adhesins.	−8.8

5.3.5 Interaction network analysis of the response of *P. aeruginosa* and *S. aureus* to SBC3

Protein interaction networks generated via STRING incorporated SSDA proteins derived from Pairwise *t*-tests. Individual nodes representing proteins (annotated with the corresponding gene name or STRING identifier) are connected by lines denoting an interaction and the width of the line denotes confidence i.e. the strength of data support.

Exposure of *P. aeruginosa* to SBC3 resulted in 366 SSDA proteins with a $\log_2$ fold difference ranging from −5.2 to 7.2. Mapping of these proteins revealed a multitude of targeted protein pathways (**Figure 5.6**). Whilst some virulence and resistance mechanisms (alginate/spermidine biosynthesis, type VI secretion and drug efflux) were elevated (**Figure 5.6A**), a large proportion of clusters were decreased and include ABC transporters, type III secretion system, respiration and amino acid metabolism (**Figure 5.6B**).

S. aureus challenged with SBC3 generated 251 SSDAs with a $\log_2$ difference of −3.1 to 2.9. STRING protein clusters associated with cell wall organisation, cell redox homeostasis and DNA replication and repair were among the most elevated (**Figure 5.6C**). A substantial number of clusters such as ribosome, lipoylation and glycolysis proteins, were decreased in treated samples (**Figure 5.6D**).

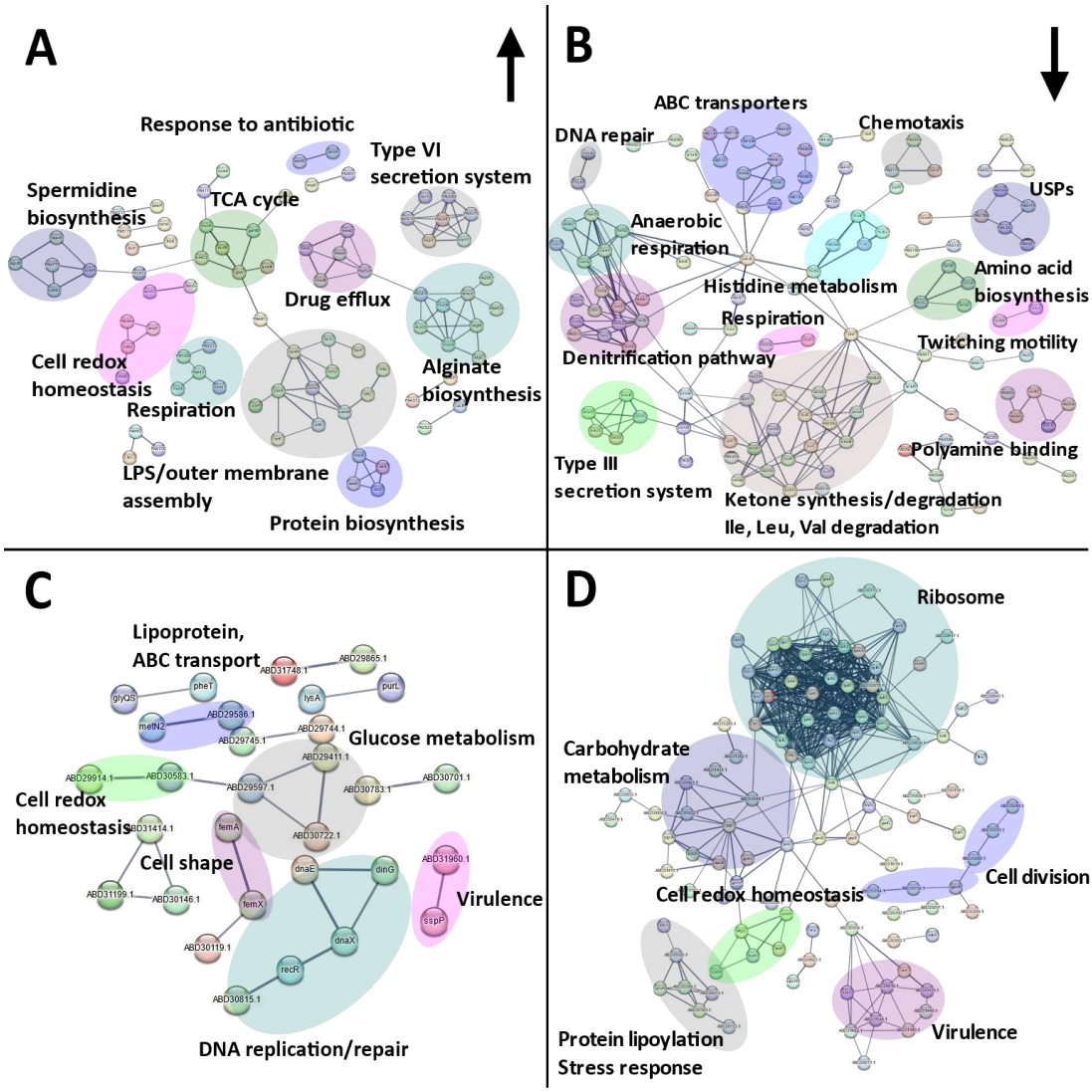

Figure 5.6. STRING network analyses of SSDA proteins derived from two-sample *t*-tests ($p < 0.05$) showing the responses of *P. aeruginosa* (A and B) and *S. aureus* (C and D) to SBC3. Nodes represent individual proteins (annotated with the corresponding gene name or STRING identifier) and are connected to represent an interaction. The thickness of the line represents the strength of data support. Interaction networks with proteins increased in abundance are displayed on the left (A and C) and those decreased are shown on the right (B and D). Protein genes/IDs were searched via the UniProt database in addition to functional enrichment analysis on STRING to determine KEGG and Gene Ontology (GO) terms.

5.4 Discussion

P. aeruginosa and *S. aureus* are widespread nosocomial pathogens, displaying high levels of multi-drug resistance (van Duin & Paterson, 2016). Susceptible individuals are at risk of contracting chronic wound infections, sepsis, endocarditis and other infections introduced via indwelling medical devices (e.g. catheter-associated urinary tract infection) (Goldsworthy, 2008). *P. aeruginosa* and *S. aureus* are the main causes of bacterial infection in cystic fibrosis (CF) patients. *S. aureus* typically precedes *P. aeruginosa* in the earlier stages of disease, however, incidents of co-infection enhance morbidity and mortality rates (Trizna et al., 2020). Biofilm formation is common between these species and offers high levels of protection and resistance against host immune clearance and antimicrobial agents (Reigada et al., 2021).

5.4.1 SBC3 compromises cell structural integrity in both *P. aeruginosa* and *S. aureus*

The onset of resistance to conventional antibiotics has prompted the development of improved silver formulations for the treatment of drug resistant pathogens (Turner, 2018). Positively charged silver (Ag^+) ions bind to negatively charged cell membranes and induce cell membrane/wall leakage and/or rupture (Dakal et al., 2016). The proteomics results presented here revealed significant alterations in the abundance of proteins associated with cell wall structure in *S. aureus*. For example the peptidoglycan biogenesis and degradation protein, glycyl-glycine endopeptidase LytM, was increased +6.2-fold following exposure to SBC3. Acyltransferase enzymes (endcoded by the *femA* and *femX* genes) utilised in cell wall organisation and peptidoglycan biosynthesis were also elevated (Sharif et al., 2009). Alterations in the abundance of outer membrane proteins and the lipopolysaccharide layer in *P. aeruginosa* provide further evidence of the roles of SBC3 in disrupting the cell structural integrity. Increased abundance of outer membrane protein assembly factor BamB (+2.9-fold) and outer membrane protein assembly factor BamD (+1.8-fold) are indicative of a stress response (Klein et al., 2019) whilst an array of lipopolysaccharide proteins identified within a STRING cluster could be indicative of the cell's response in decreasing membrane permeability and limiting entry of noxious compounds (Ebbensgaard et al., 2018; Farhana & Khan, 2020).

Elevated abundance of SpeH (S-adenosylmethionine decarboxylase proenzyme, +2-fold) and SpeE2 (Polyamine aminopropyltransferase 2, +1.9-fold) were further indications of the role of silver in damaging cell structural integrity. This compliments previous studies where treatment of *P. aeruginosa* PAO1 with sublethal concentrations of polymyxin B (an outer membrane damaging agent) resulted in increased expression of SpeE (Johnson et al., 2012).

5.4.2 The proteomic effects of SBC3 on *P. aeruginosa*

5.4.2.1 Translocation and biofilm formation

Chronic infection by *P. aeruginosa* in CF patients is characterised by phenotypic switching from a non-mucoidal to mucoidal phenotype and concurs with alginate over-production (Hentzer et al., 2001). Proteins identified within the STRING cluster were among some of the most differentially abundant. The highly conserved transcription factor AmrZ (*amrZ*) and activator of alginate biosynthesis was increased +12.2-fold whereas PA3205 (identified as LTXXQ domain protein via UniProt) was also increased +12.2-fold and this is hypothesised to detect membrane alterations and commence adherence and biofilm formation on abiotic surfaces (Jones et al., 2022). Other key regulators in alginate biosynthesis such as sigma factor AlgU negative regulatory protein (*mucA*, +1.9-fold), Sigma factor AlgU regulatory protein MucB (*mucB*, +1.5-fold), periplasmic serine endoprotease DegP-like (*mucD*, +1.6-fold), alginate biosynthesis transcriptional regulatory protein AlgB (*algB*, +1.6-fold) and phosphomannomutase/phosphoglucomutase (*algC*, +1.9-fold) are also located within this cluster.

Successful colonisation and establishment of mature biofilms depends on a chemosensory system mediated by pili for translocation towards favourable environments (Persat et al., 2015), both of which were reduced post-exposure to SBC3 (**Figure 5.6)**. Detection of chemical gradients by chemotaxis initiates adherence whilst the type IV pilus system in *P. aeruginosa* consists of cell surface appendages that elongate and retract to further enable adhesion, biofilm formation, twitching motility and DNA uptake (Shi & Sun, 2002; Beaussart et al., 2014). Pil proteins can be categorised into four sub-complexes: the outer membrane secretin pore complex (PilQ, +1.6-fold), the inner membrane alignment subcomplex (PilM, −2.3-fold), the

cytoplasmic motor subcomplex (PilU, −1.8-fold) (**Figure 5.6/Supp Dataset 5.1**). The fourth sub-complex is the pilus itself requiring PilA and minor pilins. The non-pilin protein PilY1 which showed −1.8-fold reduction in abundance has roles in pilus retraction and as a mechanosensory element which upon attachment, induces an acute virulence phenotype (Persat et al., 2015; Tammam et al., 2013; Nguyen et al., 2015; Marko et al., 2018).

5.4.2.2 Virulence

The type III secretion system of *P. aeruginosa* injects effector proteins into host cell cytoplasm and exert cytotoxicity activity and suppress host immunity (Hauser, 2009; Coburn, Sekirov & Finlay, 2007; Dillon et al., 2019). One such effector responsible for disrupting phagocytosis is the secreted exoenzyme S (*exoS*) and this was decreased in abundance by −6-fold following SBC3 treatment (Rangel et al., 2015). Translocator protein PopB (−5.8-fold) and translocator outer membrane protein PopD (−8.4-fold) comprise the pore and are linked to the needle tip type III secretion protein PcrV (Emma et al., 2022). Transcription anti-activator ExsD (−1.7-fold) is a negative regulator of the type III secretion system regulon (McCaw et al., 2002).

Upregulation of secretion systems can arise in response to antibiotic-induced stress (Liu et al., 2017a). Components of the type VI secretion system such as the type VI secretion system sheath protein TssB1 (+4.8-fold), type VI secretion system sheath protein TssC1 (+6.5-fold), type VI secretion system baseplate component TssK1 (+2.3-fold), protein Hcp1 (+3-fold), which is an effector protein and its secretion is aided by AAA+ ATPase ClpV1 (*clpV1*), +2.2-fold were increased in abundance (Chen et al., 2020b).

5.4.2.3 Anaerobic respiration/stress response

Thick mucus in the CF lung can generate an anaerobic environment to which *P. aeruginosa* must adapt for survival (Yoon et al., 2002). Anaerobic growth is mediated by two main pathways: (1) arginine fermentation and (2) the denitrification pathway whilst pyruvate fermentation is implemented in nutrient-deprived environments. Although growth and metabolism are halted, the latter sustains long-term survival (Masamba & Kappo, 2021). Universal stress proteins (USPs), such as PA3309 (encoded by *uspK*), PA4352 (*uspN*) have roles in pyruvate fermentation that are

essential for anaerobic stationary phase survival. Both proteins were decreased in abundance in response to SBC3 (−4.5-fold and −4.8-fold, respectively). In addition, PA1789 (*uspL*), PA4328 (*uspM*) and PA5207 (*uspO*) proteins were decreased −4.9-, −6.3- and −5.5-fold, respectively, and all of these are induced in oxygen-depleted conditions (Boes, Schreiber & Schobert, 2008). The transcription of these genes is controlled by the global anaerobic regulator Anr (encoded by *anr*) and counterintuitively was increased +1.8-fold (gene and protein products are located in **Supp Dataset 5.1** (Trunk et al., 2010)).

Denitrification is a multistep process involving nitrate reduction to ultimate nitrogen gas formation (Arat, Bullerjahn & Laubenbacher, 2015). Heme d_1 is an isobacteriochlorin co-factor of the nitrite reductase, NirS, which catalyses the reduction of nitrite to nitric oxide (Klünemann et al., 2019). Synthesis of heme d_1 requires enzymes encoded by the *nir* genes (Storbeck et al., 2011; Adamczack et al., 2014; Kawasaki et al., 1997), several of which (and their protein products) were decreased: *nirD* (siroheme decarboxylase NirD subunit, −10.1-fold), *nirE* (uroporphyrinogen-III C-methyltransferase, −11.7-fold), *nirG* (siroheme decarboxylase NirG subunit, −7.8-fold), *nirL* (siroheme decarboxylase NirL subunit, −3-fold), *nirN* (dihydro-heme d1 dehydrogenase, −4.1-fold), *nirQ* (denitrification regulatory protein NirQ, −13.8-fold) and *nirS* (nitrite reductase, −5.9-fold). Also shown are the enzymatic complex NarGHI nitrate reductase genes utilised in anaerobic respiration. *NarG* (nitrate reductase (quinone), −2.8-fold), *narH* (respiratory nitrate reductase beta chain, −2.4-fold) and *narJ* (respiratory nitrate reductase delta chain, −2.1-fold) along with *dnr* (transcriptional regulator Dnr, −3.3-fold) (Schreiber et al., 2007). The disruption of these growth and/or anaerobic survival strategies highlight the potential of SBC3 for the treatment of recalcitrant infections.

ABC transporters belong to a superfamily of proteins driven by ATP hydrolysis to facilitate cellular import and export. Uptake of di- and tripeptides for nitrogen sources is mediated by the DppBCDF system consisting of DppA1-5 substrate-binding proteins (in *P. aeruginosa* PA14) which play a role in biofilm formation and swarming ability (Lee et al., 2018). Homologous proteins belonging to this system were decreased in abundance following treatment with SBC3 (PA4497/dppA7 and PA4496/dppA9; −10.6- and −2.9-fold, respectively). The high-affinity branched-chain

amino acid transporters within the same cluster including high-affinity branched-chain amino acid transport ATP-binding protein BraG (−1.5-fold) and leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein (part of the high-affinity branched amino acid transport system LIV-1; −1.8-fold) were also among the same cluster of ABC transporters. Branched-chain amino acids (Ile, Leu and Val) are fundamental nutrients for protein synthesis which not only support growth, but also environmental adaptation and virulence (Kaiser & Heinrichs, 2018). Interference with amino acid metabolism is also shown through decreased abundance of proteins associated with amino acid catabolism via the *liu* gene cluster (Ile, Leu and Val degradation) in addition to ketone body regulation which serves as an alternative metabolic fuel source (Puchalska & Crawford, 2017).

Putrescine and spermidine are linked to a multitude of functions such as virulence, biofilm formation and antibiotic resistance (Johnson et al., 2012). PotABCD polyamine transporters for putrescine and spermidine uptake were altered by SBC3 treatment (potA/PA0603, −1.8-fold and potD, −2.7-fold). Another ATP-binding component of ABC transporter protein encoded by PA1807 was increased within this cluster by 2-fold. The exact function of this protein in *P. aeruginosa* is unclear but as part of the YejABEF ABC transporter system in *Brucella melitensis*, these proteins conferred resistance to polymyxin B (Wang et al., 2016).

5.4.2.4 Detoxification mechanisms

Exposure to SBC3 increased the abundance of multidrug resistance protein MexA and B, belonging to membrane fusion protein (MFP) and RND, respectively, by +2.2-fold, and +1.9-fold. *P. aeruginosa* has a homologous efflux system, MexCD-OprJ, including RND multidrug efflux membrane fusion protein MexC, +1.9-fold, and efflux pump membrane transporter (encoded by *mexD*), +1.8-fold (López et al., 2017; Gotoh et al., 1998).

Increased abundance of cell redox homeostasis proteins such as catalase (*kat B*, +3.7-fold), thioredoxin reductase (*trxB2*, +2.3-fold), alkyl hydroperoxide reductase C (PA0848, +1.9-fold) and alkyl hydroperoxide reductase subunit F (*ahpF*, +1.6-fold) are indicators of a stress response. Interestingly, the most differentially abundant *P. aeruginosa* protein identified here was thiol:disulfide interchange protein DsbG

(+142.1-fold) which is also required for the maintenance of cell redox homeostasis. It is possible that this increase is a damage control measure against misfolded proteins which reflects previous findings in response to copper stress (Wright et al., 2019).

5.4.2.5 Aerobic respiration

The ability to adapt to hostile environments is dependent on adequate energy supplies. The tricarboxylic acid (TCA) cycle is the predominant energy source for cells as part of aerobic respiration (Kwong, Zheng & Moran, 2017). SBC3 exposure increased the abundance of several TCA cycle proteins and decreased two cytochrome-c oxidases (ccoO2/ccOP1/N1) in *P. aeruginosa* (**Figure 5.6**).

5.4.3 The proteomic response of *S. aureus* to SBC3

5.4.3.1 Protein synthesis

Inhibition of protein synthesis can have detrimental effects on cell proliferation (Serbanescu, Ojkic & Banerjee, 2021). This, along with structural differences to eukaryotic ribosomes, has been exploited in conventional antibiotics (aminoglycosides, macrolides and tetracyclines) to inhibit protein synthesis however, increasing rates of antibiotic resistance demand alternative options (Arenz & Wilson, 2016). Functional enrichment analysis via STRING revealed significant reduction in *S. aureus* ribosomal proteins necessary for transcription and translation following SBC3 exposure. This is consistent with previous results which examined the proteomic response of *Candida parapsilosis* to SBC3 (Piatek et al., 2022).

5.4.3.2 Protein lipoylation

The functioning of several metabolic enzymes relies on the post-translation modification, lipoylation (Rowland, Snowden & Cristea, 2018). *S. aureus* can acquire lipoic acid via *de novo* synthesis or retrieval from their host under nutrient deficient conditions in the environment. This important cofactor is required by enzyme complexes used for metabolism and host immune suppression (Teoh et al., 2021; Grayczyk et al., 2017). One such complex is the glycine cleavage system (GCS), which catalyses the degradation of glycine, and comprises of four proteins: P, H, T and L proteins Lipoic acid synthesis commences with the transfer of octanoic acid to GcvH via an octanoyltransferase LipM. This mechanism is an adaptive strategy to overcome

nutrient scarcity (Laczkovich et al., 2018). Within this downregulated cluster, glutamine synthetase (encoded by *glnA*) was also identified. This enzyme has multifunctional roles as a transcriptional co-regulator and chaperone in ammonium assimilation in addition to growth and biofilm formation. This may open avenues as a novel therapeutic targets (Cui et al., 2019).

5.4.3.3 Glucose metabolism

The role of silver in targeting the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase in *E. coli* has been characterized (Wang et al., 2019a). Silver disruption of glyceraldehyde-3-phosphate dehydrogenase preceded more recent studies on the inactivation of glycolytic enzymes in *S. aureus* in response to silver (Wang et al., 2021). Treatment of *S. aureus* with SBC3 increased the abundance of some glycolytic enzymes (glucokinase and alpha amylase family and 6-phospo-beta-glucosidase proteins, putative) while a larger proportion of proteins associated with pentose phosphate and glycolysis pathways was decreased. It is important to note that glucose-derived energy is essential for *S. aureus* (and other pathogens) to establish infection (Tomlinson et al., 2021).

5.4.3.4 Cell redox homeostasis

S. aureus challenged with a higher dose of SBC3 (21.1 μM) to *P. aeruginosa* proved more effective in disrupting the oxidative stress response. Whilst thioredoxin reductase and YpdA family bacillithiol disulfide reductase were elevated in abundance, thioredoxin, catalase, superoxide dismutases and alkyl hydroperoxide reductase C were significantly reduced in abundance.

5.5 Conclusion

Exposure of *S. aureus* and *P. aeruginosa* to SBC3 resulted in inhibition of growth but differential proteomic responses. Both cell types showed alterations in the abundance of proteins associated with the cell wall or envelope. However, in *P. aeruginosa* a multitude of pathways were affected including alginate biosynthesis, secretion systems, drug detoxification and anaerobic respiration. This contrasted with the response of *S. aureus* where pathways such as protein synthesis, lipoylation, glucose metabolism and cell redox homeostasis were affected. The results presented here

demonstrate the broad-spectrum activity of SBC3 against Gram-positive and Gram-negative bacteria with differing proteomic responses from both cell types. SBC3 may have applications in the treatment of Gram-positive and Gram-negative mono or dual-infections.

Chapter 6

General Discussion

6.1 General discussion

Historically, medicinal chemistry was dominated by the production of organic compounds. The design and synthesis of biologically active molecules centred on drug discovery although metals have long been recognised for their antimicrobial activity (Brown & Boström, 2016; Karges, Stokes & Cohen, 2021). Medical application of metal ions and coordination complexes date back to ancient times. Arsenic-containing Salvarsan was the first structurally-defined antimicrobial metal complex isolated in the early 20th century (Karges, Stokes & Cohen, 2021). Metal compounds played an important but small role throughout the 1900s, being largely overshadowed by the introduction of penicillin and its successors. The discovery of cisplatin in 1965 was a breakthrough in medicinal inorganic chemistry (Frei, 2020). The success of these platinum-based chemotherapeutics gave rise to various other metallodrugs that have had a profound impact in healthcare with diagnostic, anticancer and antimicrobial uses (Frei, 2020). Rapidly emerging AMR demands novel agents and the wide range, structural diversity and multimodal activity of metals have shown great potential in combatting this crisis (Frei et al., 2023).

Although metals have a long history of antimicrobial use, only recently have methods and technology permitted the characterisation and identification of their drug target sites (Anthony et al., 2020). Elucidating drug mechanisms of action provide knowledge on potential clinical safety issues (Lam & Ge, 2018; Khalkhal, Rezaei-Tavirani & Rostamii-Nejad, 2019). For example, agents which interfere with cellular components present in both microbial and human cells are likely to be more toxic (Dalhoff, 2021). Furthermore, proteomics can monitor drug resistance development. A better understanding of the underlying mechanisms can enhance current and novel strategies to circumvent resistance challenges (Tsakou et al., 2020; Liu et al., 2019). Proteomic analysis was integral to the results presented in this thesis, offering an unbiased, global overview of microbial responses to drugs in terms of protein abundance changes, localisation, interaction and biochemical function (Baker et al., 2012). Techniques have come a long way from gel-based to high through-put methods such as mass spectrometry and this is paralleled with sophisticated bioinformatics tools for data interpretation (Cui, Cheng & Zhang, 2022). Proteomics is therefore becoming

increasingly recognised for its contributions in drug discovery and accelerating the identification of drug targets (Schirle, Bantscheff & Kuster, 2012).

In Chapter 2, the first research objective was to examine the anti-pseudomonal activity of GaM *in vitro* and *in vivo*. Literature has documented the therapeutic efficacy of gallium since the 1930s and the role of gallium in disrupting iron homeostasis is widely accepted however, large-scale proteomic studies of gallium efficacy are seldom reported (Frei et al., 2023). Chapter 2, and indeed subsequent chapters, utilised LFQ proteomics as a novel approach to assess metallodrug activity. GaM appears to target a number of iron-dependent and interlinked pathways in the highly drug-resistant pathogen, *P. aeruginosa*. Proteins used for sourcing and storing iron were elevated in abundance as a result of exposure of *P. aeruginosa* to GaM, which is a believed counteractive response to treatment-induced iron-limitation. Furthermore, iron-dependent QS systems were downregulated. These systems enable cells to collectively regulate gene expression in response to environmental queues and regulate the global expression of virulence genes (Ding et al., 2018). Twitching and swarming motilities permit bacterial colonisation of diverse environments and increased expression of virulence and drug-resistance genes. (Overhage et al., 2008; Coleman et al., 2020). *P. aeruginosa* undergoes planktonic growth in iron-limited conditions however, GaM reduced structural proteins for motility and adhesion, namely flagellar and fimbrial proteins. The work presented in Chapter 2 highlighted the multifaceted activity of gallium in targeting interconnected pathways for the attenuation of *Pseudomonal* growth and virulence.

The *in vitro* antibacterial activity of GaM described in Chapter 2 translated into *in vivo* efficacy using *G. mellonella* larvae. GaM (1000 µg/ml, equivalent to 66.67 – 100 mg/kg) protected ≥ 90% of larvae against a lethal *P. aeruginosa* dose for up to 24 hours. This is comparable with previous studies using a lethal *P. aeruginosa*-infected thermally injured mouse model whereby a double dose of 25 mg/kg was capable of maintaining 100% murine survival and no bacteria were detected in eschars of mice administered 100 mg/kg (DeLeon et al., 2009). This highlights the utility of *G. mellonella* in providing efficient and informative results prior to animal testing. Standalone treatment showed no adverse toxicity to larvae however, larval hemocyte densities were increased, suggesting induction of an immune response. Injection of

foreign material can have this effect although it is unknown whether this enhances GaM activity.

The evolution of gallium therapeutics have led to the development of novel siderophore-conjugates (Pandey et al., 2019; More O'Ferrall et al., 2023; Kelson, Carnevali & Truong-Le, 2013). As we approach a post-antibiotic era, more powerful strategies are needed for increasingly-resistant pathogens (Wang et al., 2020). Cefiderocol (Fetroja®), is a newly FDA-approved siderophore cephalosporin (Lee & Yeo, 2020) which shares similarities to cefepime and ceftazidime, but it bears a unique catechol moiety that mimics naturally-occurring siderophores (Sato & Yamawaki, 2019; Syed, 2021). Cefiderocol chelates and incorporates extracellular iron into the cell via iron transporter channels in the bacterial outer membrane. Inside the periplasmic space, iron is liberated and the antibiotic binds to the target site (PBPs) to disrupt peptidoglycan synthesis (Rangarajan & Venkataraman, 2020). Having greater efficacy over ceftazidime therapy, Cefiderocol is administered for complicated urinary tract infections caused by Gram-negative pathogens (Wang & Palasik, 2022). The success of this agent expands the scope for gallium siderophore therapies which may address current issues associated with low bioavailability and uncontrolled release (Stelitano et al., 2023). Polypyridyl ligands are highly versatile due to their metal chelating properties and broad medicinal applications (Hachey, Havrylyuk & Glazer, 2021; Yang, Liao & Fu, 2018; Cao, Zheng & Chen, 2015). A range of novel gallium(III) polypyridyl catecholate complexes synthesised by the Griffith group were characterised and biologically assessed against Gram-positive and -negative bacteria (More O'Ferrall et al., 2023). Despite having broad-spectrum antimicrobial activity, the antifungal effects of gallium are less frequently explored. *A. fumigatus* is a common coloniser of CF patient lungs, damaging lung tissue and function, and relies on siderophore-mediated iron uptake for virulence (Cowley et al., 2017; Burgel et al., 2016; Tyrrell & Callaghan, 2016). The objective of Chapter 3 therefore, was to acquire insight on the anti-*Aspergillus* activity of gallium polypyridyl complexes **1** - **3**. Having achieved about 90% growth inhibition and outperforming existing gallium compounds, LFQ proteomics was applied to uncover the underlying molecular mechanisms of activity. Gallium treatment had a significant impact on mitochondrial function. Besides energy generation, mitochondria are implicated in oxidative stress/drug resistance, morphological changes, innate immune interaction and iron

metabolism (Calderone, Li & Traven, 2015; Song et al., 2020). It is a key element for host adaptation and virulence and the dysfunction of which can be detrimental to cells (Calderone, Li & Traven, 2015). At equivalent treatment doses, Complex **1** induced a greater response on the *A. fumigatus* proteome compared to GaN. The selection of GaN was based on previous *A. fumigatus* studies which demonstrated antifungal activity in iron-limited conditions (Bastos et al., 2019). Our work utilised Sabouraud dextrose medium which contains iron (Melvydas et al., 2020). It is suspected that this may dampen the effects of gallium and as a result, reduced the efficacies of GaC, GaM and GaN. This was not the case for **1** – **3** possibly due to the enhanced drug uptake via the siderophore moiety. Nevertheless, mammals are devoid of free iron and so mimicking these conditions using an iron-depleted medium would be beneficial to this study. Further reducing iron availability would likely lower MIC values and inform a more suitable therapeutic dose.

Future applications of these agents show immense promise for the treatment of respiratory pathogens. The Piras Group, University of Pisa, developed solutions for nebulisation and dry powder inhaler formulations by spray drying complex **1** with varying amounts of L-leucine. These formulations retained activity against *A. fumigatus* and deposition studies and SEM confirmed aerodynamic characteristics for successful lung delivery. In addition, the nebulised solution of **1** has been applied to clinical *A. fumigatus* isolates as part of a lung infection model utilising NCl-H441 distal lung epithelial cells and artificial sputum medium to imitate the CF lung environment. The antifungal dose range was non-toxic and dose-dependently impaired fungal growth and hyphal formation. The preliminary findings are an exciting prospect for these novel gallium siderophore agents (data not shown).

Chapters 4 and 5 evaluated the antimicrobial properties of the transition metal silver. The synthesis, characterisation and biological studies were carried out on a range of NHCs complexed with silver acetate and lipophilic substituents by Tacke and co-workers. The lead compound, SBC3, has demonstrated the most potent activity to date (O'Beirne et al., 2021). *S. aureus*, MRSA, *E. coli*, *K. pneumoniae* and *P. aeruginosa* along with *C. albicans* and *C. parapsilosis* were all susceptible to treatment which also showed some additional anti-biofilm capabilities (O'Beirne et al., 2021). O'Loughlin *et al*. (2021) identified *E. coli* thioredoxin reductase as a target of SBC3 and suitability

of SBC3 for synergistic use with gentamicin for *E. coli* and *P. aeruginosa* infections (O'Loughlin et al., 2021). Further investigation was performed due to particularly high potency of SBC3 against *C. parapsilosis*. This pathogen is rapidly emerging as a serious cause of disease in susceptible patients posing particular risk to vulnerable immunocompromised individuals and neonates (West et al., 2021). The proteomic response of *C. parapsilosis* to NHCs has not previously been characterised. To this end, LFQ proteomics identified specific proteins targeted by SBC3. A large number of proteins involved in protein biosynthesis were reduced in abundance. Whether this is an energy-conserving strategy to better cope with stressful conditions or a direct results of silver treatment (for example, silver-induced denaturation of ribosomes), remains unknown (Yin et al., 2020). In either case, it is apparent that SBC3 induces a toxic environment and is further reinforced by elevated abundance of detoxifying enzymes. In addition, cell-wall associated proteins were altered in abundance, and further studies showed alterations in cellular morphology and reduced biofilm formation. Adherence and biofilm formation are paramount to *Candida* virulence thus, SBC3 may have potential in supressing persistent and/or resistant infection (Branco, Miranda & Rodrigues, 2023).

Silver ions are reported to cause structural damage of the microbial outer cell membrane and cell wall (Kędziora et al., 2018; Yin et al., 2020). Differences in cell structure in Gram-positive versus -negative bacteria raised questions on the differences in efficacy and proteomic responses of *S. aureus* and *P. aeruginosa* to SBC3. *P. aeruginosa* was slightly more susceptible to treatment, achieving 85% maximum growth inhibition compared to 71% for *S. aureus*. As such, selected doses for proteomic analyses were based on potency level. SBC3 elevated the abundance of peptidoglycan-associated proteins in *S. aureus* and outer membrane proteins in *P. aeruginosa* indicating a possible compensatory response for structural damage. Due to the lack of an outer membrane in Gram-positive bacteria, positively-charged silver ions adhere to negatively-charged peptidoglycan thus can reduce penetration and efficacy (Dakal et al., 2016; Yin et al., 2020). Binding to the negatively-charged cell membrane and the perturbation of permeability through the formation of 'pits' have been described in Gram-negative bacteria (Li et al., 2010; Dakal et al., 2016). Moreover, uptake mechanisms differ whereby silver ions enter cells through outer membrane proteins (Kędziora et al., 2018). Interestingly, and in line with earlier work,

protein synthesis was a major SBC3 target in *S. aureus*. A large proportion of ribosomal proteins were affected, which is likely due to silver-induced ribosomal denaturation (Dakal et al., 2016). The multifaceted effects of silver were very evident in the response of *P. aeruginosa* and the multitude of affected pathways such as those required for virulence (twitching motility, type III secretion system and amino acid metabolism, for example). Signs of cellular stress were evident in both bacteria with elevated abundances of cell redox homeostasis, DNA repair, and drug efflux proteins. This study identified some previously uncharacterised targets of silver therapy.

Approximately one third of drug candidates are omitted due to toxicity issues, and can magnify costs if left undetected into final clinical stages or post-marketing (Guengerich, 2011). Similarly, the development of resistance can have profound implications raising health and financial concerns (Matthew et al., 2021). As a result, toxicity and resistance are important considerations in drug development. The safety of therapeutic silver is still debated. Relative toxicity differs depending upon the solubility, binding capacity to biological sites, and mechanisms which sequester, metabolise and excrete a metal complex (Cutting, White & Edmonds, 2007). Whilst the antimicrobial silver agents are generally regarded as safe and intact skin protects against absorption, silver can gain systemic access via breached skin and mucosal sites thus increasing toxicity risks (Hadrup, Sharma & Loeschner, 2018). Toxicity is evidenced in numerous case studies however, these are linked with prolonged exposure and/or treatment of large surface areas. Additionally, the formulation of the silver compound will govern its absorption, distribution and metabolic rate(s) (Cutting, White & Edmonds, 2007). The *in vivo* efficacy of SBC3 was firstly examined in *G. mellonella* larvae with no adverse effects up to 250 μg/ml and enhanced survival in *S. aureus*- and *C. albicans*-infected hosts (Browne et al., 2014). Individual administration of SBC3 in subsequent murine studies proved non-toxic and showed some degree of MRSA clearance. Combined therapy with a trifluoroacetate derivative of SBC3 had improved antibacterial activity however, presented toxicity issues (O'Beirne et al., 2021). Applications of silver antimicrobials must therefore be exercised with caution. Topical application of SBC3 seems like the most plausible route of administration although this demands further evaluation.

In nature, complex microbial communities must continuously evolve to outcompete neighbours for nutrients and resources. The widespread use of antimicrobials has heightened this selective pressure accelerating evolution and drug resistance (Peterson & Kaur, 2018). Surveillance of resistance mechanisms can assist formulation development to reduce the likelihood of future resistance (Morel, de Kraker & Harbarth, 2021). In theory, gallium resistance by reduced uptake would in turn reduce nutrient iron uptake (Li et al., 2022a). Graves *et al*. (2019) discovered rapidly evolved gallium resistance in *E. coli*, in opposition to previous claims. Resistance was conferred against ionic silver and iron (III) but not iron (II) and conventional antibiotics (Graves et al., 2019). Gallium resistance in *P. aeruginosa* reference strains occurred due to the functional loss of the HitAB iron transporter and pyochelin siderophore system and enhancing pyocyanin levels whilst a resistant clinical isolate had a stronger antioxidant response (Tovar-García et al., 2020). Despite this, gallium has shown to induce low rates of resistance in *P. aeruginosa* (Goss et al., 2018). To the best of our knowledge, antifungal resistance to gallium remains uncharacterised.

Silver resistance can be mutational or horizontally acquired (Hosny et al., 2019). In Gram-negative bacteria, plasmids containing an operon encoding the Sil protein complex can be acquired which influences drug uptake and efflux. Binding of silver to periplasmic binding proteins prevents cytoplasmic entry whilst other *sil* operon-encoded proteins are also employed in active efflux systems via RND-type efflux transport. Mutations altering membrane permeability can also arise and are mediated by the loss of outer membrane porin expression (Randall et al., 2014). Randall and colleagues also assessed the tolerance of Gram-positive *S. aureus* but failed to select for Ag^{+} resistance among a large cohort of clinical strains (Randall et al., 2013). Perhaps this is due to a thicker, negatively-charged peptidoglycan layer and as such, greater silver ion adhesion and cell sensitivity (Wasilewska et al., 2023).

6.2 Concluding remarks and future direction

This project focused on bacterial and fungal pathogens frequently encountered in healthcare and community-based settings. These are some of the leading pathogens associated with the growing burden of AMR. The results presented here have provided additional insight into the mechanistic roles and potential for the use of novel and repurposed metal-based therapies. LFQ proteomics has proved a powerful tool in

studying antimicrobial drug responses and drug efficacy and continuous advancements in this area would no doubt further enhance our knowledge of novel drug candidates.

Interdisciplinary work can greatly strengthen our knowledge and productivity in drug discovery and development. Inorganic chemistry techniques enable the production of a plethora of potential drug candidates, the safest and most potent of which can be biologically determined. This can also better inform and guide the synthesis of enhanced therapeutic formulations. Future work demands additional insight into the properties of the discussed metallodrugs in terms of their safety/efficacy in mammalian hosts, and pharmacodynamics and pharmacokinetic properties. The good efficacy and *in vivo* tolerability demonstrated within this project provide a good basis for further examination and show potential for use in combination with existing antibiotic and/or antifungal agents.

Chapter 7

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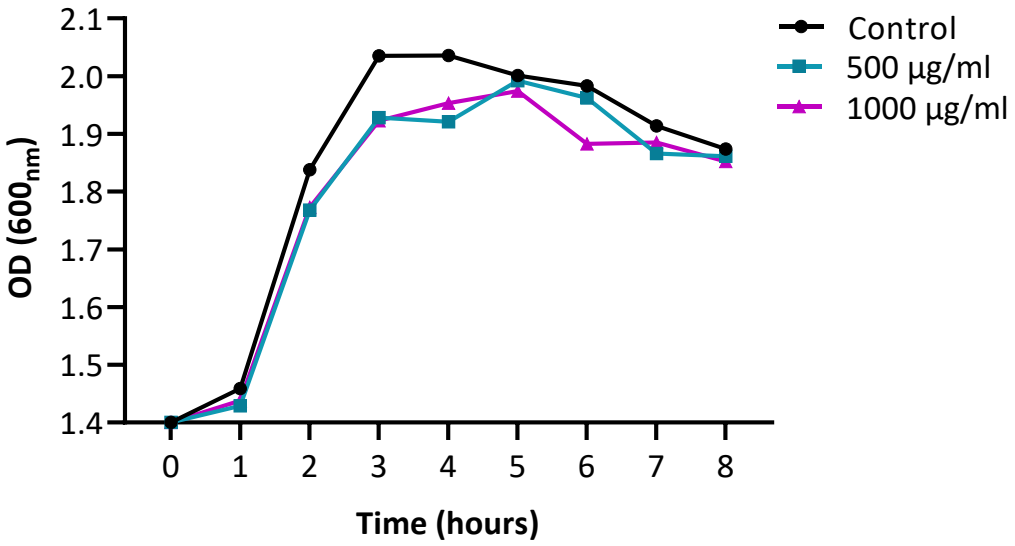
Chapter 8

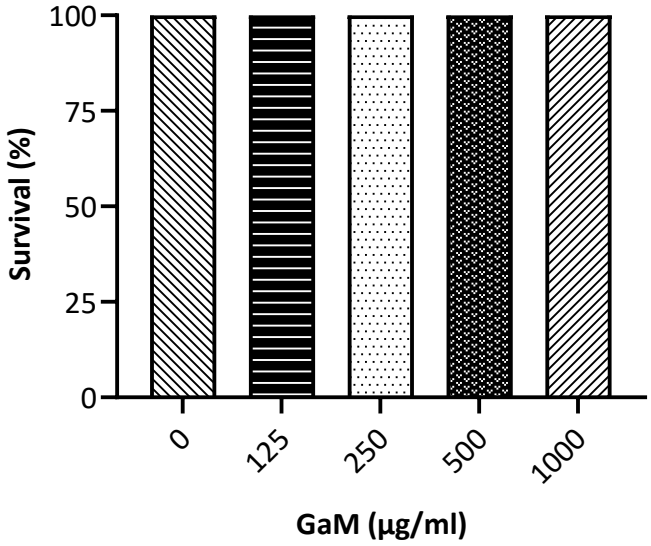
Appendix

Supplementary datasets can be accessed via the following link:

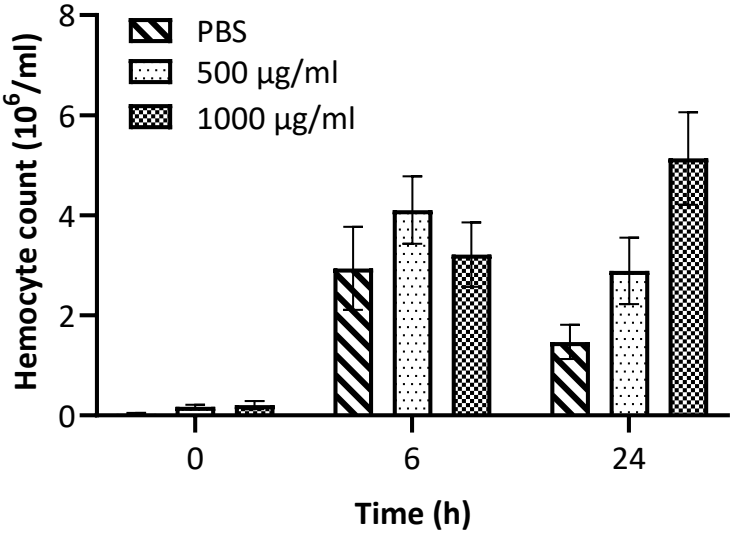
https://drive.google.com/drive/folders/1gfiI-e48wwhSCst7RForW_bJFeGHVzoS?usp=sharing



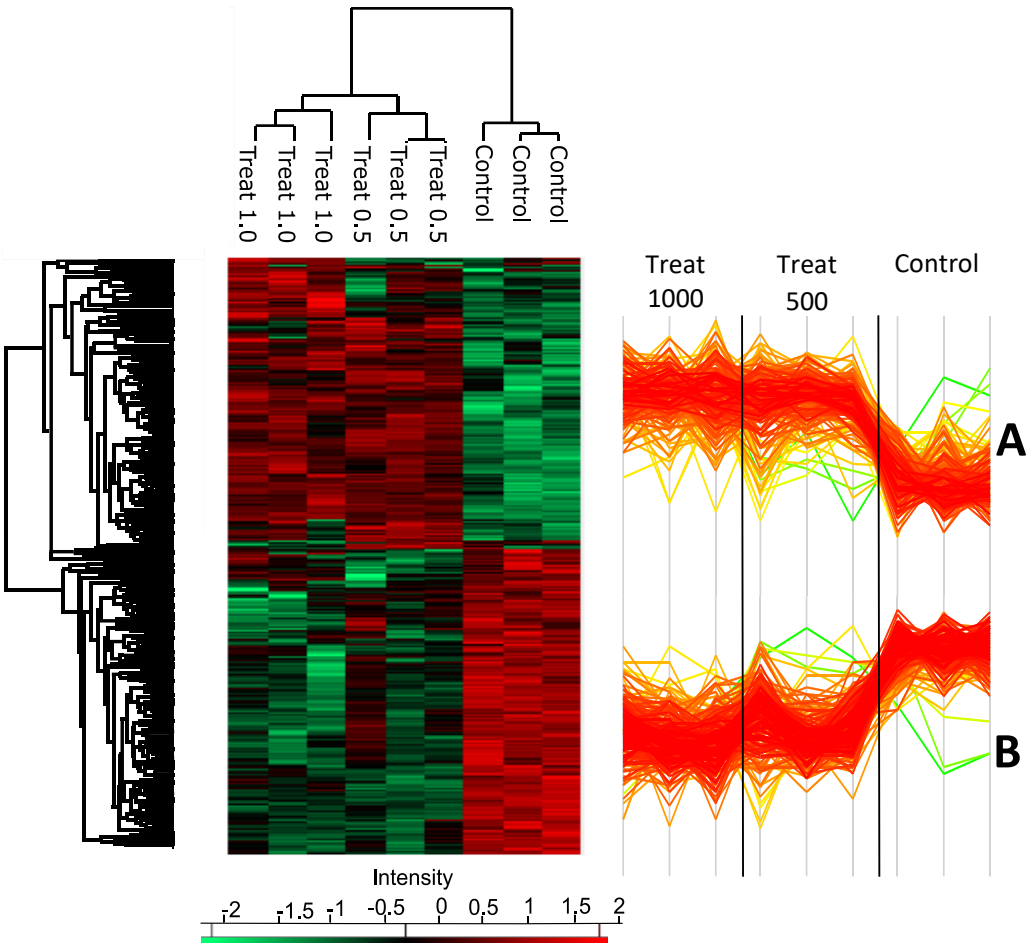
Appendix 8.1. Growth of *P. aeruginosa* in response to GaM over 8 h. Growth curve of *P. aeruginosa* grown in aerated conditions with the addition of 500 and 1000 µg/ml GaM.



Appendix 8.2. Evaluation of GaM toxicity on *G. mellonella* larvae using a range of concentrations (125, 250, 500, 1000 µg/ml) and PBS control. GaM treatment did not affect larval vitality as survival rates remained at 100% after 24 hours at 37°C.



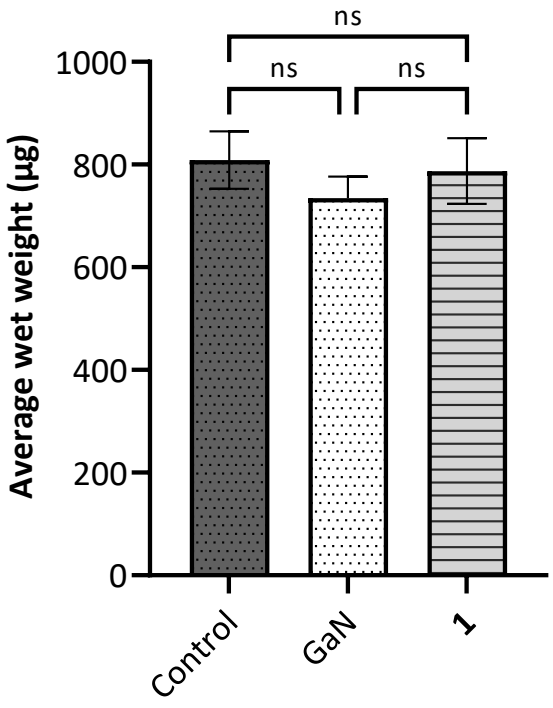
Appendix 8.3. Hemocyte cell count per ml following larval treatment with GaM. Larvae were inoculated with 20 µl of 500 and 1000 µg/ml GaM and PBS, incubated at 37°C and assessed over 24 h. Hemocytes were extracted and counted via a hemocytometer at specific time points post GaM treatment. All values are the mean ± S.E. of three independent experiments.



Appendix 8.4. Two-way unsupervised hierarchal clustering of the median expression levels of all statistically significant differentially abundant proteins (SSDA), (n = 389). On the basis of similar expression trends, three replicates are grouped together into three hierarchal clusters (columns), each representing two protein clusters (A and B).

Appendix 8.5. Top ten characterised SSDA proteins (*t*-tests, $p < 0.05$) increased and decreased in abundance in *P. aeruginosa* treated with 1000 µg/ml GaM and incubated at 37°C for 6 h as compared to non-treated *P. aeruginosa*.

Protein name	Gene name	Peptides	Sequence coverage [%]	Score	Fold change
ArsC	arsC	9	87.2	191.13	+37.99
ArsR	arsR	5	48.3	18.694	+15.13
FMN_red domain-containing protein	PA2280	8	47	45.387	+8.98
Methylated-DNA—protein-cysteine methyltransferase	PA3596	10	38.1	95.793	+8.5
Heme oxygenase PigA	pigA/HemO	10	59.1	76.989	+7.6
Biotin synthase	bioB	9	48	97.883	+7.14
Transcriptional regulator	PA3260	3	28.2	16.67	+5.75
Co-chaperone protein HscB homolog	hscB	6	59	27.164	+4.83
Hemin degrading factor	PA4709	8	34.5	64.507	+4.19
Formimidoylglutamase	hutG	4	21.2	44.768	+4.15
Ton-B dependent receptor	PA5505	19	66.5	293.39	–3.3
Cold-shock protein CspD	cspD	5	82.2	52.67	–3.32
Probable molybdoprotein-binding protein	PA3441	2	38	4.5436	–4.3
Type 4 fimbrial biogenesis protein PilY2	pilY2	6	91.3	45.269	–4.6
Dihydrolipoyl dehydrogenase	lpdV	31	83.4	261.03	–5.45
DUF1653 domain-containing protein	PA3012	2	18.5	21.927	–6.2
Dihydrolipoyl dehydrogenase	lpdG	32	74.5	323.31	–7.07
Anaerobic and virulence activator modulator AnvM	anvM/PA3880	5	64.1	45.989	–9.75
Protease LasA	lasA	14	51.7	164.09	–11.83
Elastase	lasB	16	52.4	113.05	–16.35



Appendix 8.6. Average wet weights of *A. fumigatus* grown for 24 h at 37°C, treated with GaN and **1**, and grown for a further 24 h. One-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$) show no statistically significant differences between sample groups, n = 5, per sample group.

Appendix 8.7. The top 10 statistically significant ($p < 0.05$) *A. fumigatus* proteins increased and decreased in abundance (fold change ≥ 1.5) in response to GaN (250 μg/ml) versus control.

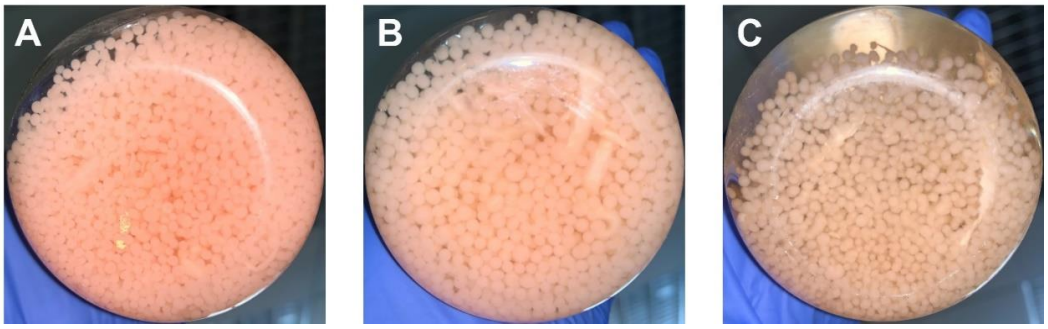
Protein name	Protein ID	Gene name	Function	Fold change
Nitric oxide dioxygenase	Q4W9X3	AFUA_4G03410	Cellular response to nitrosative stress	+52
N(5)-hydroxyornithine:cis-anhydromevalonyl coenzyme A-N(5)-transacylase sidF	Q4WF55	sidF	N',N",N"'-triacetylfusarinine C biosynthesis	+36
L-ornithine N(5)-monooxygenase	E9QYP0	sidA	Ferrichrome biosynthesis	+21
Mevalonyl-coenzyme A hydratase sidH	Q4WF54	sidH	N',N",N"'-triacetylfusarinine C biosynthesis	+19
Superoxide dismutase [Cu-Zn]	Q9Y8D9	sodC	Removal of superoxide radicals	+12
Nonribosomal peptide synthase sidD	Q4WF53	NRPS4	N',N",N"'-triacetylfusarinine C biosynthesis	+8
Iron transport multicopper oxidase fetC	E9R598	fetC	Reductive iron assimilation	+7
Uncharacterized protein	Q4WQH8	AFUA_4G12880	Unknown	+7
Acyl-CoA ligase sidI	Q4WR83	sidI	Siderophore biosynthesis	+7
Indoleamine 2,3-dioxygenase	Q4WAP3	AFUA_7G02010	Tryptophan catabolic process to kynurenine	+6
Cytochrome b5, putative	Q4X246	AFUA_2G07720	Sterol biosynthesis	−3
Cysteine synthase B, putative	Q4W9S1	AFUA_4G03930	Cysteine biosynthesis	−3
Ubiquinone biosynthesis protein, putative	Q4WNS2	AFUA_4G06760	Lipid homeostasis; Mitochondrion organisation	−3
Methyltransferase psoC	Q4WB00	psoC	Methylation	−4
FAD dependent oxidoreductase domain-containing protein	Q4WGZ6	AFUA_7G06780	Sarcosine oxidase activity	−4
L-amino acid oxidase LaoA	Q4WGZ9	AFUA_7G06810	Amino acid catabolic process	−4
Acyltransferase, putative	Q4WTS0	AFUA_5G05880	Phosphatidylinositol acyl-chain remodelling	−4
Dual-functional monooxygenase/methyltransferase psoF	Q4WAZ0	psoF	Fumagillin biosynthesis	−5
CobW domain protein	Q4WBK5	AFUA_8G02620	Unknown	−6
Glucose repressible protein Grg1, putative	Q4WW28	AFUA_5G14210	Glucose metabolism	−50

Appendix 8.8. The top 10 statistically significant ($p < 0.05$) *A. fumigatus* proteins increased and decreased in abundance (fold change ≥ 1.5) in response to **1** (250 μg/ml) versus control.

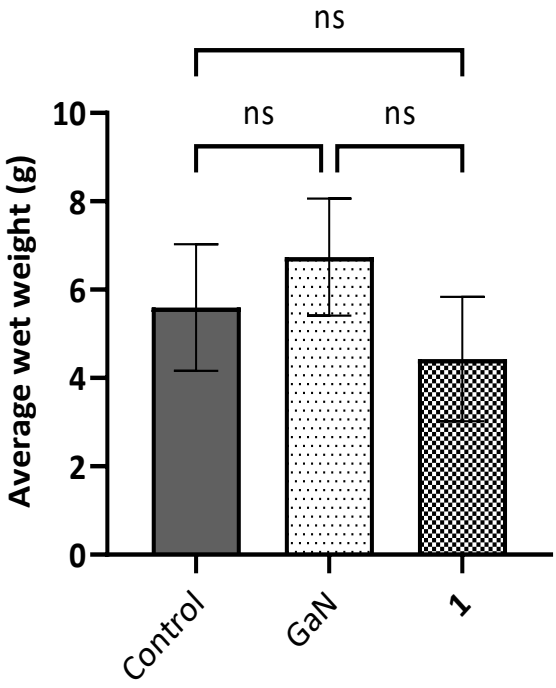
Protein name	Protein ID	Gene name	Function	Fold change
Cupin domain protein	Q4WYM9	AFUA_3G13620	Suspected pathogenesis	+398
Superoxide dismutase [Cu-Zn]	Q9Y8D9	sodC	Removal of superoxide radicals	+69
N(5)-hydroxyornithine:cis-anhydromevalonyl coenzyme A-N(5)-transacylase sidF	Q4WF55	sidF	N',N'',N'''-triacetylfusarinine C biosynthesis	+28
DUF636 domain protein	Q4WKA2	AFUA_1G03180	Metal ion binding (not fully characterised)	+25
4-hydroxyphenylpyruvate dioxygenase	Q4WHU1	hppD	Melanin biosynthesis	+20
DUF323 domain protein	Q4WZV3	AFUA_2G15650	Ergothioneine biosynthesis for antioxidant properties	+17
Methylsterol monooxygenase erg25A	Q4WBI8	erg25A	Ergosterol biosynthesis	+16
Nonribosomal peptide synthase sidD	Q4WF53	NRPS4	N',N'',N'''-triacetylfusarinine C biosynthesis	+16
Elastase inhibitor AFUEI	Q4WZ11	AFUA_3G14940	Negative regulation of peptidase activity	+15
BYS1 domain protein, putative	Q4WE72	AFUA_5G01990	Unknown	+14
Mitochondrial import inner membrane translocase subunit tim14	Q4WI88	pam18	Protein import into mitochondrial matrix	−7
3-isopropylmalate dehydratase	Q4X144	AFUA_2G11260	Amino acid biosynthesis	−7
ABC multidrug transporter mdr1	Q4WTT9	mdr1	Transmembrane transport	−8
Isocyanide synthase-NRPS hybrid crmA	Q4WYN6	crmA	Secondary metabolite biosynthesis	−11
ABC transporter, putative	Q4WV21	AFUA_5G10510	Transmembrane transport	−12
Plasma membrane low affinity zinc ion transporter, putative	Q4WHX5	AFUA_2G03860	Zinc ion transport	−13
Cytochrome c oxidase assembly factor 6	Q4WXK0	AFUA_3G09780	Mitochondrial cytochrome c oxidase assembly	−14
CobW domain protein	Q4WBK5	AFUA_8G02620	Unknown	−16
Cysteine synthase B, putative	Q4W9S1	AFUA_4G03930	Cysteine biosynthesis	−27
Glucose repressible protein Grg1, putative	Q4WW28	AFUA_5G14210	Glucose metabolism	−58

Appendix 8.9. The top 10 statistically significant ($p < 0.05$) *A. fumigatus* proteins increased and decreased in abundance (fold change ≥ 1.5) in response to **1** (250 μg/ml) versus GaN (250 μg/ml).

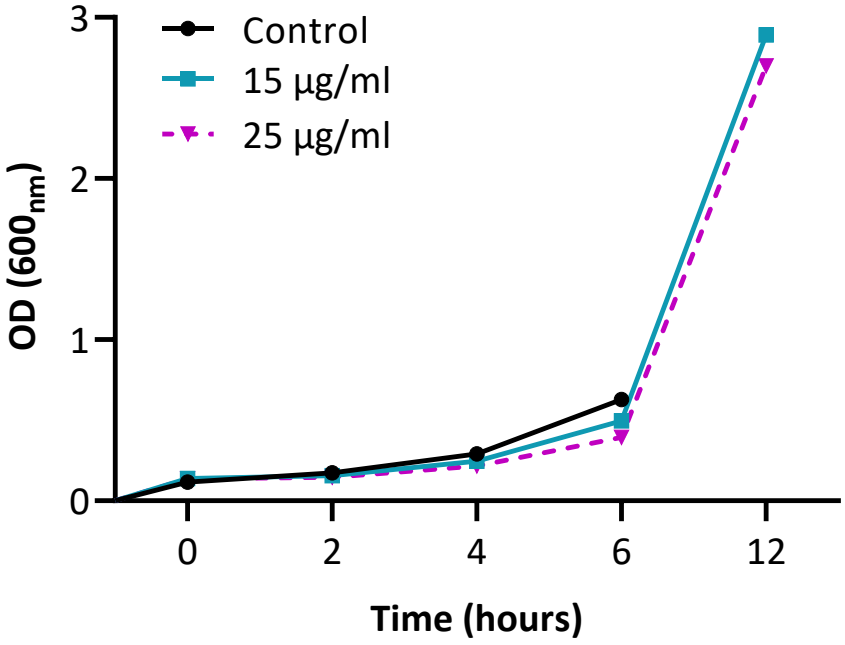
Protein name	Protein ID	Gene name	Function	Fold change
Cupin domain protein	Q4WYM9	AFUA_3G13620	Suspected pathogenesi	+230
Uncharacterised protein	Q4W8X0	AFUA_6G00180	Fibrinogen binding	+27
4-hydroxyphenylpyruvate dioxygenase	Q4WHU1	hppD	Melanin biosynthesis	+23
Uncharacterised protein	Q4WQF0	AFUA_4G12590	Lipid binding	+19
Ribonucleotide reductase small subunit RnrA, putative	Q4WVJ7	AFUA_5G12350	Deoxyribonucleotide biosynthesis	+17
DUF636 domain protein	Q4WKA2	AFUA_1G03180	Metal ion binding	+15
AAA family ATPase, putative	Q4WGY6	AFUA_7G06680	ATP binding	+15
BYS1 domain protein, putative	Q4WE72	AFUA_5G01990	Unknown	+14
DUF323 domain protein	Q4WZV3	AFUA_2G15650	Ergothioneine biosynthesis for antioxidant properties	+14
IgE-binding protein	Q4W8Z7	AFUA_6G00430	Allergin	+11
Myb-like domain-containing protein	A4D9H4	AFUA_5G14865	Growth and development	−8
proline--tRNA ligase	Q4WT30	AFUA_1G10750	Prolyl-tRNA aminoacylation	−8
Acyl-CoA dehydrogenase family protein	Q4WGS9	AFUA_7G06100	Fatty acid metabolism	−8
Glutathione S-transferase	Q4WZD9	AFUA_2G17300	Protection against oxidative stress	−9
Cysteine synthase B, putative	Q4W9S1	AFUA_4G03930	Cysteine biosynthesis	−9
Proteasome subunit beta	Q4WNZ8	AFUA_4G07510	Proteasome-mediated ubiquitin-dependent protein catabolic process	−10
Aldo-keto reductase (AKR13), putative	Q4WAB4	AFUA_7G00700	Aldo-keto reductase (NADP) activity	−12
Cytochrome c oxidase assembly factor 6	Q4WXK0	AFUA_3G09780	Mitochondrial cytochrome c oxidase assembly	−14
Aldose 1-epimerase, putative	Q4WWG2	AFUA_3G05740	Glucose metabolism	−15
Nitric oxide dioxygenase	Q4W9X3	AFUA_4G03410	Cellular response to nitrosative stress	−57



Appendix 8.10. Calorimetric change of *A. fumigatus* cultures untreated (A), treated with 250 μg/ml GaN (B) and treated with 250 μg/ml **1** (C) for 24 h and supplemented with 0.5 mg/ml triphenyltetrazolium chloride (TTC) for a further 24 h. Mitochondrial enzymes convert colourless TTC to produce red formazan crystals (presented as pink *A. fumigatus* hyphal pellets). Formazan is solubilised with acidified isopropanol and the intensity of colour in supernatant is measured in a microplate reader at 570 nm.



Appendix 8.11. Average wet weights of gallium-treated and untreated *A. fumigatus* cultures supplemented with TTC for 24 h. Wet weights were recorded prior to formazan solubilisation in HCl isopropanol. One-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$) show no statistically significant differences between sample groups, n = 4, per sample group.



Appendix 8.12. Growth curve of *C. parapsilosis* with SBC3 (15 and 25 µg/ml). YEPD supplemented with SBC3 was inoculated with *C. parapsilosis* and incubated for up to 12 h at 30°C in an orbital shaker at 120 rpm. Readings were taken at various time intervals using a spectrophotometer at an optical density (OD) of 600 nm to determine fungal growth. All values are the mean ± S.E., n = 3.

Appendix 8.13. Top 20 increased and decreased proteins in *C. parapsilosis* treated with 15 μg/ml SBC3.

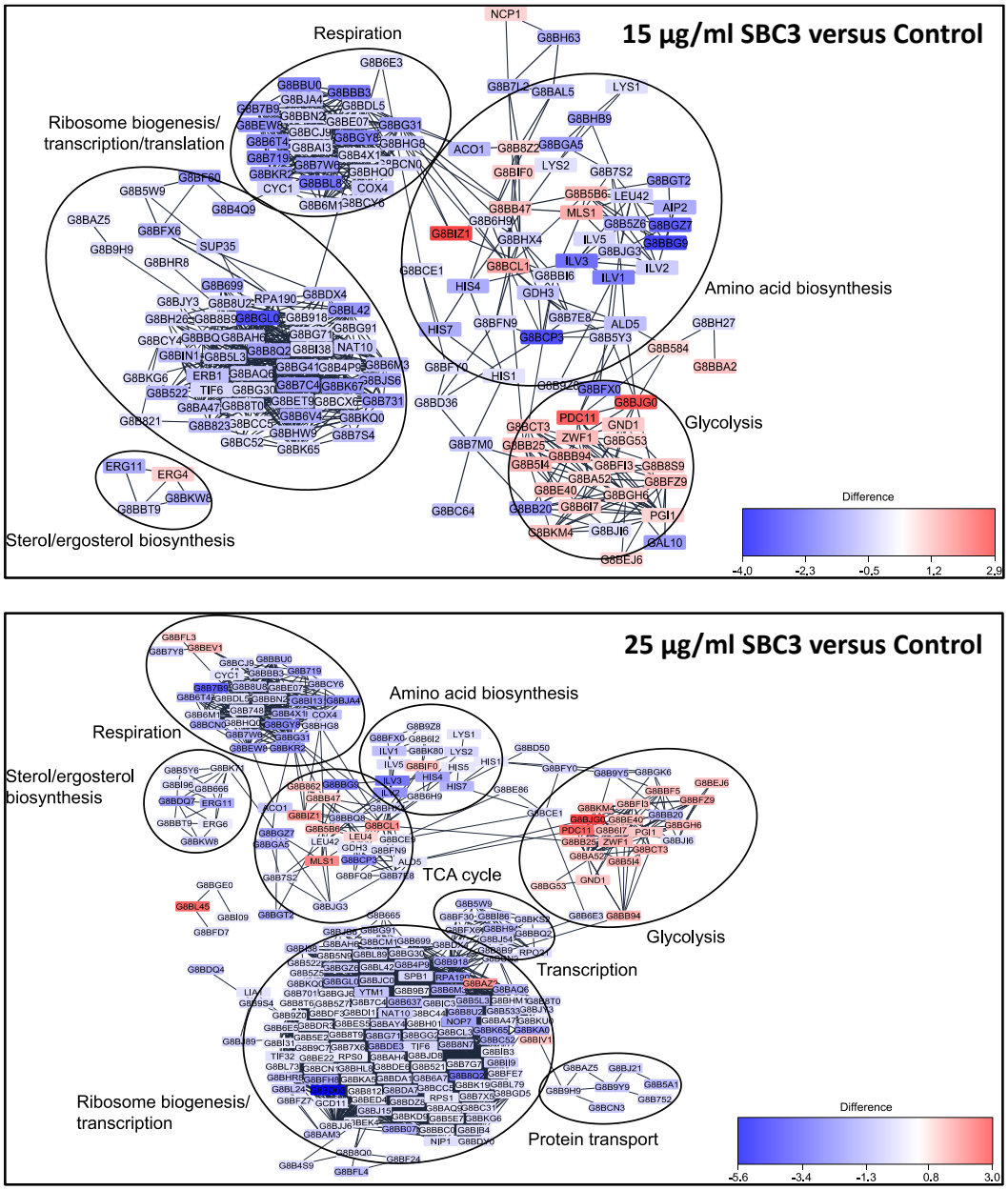
Protein name	Gene name	Peptides	Sequence coverage [%]	Score	Fold change
Chitinase	CPAR2_502120	16	24.3	115.93	+222.2
CFEM2	CPAR2_402910	1	6	14.536	+207.2
CFEM5; CFEM6	CPAR2_300120; CPAR2_300110	4	8.7	116.31	+162.5
Uncharacterised	CPAR2_304370	39	35.2	102.48	+42.5
Uncharacterised	FET3	13	26.6	129.09	+35.0
RRM domain-containing protein	CPAR2_100830	13	62.6	80.232	+35.0
Thioredoxin domain-containing protein	CPAR2_402640	12	87.4	115.02	+29.8
Uncharacterised	CDR1	39	34.7	323.31	+25.0
Uncharacterised	CPAR2_303120	8	9.6	63.535	+22.8
Chitinase	CPAR2_502130	7	15.3	34.261	+21.1
Peptidylprolyl isomerase	CPAR2_202630	5	55.3	26.502	+20.3
Uncharacterised	CPAR2_208430	9	51	323.31	+19.6
FAD-binding FR-type domain-containing protein	CFL5	11	19.5	56.965	+18.3
Uncharacterised	SCR2; ADH; SCR3	12	59.9	112.28	+17.7
FAD-binding FR-type domain-containing protein	CPAR2_210110; CPAR2_808110	9	15.4	28.301	+17.2
Pyr_redox_2 domain-containing protein	CPAR2_802640	11	33.3	57.834	+14.1
Uncharacterised	CPAR2_603800	38	30.8	25.22	+11.9
Uncharacterised	CPAR2_301610	7	19.4	59.647	+11.4
Alpha-mann_mid domain-containing protein	CPAR2_500360	11	16.3	31.539	+10.1
Phosphatidylinositol transfer protein SFH5	CPAR2_804790	9	37.2	22.669	+7.4
AMP-binding domain-containing protein	CPAR2_600460	8	26.8	76.429	−5.4
MFS domain-containing protein	CPAR2_104450	4	15.4	29.788	−5.5
Uncharacterised	CPAR2_701510	9	35.9	34.579	−5.6
4Fe-4S ferredoxin-type domain-containing protein	CPAR2_801570	5	34.9	44.97	−5.8
Mitochondrial import inner membrane translocase subunit TIM44	CPAR2_504060	15	41.8	58.004	−5.9
Uncharacterised	CPAR2_102890	2	20	14.493	−5.9
NAD(P)-bd_dom domain-containing protein	CPAR2_503600	16	60.2	126.83	−6.0
Uncharacterised	CPAR2_807040	4	29.9	27.563	−6.2

Beta_elim_lyase domain-containing protein	CPAR2_203970	9	48.2	34.776	−6.3
Threonine dehydratase	ILV1	13	32.8	50.422	−6.6
Oxidored_q6 domain-containing protein	CPAR2_808860	3	16.4	11.678	−7.0
Cytochrome b5 heme-binding domain-containing protein	CPAR2_703300	2	18	7.4867	−7.1
Uncharacterised	CPAR2_800850	6	38.8	22.069	−7.5
Uncharacterised	ILV3	16	57.4	147.15	−8.1
Uncharacterised	CPAR2_407280	2	14.2	16.392	−8.4
Candida_ALS_N domain-containing protein	ALS7; CPAR2_404780; ALS3	7	10.6	54.262	−8.6
Uncharacterised	CPAR2_503690	15	51.1	72.504	−10.0
Uncharacterised	CPAR2_206380	29	22.8	83.333	−12.7
Glutamine amidotransferase type-2 domain-containing protein	CPAR2_206810	32	24.2	115.55	−13.1
3-isopropylmalate dehydratase	CPAR2_800360	26	40	112.53	−15.5

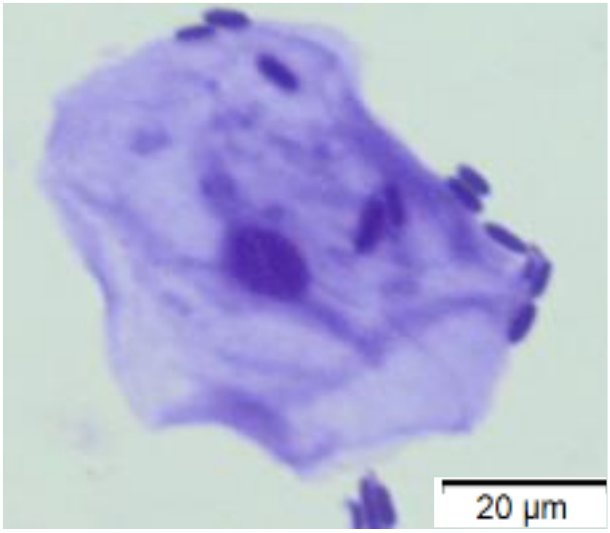
Appendix 8.14. Top 20 increased and decreased proteins in *C. parapsilosis* treated with 25 μg/ml SBC3.

Protein name	Gene name	Peptides	Sequence coverage [%]	Score	Fold change
CFEM2	CPAR2_402910	1	6	14.536	+178.5
CFEM6; CFEM5	CPAR2_300120; CPAR2_300110	4	8.7	116.31	+171.6
Chitinase	CPAR2_502120	16	24.3	115.93	+139.2
Uncharacterised	CPAR2_304370	39	35.2	102.48	+57.5
RRM domain-containing protein	CPAR2_100830	13	62.6	80.232	+42.7
Uncharacterised	CDR1	39	34.7	323.31	+32.6
Thioredoxin domain-containing protein	CPAR2_402640	12	87.4	115.02	+27.8
Uncharacterised	FET3; CPAR2_603590	13	26.6	129.09	+22.2
Uncharacterised	SCR2; ADH; SCR3	12	59.9	112.28	+20.8
Thioredoxin domain-containing protein	CPAR2_403210	6	67.8	22.101	+18.8
Uncharacterised protei	CPAR2_603800	38	30.8	25.22	+18.1
Pyr_redox_2 domain-containing protein	CPAR2_802640	11	33.3	57.834	+17.3
CFEM7	PAR2_500080	2	33.8	22.019	+16.0
Chitinas	CPAR2_502130	7	15.3	34.261	+15.5
Uncharacterised	CPAR2_208430	9	51	323.31	+15.0
Uncharacterised	CPAR2_209770	7	7.1	34.804	+14.6
Uncharacterised	CPAR2_206560	4	31.2	30.568	+13.7
Uncharacterised	CPAR2_301610	7	19.4	59.647	+13.5
Uncharacterised	CPAR2_303120	8	9.6	63.535	+13.5
PKS_ER domain-containing protein	CPAR2_211280; CPAR2_211230	13	58.3	79.107	+13.0
Uncharacterised	CPAR2_400800	4	55.9	11.006	−7.1
Uncharacterised	CPAR2_108260	17	56.3	105.03	−7.1
Uncharacterised	CPAR2_200690	30	57.8	318.26	−7.2
H/ACA ribonucleoprotein complex subunit NOP10	CPAR2_701770	4	62.7	121.06	−7.2
Squalene monooxygenase	CPAR2_210480	10	24.9	38.203	−7.4
MFS domain-containing protein	CPAR2_600460	8	26.8	76.429	−7.8
DNA-directed RNA polymerase subunit	RPA190	26	26.2	124.03	−7.8
Complex1_49kDa domain-containing protein	CPAR2_703410	16	43.3	144.06	−8.2
Uncharacterised	CPAR2_407940	39	20.7	150.2	−8.3
Mitochondrial import inner membrane translocase subunit TIM44	CPAR2_504060	15	41.8	58.004	−8.7
Acetolactate synthase	ILV2	25	44.8	190.6	−9.0

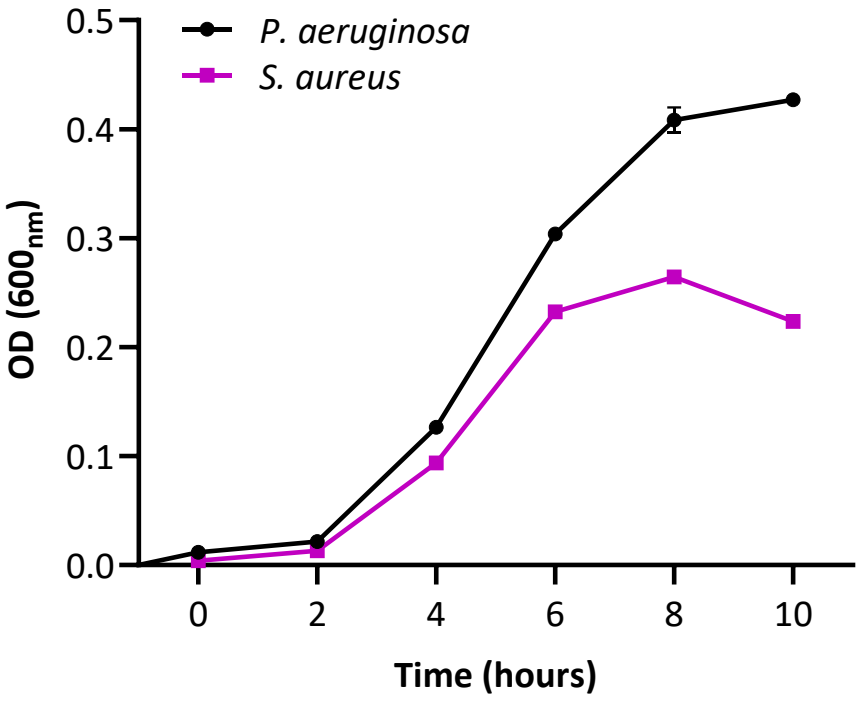
Glutamine amidotransferase type-2 domain-containing protein	CPAR2_206810	32	24.2	115.55	−9.7
zf-CHCC domain-containing protein	CPAR2_405230	10	71.8	58.2	−10.0
NAD(P)-bd_dom domain-containing protein	CPAR2_503600	16	60.2	126.83	−11.0
3-isopropylmalate dehydratase	CPAR2_800360	26	40	112.53	−15.6
Uncharacterised	CPAR2_108760	24	44.7	323.31	-16.1
Uncharacterised	ILV3	16	57.4	147.15	-16.4
Uncharacterised	CPAR2_601420	5	47	127.71	-20.9
NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	CPAR2_103910	21	64	99.345	-24.7
Ribosomal protein L37	CPAR2_207910	3	26.7	10.947	-48.6



Appendix 8.15. Cytoscape network analysis of differently expressed proteins in *C. parapsilosis* treated with SBC3 15 µg/ml versus control, 25 µg/ml versus control and 25 µg/ml versus 15 µg/ml SBC3. SSDA proteins were uploaded onto Cytoscape software using a high confidence score (0.9) to create interaction networks. Nodes represent individual proteins that are interconnected to establish pathways. Differential expression is highlighted in red (increased abundance) and blue (decreased abundance). Functions associated with protein clusters are annotated according to functional enrichment in UniProt key words.



Appendix 8.16. Microscopy image of *C. parapsilosis* cells attached to a buccal epithelial cell (BEC).



Appendix 8.17. Growth curves of *P. aeruginosa* and *S. aureus* over a 10-hour period. All values are the mean ± S.E. of three replicate experiments.